ORAL PRESENTATIONS

SUNDAY, DECEMBER 4

Symposium 1: Molecular Mechanisms

1

Dicing and Beyond: Regulatory RNA in Humans and Bacteria.

J. Doudna[1]; [1]Department of Molecular and Cell Biology, Univ California-Berkeley/HHMI, Berkeley, CA

In eukaryotic cells, small RNA molecules regulate the expression of many genes. The enzyme Dicer plays a central role in producing these RNAs and ensuring that they assemble with other proteins into effector complexes. Recent findings show that Dicer and its partner RNA binding proteins can alter miRNA processing to change the set of mRNAs targeted for silencing. Although Dicer homologs do not occur in prokaryotic cells, bacteria possess distinct sets of enzymes that produce short RNA molecules to block the propagation of viral and plasmid sequences. In bacteria, these regulatory RNAs are produced from clustered regularly interspaced short palindromic repeats (CRISPRs). I will discuss our recent work to uncover the molecular basis for small RNA production and targeting. Molecular structures of the CRISPR machinery for targeting nucleic acids will be presented, revealing how the RNA is used to identify foreign sequences for destruction. Based on these structures and related biochemical studies, we find interesting similarities in target recognition mechanisms between RNAi-type and CRISPR-type systems.

2

Molecular Origami: chaperone-asisted protein folding and misfolding in health and disease.

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Achieving correct protein folding and quality control is essential for normal cellular function. The accumulation of misfolded proteins is emerging as central to a wide range of disease states, including many neurodegenerative disorders such as Huntington's and Prion Disease. Molecular chaperones are a diverse family of enzymes that assists the folding of newly translated and stress-denatured proteins, as well as affects protein quality control. The role of chaperones in these diverse functions will be discussed.
Biochemical analyses together with systems approaches are used to define the chaperone networks and mechanisms in the eukaryotic cytosol. We find that a chaperone network linked to the protein synthesis apparatus assists protein biogenesis. The emergence of this translation-linked chaperone network likely underlies the elaborate co-translational folding process necessary for the evolution of larger multidomain proteins characteristic of eukaryotic cells. A stress-inducible chaperone network protects cells from environmental stress and assists quality control. These chaperones also communicate with the ubiquitin-proteasome pathway to clear misfolded proteins from the cell. We identify two intracellular compartments for the sequestration of misfolded cytosolic proteins and discuss how our findings provide a framework to understand the link between protein misfolding and human disease.

3

Quantitative Systems Biochemistry of Microtubule Cytoskeleton Organisation and Function.

T. Surrey[1]; [1]Cancer Research UK London Research Institute, London, England

The microtubule cytoskeleton performs essential mechano-chemical tasks in eukaryotic cells. It is crucial for the internal organisation of the cell, intracellular trafficking and for the separation of the genetic material during cell division. These complex processes require the coordinated activity of dynamic microtubules, molecular motors, their regulators, and other proteins linking microtubules to intracellular substructures. All these proteins form a dynamically interconnected microtubule cytoskeleton whose distinct biological function is intimately linked to its overall organisation and dynamic state. How this network operates as an integrative system and how its large-scale behaviour depends on the combinatorial action of its nano-scale biochemical constituents is a major open question. Biochemical reconstitutions of cytoskeletal subsystems mimicking in vivo behaviour, in combination with quantitative fluorescence microscopy and cryo-electron microscopy, have become a powerful approach to extract the underlying rules of how the microtubule cytoskeleton acts as a dynamic system. For example, through this approach the hierarchical functioning of a regulatory protein interaction network at growing microtubule ends, formed around so-called end binding (EB) proteins, can now be understood from the atomic to the micrometer scale. Similarly, the minimal mechanism for the establishment of distinct topological features of bipolar spindles, e.g. central anaphase microtubule overlaps mediated by PRC1 and a kinesin regulating microtubule dynamics, can now be understood from the knowledge of the biophysical properties of the interacting proteins. In the future, the challenge will be to reconstitute even more complex systems to be able to test directly our understanding of higher-order cytoskeletal functions.

Bruce Alberts Award for Excellence in Science Education

4

CourseSource, a New Partnership for Higher Education.

P. Bruns[1]; [1]HHMI, Chevy Chase, MD

High quality undergraduate science instruction is being challenged by a changing world. Knowledge is expanding at an increasing rate. Students are more diverse in age, background and place of instruction, and use technology to gather information in new ways. Education research is uncovering aspects of student learning that can advance the practice of teaching. And although the internet is a growing source of information, it is failing to enhance instruction; it is noisy, complex and unreliable. Faculty members want to respond but they do not want to reinvent the wheel every time they contemplate revising an existing course or creating a new one. The growing inclusion of educational issues in the organization and workings of scientific societies such as ASCB shows that the scientific community takes improved teaching seriously and recognizes the need for change, but professional society responses are independent efforts with few interconnections between disciplines and no uniformity in type of materials offered. Finally, a recent national effort organized by the American Association for the Advancement of Science (“Vision and Change in Undergraduate Biology Education”) strongly supports the adoption of student centered education strategies and decisively calls for a web tool that would be easy to use, address undergraduate teaching needs, and be reliably vetted.

I will describe a web portal, the CourseSource, designed to respond to these issues. The site will be organized by courses and will be based on highly successful design principles

recommended by Vision and Change and promoted by efforts such as the National Academy of Sciences Summer Institute for Undergraduate Education in Biology. Each course will be represented by a series of modules; each module will be anchored by a specific learning goal and contain appropriate assessment and learning materials. Initial collections of modules for specific courses will be made in partnership with appropriate scientific societies. Content will be included by references and links. CourseSource will include a navigation system that will enable users to follow learning goals and underlying concepts through multiple courses; faculty will be able to find and combine learning goals and associated materials from many different, sometimes seemingly unrelated courses. The primary audience for the portal will be faculty, ranging from those who want the basic design of a whole course ready for use, to people who want to enhance existing courses, to users who want to develop entirely new courses. Although the site will be in English, it will serve faculty from all sectors of higher education, anywhere in the world.

Symposium 2: Function of Multi-Molecular Machines

5

Single Molecule Microscopy of Macromolecular Machines: The Spliceosome.

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Excision of introns from pre-mRNAs is mediated by the spliceosome, a large, dynamic complex consisting of five small ribonucleoprotein particles (snRNPs) and scores of associated proteins. Our current understanding of spliceosome assembly is based largely on the procession of stable complexes that can be resolved from in vitro splicing reactions. Such ensemble experiments have suggested a highly ordered, linear assembly pathway in which initial binding of U1 snRNP to the 5' splice site is followed by stable U2 association with the branch site and subsequent U4/U5/U6 tri-snRNP and Nineteen Complex (NTC) addition to form the fully assembled spliceosome. Previously unknown, however, were the detailed forward and reverse kinetics of each assembly step, the extent to which branched and/or dead-end assembly pathways exist, and whether or not different introns utilize the same or alternate assembly pathway(s). We are now addressing these questions by combining yeast genetic engineering, chemical biology, and multi-wavelengthfluoresence microscopy to follow assembly of single spliceosomes in real time. Because no protein purification or reconstitution is required for such Colocalization Single Molecule Spectroscopy (CoSMoS), this experimental strategy should prove widely useful for mechanistic analysis of many other macromolecular machines in environments approaching the complexity of living cells.

6

Mechanisms and regulation of cullin-RING ubiquitin ligation machines.

R. J. Deshaies[1]; [1]Division of Biology, California Inst Technol/HHMI, Pasadena, CA

The ubiquitin-proteasome system (UPS) comprises a set of macromolecular machines that recognize substrates with a high degree of specificity, catalyze the polymerization of chains of ubiquitin molecules on these substrates, extract them from the complexes in which they reside, and unfold them to enable their degradation. Although it is now appreciated that the protein machines of the UPS play critical roles in normal and diseased cells and are the targets of both disease-causing mutations and therapeutic drugs, we know relatively little about how these

machines operate. My talk will discuss our recent progress in understanding the mechanism of actions and regulation of cullin-RING ubiquitin ligase (CRL) enzymes and an ubiquitin-selective chaperone, p97, that acts downstream of CRLs. I will also touch on the prospect of developing drugs that target these enzymes as potential tools for the treatment of cancer.

7

Spatio-Dynamics of Clathrin-Mediated Endocytosis in Yeast and Mammals.

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Clathrin-mediated endocytosis (CME) is the best-studied pathway by which cells selectively internalize molecules from the plasma membrane and surrounding environment. We study this process by live-cell microscopy in yeast and mammalian cells. The yeast studies have revealed a regular sequence of events necessary for endocytic vesicle formation involving some 60 proteins, which induce a highly choreographed series of changes in membrane geometry, ultimately resulting in scission and vesicle release. To analyze endocytic dynamics in mammalian cells in which endogenous protein stoichiometry is preserved, we previously targeted zinc finger nucleases (ZFNs) to the clathrin light chain A and dynamin-2 genomic loci and generated cell lines expressing fluorescent protein fusions from each locus (1). The genome-edited cells exhibited enhanced endocytic function, dynamics and efficiency when compared with previously studied cells. The high regularity of CME dynamics in these genome-edited cells has now been exploited to interrogate by RNAi and chemical inhibition how endocytic proteins and the actin cytoskeleton contribute to endocytic vesicle formation and to the regulation and dynamics of the process. These studies demonstrate the importance of actin assembly for robust endocytic dynamics in mammalian cells.

1. Doyon JB, Zeitler B, Cheng J, Cheng AT, Cherone JM, Santiago Y, Lee AH, Vo TD, Doyon Y, Miller JC, Paschon DE, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Drubin DG. (2011) Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited mammalian cells. Nat Cell Biol. 13(3):331-7.

E.E. Just Lecture

8

Thymic Nurse Cell Function: The "Proto-Thymus"?

J. Guyden[1]; [1]Department of Biology, City Coll New York, New York, NY

MHC restriction is a selection process that removes potentially autoreactive thymocytes during T cell development. The thymocyte subset that participates in the process of MHC restriction expresses the ??TCR+CD4+CD8+ cell surface phenotype. Thymocytes that survive MHC restriction continue to develop into mature functional T cells. Over ninety-five percent of developing thymocytes do not survive this process and are induced to undergo apoptosis. Thymic nurse cells (TNCs) are specialized epithelial components of the thymic stroma that contain developing T cells completely enclosed within intra-cytoplasmic vacuoles. As many as 200 thymocytes are found within this unique multicellular complex. For twenty years after their discovery very little information about their function was reported due to the lack of pure TNC populations in quantities necessary for thorough analyses. Our laboratory was the first to immortalize TNCs and to make a TNC-specific monoclonal antibody (pH91). Cells from our TNC lines have been shown to internalize thymocytes in tissue culture. This was the first report of the

isolation of a cell able to internalize and maintain viable another cell. Since that time, we have shown that TNCs exclusively bind and internalize ??TCR+CD4+CD8+ cells (the thymocyte subset involved in MHC restriction). We captured the internalization process using video microscopy. A subset of internalized thymocytes dies through the process of apoptosis and is degraded through lysosomal fusion. The remaining thymocytes survive and are released back into the general microenvironment of the thymus. We propose that TNCs house thymocytes undergoing the process of MHC restriction.

Most recently we performed a literature search and discovered that thymic nurse cells exist in the thymus of all jawed vertebrates (Chondrichthyes). Even shark thymi (almost 500M years old) have TNCs. On the other hand Good and Finstad (1964) discovered that lampreys, jawless vertebrates (Agnatha) that are the most immediate extant ancestors to sharks, have no thymus. This precipitated the question of what provided thymic functions before a thymus existed. Using lamprey larvae, Good and Finstad described what they termed the “proto-thymus” as 5-25 lymphocytes in intimate contact with epithelial cells located in the pharyngeal gutter. This cellular complex was described as having no barriers between the lymphocytes and the epithelial cell. They propose these complexes to be the primitive forerunners of the lymphoepithelial thymus. In 1995, Zurbrigg and Beamish found a subset of lymphocytes to stain with anti-Thy-1 antibody in the typhlosole of larval lamprey. Recently, we stained the typhlosole with the TNC-specific monoclonal antibody pH91 and found pH91+ stromal cells in association with Thy-1 positive lymphocytes. We have now isolated structures with the morphology of thymic nurse cells from the typhlosole of lamprey larvae. Are thymic nurse cells evolutionary descendants of the proto-thymus? Or, do thymic nurse cells represent the evolutionary bridge from the lamprey VLR-based immune system to one involving the IgG superfamily?

Minisymposia 1: Actin Dynamics

9

The formin Cappuccino contains a C-terminal actin binding domain that is essential for nucleation and is regulated by Spire.

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The *Drosophila* formin Cappuccino (Capu) is essential for body axis establishment during oogenesis. Capu interacts directly with the WH2-based actin nucleator Spire via a short (~30 aa) sequence at the C-terminus of Capu and the N-terminal kinase non-catalytic C-lobe domain of Spire (Vizcarra, 2011), analogous to the interaction described in their mammalian orthologs (Pechlivanis, 2009). This interaction potently inhibits Capu’s actin nucleation activity (Quinlan, 2007), which lead us to further investigate the role of Capu’s C-terminal tail in actin assembly. We found that truncation of the tail markedly reduced Capu’s ability to stimulate polymerization in pyrene-actin assays. Additionally, substitution of the tail with the DAD domains from the more potent nucleators mDia1 and mDia2 increased the actin assembly activity of Capu, while substitution with the DAD domain from the less active FRL1 suppressed its activity. Monomer binding was recently reported for the DAD domains of Diaphanous-related formins. The Capu tail also binds actin monomers as well as actin filaments. Interestingly, the interaction between the Capu tail and actin monomers was observed for actin purified from *Acanthamoeba castellanii* and rabbit skeletal muscle but not for actin purified from *Saccharomyces cerevisiae*. However, a single point mutation in *S. cerevisiae* actin, A167E, was sufficient to recover actin

binding by the Capu tail to affinities comparable to those measured for other actins. The fact that the Capu tail is able to bind to the A167E mutant of yeast actin leads us to hypothesize that the tail binds in or near the hydrophobic cleft of actin. Consistent with this, WH2 domains compete with the Capu tail for actin binding. Taken together, our data indicate that the Capu tail is an actin binding domain that coordinates with the FH1 and FH2 domain to assemble actin filaments. The fact that actin binding by the Capu tail is essential for nucleation provides a mechanistic explanation for regulation of Capu by Spir. Moreover, we propose that the C-termini of most or all formins help determine the nucleation rates of their respective FH2 domains.

Pechlivanis M., Samol A., Kerkhoff E. (2009) *J. Biol. Chem.* **284**, 25324-25333.
Quinlan M.E., Hilgert S., Bedrossian A., Mullins R.D., Kerkhoff E. (2007) *J. Cell Biol.* **179**, 117-28.
Vizcarra C.L., Kreutz B., Rodal A.A., Toms A.V., Lu J., Zheng W., Quinlan M.E., Eck M.J. (2011) *Proc. Natl. Acad. Sci. U.S.A.* **108**, 11884-11889.

10

Actin network architecture determines myosin motor activity.

A-C. Reymann[1], R. Boujemaa-Paterski[1], J-L. Martiel[1], W. Cao[2], H. Chin[2], E. De La Cruz[2], M. Théry[1], L. Blanchoin[1]; [1]Institut de Recherches en Technologies et Sciences pour le Vivant, CNRS/CEA/INRA/UJF, Grenoble, France, [2]Department of Molecular Biophysics and Biochemistry,Yale University, New Haven, CT

The organization of actin filaments into higher-ordered networks governs overall eukaryotic cell shape, mechanical integrity and directed movement. The global architecture of the actin cytoskeleton is determined by coordinated actions of a large number of actin regulatory proteins that modulate filament assembly and disassembly dynamics. Myosin motors also plays a critical role in these processes and reorganize filament structures through sliding (e.g. contractility) and/ or depolymerization. Understanding the molecular mechanism of such complex spatiotemporal orchestration is extremely challenging in cells, where hundreds of different proteins act simultaneously on overlapping actin sub-structures. Here, we use geometrically controlled and polarized in vitro actin networks to evaluate how myosin motors influences filament architecture. Direct visualization of filaments indicates that myosins selectively disassemble randomly-oriented and anti-parallel actin filament structures while parallel actin filament bundles are unaffected by myosin contractility. This “orientation selection” reveals how the overall organization and dynamics of the actin cytoskeleton is controlled by actomyosin contractility. General principles governing the spatial organization of actin filaments in cells emerge from this work.

11

Bar-coding of the actin track and myosin-II motor activity by tropomyosins.

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How alternative assemblies self-organize represents a key problem in cell biology. With respect to the different actomyosin structures found in cells, it remains unclear how the distinct classes and isoforms of myosin motors are specified to their relevant structures. Given the number of different actin structures in cells, properties of the actin track likely contribute to spatial regulation of myosin motor activity.

Using the relatively simple and tractable fission yeast model, we found that the actin filament-binding protein tropomyosin plays a central role in the differential regulation of myosins at distinct actin structures. The presence of fission yeast tropomyosin on the actin track stabilizes

certain actomyosin interactions, favoring myosin-II (Myo2p) and myosin-V (Myo52p) motility in vitro. Tropomyosin in turn promotes Myo2p-driven contractile ring assembly and Myo52p intracellular transport along actin cables in vivo. In contrast, tropomyosin inhibits myosin-I (Myo1p) activity, thereby restricting Myo1p motor function to the endocytic actin patches (which unlike rings and cables are devoid of tropomyosin).

Ultimately we wish to test how well this actin track-based regulation outlined in fission yeast extends into mammalian cells, where there are many more myosins and tropomyosins in play. We are currently taking a global approach using actin-activated ATPase and motility assays to survey and compare the ability of yeast and mammalian tropomyosins to regulate yeast and mammalian (non-muscle and muscle) myosin-II motors. Our analysis is uncovering distinct patterns of regulation that depend on both the tropomyosin and myosin isoform. For example, while fission yeast tropomyosin and two mammalian tropomyosins (Tm4 and Tm5NM1) significantly increase the activity of fission yeast Myo2p, they potently inhibit skeletal muscle myosin-II; on the other hand another mammalian tropomyosin (TmBr3) is passive toward myosin-IIs. We predict that certain tropomyosins can differentially regulate myosin-II isoforms. Such ‘bar-coding’ of the actin track by tropomyosins offers a way of sorting the activity of myosin-II isoforms to specific actin structures in the complex environment of the cytoplasm. This represents a major divergence from current thinking regarding steric regulation of myosin-II, where tropomyosin dictates an ‘open’ or ‘closed’ state. Our studies indicate one state can mediate different outputs depending on the myosin-II isoform.

12

Quantifying the Dynamic Interactions of the Actin Cytoskeleton with T-Cell Receptor Clusters.

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Actin's effects on cell surface receptors have recently received significant attention, and it is becoming clear that virtually every property of receptors can be influenced by the cytoskeleton, including spatial organization, receptor-ligand interactions, and signaling thresholds. Somewhat less characterized are the reciprocal effects of the receptors on the local actin environment. We have studied the behavior of actin as it interacts with microclusters of T-cell receptors (TCRs), which form in response to antigen presentation on an apposing membrane and converge into a micrometer-scale central domain in an actin-dependent manner. Using the hybrid live cell-nanopatterned supported lipid bilayer system, we find that the cytoskeleton shows characteristic responses to TCR clusters that are specifically limited in their ability to laterally diffuse. These responses are spatially and temporally dynamic, and we have developed a quantitative analysis method based on time-autocorrelation to describe them. Such a method may prove useful for identifying and quantifying similar actin behavior during cytoskeleton interactions with other cell surface receptors and in other cell systems, and it should be broadly applicable to any dynamically fluctuating protein-protein interaction that can be observed in real-time.

13

Dual roles of Arp2/3 complex in maintenance of the asymmetric position of meiotic II spindle in mammalian oocytes.

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Mammalian female meiosis is highly asymmetric in that it generates one large-volume mature oocyte and three tiny polar bodies through two consecutive meiotic divisions. Cell division apparatus, the spindle, is formed in the center of the cell and then trans-located to the cortical

region in meiosis I, whereas in meiosis II, the spindle is formed de novo near the cortex. It was clear meiotic I spindle migration is an actin dependant process; however, how the spindle is positioned in meiosis II oocyte remains mysterious. Here, we show that the Arp2/3 complex plays dual roles in the maintenance of asymmetric spindle positioning in meiosis II oocyte. The Arp2/3 complex is localized to the cortical cap through a Ran-dependant mechanism, where it nucleates the assembly of a continuous actin flow. With the assistance of high resolution live imaging, spatiotemporal image correlation spectroscopy (STICS) and mathematic simulation, we show that this actin flow then powers a cytoplasmic streaming that exerts a net pushing force to the spindle toward the cortical cap. Inhibition of the Arp2/3 complex-nucleated actin assembly completely abolished the actin flow and the cytoplasmic streaming. Under this condition, it was further revealed the myosin based contractile force in actomyosin-rich cortical region could be active even before cytokinesis onset. When Arp2/3 nucleated actin assembly is inhibited, this contractile force facilitates a cytoplasmic streaming in reverse direction, resulting in spindle movement away from the cortex. Therefore, our results indicate that, in meiotic II oocyte, the role of Arp2/3 nucleated actin assembly is two-fold: initiating continuous actin flow from the cortical cap and preventing premature actomyosin contraction of the same cortical domain. The asymmetric positioning of MII spindle is maintained dynamically through the balance of these actin-based forces.

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Regulation of stereocilia length by myosin XVa and whirlin depends on the actin-regulatory protein Eps8.

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Proper hearing and balance depend on the staircase shaped bundle of inner ear hair cell stereocilia, which are the actin-based protrusions or “hairs” inside our cochlear and vestibular organs which move in response to sound vibrations and head movement. In comparison to other actin protrusions (e.g. microvilli), stereocilia are extraordinary in at least 2 ways: 1) Differential regulation - in each hair cell there are rows of stereocilia with lengths that increase in height by several micrometers, but stereocilia within the same row vary in height by no more than several nanometers. 2) Length – normal epithelial microvilli are ~500 nanometers long, while stereocilia are up to 120 micrometers long. A large number of mutations that cause deafness affect proteins involved in regulating stereocilia length. Our recent work has revealed that stereocilia are dynamic structures undergoing constant renewal and regulation via the activities of numerous myosin motor proteins and their actin-regulatory cargoes. Myosin XVa (MyoXVa) and its cargo whirlin are implicated in deafness (DFNB3 and DFNB31, respectively) and have been shown to localize at stereocilia tips and to be essential for stereocilia elongation. Given that whirlin is a scaffolding protein with no actin-regulatory activity, it remains unclear how these proteins work together to elongate stereocilia. Here we show that the actin-regulatory protein Eps8 interacts with MyoXVa and whirlin, and that mice lacking Eps8 have very short stereocilia similar to MyoXVa- and whirlin-deficient mice. We also show that Eps8 localizes to stereocilia tips in concentrations directly proportional to length, showing for the first time a relationship between the amounts of an actin-regulatory protein and stereocilia length, revealing a biochemical mechanism for differential stereocilia elongation. We show that Eps8 fails to accumulate at the tips of stereocilia in the absence of MyoXVa, that overexpression of MyoXVa results in both elongation of stereocilia and increased accumulation of Eps8 at stereocilia tips, and that the exogenous expression of MyoXVa in MyoXVa-deficient hair cells rescues Eps8 tip

localization. We also found that both MyoXVa and Eps8 appear in reduced amounts at the tips of whirlin-deficient stereocilia, which suggests that whirlin plays an integral role in the efficient accumulation of the MyoXVa:Eps8 complex at stereocilia tips, perhaps via its scaffolding activity. Our data demonstrates that MyoXVa, whirlin, and Eps8 are integral components of a stereocilia tip complex, where Eps8 is a central actin-regulatory element transported by MyoXVa to stereocilia tips for elongation of the stereocilia actin core. This work provides insight towards DFNB3 and DFNB31 pathologies, and identifies EPS8 as a candidate deafness gene.

Minisymposium 2: Cell-Cell and Cell-Matrix Interactions

15

The differential recruitment of vinculin to adherens junctions and focal adhesions dictates biological response.

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Integrin and cadherin adhesion complexes exhibit an antagonistic relationship that determines whether cells maintain contact with their neighbors or uncouple and migrate. Signal events that regulate this antagonism have received considerable attention, but how these signal pathways differentially maintain cell adhesion at one site while promoting its loss at another are not known. Here we demonstrate that vinculin is tyrosine phosphorylated by Abelson tyrosine kinase in cell-cell, but not cell-matrix, adhesions. Phosphorylation at tyrosine 822 is required for adherens junction assembly and integrity and collective cell migration. Substitution of tyrosine 822 with phenylalanine prohibited vinculin recruitment and function in adherens junctions. Furthermore, preventing vinculin tyrosine phosphorylation, either by substitution with phenylalanine or by induction of an epithelial to mesenchymal transition, resulted in elevated integrin-mediated cell adhesion and cell spreading and single cell migration. Taken together these data provide a mechanism for determining if a cell maintains contact with their neighbors or uncouples and migrates. This work provides insight into how antagonism between integrins and cadherins is achieved.

16

A novel proteolytic processing of talin is associated with cadherin-mediated cell-cell adhesion.

F. Zhang[1], A. Kashina[1]; [1]Animal Biology, University of Pennsylvania, Philadelphia, PA

Talin is an essential cell adhesion molecule that plays a key role in integrin-mediated cell adhesion to the extracellular matrix, but has never been found to be involved in cadherin-mediarted cell-cell adhesions. Talin is a large rod-like molecule that scaffolds multiple components of the actin cytoskeleton. It is regulated by limited calpain-mediated proteolysis with a release of its N-terminal regulatory domain during integrin adhesion maturation and turnover. Other types of cleavages in vivo with functional significance have not been reported. Here we studied talin regulatory proteolysis in response to changes in cell density and found that the formation of cell-cell adhesions in a dense culture is accompanied by calpain-dependent generation of a novel 70 kDa talin fragment, which partially co-localizes with cadherin and accumulates in a dose-dependent manner in cells establishing cell-cell adhesions. Analysis of arginyltransferase (Ate1) knockout cells deficient in cell-cell adhesions revealed that these cells have little or no 70 kDa talin fragment, suggesting that arginylation knockout inhibits this fragment's generation, which in turn may be responsible for the adhesion defects in these cells. Phentype rescue experiments showed that reintroduction of the 70 kDa talin fragment into Ate1

knockout cells rescues their cell-cell adhesion defects, and that arginylation of this fragment further enhances the rescue effect in addition to regulating its turnover. Thus, we have discovered a novel proteolytic cleavage of talin that participates in its novel function in cadherin-mediated cell-cell adhesion and is regulated by a multi-level mechanism that involved cell density, calpain, and arginylation.

17

Myosin-X is required for proper junction assembly and epithelial morphogenesis.

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Myosin-X (Myo10) is an unconventional myosin that localizes to the tips of actin-rich protrusions such as filopodia and invadopodia and is necessary for filopodia formation. Although Myo10 has been studied primarily in fibroblast-like cells, little is known about its functions in polarized epithelial cells and junction formation. Here we use MDCK cells as a model system to investigate the functions of Myo10 in polarized epithelial cells and junction formation.

In MDCK cells triggered to form junctions using a calcium switch assay, TIRF imaging shows that GFP-Myo10 localizes to the tips of dynamic, filopodia-like protrusions. As cells contact one another and junctions begin to assemble, GFP-Myo10 transiently localizes to lateral membranes labeled by E-cadherin. In fully polarized cells, GFP-Myo10 exhibits little localization to apical microvilli and instead localizes to puncta on the basolateral surface that appear to represent the tips of basolateral filopodia. Deletion mapping studies indicate that the basolateral localization of Myo10 is dependent on its FERM and PH domains, which are known to bind to integrin and PIP3, respectively.

Knockdown of Myo10 resulted in a delay in the recruitment of junctional proteins, E-cadherin and ZO-1, as well as a delay in the development of peak transepithelial resistance (TER). Importantly, knockdown of Myo10 does not perturb apicobasal polarity or steady state TER. In three-dimensional cyst culture, knockdown of Myo10 resulted in a multiple lumen phenotype, indicating that Myo10 is required for normal lumen formation during epithelial morphogenesis. Together, this work reveals unexpected functions for Myo10 in junction formation and epithelial morphogenesis. The localization of Myo10 to basolateral puncta also raises intriguing questions about the functions of basolateral filopodia in cell-cell and cell-substrate interactions.

18

Invasive adhesion refines inductive signaling in the Ciona heart progenitor lineage.

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Discrete populations of progenitor cells give rise to the heart and other organs. To ensure proper organ size and function, inductive signals that pattern these progenitor fields must be tightly regulated. We are exploring the role of extrinsic, micro-environmental cues in refining inductive signaling. In the basal chordate, *Ciona intestinalis*, fibroblast growth factor (FGF) drives induction of the heart progenitor lineage. Cardiac founder cells are exposed to a uniform FGF inductive gradient. However, founder cells respond differentially to this inductive signal, dividing to produce one heart progenitor daughter and one tail muscle progenitor. Differential induction is associated with a highly polarized invasion of adjacent epithelia. Through targeted transgenic manipulations, we have found that localized Cdc42 activity and actin polymerization

associated with invasive protrusions restrict inductive signaling. We are now investigating the precise mechanism by which invasive protrusions localize induction. Our initial results indicate that invasion leads to regional changes in cell-matrix adhesion that potentiate localized FGF signaling. We are currently investigating the precise in-vivo role of adhesion in the sub-cellular localization of FGF signaling components.

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Syndecan-4 in breast carcinoma cells is an invadopodia component that regulates invasive behavior.

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Syndecan-4, a transmembrane heparan sulfate proteoglycan, has roles in integrin-mediated adhesion. It signals to the actin-associated cytoskeleton, and directly interacts with alpha-actinin. However, not much is understood of its roles in tumor cells. We have demonstrated that syndecan-4, in MDA-MB231 cells has a punctate distribution, shown by confocal microscopy to co-localize with invadopodia components, including matrix metalloproteinase 14 (MT1-MMP). However, experiments with siRNA or over-expression showed that syndecan-4 opposes both cell migration and collagen matrix invasion. These properties were dependent on the heparan sulfate chains, but not, surprisingly, on its cytoplasmic domain. Moreover, addition of heparin or heparan sulfate, but not chondroitin or dermatan sulfate to the breast carcinoma cells, led to decreased migration, with increased spreading, microfilament bundle formation, and cadherin 11-containing adherens junctions. These effects were independent of integrin or MMP14 status on the cell surface, although collagen gel invasion was a MMP14-dependent process. Recent experiments suggest that syndecan-4 and its heparan sulfate chains may inhibit thrombin on the cell surface, with downstream effects on the cytoskeleton.

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L-lectin Domain-dependent Associations Mediate Extracellular Matrix Incorporation of Thrombospondins: a Novel Process in Matrix Organisation.

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The extracellular matrix is a key mediator of multi-cellularity in the metazoa. Comparative genomics indicate that a core set of ECM components and associated proteases arose on the metazoan stem lineage and have been highly conserved subsequently (Ozbek et al., (2010) Mol. Biol. Cell 21:4300-4306). Within this core ECM set are the Thrombospondins (TSPs), a family of multi-domain, calcium-binding glycoproteins. The 5 TSPs of mammals have multiple roles in tissue organisation, and disorganisation of collagen fibrils is a phenotype shared by several *Thbs* gene knockout mice. Despite many extracellular matrix (ECM)-associated roles, mechanisms by which TSPs incorporate into and impact on ECM organisation are poorly understood. Through a model assay for isolation of insoluble ECM we identified that the C-terminal region of TSP1 in trimeric form is sufficient to mediate its ECM incorporation. Mechanistically, a novel, conserved site on the outer face of the TSP L-lectin like domain is the major mediator of ECM incorporation of TSP-1 and controls ECM deposition of TSP-5/COMP. The site acts in trans to mediate homophilic (eg, TSP1-TSP1) and heterophilic (eg, TSP1-TSP5) recruitment of trimeric or pentameric TSPs into insoluble ECM. This specificity is not replicated in conditioned media, indicating additional cell-surface and/or ECM-based steps in the process. Considering the many physiological and pathological contexts that involve modulation of levels

of extracellular TSPs, we propose the novel concept that concentration-dependent "matrix-trapping" of TSPs is a fundamental mechanism for ECM incorporation of TSPs, and modulates collagenous ECM organisation.

Minisymposium 3: Chemical Biology : Probes and Therapeutics

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Optical recording of action potentials in mammalian neurons with a voltage indicating protein.

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Reliable optical detection of single action potentials in mammalian neurons has been one of the longest-standing open challenges in neuroscience. Here we achieve this goal in cultured neurons using a new voltage indicating protein (VIP1) which has approximately 10-fold improvements in sensitivity and speed over other genetically encoded voltage indicators. The endogenous fluorescence of our microbial rhodopsin-based VIP1 showed 2-fold variation in brightness over physiological voltage range, with a sub-millisecond response time. Single action potentials yielded bursts of fluorescence with optical signal-to-noise ratio > 10. The mutant VIP2 showed 50% greater sensitivity than VIP1 and lacked endogenous proton pumping, but had a slower response (41 ms). VIP2 was still capable of resolving individual action potentials. Microbial rhodopsin-based voltage indicators promise to enable optical interrogation of complex neural circuits, and electrophysiology in systems for which electrode-based techniques are challenging.

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Chemical tools for probing protein function with spatial and temporal resolution in living cells.

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I will describe new methods from our lab to (1) image specific protein function in living cells, and (2) determine the proteomic composition of subcellular compartments of living cells.

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Expanding the scope of bioluminescence imaging: synthetic luciferins and mutant luciferases.

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Firefly luciferase-catalyzed light emission from D-luciferin is widely used as a reporter of gene expression and enzymatic activity both in vitro and in vivo. Despite the power of bioluminescence for non-invasive tumor imaging and anti-cancer drug discovery, light emission from luciferase is fundamentally limited by the photochemistry of the luciferin substrate and its ability to access the luciferase (modulated by affinity, cell permeability, and cellular efflux). We have recently described synthetic aminoluciferin derivatives such as CycLuc1 and CycLuc2 which exhibit light emission at longer wavelengths than D-luciferin (> 600 nm) and have increased affinity for luciferase. However, these desirable properties come with the caveat that aminoluciferin substrates exhibit marked burst kinetics with wild-type firefly luciferase due to

product inhibition. To help overcome this limitation, we have screened for and identified mutant luciferases that can efficiently emit light with aminoluciferins, both in vitro and in live mammalian cells. We have also synthesized new aminoluciferins that are substrates for these mutant luciferases and yield bioluminescent light emission at even longer wavelengths that are more suitable for whole animal imaging (> 630 nm). Together, these synthetic luciferin substrates and mutant luciferases have the potential to expand the scope of bioluminescent detection, allowing bioluminescence at wavelengths where tissue is more transparent to light, and the construction of orthogonal luciferase-luciferin pairs for multiplexed bioluminescent imaging.

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Drugging the Undruggable: Small-Molecule Inhibition of the Ras Oncoprotein.

T. Maurer[1], L. Garrenton[1], A. Oh[1], K. Pitts[1], N. Skelton[1], B. Fauber[1], B. Pan[1], S. Malek[1], D. Stokoe[1], K. Bowman[1], J. Wu[1], A. Giannetti[1], M. Starovasnik[1], I. Mellman[1], P. Jackson[1], J. Rudolph[1], W. Wang[1], G. Fang[1]; [1]Genentech, Inc., South San Francisco, CA

The Ras protein is the most frequently mutated oncogene and has been considered by the scientific community one of the most important oncology drug targets. Despite the enormous interest and extensive exploratory efforts in industry and academia, small molecules that bind to Ras in a well-defined manner and exert inhibitory effects have not been uncovered to date.

To explore a new means of directly targeting Ras, we took a fragment-based lead discovery approach in an NMR-based screen. We identified a group of small molecules that bind to a unique site adjacent to the switch I/II regions in the Ras protein. X-ray crystallography studies of three compound-Ras complexes indicate that the binding site can be expanded upon ligand binding. Nucleotide exchange factors, notably SOS, are required to convert inactive RasGDP to active RasGTP. We determined that the compound binding site is located at the interface of Ras and SOS. A subset of our Ras-binding molecules indeed inhibited SOS-mediated nucleotide exchange. Further mechanistic studies revealed that the compounds block the formation of the Ras-SOS complex, a key intermediate of the exchange reaction. At the cellular level, our compounds inhibit the formation of active RasGTP and prevent Ras signaling to downstream effectors. We conclude that the compounds act as competitive inhibitors of nucleotide exchange to prevent the activation of Ras. The discovery of a binding pocket on Ras with functional significance represents a breakthrough finding that will offer a new direction for therapeutic intervention of the Ras oncoprotein. Our findings provide new opportunities to target the “undruggable” Ras oncoprotein.

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3000 chemogenomic screens reveal novel targets and inhibitors: compound prioritization for academic chemical biologists.

C. Nislow[1], M. Urbanus[2], L. Heisler[2], R. St. Onge[3], G. Giaever[2]; [1]Donnelly Centre and Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada, [2]Donnelly Centre, University of Toronto, Toronto, ON, Canada, [3]Biochemistry, Stanford University, Stanford, CA

Our lab has pioneered the use of genome-wide pooled screens for drug target and target pathway identification by employing two validated assays; HaploInsufficiency Profiling (HIP) and Homozygote Profiling (HOP) using the barcoded yeast deletion collections. One of the most vexing challenges has become the selection of compounds for screening in a manner that is both cost-effective and which explores maximal chemical diversity. We describe how pre-selection of compounds that are more likely to induce a phenotype can increase the efficiency and reduce the costs for model organism screening. To identify such molecules, we screened

~81,000 compounds in *S. cerevisiae* and identified ~7,500 that inhibit cell growth. Screening these growth-inhibitory molecules across a diverse panel of model organisms resulted in an increased phenotypic hit-rate. This data was used to build a model to predict compounds that inhibit yeast growth. Empirical and *in silico* application of the model enriched the discovery of bioactive compounds in diverse model organisms. To demonstrate the potential of these molecules as lead chemical probes we used chemogenomic profiling in yeast and identified several dozen novel protein targets and their cognate inhibitors from over 3000 full genome-screens. This library of growth-inhibitory molecules has been made commercially available and the computational model and filter used are available as a chemical biology resource.

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Predicting Synergy: Drug combination screening to identify predictive biomarkers for combination drug therapy.

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Navitoclax (formerly ABT-263) is a potent, selective inhibitor of Bcl-2 and Bcl-xL that has shown clinical activity in chronic lymphocytic leukemia (CLL), a disease with frequent high expression of Bcl-2. Although there is little evidence of single agent navitoclax activity in pre-clinical models of epithelial solid tumors, data suggest that Bcl-xL protects against chemotherapy induced cellular stress in many cancer cells. To determine if Bcl-xL inhibitors synergize with chemotherapy or targeted anti-cancer agents, we performed drug combination screens of navitoclax and gemcitabine, paclitaxel, carboplatin, erlotinib, as well as experimental agents that inhibit MEK, and phosphatidylinositol-3-kinase in a panel of cell lines derived from NSCLC, breast, ovarian and pancreatic cancer. We find that navitoclax is broadly synergistic with most of the agents tested. However, there were significant differences in the level of synergy among cell lines, suggesting it may be critical to identify biomarkers to identify the cancers that are more likely to exhibit strong synergy in response to drug combinations. We performed correlative marker analysis of mutations, gene expression and protein levels of relevant pathways to identify predictive biomarkers of drug synergy and found that cell lines with a high ratio of Bcl-xL/Mcl-1 exhibit a stronger synergistic response to the combination of paclitaxel and navitoclax. Single cell analysis of time-lapse microscopy revealed a significant decrease in the time from mitotic entry to cell death in cells exposed to paclitaxel plus navitoclax compared to paclitaxel alone, resulting in more cell killing and a reduction in clonogenic survival. In contrast, the best correlative markers for the MEK inhibitor navitoclax combination were activating mutations of EGFR or KRAS. However, we observed variable degrees of synergy even with the KRAS mutant cell lines, suggesting additional contributing factors. We find variation in the regulation of Mcl-1, Bad and Bim in response to MEK inhibition that could account for the differences in synergy. In conclusion, we find 1) it is possible to identity predictive markers for synergy through drug combination screening and correlative marker analysis, and 2) the predictive markers depend upon the mechanism of action of the combining agent.

Minisymposia 4: Innovations in Cell Biology Graduate Education

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The role of graduate education in bridging the gap between basic sciences and human health.

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Since the 70's, progressive decline in physician-scientists and increased technology created a gap between basic science and medicine which delays application of basic science to human health. Interest surveys and outcome data support the effectiveness of teaching pathobiology to PhD students/fellows. Since an initial proposal (NEJM,'89), the number and variety of successful programs have increased. Programs vary in duration, frequency and degree relatedness, and are supported by NIH, foundations and institutions worldwide. Teaching pathobiology to PhDs has become a global concern. Because there are more than 4000 PhD students/fellows at NIH, a different format was required. A weekly one semester course entitled "Demystifying Medicine for PhDs" was established 10 yrs ago. Attendance exceeds ~900 per year. The format includes a specific disease,live patient(s), discussion by "translational" investigator followed by basic scientist who presents state-of-the-art research and challenges. All materials are available on a NIH website (Demystifying Medicine) and videoarchive. Over 1200 Course DVDs have been distributed worldwide. The basic concept has been replicated in basic science departments in many American institutions and 16 countries. Particularly in the present era of cell, molecular and genetic biology, teaching pathobiology to undergraduates and PhD students/fellows is timely,challenging,supported by outcomes data,and,for some, preparatory for academic positions in clinical departments. The goal is to enable basic scientists to share ideas with clinical investigators and other basic scientists. These programs contribute to bridging advances in biological and engineering sciences and human health, challenge longstanding educational paradigms and offer exciting, productive and novel research careers.

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The Mechanisms of Disease & Translational Science Track at UT Southwestern.

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“Mechanisms of Disease & Translational Science “(MoDTS) is a PhD enrichment track that provides students with new opportunities to study human diseases at the interface between basic and clinical sciences. MoDTS students will be empowered to spearhead the bidirectional translation of discoveries between the bench and bedside to harness the explosion of scientific knowledge to improve human health. MoDTS was launched in 2008 with institutional seed money. It obtained a 5-year grant from the Texas Innovative Graduate Program Initiative in 2009 and a 4-year grant from the HHMI Med into Grad Initiative in 2010. Matriculated students apply for admission in the spring of graduate year 1 (G1). MoDTS now admits 8 students per year. There has been a large increase in the number of applicants and selectivity. In 2001, the rate of acceptance was 50% and 7 of the 8 admitted students graduated from top-tier national research universities. The MoDTS curriculum is overlain on the traditional graduate school curriculum, and involves the following: (1) Summer Immersion in Clinical and Translational research in G1/G2. It provides first hand clinical research experience, observations in hospital ward teaching rounds and Institutional Review Board sessions; (2) new mini-medical school style courses on Human Physiology and Pathology, new Introduction to Biostatistics, and an existing Human Biology and Disease course; (3) a Bench to Bedside elective in G3 spring, in which the student designs a program of independent study with a team of clinical experts to obtain an individualized, in-depth experience about the diseases directly aligned with his/her

dissertation research topic. The dissertation research topic must have relevance to human biology and diseases, although there is no absolute requirement for actual translational research. However, the qualifying exam, which is based on the student's thesis research, must propose a translation aim using primary human derived samples. In spite of the increase in coursework, the 4 initial students will graduate with a time to degree of 3.9 years, compared with the graduate school average of 5.1 years. There are opportunities for PhD student interactions with MD/PhD and Doris Duke medical students through the monthly joint Grand Rounds, and small group discussions. We have forged an alliance with the Clinical and Translational Science Award (CTSA) and increased the number of clinicians participating for the first time in PhD graduate education.

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IICCBB - Building an International Network for Student Training and Research Collaboration in the Biological Sciences.

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Students, academic scientists, and private sector scientists in South America have historically had limited opportunities to present and discuss their work in an international setting, thereby diminishing the broader impact of their work and training. For nearly two decades, a team of South and North American scientists have joined efforts to establish an international "school" - International Institute for Collaborative Cell Biology and Biochemistry (IICCBB) - an organization without borders or physical location. Our mission is to inspire future scientists in biochemistry and cell biology. IICCBB offers annual interdisciplinary courses in biochemistry, cell biology, and molecular biology to international students in the biosciences. With a history of over twenty organized courses and symposia, more than 1500 students from 28 countries, and over 100 faculties from over 15 countries, we have succeeded in establishing a stable consortium of scientists dedicated to training the next generation of scientists and promoting research collaboration at an international level. We will provide information on the diversity of talks and interests, new collaborations, and other interactions, students exchange and postdoctoral positions that have resulted from our courses.

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Innovations in Graduate Education: the Professional Science Master's Degree.

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The Professional Science Master's (PSM) is an innovative, graduate degree designed to allow students to pursue advanced training in science or mathematics, while simultaneously developing workplace skills highly valued by employers. PSM programs consist of academic training in an emerging or interdisciplinary area, along with a professional component that may include internships and "cross-training" in workplace skills, such as business, ethics, communications, and regulatory affairs. Programs are developed in concert with employers and are designed to dovetail into present and future professional career opportunities. PSM degrees date from 1997, when the Sloan Foundation began funding research universities to develop clusters of such programs, and the Keck Foundation endowed the Keck Graduate Institute to offer degrees in applied biomedical sciences following the PSM model. In 2001 the Council of

Graduate Schools (CGS) partnered with the Sloan Foundation to expand PSMs to select “master’s focused” institutions, and in 2006 CGS assumed primary responsibility for the promotion of the PSM degree. PSM programs now number about 250 at about 115 institutions. They attract students who are not likely to pursue a doctorate, but want a degree that leads to a science-based career in innovative organizations and that prepares them for advancement into leadership positions. PSM graduates experience almost 100% employment and many programs – especially in biology – report more demand for graduates than they can supply. Examples will be given from specific PSM programs in biology of student experiences in the program and employment of graduates.

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Crossing Silos: Graduate Training in Cell Analysis and Modeling.

R. M. Holmes[1,2]; [1]Center for Cell Analysis and Modeling, U. Conn. Health Center, Farmington, CT, [2]Center for Computational Science, Boston University, Boston, MA

Modern cell biology draws on multiple areas of expertise including biology, mathematics, physics and computational sciences. Developing researchers capable of working in this increasingly interdisciplinary field requires strategies to support communication, collaboration and innovation among current and future researchers. At the Center for Cell Analysis and Modeling (CCAM), home to the newly established Cell Analysis and Modeling (CAM) area of concentration with the Biomedical Sciences graduate program, we have implemented both structural components that are common to interdisciplinary research and training programs, as well as activities to address the qualitative and creative characteristics of collaborative, interdisciplinary research. The CAM graduate program is geared to students who are interested in understanding the biophysical and biochemical mechanisms that underlie cell function. Our faculty research is broadly multi-disciplinary, which allows students to work closely and concurrently with experimentalists and modelers with existing collaborations, and on projects that incorporate both types of approaches. CCAM faculty are from diverse disciplines, are physically co-located and collaborate on jointly funded multi-disciplinary projects. Combined with multi-disciplinary seminars these features are important structures for a rich interdisciplinary environment. Students and postdocs joining the research environment typically come from traditional biology backgrounds that require training that incorporates physics, math, and computer science, or from physics, engineering, math, and computer science backgrounds that lack sufficient training in biology. Individually-tailored short courses are used to address each student’s needs, while a minimal set of core classes cover a breadth of topics in modern cell biology. We take a sociocultural approach (Vygotsky, 1976; Newman and Holzman, 1993; Lobman and Lundquist, 2007; Alon, 2009) to developing students’ abilities to collaborate, communicate and organize their scientific careers. Along side our academic courses, we have held improvisational theater workshops for scientists, which support active listening, risk taking, and the breaking down of barriers due to language, culture, or disciplines. Meetings are held by directors of the program with students to invite suggestions to improve their training. The CAM Student Association, initiated and organized by students, produces a CAM Newsletter which provides science highlights, “reports from the lab” and invitations to social events to foster rapport among colleagues. The combination of a rigorous scientific environment with rich social interactions creates a context for students to develop as innovative, collaborative researchers in cell analysis and modeling. Variations of the improvisation workshop sessions have been incorporated into graduate student orientations, postdoctoral trainings at UCHC and are active components of national programs building inclusive scientific communities.

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Professional Training Experiences of Science Faculty with Education Specialties (SFES) in the US.

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Efforts to improve K-16+ science education in the U.S. have included the hiring of Science Faculty with Education Specialties (SFES) – scientists who take on specialized roles in science education within their departments, including biology departments (Bush, 2006). Though SFES occupy a potentially pivotal role in reform efforts, there has been little systematic investigation of these positions. In the first cross-disciplinary research study of SFES in the U.S. – conducted in the nation's largest university system, the California State University (CSU), we previously demonstrated that SFES populations reported more formal training in basic science than in science education (Bush, 2008, Bush, 2010). Most CSU SFES (88%) across all science disciplines had earned science PhDs, whereas many had completed science postdocs (37%) and/or science master's degrees (48%). In contrast, only 32% of CSU SFES reported having any type of formal post-baccalaureate training in science education. In the CSU, Chemistry SFES had the greatest (43%) proportion of faculty with any formal science education training; proportions for Biology (30%), Geoscience (25%), and Physics (27%) were lower. Patterns of the types of formal training in science education varied among disciplines. For example, 20% of Biology CSU SFES had earned teaching credentials, 10% had conducted postdoctoral work in science education, and only 5% had earned science education doctorates. More recently, preliminary analyses of data collected in a National Study of SFES in the U.S. suggest similar trends nationwide. Nearly all of US SFES report formal training in science through MS, PhD, and/or postdoctoral training. However, only about a third of US SFES reported formal training in science education. Many fewer Biology and Geosciences SFES reported formal training in science education compared to SFES in Physics and Chemistry. These research findings can inform aspiring SFES of potential training pathways and biology departments considering SFES faculty hires, as well as policymakers and grant agencies that aspire to establish training pathways for future SFES.

Minisymposium 5: Membrane Fission and Fusion

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Regulation of COPII recruitment to ER exit sites.

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Export of proteins from the endoplasmic reticulum (ER) in COPII-coated vesicles occurs at defined sites, which contain the scaffolding protein Sec16. Although Sec16 has been shown to interact with multiple COPII subunits to mediate vesicle biogenesis at the ER, mechanisms by which COPII recruitment is regulated remain poorly defined. Using both functional genomics and biochemical approaches, we identify a new, conserved Sec16-interacting protein named TFG that is required for COPII subunit accumulation at ER exit sites. Consistent with this finding, depletion of TFG inhibits secretion of multiple cargoes from the ER. Furthermore, using immuno-gold EM techniques, we demonstrate that TFG localizes to a matrix between ER exit

sites and the Golgi. We hypothesize that a TFG matrix may serve as a molecular sink, which helps to retain COPII components locally and facilitate efficient vesicle egress from the ER.

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Regulating AP-2 interactions to control vesicle size and number during clathrin-mediated endocytosis.

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The clathrin adaptor protein AP-2 is the core organizing element in clathrin-mediated endocytosis; it concentrates cargo and clathrin at the site of vesicle formation and it orchestrates a myriad of endocytic accessory proteins to control aspects of vesicle formation ranging from membrane deformation to vesicle fission. Thus, clathrin and accessory proteins need to interact with AP-2 in a temporally and spatially controlled manner, yet it remains elusive as to how access of these binding partners to AP-2 is regulated. Using knock down and functional rescue studies, we now demonstrate that the AP-2-binding protein NECAP 1 is a critical regulator of AP-2 interactions. In fact, NECAP 1 needs to be present in a complex with AP-2 to enhance the stability of FCHo2-marked vesicle nucleation sites, thereby controlling the number of clathrin-coated vesicles that form. During vesicle formation, NECAP 1 and AP-2 function cooperatively in the efficient recruitment of other accessory proteins that in turn control vesicle size. Moreover, we use binding assays and nuclear magnetic resonance studies to reveal NECAP 1 as the first known factor that competes with clathrin for access to the AP-2 beta2-linker, positioning NECAP 1 to regulate the ability of AP-2 to recruit and polymerize clathrin. Thus, our data demonstrate that NECAP 1 is a key modulator of the AP-2 interactome needed for productive vesicle formation and reveal a new layer of organizational control within the endocytic protein machinery.

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Coupling the membrane to the actin cytoskeleton during clathrin-mediated endocytosis.

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Polymerization of actin filaments at the site of clathrin-mediated endocytosis is thought to provide force for the membrane shape changes that take place during vesicle formation. However, the molecular mechanism by which the force from the actin cytoskeleton is transmitted to the membrane has not been known. We show that in budding yeast, Saccharomyces cerevisiae, Ent1 (epsin) and Sla2 (homolog of mammalian HIP1R) proteins couple the membrane to the actin cytoskeleton during endocytic vesicle formation. Deficiency of either Sla2 or the redundant pair of two endocytic epsins Ent1/Ent2 leads to an uncoupling phenotype where actin is still polymerizing at the endocytic site, but the membrane does not invaginate. In vivo and in vitro experiments revealed that the N-terminal lipid-binding domains of Sla2 and Ent1 interact with the membrane in a cooperative manner. Furthermore, the C-terminal parts of both Sla2 and Ent1 interact with the actin cytoskeleton. Sla2 binds actin filaments through a well-characterized THATCH domain, and Ent1 by a novel actin-binding domain in its natively unfolded C-terminal region. One of these actin-binding domains is sufficient for endocytosis, while deletion of both domains uncouples actin and prevents membrane invagination. The actin-binding domains of Sla2 and Ent1 can be functionally replaced with a heterologous actin-binding domain fused to Ent1. By their synergistic binding to the membrane and redundant interaction with the actin cytoskeleton, Ent1 and Sla2 form a unique molecular

linker that transmits the force generated by the actin cytoskeleton to the invaginating membrane.

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Curvature and tension control of dynamin fission reaction.

A. Roux[1]; [1]Biochemistry, University of Geneva, Geneva, Switzerland

Dynamin is a GTPase that polymerizes into helical collars at the neck of endocytic buds, and constricts upon GTP hydrolysis in order to release free vesicles. Several hypotheses are currently being debated to understand how this polymerization and conformational change are promoting membrane fission in a controlled manner.
I'll show recent results where we have investigated the lipid membrane parameters influencing the fate of dynamin reaction. By using templates consisting of thin lipid tubules extracted by optical tweezers from Giant Liposomes maintained in micropipettes, we studied the mechanical parameters controlling membrane fission. We showed that if the curvature of the tube triggers polymerization of dynamin (spontaneous nucleation is observed at radii below 20nm), the saddle-like shape of endocytic buds strongly favors the dynamin reaction. As well, high membrane tension intensively reduces both the stochasticity and the average time it takes for fission. Accordingly, when membrane tension is changed in cells by osmotic changes, dynamin is blocked when tension decreases but not when it increases. Our findings support the idea that the high curvature of the neck and its saddle-like shape are respectively triggering the nucleation of the polymer and the membrane break. Also, as expected from our previous findings, membrane tension is a primordial parameter of fission efficiency.

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Cdc42 Controls the Balance between Kiss-and-run and Full Fusion of Secretory Vesicles by Regulating Membrane Tension.

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Membrane fusion underlies several physiological processes including exocytosis of hormones and neurotransmitters. Fusion pore dynamics controls the release of vesicle content but is poorly understood. Here we show that the small GTPase Cdc42 controls the dilation of the fusion pore during exocytosis. We found that Cdc42 silencing in neuroendocrine cells had no effect on the probability of vesicle exocytosis but compromised the enlargement of the fusion pore, as measured by carbon fiber amperometry and evanescent wave microscopy. As a consequence, the mode of vesicle exocytosis was shifted from full-collapse fusion to kiss-and-run in Cdc42 knockdown cells. Remarkably, using the tether pulling technique, we found that Cdc42 silencing reduced the membrane tension. Finally, the artificial increase of membrane tension restored fusion pore enlargement in these cells. We conclude that membrane tension is the driving force for fusion pore dilation and that Cdc42 is a key regulator of this force.

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Dynamics of SNARE assembly in homotypic and heterotypic vacuole fusion.

A. J. Merz[1], B. T. Lobingier[1], M. L. Schwartz[1], C. G. Angers[1]; [1]Department of Biochemistry, University of Washington School of Medicine, Seattle, WA

Our laboratory has used a combination of genetic analyses, cell-free reconstitution approaches, and protein biochemistry to dissect the pathway of SNARE zippering during the docking and

fusion of intact yeast vacuoles. We find that SNARE assembly relies on SNARE interactions with multiple components of the HOPS tethering complex, including the SM protein Vps33. We also show that a single α-helix between layers +4 and +5 of the SNARE core complex defines the transition between the efficient formation of a stable but non- fusogenic trans-SNARE complex, and a complex that is almost fully fusion competent. Using partially-zipped complexes in cell-free assays and in vivo, we demonstrate that we can trap and analyze pre-fusion complexes. We employ these tools to analyze the docking stages of heterotypic docking and fusion of AP-3 vesicles, which in yeast mediate transport from the late Golgi to the vacuolar lysosome. SNARE-trapping experiments and other approaches show that the AP-3 vesicle coat couples to the HOPS tethering complex at the vacuole, and that at least a portion of the AP-3 complex remains membrane associated through the docking and trans-SNARE complex assembly sub-reactions.

Minisymposium 6: Synthetic Cell Biology

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Designing Biological Systems.

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Biology presents us with an array of design principles. From studies of both simple and more complex systems, we understand some of the fundamentals of how Nature works. We are interested in using the foundations of biology to engineer cells in a logical and predictable way to perform certain functions. By necessity, the predictable engineering of biology requires knowledge of quantitative behavior of individual cells and communities and the ability to construct reliable models. By building and analyzing synthetic systems, we learn more about the fundamentals of biological design as well as engineer useful living devices with myriad applications. For example, we are interested in building cells that can perform specific tasks, such as remembering past events and thus acting as a biological computer. Moreover, we design cells with predictable biological properties that serve as cell-based sensors, factories for generating useful commodities and improved centers for carbon fixation. We have recently constructed synthetic protein/RNA structures to increase the efficiency of biological reactions. In doing so, we have made new findings about how cells interact with and impact on their environment.

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Synthetic biology: from parts to modules to therapeutic systems.

R. Weiss[1]; [1]Department of Biological Engineering, Massachusetts Inst Technol, Cambridge, MA

Synthetic biology is revolutionizing how we conceptualize and approach the engineering of biological systems. Recent advances in the field are allowing us to expand beyond the construction and analysis of small gene networks towards the implementation of complex multicellular systems with a variety of applications. In this talk I will describe our integrated computational / experimental approach to engineering complex behavior in living systems ranging from bacteria to stem cells. In our research, we appropriate design principles from electrical engineering and other established fields. These principles include abstraction, standardization, modularity, and computer aided design. But we also spend considerable effort towards understanding what makes synthetic biology different from all other existing engineering disciplines and discovering new design and construction rules that are effective for

this unique discipline. We will briefly describe the implementation of genetic circuits and modules with finely-tuned digital and analog behavior and the use of artificial cell-cell communication to coordinate the behavior of cell populations. The first system to be presented is an RNAi-based logic circuit that can detect and destroy specific cancer cells based on their microRNA expression profiles. We will also discuss preliminary experimental results for obtaining precise spatiotemporal control over stem cell differentiation for tissue engineering applications. We will conclude by discussing the design and preliminary results for creating an artificial tissue homeostasis system where genetically engineered stem cells maintain indefinitely a desired level of pancreatic beta cells despite attacks by the autoimmune response, relevant for diabetes.

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Studying Multiple Motor Ensembles using DNA Nanotechnology.

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In eukaryotic cells, many cargos are transported by groups of microtubule-based molecular motors. Some of these cargos move bidirectionally and can be observed switching directions during movement, but ultimately, cellular contents are sorted with spatial and temporal precision. The biophysical mechanisms that allow ensembles of same- or opposite-polarity motors to coordinate their behaviors are not known. The major technical challenge for dissecting this problem in vitro has been the inability to control the spacing, number, and type of motor present on individual cargo molecules. Using three-dimensional DNA origami, we have designed a synthetic cargo, to which precise numbers and geometries of DNA-linked dynein and kinesin motors can be attached in varying combinations of 1 to 7 motors. We find that synthetic cargo bearing multiple dynein motors can move farther, but not faster than single dynein molecules. Under physiological conditions, multiple dynein motors can move cargo processively, while single dynein motors cannot. Our results suggest that dynein motors may coordinate to move cargo efficiently. Studies of “tug-of-war” scenarios are underway to determine whether cargos driven by both dynein and kinesin exhibit stochastic bidirectional movements.

*Brian Goodman and Nathan Derr contributed equally to this work.

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Rewiring the histone code using synthetic effectors.

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Synthetic transcription factors that control specific regulatory genes are of great interest for basic research, tissue engineering, and cancer therapy. To date, transcription factors have been engineered to bind DNA sequences, but do not recognize epigenetic marks that distinguish developmental and disease-related cell states. We set out to use a novel gene targeting approach that exploits chromatin signatures to target a subset of genes for reactivation. We used the human Polycomb protein CBX8 and homologues from other species to design artificial transcription factors that translate the repressive tri-methyl histone H3 lysine 27 (H3K27me3) signal into gene activation in human cells. Protein modules including the herpes simplex virus VP64 transcription activation domain, H3K27me3-binding Polycomb chromodomains (PCDs), red fluorescent mCherry, and the SV40 nuclear localization signal were combined to create a set of fluorescently tagged Polycomb-based synthetic transcription factors (Pc-TFs). Induced expression of Pc-TF leads to increased transcription of the senescence locus p16 (CDKN2a)

and other loci in a PCD- and VP64-dependent manner. We also observe induction of senescence-associated beta-galactosidase activity upon expression of Pc-TF in an osteosarcoma cell line. Our results show that a subset of genes can be regulated by an artificial transcription factor that recognizes histone methylation, rather than a specific DNA sequence. This work demonstrates the flexibility of synthetic transcription factor engineering and is the first example of chromatin-mediated TF targeting in human cells.

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Generation of Intracellular Logic Gates with Two Orthogonal Chemically Inducible Systems.

R. DeRose[1], T. Miyamoto[1], A. Suarez[1], T. Ueno[1], M. Chen[1], T-P. Sun[2], C. Mukherjee[3], D. Meyers[3], T. Inoue[1]; [1]Cell Biology, Johns Hopkins Univ, Baltimore, MD, [2]Biology, Duke Univ, Durham, NC, [3]Pharmacology, Johns Hopkins Univ, Baltimore, MD

In the creation of non-silicon-based computers, various biomolecules have been used to construct Boolean logic gates. However, their □ecomputational□f timescale is relatively slow (tens of minutes to hours), as most of these biological logic gates utilize gene expression that requires time-consuming processes such as transcription and translation. For faster processing, we employed chemically inducible protein dimerization systems. To rapidly trigger two chemically inducible signals, we developed an efficient chemical dimerization system using a newly synthesized gibberellin analog (GA3-AM) and its binding proteins, a system that is completely orthogonal to rapamycin-mediated protein dimerization. With the two chemical inputs (rapamycin and GA3-AM), we achieved AND as well as OR gates, that produced output signals such as fluorescence and membrane ruffling on a timescale of seconds. The use of two orthogonal dimerization systems in the same cell also allows for finer modulation of protein perturbations than is possible with a single dimerizer. (The content of the study is now under review by Nature Chemical Biology.)

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Quantitative assessment of Ras over-expression via shotgun deployment of vectors utilizing synthetic promoters.

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We sought to characterize and compare wild-type and oncogenic Ras over-expression in pre-B cells immortalized by the viral Abelson kinase. Because different levels of Ras over-expression can have different cellular outcomes, it was important to evaluate a wide range of expression. First, we generated a library of synthetic promoters capable of a 40-fold expression range. Different expression levels were then achieved by using retroviral vectors equipped with various synthetic promoters. Cells were “shotgun” transduced with a mixture of these vectors to generate heterogeneous populations exhibiting a range of expression levels. We used flow cytometry to analyze the populations and generate continuous, high resolution, Ras dose-response curves. By inactivating all isoforms of endogenous Ras using the inhibitor salirasib and transducing cells with Ras engineered to be resistant to salirasib (myristoylated Ras), we were able perform experiments in the presence of the inhibitor and characterize a complete Ras dose response ranging from nearly zero Ras activity to 10-fold over-expression. Experiments revealed that a single-copy level of oncogenic Ras generated maximal imatinib resistance and activated MAPK pathway signaling as effectively as six-fold amplification of wild-type Ras. Although further increased expression lead to even greater signal transduction, this increased expression had minimal or decreasing effects on the proliferation rate. In general, this study

introduces a method to quantify genetic dose-response relationships and identify gene expression ranges that produce an optimized phenotypic response.

Minisymposium 7: The Nuclear Periphery

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Radial organization of the nuclear pore complex imaged by super-resolution microscopy.

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Integration of atomic structures of individual proteins into macromolecular models of multiprotein assemblies is a long standing challenge in the understanding of the mechanistic details and function of cellular machinery. One of the most prominent examples is the nuclear pore complex (NPC), a ~100 MDa assembly of several hundred polypeptides that functions as the sole channel for nucleocytoplasmic transport in all eukaryotes. Cryo-electron tomography has provided an overall structure of NPC, but due to technical limitations its resolution does not allow to unambiguously map the position, stoichiometry and orientation of individual subcomplexes or atomic structures of single nucleoporins within the assembly. How the individual molecular building blocks assemble to make the nuclear pore therefore remains an open question.
Here, we have used a super-resolution light microscopy method, ground state depletion followed by individual molecule return (GSDIM), to study the substructure of the NPC in human cultured cells. We initially focused on the organization of the Nup107-160 complex, which is the major component of the vertebrate NPC's scaffold and is responsible for stabilization of the highly curved pore membrane. Using optimized immunofluorescence protocols with GSDIM compatible dyes we are able to resolve individual members of the Nup107-160 complex and measure their radial distance from the central 8-fold symmetry axis of the pore. To our knowledge this is the first time that the radial substructure of the NPC was directly visualized by light microscopy.

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Formation of the post-mitotic nuclear envelope from extended endoplasmic reticulum cisternae precedes nuclear pore assembly.

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During mitosis, the nuclear envelope membrane merges with the endoplasmic reticulum while the nuclear pore complexes are disassembled. In a current model, the nuclear envelope forms at the end of mitosis by re-shaping of endoplasmic reticulum tubules. Two major models have been proposed regarding the assembly of nuclear pore complexes. In the insertion model, nuclear pore complexes are assembled and embedded in the nuclear envelope following its formation. In the pre-pore model, nucleoporins, such as Nup107-160 complexes, assemble on the chromatin as an intermediate nuclear pore complex prior to nuclear envelope formation. Using multi-dimensional live cell imaging and electron microscope tomography, we find that the assembly of the nuclear envelope occurs by direct adhesion and migration of mitotic endoplasmic reticulum cisternae around the chromosome mass. Furthermore, we find that the

nuclear pore complexes only assemble on the newly formed nuclear envelope membrane. By a single molecule approach developed for this study, we demonstrate that all the chromatin associated Nup107-160 complexes are in single units instead of assembled pre-pores. Our observations thus support a model in which the post-mitotic nuclear envelope assembles directly from endoplasmic reticulum cisternae, followed by membrane-dependent insertion of nuclear pore complexes.

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Cell Cycle-dependent Nuclear Envelope Dynein Recruitment Factors Mediate Interkinetic Nuclear Migration in Developing Brain.

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Radial glial neural progenitors (RGPCs) are highly elongated cells that span the developing neocortex from the ventricular to the pial surface. These cells exhibit long-range cell cycle-dependent "interkinetic nuclear migration" (INM; Sauer, J. Comp. Neurol., 1935, 62:377-405), the purpose and mechanism of which have long been mysterious. We have found MTs to be uniformly oriented throughout the cytoplasm of the RGPCs, while the centrosome remains at the ventricular surface throughout INM (Tsai et al. Nat. Neurosci. 2010, 13:1463-71). Our data have further revealed nuclear migration to be mediated by Kif1A in the MT plus end direction during G1 (basal migration), and by cytoplasmic dynein in the minus end direction during G2 (apical migration), followed by mitosis, which occurs only at the ventricular surface. Our results suggest that the motor proteins act from the nuclear envelope. We have now tested this hypothesis by analysis of factors previously implicated in dynein recruitment to the nuclear envelope during G2 in nonneuronal cells (BicD2, Splinter, D. et al., 2010, PLoS Biol.8: e1000350; Nup133-CENP-F, Bolhy S. et al., JCB, 2011, 192:855-71). Using in utero electroporation in E16 rat brain we find that RNAi for BicD2, Nup133, or CENP-F dramatically inhibits neuronal redistribution. Live imaging reveals specific inhibition of MT minus end-directed apical nuclear migration in RGPCs. BiCD2 RNAi produces a broad distribution of nuclei throughout the ventricular zone, whereas Nup133 RNAi arrests most RGPC nuclei within 10µm of the ventricular surface, consistent with early vs. late G2 roles for these factors. Nup133 RNAi arrested cells are phospho-histone H3-negative, identifying a novel premitotic arrest state. Together these data strongly support a role for dynein recruitment to the NE in apical nuclear migration in these cells. BicD2 and Nup133 now appear to be required for long-range nuclear migration in RGPCs, in addition to their role in centrosome positioning and force generation during NEB. Moreover, our data provide new insight into cell cycle control of INM. Supp. by HD40182 to RBV; AHA and NIH fellowships to TN; ANR-07-blan to VD; and NWO ALW-VICI to AA.

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The nuclear pore complex protein Ndc1 is involved in nuclear positioning in migrating fibroblasts.

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Positioning of the nucleus within cells has a role in different processes such as cell migration and muscle differentiation. In migrating fibroblasts the nucleus moves backwards prior to cell migration and this active movement is dependent on the actin cytoskeleton and on nuclear envelope proteins members of the LINC complex such and Nesprin and SUN proteins. In recent

years several mass spectrometry studies in mammalian cells have identified many putative transmembrane nuclear envelope proteins that may be involved in establishing or regulating the interaction between nucleus and cytoskeleton thereby being required for nuclear positioning. In order to identify novel proteins involved in this process we preformed a live-imaging screen for inhibition of nuclear movement through short interfering RNA (SiRNA) against a set of 230 potential/confirmed nuclear envelope proteins from published organellar proteomics studies. Ndc1, a conserved transmembrane protein of the nuclear pore complex (NPC) was one of our hits. The screening results were further validated by the inhibition of centrosome reorientation and nuclear positioning in a fixed cell assay. Furthermore the knockdown of Ndc1 caused cell migration defects similar to what observed in Nesprin-2 SiRNA treated cells. Although the full knock down of Ndc1 resulted in general disruptions of the nuclear envelope, consistent with its role in nuclear pore assembly, a partial knockdown was sufficient to inhibit nuclear movement without significantly affecting nuclear envelope proteins and structure. Moreover we identified a specific interaction between the N-terminal region of Ndc1 and SUN2, distinct from the proposed NPC interacting region on the conserved C-terminus of Ndc1. Expression of full length siRNA resistant Ndc1 or Ndc1 lacking the c-terminus were able to rescue centrosome reorientation in Ndc1 siRNA cells, suggesting that the interaction of Ndc1 with the nuclear pore is not required for nuclear movement. Furthermore, SUN2 and the NPC do not seem to co-localize on the nuclear envelope. We propose a model where a non-NPC subpopulation of Ndc1 is involved in the regulation of SUN2 during nuclear movement.

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The KASH Domain Protein Nesprin 4 is essential for cochlear outer hair cell maintenance and hearing.

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The mechanosensory process of hearing involves highly specialized epithelial cells in the inner ear known as hair cells. Through apical stereocilia, these cells detect vibrations generated by sound waves, triggering a cellular depolarization that sends a nerve impulse to the hearing center of the brain. Hair cells are divided into two groups, inner and outer. Outer hair cells (OHCs) are critical to the cochlear sensory epithelium: impaired function or loss of OHCs results in deafness. Nesprin4 (Nesp4) is an outer nuclear membrane KASH-domain protein we previously identified in secretory epithelial cells. Nesp4 functions as a nuclear envelope (NE)-associated adaptor for Kinesin-1. Like other nesprin family members, it is a component of nuclear envelope-associated LINC (LInker of Nucleoskeleton and Cytoskeleton) complexes. These structures, consisting of inner nuclear membrane SUN proteins in association with outer nuclear membrane KASH domain proteins, provide a direct physical connection between nuclear components and the cytoskeleton. We now show that Nesp4 is expressed in OHCs, where it localizes exclusively to the NE. By deriving mice deficient for Nesp4, we are able to demonstrate that expression of this protein is essential for OHC maintenance. At birth (P0), the cochlear sensory epithelium from *Nesp4*-null animals appears indistinguishable from that of wild-type littermates with an overtly normal OHC morphology. However, by day 11 (P11), the sensory epithelium of *Nesp4*-null animals begins to lose OHCs through a process consistent with apoptotic cell death, as evidenced by TUNEL staining. No such loss was detected in wild-type littermates. By P30, this loss of OHCs is widespread within the cochlea, and *Nesp4*-null mice are deaf by the age of 8 weeks. Our conclusion is that Nesp4 is essential for the maintenance of OHC viability.

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B-type lamins regulates neither their bound genes nor embryonic stem cells but are essential for organogenesis.

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B-type lamins are believed to regulate gene silencing of their tethered genes. They are also thought to be essential for several basic functions including cell survival, DNA replication, and cell proliferation. Using genome-wide chromatin-lamin B interaction and gene expression analyses, we show that chromatin-lamin-B interaction correlates with reduction of gene expression during embryonic stem cells (ESC) differentiation toward the trophectoderm (TE) lineage. To further investigate the significance of the chromatin-lamin-B interaction, we created lamin-B1 and -B2 double knock out (DKO) ESCs. These lamin-B DKO ESCs do not express any type of lamins including lamin-A/C, -B1, -B2 and -B3. Unexpectedly, their nuclear morphology, growth rates, ploidy, expression of pluripotent markers, and potential to differentiate to TE cells are indistinguishable from wild-type ESCs. Gene expression array analysis of these DKO ESCs and TE cells conclusively demonstrates that B-type lamins are not required for the silencing of their bound genes during ESC differentiation toward TE cells. Furthermore we report that the lamin-B DKO mice can develop to term. However they die soon after birth with a small body size and defects in multiple organs including the lung, diaphragm, phrenic nerves, and the brain. We further define the requirement for B-type lamins in regulating spindle orientation in neural progenitor cells (NPC), migration of neurons, and survival of both NPCs and neurons. While refuting several general requirements for B-type lamins, our findings redefine the essential role of these nuclear proteins in organogenesis.

Keith R. Porter Lecture

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Navigating the cellular landscape with new optical probes, imaging strategies and technical innovations.

J. Lippincott-Schwartz[1]; [1]NIH/NICHD, Bethesda, MD

Emerging visualization technologies are playing an increasingly important role in the study of numerous aspects of cell biology, capturing processes at the level of whole organisms down to single molecules. Recent developments in probes, techniques, microscopes and quantification are dramatically expanding the areas of productive imaging. Photoactivatable fluorescent proteins (PA-FPs) have been particular fruitful in this regard. They become bright and visible upon being exposed to a pulse of UV light. This allows selected populations of proteins to be pulse-labeled and tracked over time. Used for *in cellulo* pulse chase experiments, the PA-FPs have helped clarify mechanisms for biogenesis, targeting, and maintenance of organelles as separate identities within cells. PA-FPs have further permitted the development of single molecule-based superresolution (SR) imaging, which dramatically improves the spatial resolution of light microscopy by over an order of magnitude (~10-20 nm resolution). Involving the controlled activation and sampling of sparse subsets of photoconvertible fluorescent molecules, single molecule SR imaging offers exciting possibilities for obtaining molecule scale information on biological events occurring at variable time scales. Here, I discuss the new

fluorescent imaging techniques and the ways they are helping researchers navigate through the cell to unravel long-standing biological questions.

MONDAY, DECEMBER 5

Symposium 3: Cellular Networks and Information Processing

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Rho GTPase signalling modules in tumour cell migration and invasion.

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Cell migration requires coordination of many events such as membrane protrusion, rearrangement of the cytoskeleton and the generation of force by actomyosin contractility. Migration of tumor cells is an essential component of invasion and metastasis. Rho-family GTPase signalling coordinates the dynamic cytoskeletal changes that are required for cell migration. To delineate Rho-family GTPase signalling in cell migration we are carrying out a systematic analysis of Rho-family GTPases their regulators and signalling pathways. Using RNA interference to target all Rho-family GTPases, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) we have described pathways controlling two distinct forms of movement in melanoma cells. Elongated, mesenchymal-type movement is driven by Rac activation mediated by a pathway containing the adaptor protein NEDD9 and the exchange factor DOCK3. In contrast rounded/amoeboid movement is driven by Rho and Cdc42 signalling to actomyosin contractility and suppressed by Rac activation. To investigate how Rho GTPase signalling is regulated we have studied upstream signalling events regulating the activation of Rac and actomyosin contractility. These studies reveal new insights into transmembrane signalling to Rho GTPases and demonstrate a tight interplay between Rho and Rac signalling in determining modes of cell movement.

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Organizing genetic information and its processing without membrane compartmentalization.

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It has long been known that eukaryotic cells highly organize genetic information and its processing in space, notably through extensive cellular compartmentalization. More primitive organisms such as bacteria lack nuclei and other membrane-enclosed organelles. Therefore, all processes involved in the transfer of genetic information (such as transcription, translation and RNA processing and decay) occur in one single compartment: the cytoplasm. The bacterial cytoplasm is furthermore extremely crowded, with macromolecule concentration reaching 340 g/L. Such crowding implies that significant contributions from excluded volume effects reduce mobility of cytoplasmic components. Recent evidence shows that despite its apparent simplicity, the bacterial cell spatially organizes its genetic information with genes remaining within confined subcellular locations that are dictated by chromosomal gene arrangement. An active DNA segregation process and, in some bacteria, the presence of proteins localized at the cell poles help propagate this spatial information through generations. Spatial organization is also seen for specific messenger RNAs and RNA processing enzymes. These spatial constraints ultimately affect ribosome dynamics and translation.

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Cell signaling at the single-cell level.

M. Elowitz[1]; [1]Division of Biology, California Inst Technol/HHMI, Pasadena, CA

What's special about the repertoire of signaling pathways we find in living cells? Despite increasingly comprehensive knowledge of the molecules and interactions involved in these signaling pathways, it often remains unclear how and why cells encode and represent signals in particular ways. I will discuss the quantitative analysis of signaling pathways using single-cell time-lapse microscopy, including recent work in bacteria, and in eukaryotes. Our results show that cells encode signals in unexpected and often highly dynamic ways, and that these encoding schemes enable cells to perform critical regulatory and developmental functions.

Symposium 4: Self-Organization of Cellular Structures

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Self-Organization of Secretory Compartments.

Y. Liu[1], N. Bharucha[1], E. Papanikou[1], B. S. Glick[1]; [1]Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL

During the past decade, the concept of self-organization has been increasingly used to describe the biogenesis of secretory compartments. We have applied this concept to analyze transitional ER (tER) sites and the Golgi apparatus. These studies have focused on two budding yeasts: *Pichia pastoris*, which has a few large tER sites that are linked to Golgi stacks, and *Saccharomyces cerevisiae*, which has numerous small tER sites and a nonstacked Golgi. We found that *P. pastoris* tER sites are long-lived structures that form *de novo*, fuse upon collision, and grow or shrink to attain a steady-state size. This behavior can be explained by a self-organization model. Specifically, we postulate that capture of new tER components is balanced by shrinkage driven by the budding of COPII coated transport vesicles. To test this model, we used a genetic screen to identify Sec16 as a key player in tER organization. Sec16 is a large peripheral ER membrane protein that interacts with multiple COPII components. Our results suggest that Sec16 partitions between the ER membrane and the cytosol, and that the membrane-associated form acts as a negative regulator of ER export. According to this view, Sec16 functions primarily to control tER dynamics rather than to nucleate tER site formation. Interestingly, the redistribution of Sec16 to the cytosol is influenced by association of the central conserved domain of Sec16 with the COPII component Sec13. The combined data imply that Sec16 defines a new level of regulation for the ER export system. To study Golgi organization, we took advantage of the nonstacked Golgi cisternae in *S. cerevisiae*. Our 4D confocal microscopy studies revealed that Golgi cisternae mature by progressively acquiring and then losing early resident Golgi proteins followed by late resident Golgi proteins. Recently, we have found that early Golgi compartments are closely associated with tER sites even in *S. cerevisiae*, consistent with the idea that Golgi cisternae assemble by the coalescence of ER-derived vesicles. However, the mechanisms of subsequent Golgi maturation are more obscure. One possibility is that COPI coated vesicles drive the recycling of resident Golgi proteins from older to younger cisternae, but the experimental evidence for the role of COPI has been ambiguous. We are now using new approaches to achieve rapid and selective inactivation of COPI and other key trafficking components in *S. cerevisiae*. The long-term goal is to elucidate the cascade of interactions that enables the cell to rebuild the Golgi apparatus with each round of transport.

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Spatiotemporal Integration of Chemical and Mechanical Signals in Cell Migration.

G. Danuser[1]; [1]Department of Cell Biology, Harvard Medical School, Boston, MA

Cell migration is driven by the self-organization of innumerable, coupled chemical and mechanical processes. Chemical signals activate and deactivate the assembly and disassembly of cytoskeleton polymer networks. Both assembly and disassembly generate mechanical forces that push parts of the cell forward and retract others. They also define the mechanical properties of the cytoskeleton, which in turn determine how forces translate into cell shape deformation and movements. Chemical signals modulate the activity of molecular motors that produce contractile forces. By pulling on the polymer networks, these same motors organize the architecture of the cytoskeleton. This affects again the mechanical properties of the cytoskeleton. Motors may even mediate polymer disassembly, inducing a secondary contractile response of the cytoskeleton. All these entangled mechanical outputs feed back into the activation of the upstream chemical signals, a process that can be referred to as cell intrinsic mechanotransduction. Decades of biochemical, genetic and molecular analyses have generated the parts lists for most of these chemical and mechanical processes as well as hypotheses of process interactions. However, there is still very limited understanding as to how the processes are spatially and temporally organized in a system with emergent properties. There are multiple challenges associated with addressing this question: First, the process hierarchies are transient and distributed over multiple time and length scales. Thus, new technology is required to monitor multiple processes working in parallel but at different time points and cellular locations; and analytical tools are needed to extract from these data the spatial, temporal, and functional linkages between processes. Second, because of the nested nonlinear interactions among processes, the stimulation or perturbation of a particular process component often propagates in complex ways, obscuring the relation between system response and component function. For the past ten years my lab has made attempts to implement approaches that tackle these issues. We have invested in the development of quantitative multi-parametric live cell imaging to acquire simultaneous measurements of cell morphodynamics, cytoskeleton dynamics, resulting intracellular forces, and chemical signals. We also have worked on multiplexing methods to couple these measurements across different experiments, allowing us to “stitch together” larger process models. Very recent work in the lab has focused on novel mathematical tools to predict complex nonlinear process hierarchies. Central to our approach is the use of minimally perturbing experimental strategies that avoid the ambiguities of nonlinear system responses induced by stronger interventions. This talk will highlight some of the most surprising discoveries we have made with this work and will give an outlook to the many challenges we are still facing in order to establish a comprehensive understanding of cell migration.

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Modeling cytoskeletal structures with Cytosim.

F. Nedelec[1]; [1]Cell Biology and Biophysics, EMBL, Heidelberg, Germany

Living cells have a system of fibers and associated proteins, called the cytoskeleton, that provides an essential mechanical support during migration, polarization, division, etc. Cytoskeletal fibers form spontaneously by self-assembly of monomers, and multiple fibers can further self-organize into assemblies of cellular scale. In this talk, we will introduce cytosim, a simulation software with the aim to study systems of many fibers. We will explain how the user can easily configure the simulation with simple examples. We will also illustrate techniques that can be used to systematically explore the possibilities embedded in a cytoskeletal system.

Minisymposium 9: Bioengineering and Mechanobiology

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Lamin A/C mutations linked to muscular dystrophies, but not lipodystrophies, cause impaired nuclear structure and mechanics in cells and tissue.

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Objective: Mutations in the *LMNA* gene that encodes the nuclear envelope proteins lamin A and C cause a plethora of human diseases (laminopathies), including muscular dystrophies, cardiomyopathies, and familial partial lipodystrophy. It remains unclear how mutations in a single gene can result in such a variety of diseases, many of them specifically affecting muscle tissues, while other mutations cause little or no muscle involvement. Since lamins A and C are the main contributors to nuclear stiffness, we hypothesized that lamin mutations associated with muscular phenotypes could impair the structural properties of the nuclear lamin network, weakening the nuclear integrity and resulting in cells more susceptible to mechanical stress.

Methods: We measured nuclear stiffness in fibroblasts derived from patients with diverse laminopathies and in lamin A/C–deficient mouse embryonic fibroblasts (MEFs) engineered to stably express physiological levels of specific lamin A mutants. In a subset of cells, we also probed nucleo-cytoskeletal coupling with a custom-developed microneedle assay. To assess the in situ effect of lamin mutations in muscle, we measured nuclear stiffness in body wall muscle of Drosophila melanogaster expressing lamin C mutations associated with human laminopathies or wildtype lamin.

Results: Patient fibroblasts carrying *LMNA* mutations associated with muscular dystrophies had significantly softer nuclei than cells from healthy controls ($P<0.001$), while fibroblasts from lipodystrophy patients had normal nuclear mechanics. Extending our studies to MEFs expressing a broad panel of lamin A mutations, we found that 4 of the 15 tested lamin A mutations caused decreased nuclear stiffness. Importantly, all four mutations were associated with laminopathies affecting muscle tissue, whereas mutations linked to lipodystrophy had no effect on the structural function of lamin A. Of note, most mutations linked to muscular disease also showed disturbed nucleo-cytoskeletal coupling. Experiments with freshly isolated Drosophila melanogaster body wall muscle subjected to mechanical strain confirmed that specific lamin A mutations affect nuclear mechanics not only in isolated cells, but also in intact muscle tissue.

Conclusions: *LMNA* mutations associated with muscular laminopathies can cause impaired nuclear mechanics, which may lead to increased cellular sensitivity to mechanical stress and contribute to the muscle specific phenotype in these diseases. In conclusion, our results demonstrate the importance of lamins A and C on nuclear mechanics in laminopathies, but also indicate that additional factors such as altered nucleo-cytoskeletal coupling influence the disease outcome.

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Vinculin is important for p130Cas-mediated mechanotransduction.

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Fibroblasts, when subjected to mechanical stress, reinforce their focal adhesion contacts by activating the focal adhesion protein p130Cas, followed by binding of p130Cas with focal adhesion kinase (FAK) and talin, and subsequent activation of downstream pathways such as extracellular-signal-regulated kinase, $ERK^{1/2}$ phosphorylation. A newly discovered phosphorylation site (Y12) on p130Cas has been suggested to play an important role in modulating the binding with FAK, and potentially with vinculin. In particular, phosphorylation of position Y12 or mutation with phospho-mimicking glutamate (Y12→E) suppresses binding to vinculin. Our question was how Y12 phosphorylation and vinculin binding affects the distribution of p130Cas in cells and its activation upon mechanical stress. The presence of p130Cas in the focal adhesion complexes was not altered in vinculin-deficient fibroblasts. Baseline and stretch-induced phosphorylation of p130Cas, however, was reduced compared to vinculin wildtype cells. Moreover, phospho-mimicking mutation (Y12→E) on p130Cas, which prevents vinculin binding, increases downstream phosphorylation of $ERK^{1/2}$ in stretched cells. Taken together, these data demonstrate that vinculin is an important modulator of the p130Cas-mediated mechanotransduction pathway in cells.

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A mechanosensory system governs myosin II cleavage furrow accumulation.

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The mitotic spindle is generally considered to be the initiator of furrow ingression. However, recent studies have shown that furrows can form in the absence of spindles, particularly during asymmetric cell division. In *Dictyostelium*, the mechanoenzyme myosin II and the actin crosslinker cortexillin I form a mechanosensor that responds to mechanical stress, which could account for spindle-independent contractile protein recruitment. Here, we show that the cleavage furrow regulatory and contractility network, composed of myosin II, cortexillin I, IQGAP1, IQGAP2, kinesin-6 (kif12) and INCENP, is a mechanical stress-responsive system. Myosin II and cortexillin I form the core mechanosensor, and mechanotransduction is mediated by IQGAP2 through kif12 and INCENP. Additionally, IQGAP2 is antagonized by IQGAP1 to modulate the mechanoresponsiveness of cortexillin I, suggesting a possible mechanism for discriminating between mechanical and biochemical inputs. Furthermore, IQGAP2 is important for maintaining normal spindle morphology and recruitment of kif12 and myosin II to the cleavage furrow. Finally, the mitotic spindle is dispensable for the system. Overall, we suggest that this mechanosensory system is structured like a control system characterized by mechanochemical feedback loops that regulate the myosin II levels at the cleavage furrow.

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Matrix compliance regulates epithelial-mesenchymal transition.

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Epithelial-mesenchymal transition (EMT) is a phenotypic switch wherein cells detach from a polarized epithelium and acquire a motile, fibroblastic phenotype. Matrix metalloproteinases (MMPs) have been shown to be key factors associated with pathological EMT. We found that microenvironmental context, specifically substratum stiffness, plays a pivotal role in the regulation of EMT by MMP3. Soft substrata, with compliances comparable to that of normal mammary tissue, are protective against MMP3-induced EMT, whereas stiffer substrata, with compliances characteristic of breast tumors, promote EMT. Matrix rigidity governs EMT by controlling the subcellular localization of Rac1b, a highly activated splice variant of Rac1 found in breast and colorectal tumors. Culture on soft substrata inhibits focal adhesion formation and the membrane localization of Rac1b, and thereby interrupts its interaction with NADPH oxidase. This subsequently inhibits MMP3- and Rac1b-induced production of reactive oxygen species (ROS), expression of Snail, and activation of the EMT program. Altering Rac1b prenylation or integrin clustering using mutated forms of these proteins can bypass the signals induced by substratum compliance. These results suggest the role of a novel mechanotransduction pathway in the regulation of EMT.

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Bleaching/blinking assisted localization microscopy (BALM) for super-resolution imaging using standard fluorescent molecules.

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Super-resolution imaging techniques circumvent the diffraction-limit of light microscopes and are useful in cell biological research because they bridge the gap between the image-resolution possible from diffraction-limited light microscopy (hundreds of nanometers) and the resolution possible from electron microscopy (less than 1 nanometer). Point localization techniques, such as Photoactivated Localization Microscopy (PALM) and Stochastic Optical Reconstruction Microscopy (STORM), achieve a particularly high resolution by fitting single fluorescent molecules with a theoretical Gaussian to localize them with a precision on the order of tens of nanometers. PALM/STORM rely on photoactivatable proteins and photoswitching dyes, respectively, making them technically challenging and preventing them from being easily added to a cell biologist's "tool box". Here, we present a simple and practical way of producing point localization-based-super-resolution images, bleaching/blinking localization microscopy (BALM), that relies on the intrinsic bleaching (irreversibly turn off) and blinking (reversibly turn off and on) behaviors characteristic of all commonly used fluorescent molecules. To do BALM, the researcher first acquires a streaming recording of a fixed sample labeled with a fluorescent probe, which will bleach over time. Single molecule-turn off (bleach or blink off) events can be detected by subtracting from each image of the acquisition the next subsequent image. Similarly, blink on events are detectable by subtracting from each frame the previous one. To validate that BALM works using a standard EMCCD camera we first imaged microtubules in COS7 cells labeled with Alexa-488. After image subtractions, the localization precision of single Alexa-488 molecules was subsequently calculated (σ=26.6 +/- 14 nm) which is similar to that achieved by PALM. We then verified the increase in structural resolution by comparing microtubules reconstructed with BALM with those reconstructed with PALM. We then performed BALM on Myosin IIC-GFP to show that it works with exogenously expressed fluorescent

proteins. Finally, we performed BALM on microtubules labeled with Alexa-488, Alexa-561, and Alexa-647 to show that BALM can be used to localize several different fluorescent probes in the same cell. Thus, BALM is a practical and flexible super-resolution technique, which is only limited by the users ability to separate the emission of different fluorophores.

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Adipose-Derived Stem Cells Form Functional Myotubes Via Mechanical Induction whereas Marrow-derived cells Do Not.

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Undifferentiated stem cells integrate cues around themselves to differentiate, but often for fibrotic muscle diseases that stiffen the microenvironment, e.g. muscular dystrophy and myocardial infarction, differentiation is misdirected. When co-delivered with exogenous growth factors, bone marrow-derived stem cells (BMSC) commit to their appropriate mesodermal-derived lineages, but efficiency is not sufficiently high enough to restore function. Biochemical methods overlook biophysical interactions between cells and extracellular matrix (ECM), which is stiffened by fibrosis and clearly impacts cell function in vivo. Though they express a similar molecular signature and can commit to mesodermal lineages via growth factors, differences between adipose-derived stem cell (ASC) and BMSCs may likely impact their differentiation efficiency in diseased muscle. Here we found that ASCs reflect the same qualitative stiffness sensitivity as BMSCs with morphological changes reflecting differentiated phenotypes (Engler et al, Cell 2006). However, quantitative analysis of lineage expression showed ASCs correctly express the appropriate temporal sequence of muscle transcriptional regulators, e.g. MyoD, Myogenin, MEF2C, etc., and do so at levels at least 10-fold higher than BMSCs. Moreover by 7 days in culture, 2.1% of ASCs form multi-nucleated myotubes with a continuous network that is not the result of misdirected cell division. This process mirrors that in primary muscle cells, and most importantly, BMSCs were never observed to undergo this process. Treatment with myoseverin, a microtubule depolymerizing drug that severs myotubes, could disrupt ASC-derived myotubes, but 7 days after drug washout, ASCs refused and formed myotubes at a rate similar to their pretreated value. To understand why ASC are more myogenic when stimulated by ECM, it was determined that they appear more contractile and form adhesions faster than BMSCs. Their fate is also alpha5 and alphaV integrin-dependent as they do not undergo myogenesis in their absence. Interestingly, ASC replated on non-permissive substrates maintain their state, which implies that they are less plastic and may likely be more successful in engrafting and restoring function in fibrotic muscle.

Minisymposium 10: Cell Polarity

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PAR proteins regulate the cortical localization of LET-99 during asymmetric division.

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Positioning of the mitotic spindle is essential for a number of developmental processes, including asymmetric divisions that generate cell diversity. During such divisions, the spindle must align with the cell polarity axis, and the spindle is often displaced so that division is unequal. The PAR proteins establish polarity in many cell types. PAR proteins also regulate spindle movements, in part via a cortical complex that includes Gα, GPR (LGN/Pins) and LIN-5

(NuMA/Mud). This complex is thought to activate dynein to pull on astral microtubules and move the spindle. During the first asymmetric division of the *C. elegans* embryo, GPR and LIN-5 are localized asymmetrically. We previously showed that LET-99 is an intermediate in this spindle-positioning pathway and is required for both spindle orientation and displacement. LET-99, a DEPDC1 family protein, is localized at the cortex in a lateral-posterior band, where it prevents GPR accumulation, thus generating asymmetry. PAR-3 inhibits LET-99 localization at the anterior, while PAR-1 inhibits LET-99 at the posterior-most cortex. In other systems, phosphorylation of PAR-3 by the PAR-1 kinase generates binding sites for 14-3-3 proteins, which prevents cortical accumulation. We found that the PAR-5 14-3-3 protein and PAR-1 associate with LET-99, and have identified two serine residues in LET-99 essential for PAR-5 binding. To determine the in vivo relevance of these sites, two S to A mutations were introduced into a LET-99 transgene (LET-99-AA). LET-99-AA localized to the entire posterior cortex of the one-cell embryo instead of a band. These and other results support the model that PAR-5 binds to LET-99 to dissociate it from the cortex, and that this interaction is regulated by phosphorylation of LET-99 by PAR-1 at the posterior. In contrast, LET-99-AA is inhibited from the anterior cortex, which suggests that PAR-3 regulates LET-99 through a PAR-5 independent mechanism. We are currently determining if other anterior PAR proteins are required for LET-99 asymmetry, as well as investigating the mechanism by which LET-99 inhibits GPR localization.

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Oscillatory dynamics and spatial self-organization of Cdc42 GTPase in the control of polarized cell growth.

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The conserved Cdc42 GTPase has a crucial role in the establishment of cell polarity by promoting cytoskeletal asymmetry. Previous studies have shown that Cdc42 breaks symmetry by clustering on a single membrane spot via a complex mechanism mediated in part by “winner-take-all” Cdc42 autocatalytic amplification. This however cannot explain how multiple growing zones form simultaneously in a cell. Fission yeast switches from monopolar to bipolar growth during cell cycle progression in a process known as “new end take off” (NETO), making it an ideal system to study how bipolarity arises and is regulated in cells. Here we show that tip-bound active Cdc42 concentrations correlate with changes in growth pattern and exhibit large oscillations with an average period of five minutes. A mathematical model invoking Cdc42 autocatalytic amplification, competition between growth sites, and delayed negative feedback reproduces the observed dynamics and its predictions are confirmed by experimental tests. We find that Cdc42 oscillations facilitate transitions between polarization states and are critical for active redistribution of growth. Our findings are consistent with observations in mammalian and plant cells, suggesting that related mechanisms of self-organization underlie the emergence of cell form.

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The centrosome and PAR-3 are required during epithelial polarization for the normal hand-off of microtubule organizing center function.

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Although the centrosome is the major microtubule organizing center (MTOC) of the cell, MTOC function often is reassigned to the apical membrane in epithelial cells. In the development of the *C. elegans* intestine, for example, microtubule-nucleating proteins such as γ-tubulin and CeGrip-1/dgrip91/Spc98p that normally are found at the centrosome are present at the apical

membrane; the apical cortex becomes highly enriched in microtubules, but few or no microtubules appear associated with the centrosome. The apical deposition of γ-tubulin and CeGrip-1 occurs when intestinal cells are post-mitotic and starting to polarize. During this stage, centrosomes that were near either anterior or posterior lateral membranes following cell division migrate orthogonally toward the future apical membrane. Centrosomes and centrosome positioning appear to influence apical deposition of γ-tubulin and CeGrip-1: First, embryos lacking the polarity protein PAR-3 fail to apically position their centrosomes, and have aberrant localization of γ-tubulin and CeGrip-1. Second, γ-tubulin fails to accumulate apically in wild-type cells following laser ablation of the centrosome. Using live imaging, we show that centrosomes localize apically by first moving toward lateral foci of PAR-3 and PAR-6. These foci move together with the centrosomes toward the future apical surface. Remarkably, γ-tubulin appears to redistribute directly from the migrating centrosome onto the lateral/apical membrane. Together, these data suggest that PAR-3 contributes to apical polarity in part by positioning centrosomes, and that the hand-off of MTOC function from centrosomes to the apical membrane is associated with a physical hand-off of nucleators of microtubule assembly. γ

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Spatial Guidance of Microtubule Organization in Polarizing Epithelia.

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Establishment of epithelial polarity requires the reorganization of the microtubule (MT) cytoskeleton from a radial array into a network positioned along the apicobasal axis of the cell. Little is known about the mechanisms that spatially guide the remodeling of MTs during epithelial polarization. Septins are filamentous GTPases that associate with MTs, but the function of septins in MT organization and dynamics is poorly understood. Using the Madin-Darby Canine Kidney (MDCK) model system of epithelial polarity, we have found that septin filaments provide a navigation mechanism for the positioning of MTs along the emerging axis of apicobasal polarity. We show that cell-cell adhesion triggers the formation of perinuclear septin filaments, which colocalize exclusively with MT bundles that transverse the apex and sides of the nucleus. Using septin RNAi and dominant negative constructs and in vitro biochemistry, we show that septins mediate the formation of MT bundles by suppressing MT catastrophe. As epithelial cells grow taller and become more columnar, septin-MT bundles arch back and begin to extend vertically along the developing apicobasal axis. Our results indicate that these septin-MT bundles provide a directional cue for the establishment of the apical MT meshwork. We show that MT-septin bundles are often targeted by MT ends, which dock and change their directionality of growth along septin filaments. Upon septin knock down, MTs meander stochastically and the MT network becomes highly entangled. Importantly, the percentage of MT plus ends found in the apical cytoplasm is significantly reduced, causing a shift in MT distribution toward the basal and medial cytoplasm. Taken together, these data indicate that septins navigate the growth and positioning of epithelial MT during the establishment of apicobasal polarity.

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Self-organization of stress-fibers and focal adhesions into polar arrays.

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Fibroblasts attached to a flat rigid isotropic substrate spread first isotropically and form isotropic system of radial and circumferential (tangential) actomyosin bundles (stress fibers, SFs)

associated with the radial focal adhesions (FAs). In a couple of hours, however, these cells undergo polarization manifested by cell elongation, segregation of cell periphery into active (lamellipodia forming) and stable regions, and formation of the array of SFs oriented along the long axis of the cell. Here we show that orientation of the FAs and organization of the array of parallel SFs precedes cell elongation and formation of active and stable cell edges. Moreover, parallel orientation of SFs/FAs develops eventually even in cells in which elongation was artificially suppressed by plating on circular adhesive islands. In such cells, formation of parallel SFs array was often preceded by synchronous turn of the radial SFs ("cytoplasmic swirl" phenomenon). We present a model for the dynamic re-distribution of tangential and radial SFs that precedes and promotes polarization of the cell body. Our model hypothesis that the tangential SFs, emerging at lamellipodium-lamellum interface, are at first anchored to the mature Fas and remain bound at their both ends to the radial SFs that project from the FA towards the cell center. Energy consuming contraction of acto-myosin complexes within the actomyosin SFs generates stresses, which are translated to effective forces acting on the bound ends of the tangential SFs. These effective forces result in a global centripetal motion of the tangential SFs. The orientation of the radial SFs, the motion patterns, and the forces acting on the FAs are coupled together. Using numerical simulations we demonstrate that noise in the system can cause a spontaneous symmetry breaking of the SFs arrangement, resulting in such experimentally observed phenomena as cytoplasmic swirl and polarization of the SFs.

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Front-Rear Polarization of the Cytoskeleton develops in Cell migration from Soft to Stiff Matrix.

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It has become increasingly clear that cytoskeletal polarity is strikingly different in cells functioning in an in vivo environment compared to cells studied in vitro. Reasons for this discrepancy are unknown and whether matrix rigidity plays a role in affecting cell polarity is has yet to be studied. Tissues have been measured to have varying stiffnesses or deformabilities, and examples of matrix stiffening include scarring in the heart after a myocardial infarction or scarring of the liver in cirrhosis – which lead to rigidification of tissue through extensive collagen crosslinking, and it has been reported that adherent cells, including mesenchymal stem cells (MSCs) accumulate or 'home' to such scarred tissue. It is unknown how MSCs localize and, in particular, whether cell polarity plays a role in directing their migration from soft to stiff matrix. Our data show that the position of the microtubule organizing center (MTOC) with respect to the nucleus is influenced by matrix rigidity and thus microtubule density within the cell also depends on surface stiffness. Because myosin is responsible for cell contractility which deforms and probes matrix stiffness, we investigated the role non-muscle myosin (both MIIA and MIIB isoforms) may have in cell polarity and directed cell migration to stiff matrix. Using siRNA knockdown we determined that directed migration toward stiff matrix is more sensitive to MIIB and less so to MIIA, even though mass spectrometry determines there is ~10-fold more expressed MIIA in MSCs than MIIB on the protein level. Reports have shown that MIIB localizes to the cell rear in polarized migrating cells, but as we alter the matrix stiffness we demonstrate that this polarization diminishes when matrix stiffness becomes softer than 7 kPa. Our studies also show that the MTOC begins to polarize to the cell front at matrix stiffness above 7 kPa, suggesting a similar mechanism for stiffness induced polarization of MTOC and MIIB. Scratch wound assays induced polarization of MTOC toward the wound edge on stiff substrates but failed to do so when performed on soft matrix. Interestingly, we also found the same stiffness dependent rearward polarization in MIIB in anuclear cell fragments. We find that the polarity of

the cytoskeleton is not required for cell migration but for directing cells toward stiffer matrix. Based on our data, we believe cytoskeletal polarization thus acts as an elasticity-sensitive compass in directing migration toward stiff matrix.

Minisymposium 11: Cellular Functions of Ubiquitin and Ub-related Proteins

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Neurodegeneration and the Listerin (Ltn1) pathway of ribosome-associated protein quality control.

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mRNA lacking stop codons ('non-stop mRNA') can arise from errors in gene expression, and encode aberrant proteins whose accumulation could be deleterious to cellular function. In bacteria, these 'non-stop proteins' become co-translationally tagged with a peptide encoded by *ssrA*/tmRNA (transfer-messenger RNA), which signals their degradation by energy-dependent proteases. How eukaryotic cells eliminate non-stop proteins remained unknown. We have recently reported that the *S. cerevisiae* Ltn1 RING-domain-type E3 ubiquitin ligase acts in the quality control of non-stop proteins (Bengtson & Joazeiro 2010. Nature 467:470-3). The Ltn1-mediated process is mechanistically distinct but conceptually analogous to that performed by *ssrA*: Ltn1 is predominantly associated with ribosomes, and marks nascent non-stop proteins with ubiquitin to signal their proteasomal degradation. Ltn1-mediated ubiquitylation of non-stop proteins seems to be triggered by their stalling in ribosomes on translation through the poly(A) tail. The biological relevance of this process is underscored by the finding that loss of Ltn1 function confers sensitivity to stress caused by increased non-stop protein production. We speculate that defective protein quality control may underlie the neurodegenerative phenotype that results from mutation of the mouse Ltn1 homologue, Listerin. In my talk, I will review these data and will present recent findings and further characterization of the Listerin/Ltn1 pathway.

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A novel linear ubiquitin ligase complex regulating TNFα-induced NF-κB activity and apoptosis.

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SHARPIN is a ubiquitin-binding and ubiquitin-like domain-containing protein which, when mutated in mice, results in immune system disorders and multiorgan inflammation. We found that SHARPIN functions as a novel component of the Linear Ubiquitin Chain Assembly Complex (LUBAC) and that the absence of SHARPIN causes disregulation of NF-κB and apoptotic signalling pathways, explaining the severe phenotypes displayed by chronic proliferative dermatitis in SHARPIN deficient mice. Upon binding to the LUBAC subunit HOIP, SHARPIN stimulates the formation of linear ubiquitin chains in vitro and in vivo. Co-expression of SHARPIN and HOIP promotes linear ubiquitylation of NEMO, an adaptor of the IκB kinases (IKKs) and subsequent activation of NF-κB signalling, while SHARPIN deficiency in mice causes an impaired activation of the IKK complex and NF-κB in B cells, macrophages, and mouse embryonic fibroblasts (MEFs) by CD40L, LPS and TNFα, respectively. This effect is further enhanced upon concurrent downregulation of HOIL-1L, another HOIP-binding component of LUBAC. In addition, SHARPIN deficiency leads to rapid cell death upon TNFα stimulation via

FADD- and Caspase-8-dependent pathways, which was not induced by HOIL-1L deficiency. SHARPIN thus activates NF-κB and inhibits apoptosis via distinct pathways in vivo.

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ICAP-1 monoubiquitylation regulates cell migration. A-P. Bouin[1], A-S. RIBBA[1], M. Regent[1], E. Planus[1], C. Albiges-Rizo[1]; [1]Institut Albert Bonniot, Grenoble, France

Cell migration is a complex biological process that requires spatiotemporally regulation of integrin activation, assembly/disassembly of focal adhesion and dynamic organization of actin cytoskeleton. Extracellular matrix rigidity is an external factor influencing cell migration. ICAP-1, a beta1-integrin tail-binding protein, is essential for ensuring integrin activation cycle and focal adhesion formation by interfering with talin binding. In this study, we provide evidence that ICAP-1 function can be regulated by monoubiquitylation. We identified the monoubiquitylation site on ICAP-1. This modification is not involved in protein degradation via proteasome but rather might regulate the assembly and organisation of adhesion sites likely through modulation of protein interactions. Indeed, the monoubiquitylation of ICAP-1 prevents its beta1 integrin binding whereas the non-ubiquitinable form of ICAP-1 interacts with beta1 integrin. Expression of the non ubiquitinable form of ICAP-1 in ICAP-1 deficient cells impacts on beta1 integrin localisation and focal adhesion organization. By testing different matrix rigidities we show that cells expressing the non ubiquitinable form of ICAP-1 exhibit a modification in the matrix density and rigidity sensing which affect cell migration. The monoubiquitylation of ICAP-1 might play a key role in integrin-mediated cell migration through matrix rigidity perception. The using of ROCK inhibitor suggests that this specific role of ICAP-1 is linked to ROCK signalling pathway. Our results show that the ubiquitylation system is integrally involved in the process of cell migration acting as a nodal regulator between partners of beta1 integrin.

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Ubiquitin and cell cycle regulation of Golgi membrane dynamics.

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Partitioning of the Golgi membrane into daughter cells during mammalian cell division occurs through a unique disassembly and reassembly process. Several converging lines of evidence have suggested that monoubiquitination plays an essential role in the regulation of post-mitotic Golgi membrane fusion. Monoubiquitination, as a regulatory signal, occurs during mitotic Golgi disassembly and is required for subsequent Golgi reassembly. The p97/p47 complex binds to monoubiquitin through the UBA domain of the adaptor protein p47. Inhibition of the p47-ubiquitin interaction suppresses p97-mediated Golgi membrane fusion. VCIP135, a cofactor of the p97/p47 complex, is a deubiquitinating enzyme whose activity is required for post-mitotic Golgi reassembly. Proteasome activity is not involved in either Golgi disassembly or reassembly. These data suggest that cycles of addition and removal of ubiquitin to and from substrates is necessary for Golgi reassembly. Here, we report the identification of the ubiquitin ligase and the ubiquitinated substrates on the Golgi membranes and provide further evidence that ubiquitin plays a critical role in Golgi biogenesis during the cell cycle.

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ATG12 In Mitochondrial Homeostasis and Hypothalamic Function.

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Until recently, ATG12, a UBL required for autophagy, was proposed to conjugate a single target, ATG5. We discovered ATG3, the E2-like enzyme required for ATG8/LC3 lipidation during

autophagy, as a novel ATG12 conjugation target. ATG12-3 complex formation requires ATG3 E2 autocatalytic activity, resulting in the covalent linkage of ATG12 onto a single lysine on ATG3. Mutation of this lysine abolishes ATG12-3 complex formation but surprisingly, does not impact nonselective autophagy. Rather, cells lacking ATG12-3 display increased mitochondrial mass and fragmentation, arising from combined defects in mitophagy and mitochondrial fusion. Cells lacking ATG12-3 are also resistant to intrinsic apoptosis. Importantly, these unique functions of the ATG12-3 complex are completely separable from the established roles of either individual ATG in autophagy. Furthermore, our biochemical analysis demonstrates that multiple ATG12 conjugation targets exist (in addition to ATG5 and 3). Overall, these results reveal unexpected roles for ATG12-3 in mitochondrial homeostasis and implicate the ATG12 conjugation system in novel cellular functions. To further test this hypothesis in vivo, we created complete or conditional *atg12* deletion mice. Similar to *atg5* deficiency, complete *atg12* null mice die on postnatal day 1, corroborating an essential role for ATG12-5 for autophagy during the neonatal period. However, *atg12* (but not *atg5*) transcripts are highly enriched in the hypothalamus, which plays critical roles in mammalian food intake and energy balance. Upon *atg12* deletion in a subset of hypothalamic neurons (POMC neurons), mice display significantly reduced body weight and adiposity, as well as improved glucose tolerance, but no significant changes in food intake or leptin sensitivity. Currently, we are dissecting how ATG12 in POMC neurons directs energy expenditure and regulates diet-induced obesity, and creating POMC *atg5* deficient mice to parse if this phenotype is due to a general defect in autophagy versus a unique function mediated by ATG12.

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Regulation of SUMO enzymes by oxidative stress.

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Sumoylation is an essential posttranslational mechanism that affects hundreds of proteins in all eukaryotic species. The mechanism of sumoylation resembles the ubiquitination cascade: modification with SUMO requires ATP, a SUMO activating enzyme, a SUMO E2 conjugating enzyme, and usually one of several E3 ligases. Isopeptidases ensure reversibility of the modification. Modification of most targets is a highly dynamic process that requires additional cues such as a specific time in the cell cycle, developmental stage, DNA damage, or heat shock. A number of years ago, we found that the reactive oxygen species hydrogen peroxide causes rapid disappearance of SUMO conjugates when added to tissue culture cells. This is due to direct and reversible inhibition of SUMO conjugating enzymes through the formation of a disulfide bond between the catalytic cysteines of the SUMO E1 subunit Uba2 and the E2-conjugating enzyme Ubc9. Importantly, enzyme inactivation by disulfide bond formation was also observed upon induction of endogenous ROS production, i.e., the respiratory burst in macrophages (Bossis and Melchior, Mol Cell 2006). These findings add SUMO conjugating enzymes to the small list of specific direct effectors of H2O2 and implicate ROS as key regulators of the sumoylation pathway. To begin to understand the role of SUMO enzyme oxidation, we embarked on a search for redox insensitive Ubc9. Using random mutagenesis and in vitro sumoylation, we identified a single point mutant in the E2 conjugating enzyme Ubc9 that is as active as wt Ubc9 on E3 ligase dependent and independent targets, but insensitive to oxidation. Analysis of this mutant in mammalian tissue culture cells suggests that reversible SUMO E1-E2 disulfide bond formation contributes to oxidative stress response.

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Minisymposium 12: Chromosome Structure and Epigenetics

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Folding principles of genomes.

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We study how chromosomes are organized in three dimensions. The three-dimensional (3D) organization of chromatin is critical for regulating gene expression by bringing genes in close spatial proximity to distal regulatory elements such as enhancers. We used 5C to map long-range interaction networks throughout 1% of the human genome (the ENCODE pilot regions) across a panel of cell lines. Specifically, we interrogated 1,348,361 unique pairwise interactions between annotated transcription start sites and distal loci to discover networks of long-range interactions that connect regulatory elements to their target genes. Analysis of the data reveals that long-range interactions are abundant and widespread. Long-range acting elements are found to frequently overlap with other ENCODE annotations, including DNaseI hypersensitive sites, predicted enhancers and sites bound by CTCF. The data provide new insights into the surprisingly complex and interwoven architecture of long-range gene regulation.

We also developed novel modeling approaches to build 3D models of chromosomes using 5C data as input. Using this approach we derived 3D models of the alpha-globin gene domain in human cells. We also generated 3D models of the complete genome of the bacterium Caulobacter crescentus, which has led to the identification of DNA elements that are critical for organizing the genome inside the cell.

Combined our approaches allow the uncovering of the folding principles, and DNA elements that drive folding of chromosomes in relation to gene regulation and other genomic processes.

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The SCF^{Slimb} ubiquitin-ligase regulates Cap-H2 levels to control interphase chromosome condensation and spatial organization.

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Chromosome condensation is essential during mitosis, however little is known about how or if interphase chromosome compaction is also modulated. Two conserved complexes (condensins I and II) facilitate chromosome condensation in mitosis. Recent work has revealed that condensin II also functions as an anti-pairing factor, preventing polytene chromosome assembly in non-dividing *Drosophila* nurse cells and inhibits homolog pairing essential for transvection. These interphase functions suggest that condensin is regulated throughout the cell cycle and, at present, it is not clear how interphase condensin is different from its mitotic activities. Since control of protein turnover is a prominent mechanism cells use to regulate cell cycle-dependent events, we examined whether the ubiquitin machinery could play a role in chromosome condensation. By examining a previous RNAi screen of the Cullin-based E3 ubiquitin-ligase family, we found that depletion of the SCF components (Cullin-1 or SkpA) or the F-box protein, Slimb, resulted in a chromosome hyper-condensation phenotype in cultured *Drosophila* S2 cells. Specifically, SCF^{Slimb} RNAi causes a dramatic change in the morphology of chromatin in non-mitotic cells; compacting chromosomes into a cluster of 8-12 spheres per nuclei that we call

the 'gumball' phenotype and are likely to be individualized chromosome territories. Strikingly, we find that double RNAi of Slimb and either SMC-2, Cap-D3, or Cap-H2 completely rescues the gumball phenotype, suggesting that condensin II activity is negatively regulated by SCF^{Slimb}. Indeed, we also find that the non-SMC subunit, Cap-H2, is a novel Slimb ubiquitin-target, as Cap-H2 possesses a conserved Slimb-binding domain, co-immunoprecipitates with Slimb, and is stabilized upon Slimb depletion. Furthermore, mutation of the Slimb-binding domain stabilizes Cap-H2 and expression of this mutant in S2 cells is sufficient to induce the hyper-condensation gumball phenotype. Here, we also present *in vivo* evidence supporting a Slimb/Cap-H2 genetic interaction in modulating polytene chromosome assembly and pairing. Our results reveal a previously unknown mechanism for regulating condensin that utilizes ubiquitin-mediated proteolysis of a key regulatory subunit to attenuate chromosome compaction and spatial organization during interphase of the cell cycle. We propose a model where SCF^{Slimb} controls Cap-H2 proteins levels thereby linking mitotic exit and chromosome decondensation to interphase specific compaction and chromosome organization.

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DNA Topoisomerase II acts as a mitotic scaffold protein in chromosome assembly in *C. elegans.*

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Assembly of mitotic chromosomes is a dynamic event, occurring rapidly during prophase of each cell cycle. Given the complexity of this process, genetic and biochemical approaches have identified surprisingly few factors involved in condensation, notably histone H1, the condensin complex, DNA TOPOisomerase II (TOPO II) and CENtromere-Protein A (CENP-A). TOPO II can cut and reseal DNA and has been hypothesized to act in a structural capacity, but how it acts in mitotic chromosome condensation remains unknown. To define the roles of known and novel proteins in chromosome condensation, we are performing high-resolution live imaging of the *C. elegans* zygote. Images of fluorescently-tagged core histones are thresholded and segmented to quantify the distribution of chromatin within the prophase nucleus over time. When TOPO II is depleted by RNAi, chromosomes collapse prematurely during prophase. When the catalytic activity of TOPO II is inhibited with Dexrazoxane, chromosomes do not collapse prematurely; rather, chromatin remains diffuse throughout prophase. These results indicate that independent of its catalytic activity, TOPO II acts to scaffold condensation. Its decatenating activity is also important for chromosome resolution during condensation. Interestingly, simultaneous depletion of Topo II and centromeric chromatin partially rescues the TOPO II depletion phenotype, but simultaneous depletion of condensin and TOPO II does not. This suggests that TOPO II with CENP-A have opposing contributions to mitotic chromosome assembly. Endogenous TOPO II localizes to an axis along metaphase chromosomes distinct from that formed by CENP-A, further indicating that their mechanisms of action are independent. We conclude that metaphase chromosomes are built by the distinct scaffold activities of three proteins: TOPO II, CENP-A and Condensin. Importantly, our analysis revealed that depletion of candidate proteins results in quantitatively distinct phenotypes, indicating that they function in discrete events during mitotic chromosome assembly.

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An Inverse Relationship to Germline Transcription Defines Centromeric Chromatin in *C. elegans.*

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Centromeres direct segregation of the genome during cell division. The histone H3 variant CENP-A/CenH3 defines centromeres in both monocentric organisms, in which the centromere is localized at a discrete site on the chromosome, and holocentric organisms, in which centromere activity is distributed along the chromosome length. Stable inheritance of CENP-A nucleosomal chromatin, with replenishment once per division that is guided by the inherited nucleosomes, is postulated to epigenetically propagate centromere identity. As the highly repetitive DNA found at most centromeres is neither necessary nor sufficient for centromere function, the genomic features conducive to the establishment of CENP-A chromatin remain largely uncharacterized. We show that CENP-A is not inherited on chromatin through fertilization in the holocentric nematode C. elegans, indicating that pre-existing CENP-A nucleosomes are not required to target assembly of new CENP-A nucleosomes. Genome-wide mapping of CENP-A in embryos and counting of CENP-A molecules in embryonic nuclei indicated that CENP-A is incorporated at a low density in discrete domains that cumulatively occupy ~47% of the genome. To identify the property that makes a genomic region permissive for CENP-A incorporation, we compared the distribution of CENP-A to other chromatin and genomic features. This analysis revealed a genome-wide inverse correlation between CENP-A and RNA Polymerase II (Pol II) occupancy, suggesting that transcriptional activity in the embryo defines the CENP-A distribution. However, inhibition of transcription did not affect either loading of CENP-A or chromosome segregation in early embryos, and the CENP-A distribution did not change during embryonic development, arguing against this idea. Analysis of CENP-A and Pol II occupancy on various gene sets defined by their expression profiles revealed that genes that are expressed in the germline but transcriptionally inactive in embryos, did not incorporate CENP-A. This suggested that memory of germline transcription in the mother, as opposed to active transcription, defines the CENP-A distribution in embryos. Consistent with this, a mutant that ectopically triggers epigenetic memory of germline transcription at a small subset of genes shows loss of CENP-A at those genes in embryos. This change is observed without transcriptional activation in embryos, as assessed by Pol II occupancy. Thus, memory of germline transcription defines the genomic regions in embryo progeny permissive for CENP-A incorporation to build the C. elegans holocentromere. These findings link centromere identity to epigenetic memory of gene expression and shed light on the evolutionary plasticity of centromeres.

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In vitro centromere and kinetochore assembly on defined chromatin templates.

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During cell division, chromosomes are segregated to nascent daughter cells by attaching to the microtubules of the mitotic spindle through the kinetochore. Kinetochores are assembled on a specialized chromatin domain, called the centromere that is characterized by the replacement of nucleosomal histone H3 with the histone H3 variant centromere protein A (CENP-A). CENP-A is essential for centromere and kinetochore formation in all eukaryotes but it is unknown how

CENP-A chromatin directs centromere and kinetochore assembly. Here we generate synthetic CENP-A chromatin that recapitulates essential steps of centromere and kinetochore assembly *in vitro*. We show that reconstituted CENP-A chromatin when added to cell free extracts is sufficient for the assembly of centromere and kinetochore proteins, microtubule binding and stabilization, and mitotic checkpoint function. Using chromatin assembled from histone H3/CENP-A chimeras, we demonstrate that the conserved C-terminus of CENP-A is necessary and sufficient for centromere and kinetochore protein recruitment and function but that the CENP-A targeting domain (CATD), required for new CENP-A histone assembly, is not. These data show that two of the primary requirements for accurate chromosome segregation, the assembly of the kinetochore and the propagation of CENP-A chromatin are specified by different elements in the CENP-A histone. Our unique cell-free system enables complete control and manipulation of the chromatin substrate and thus presents a powerful tool to study centromere and kinetochore assembly in higher eukaryotes.

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Analysis of Native Kinetochore Particles.

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The precise regulation of chromosome segregation is critical to processes such as self-renewal, proliferation and development. Chromosomes segregate using their kinetochores, the specialized protein structures that are assembled on centromeric DNA sequences. The most fundamental activity of kinetochores is to maintain load-bearing attachments to the assembling and disassembling tips of spindle microtubules. A complete mechanistic understanding of the kinetochore requires reconstitution of kinetochore-microtubule attachments in vitro, yet these studies have not been previously possible due to the lack of a method to isolate kinetochores. We recently developed a technique to purify functional native kinetochore particles from budding yeast and are further characterizing their properties by biochemical and biophysical techniques. To elucidate the mechanism by which kinetochores bind to microtubules, we have also visualized the particles by electron microscopy. Strikingly, we find that kinetochores make multiple contacts to microtubules and often contain a ring that encircles the microtubule. These data suggest that kinetochores bind to microtubules through multivalent attachments as previously proposed. These studies lay the foundation for uncovering the mechanisms that underlie the key mechanical and regulatory functions of kinetochores.

Minisymposium 13: Meiosis and Oogenesis

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Luteinizing Hormone Reduces Guanylyl Cyclase Activity in the Granulosa Cells of the Mouse Ovary, Leading to the Resumption of Meiosis in the Oocyte.

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In the mouse ovarian follicle, the granulosa cells keep the oocyte arrested in meiotic prophase. The inhibitory signal is cGMP, which diffuses from the granulosa cells to the oocyte through gap junctions. The cGMP is generated by the guanylyl cyclase NPR2 (Zhang et al., 2010, *Science* 330, 366-369). Luteinizing hormone (LH) then acts on the granulosa cells to stimulate meiosis to resume, by closing the gap junctions and by lowering granulosa cell cGMP (Norris et al., 2009, *Development* 136, 1869-1878). Here we investigate whether LH causes a decrease in guanylyl cyclase activity that contributes to the decrease in cGMP. In membranes prepared from ovarian follicles, guanylyl cyclase activity can be stimulated by the NPR2 agonist, C-type natriuretic peptide (NPPC). In response to LH application to follicles, this NPPC-dependent guanylyl cyclase activity decreases, falling to about half by one hour after LH application. In addition, the NPPC content of the ovary decreases in response to LH, reaching 39% of the basal level by 2 hours, 23% by 4 hours, and 7% by 8 hours. Correspondingly, nuclear envelope breakdown in the oocyte, which marks the resumption of meiosis, begins at ~2 hours after LH exposure. Thus the guanylyl cyclase activity of the granulosa cells is reduced by two mechanisms: a decrease in the NPPC-dependent NPR2 activity, and a decrease in the amount of NPPC. Both contribute to reducing cGMP and triggering meiotic resumption.

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Greatwall Kinase Is an Essential Component of MPF.

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Cyclin B-Cdk1 is sometimes called MPF (maturation or M-phase promoting factor), and it is currently well accepted that both are synonymous. Originally, however, MPF was identified by a functional assay in oocyte systems. That is, MPF was first described as a cytoplasmic activity of hormone-treated maturing oocytes that upon tranfer causes recipient immature oocytes to undergo meiotic resumption without hormonal stimulation (Masui and Markert, 1971. J. Exp. Zool. 177:129-145; Kishimoto and Kanatani, 1976. Nature 260:321-322). Based on such a functional assay in the oocytes of starfish and frog, here we show that MPF is not identical to cyclin B-Cdk1 but composed both of cyclin B-Cdk1 and Greatwall kinase (Gwl), which is known to indirectly inhibit the phosphatase PP2A-B55 that antagonizes cyclin B-Cdk1. In quantitative comparison, the level of cyclin B-Cdk1 activity required for meiotic resumption was roughly one order of magnitude higher when using purified cyclin B-Cdk1 than when using cytoplasmic MPF, implying that the physiological level of cyclin B-Cdk1 is not sufficient for MPF. In the absence of Gwl, MPF was undetectable from oocyte cytoplasm, even though cyclin B-Cdk1 was fully active. When recombinant Gwl was put back, MPF activity was restored. Although Gwl alone exhibited no activity for inducing meiotic resumption, addition of Gwl greatly reduced the amount of purified cyclin B-Cdk1 required for induction of meiotic resumption. These observations establish that MPF includes at least two separate kinases, cyclin B-Cdk1 that directs mitotic entry and Gwl that suppresses the phosphatase opposing cyclin B-Cdk1.

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Chromosome transport by a homogeneously contracting F-actin meshwork in starfish oocytes.

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Actomyosin-based contractility mediates cell shape changes underlying cellular functions including cell division, cell migration or would healing that have been extensively studied and the mechanisms beginning to be understood. In contrast, the role of actin in intracellular transport is much less clear with the prevailing view that filamentous actin (F-actin) cables serve as tracks for motor-driven transport of cargo. We recently discovered an alternate mode of actin-driven intracellular transport in starfish (*Patiria miniata*) oocytes involving a contractile F-actin meshwork that mediates the long-range, directed transport of chromosomes to the forming meiotic spindle.
Here we used live-cell imaging combined with quantitative analysis of chromosome trajectories and meshwork velocities to address the mechanism by which this meshwork translates its contractile activity into directional force to transport chromosomes. Our data indicate that the 3D F-actin meshwork contracts homogeneously and isotropically throughout the nuclear space. By precisely timed drug perturbations we could show that contraction is generated internally in the meshwork that is therefore under tension. This tension is released by continuous polymerization of new actin filaments at membranes at the boundary of the meshwork. Thus, the actual rate of contraction is determined by the constant rate of filament production. Centrifugation experiments further reveal that this homogeneous contraction is translated into asymmetric, directional transport by anchoring of the meshwork to the cell cortex. Finally, by injecting inert beads and bead aggregates of different sizes, we show that this directional transport activity is size-selective and transduced to chromosomal cargo at least in part by passive sieving. Thus, dissecting the mechanism of actin-driven chromosome transport in starfish oocytes revealed design principles of a novel and potentially versatile mode of intracellular transport based on sieving by an anchored homogeneously contracting F-actin meshwork.

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Characterization of a novel subcortical actin layer regulating spindle positioning and cortical plasticity in mouse oocytes.

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Meiotic maturation is the last step of female meiosis. It leads to the formation of one oocyte and two polar bodies after two divisions very asymmetric in size. Therefore the oocyte keeps most of the maternal stores required for embryonic development. These divisions are asymmetric in size because of spindle off-center positioning. During the first division, the spindle forms where the nucleus was in prophase I, in the center of the oocyte, and then migrates to the periphery, allowing after cytokinesis the formation of a big cell, the oocyte, and a small cell, the first polar body. This movement is actin dependent. Actin forms a cytoplasmic meshwork, which requires Formin-2 and Spire 1 and 2, and which densifies during the first meiotic division. This meshwork is essential for female fertility.
We observed that another actin meshwork is present in mouse oocytes, a subcortical actin layer which is Formin-2 independent. This layer thickens progressively below the cortex at the time of spindle migration until polar body extrusion. Arp2/3 is a nucleator of this actin layer and the Mos/…/ MAPK pathway regulates its formation. The inhibition of Arp2/3 abolishes spindle migration, leading to a symmetric division. Thus this layer is essential for the asymmetry of the

division. Eventually, we performed cortical stiffness assays in various contexts and we demonstrate that this layer regulates oocyte plasticity. Altogether our work suggests that oocytes, which are non-adherent cells, require the formation of a subcortical actin layer to support efficient spindle positioning. In culture cells, actin fibers mediated adhesion of the cell prior to cell rounding has been shown to control the positioning of the mitotic spindle. Thus, actin remodelling at the cortex might be a universal feature of spindle positioning.

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A post-replicative role for Deco in meiotic cohesion and chiasma maintenance.

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Human meiosis suffers high error rates and most aneuploid gametes arise from chromosome segregation errors during female meiosis I. These errors are the leading cause of birth defects and miscarriages, and as women age, the risk of aneuploid pregnancy increases exponentially. Accurate chromosome segregation in human oocytes requires that meiotic sister-chromatid cohesion remain intact for decades and work in model organisms indicates that deterioration of meiotic cohesion over time may be a major determinant of age-related aneuploidy. One unresolved question is whether oocytes rely exclusively on cohesive linkages that are established during meiotic S phase or if maintenance of meiotic cohesion is an active process that requires rejuvenation of cohesion throughout the extended period of prophase I.

We are using Drosophila oocytes to investigate the mechanisms controlling cohesion maintenance during meiotic prophase. Deco is the Drosophila homolog of the yeast cohesion establishment factor, Eco1, an acetyltransferase that is essential for formation of cohesive linkages between sisters during S phase. To ask whether Deco activity contributes to the maintenance of meiotic cohesion during prophase I, we used a Gal4/UAS inducible system to knock down Deco in Drosophila oocytes after meiotic S phase. Because the synaptonemal complex (SC) depends on normal meiotic cohesion, we used the SC protein C(3)G as a cytological marker to monitor cohesion and found that severe SC defects arise during mid prophase, after the RNAi driver is turned on. Moreover, when Deco is knocked down during prophase I, we observed a significant increase in meiotic nondisjunction (NDJ). Using a genetic assay to determine the recombinational history of missegregating chromosomes, we found that chiasmata are formed but not maintained when Deco is knocked down after meiotic S phase. Our results demonstrate that the cohesion establishment factor Deco plays a critical post-replicative role in maintaining the cohesive linkages between sister chromatids during meiotic prophase I. In addition, our observation that Smc3 and SA knockdown also result in premature SC disassembly supports the model that turnover of chromatin-associated cohesin during prophase I contributes to cohesion maintenance. Together these data argue that cohesion rejuvenation takes place during the extended prophase I of metazoan oocytes. Such activity may represent an evolutionarily conserved mechanism that functions to help counteract the deterioration of meiotic cohesion in aging oocytes.

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Cohesin Composition Determines Mechanisms of Cohesin Loading, Cohesion Establishment, and Cohesin Removal during *C. elegans* Meiosis.

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Faithful transmission of the genome through sexual reproduction requires reduction of genome copy number during meiosis to produce haploid sperm and eggs. Three processes unique to meiosis are crucial for reducing ploidy. First, homologous chromosomes become linked through crossover recombination. Second, the two sister chromatids of each homolog attach to

microtubules (MTs) from the same spindle pole (co-orient) in meiosis I and attach to MTs from opposite spindle poles (bi-orient) in meiosis II. Third, sister chromatid cohesion (SCC) is released in two steps to allow separation of homologs and then sisters during meiosis I and II. Work in yeast has shown that establishing the meiotic pattern of chromosome segregation requires that Scc1, the “kleisin” subunit of cohesin complexes that mediate mitotic SCC, is replaced by the meiosis-specific paralog Rec8. Subsequently, *rec8* mutations have been used to define meiotic cohesin functions in many organisms. We have shown that REC-8 is not the sole meiotic kleisin in *C. elegans*, and based on our work and other published data we propose that involvement of multiple kleisins is common in meiosis. *C. elegans* REC-8 and two redundant kleisins called COH-3 and COH-4 (COH-3/4 hereafter) perform specialized meiotic functions. For example, although both REC-8 and COH-3/4 are required for meiotic SCC and meiotic recombination, only REC-8 cohesin can co-orient sister chromatids and mediate SCC beyond anaphase I. Thus, cohesin complexes that differ in their kleisin subunit can perform distinct tasks in a single nucleus, and cohesin function is determined by kleisin identity. The kleisin also influences the mechanism of cohesin loading and of SCC establishment. The axial element protein HTP-3 is required for loading of REC-8 cohesin but not COH-3/4 onto meiotic chromosomes. Once loaded, COH-3/4 cohesin is triggered to become cohesive by SPO-11-dependent double strand breaks, while REC-8 cohesin generates SCC independently of SPO-11. Finally, REC-8 and COH-3/4 become asymmetrically distributed on meiotic chromosomes late in prophase of meiosis I, consistent with their distinct roles in meiotic chromosome segregation: COH-3/4 becomes enriched where SCC is released at anaphase I and REC-8 becomes enriched where sister chromatids co-orient and SCC persists until anaphase II. Because REC-8 alone can co-orient sisters and mediate SCC that persists following anaphase I, this reciprocal pattern of cohesin localization may underlie the stepwise separation of homologs and sister chromatids.

Minisymposium 14: Modeling and Simulation of Cellular Functions

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Kinetic Aspects of Clathrin Coated Pit Formation.

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The dynamics of clathrin coated pit (CCP) formation, observed through TIRF measurements, shows considerable diversity. Foremost is fate heterogeneity, which leads to “abortive” and “productive” pits, i.e., structures which, respectively, do or do not mature into clathrin coated vesicles (CCVs). Also, there is notable heterogeneity in the lifetimes of abortive pits and the apparent times to completion of productive CCPs. We explore the extent to which the stochastic nature of CCP growth can explain these observations by building a simple model for CCP dynamics which includes the free energy of CCP formation and a related kinetic scheme. We investigate (using Monte Carlo simulations) the time development of CCP size and use the model to explain the origin of abortive pits and features of their lifetime distribution. The lifetime distribution calculated from our model agrees very well with that obtained from laboratory experiments. We also show how the binding of cargo might modify the coat parameters and thereby facilitate CCV formation. Finally, we observe how heterogeneity in CCV lifetimes is linked to the stochastic associations and disassociations of the coat components.

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Mechanical stresses and biochemical patterning: a regulation-transport feedback loop.

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Chemical reactions are inherently local phenomena, occurring when individual molecules are proximal. Nevertheless, biochemical species exhibit spatial patterns on length scales much larger than the size of individual molecules. Here, we discuss a new mechanism for pattern formation in which biochemical species regulate mechanical stress in cells, e.g., by manipulating cytoskeletal components. Stress imbalances drive movement of cytoplasmic and cytoskeletal material, resulting in transport of stress regulators. This results in a build-up of regulators, thereby forming a regulation-transport feedback loop. We develop a hydrodynamic theory describing this feedback, also including the possibility of chemical reactions. We show that mechanical stress-based patterning can be more rapid and more robust than classic reaction-diffusion mechanisms. Application of the theory suggests that known interactions between polarity-determinant proteins (the PAR proteins) and their regulation of cortical stress are sufficient to produce the observed polarization and cortical flow of the one-cell C. elegans embryo.

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Modeling mechanics of cell turning.

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Our current fair understanding of the cell migration's physics is largely derived from experiments, models and intuition developed for cells crawling straight forward. On the other hand, mechanics of cell turning remains unclear. We used observations of asymmetric geometry and key protein distributions of steadily turning fish keratocyte to develop a mechanical model according to which stick-slip property of adhesions, effective myosin drift in the moving cell framework and myosin-dependent actin disassembly lead to the cell turning. Specifically, our calculations indicate that these three processes combine to provide positive mechanical feedbacks causing long-lasting transient imbalances of myosin and adhesion distributions, actin flows and traction forces at two 'wings' of turning keratocyte. Computer simulations of the model reproduce characteristic cell shapes and movements and provide clues for understanding of unsteady cell motility underlying chemotactic response and other physiologically important cell behaviors.

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Asymmetry is an emergent property of the cytokinetic ring.

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Cytokinesis is a complex cellular event requiring spatiotemporal regulation of the actomyosin and microtubule cytoskeletons and membrane dynamics. Cell shape change in cytokinesis is accomplished by a contractile ring rich in F-actin and myosin. For almost forty years, the prevailing model for ring closure has been the “purse-string hypothesis” of actomyosin sliding

filament activity that uniformly and concentrically contracts at the cell equator. Perhaps due to the transient nature of this event and the apparent differences among cell types, almost no progress has been made to refine the purse-string hypothesis.

We devised a minimal model of the mechanics of ring initiation and closure and tested these ideas in silico. Briefly, we model that filament alignment facilitates contractility, contractility provides force to deform the cell, and the shape of the ingressed membrane favors circumferential filament alignment. We are modeling cytokinesis in the C. elegans zygote, in which cell division is highly stereotypical, essential proteins can be well depleted, and the cytokinetic ring closes non-concentrically. In order to quantitatively compare simulation output to experimental results, we developed software for semi-automated quantitative image analysis of our high-resolution live cell imaging data.

Our computational model recapitulates not only the kinetics of ring initiation and closure, but also the non-concentric geometry of closure, a prevalent but poorly understood feature of cytokinesis. With experimentally justified adjustments, the model also recapitulates the outcome of perturbations of conserved contractile ring proteins and of the spindle midzone.

Finally, we took advantage of our computational model to ask whether there is a cellular advantage to non-concentric ring closure. Our model predicts that non-concentric ring closure is indeed energetically favorable, thus suggesting a mechanism for conservation of proteins that ensure this mysterious feature of metazoan cytokinesis. Interestingly, further search of the parameter space outside that occupied by the C. elegans zygote revealed an even more efficient mode, which is more concentric, and involves a poorly organized contractile ring, but takes longer than the embryonic cell cycle. Thus, our model suggests that a trade-off between speed and energy efficiency dictates the mode of cytokinesis used by a cell.

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Polar acto-myosin contractility and cleavage furrow stability during cytokinesis.

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Cytokinesis relies on tight regulation of the mechanical properties of the cell acto-myosin cortex. Although most studies of cytokinetic mechanics focus on force generation at the equatorial acto-myosin ring, a contractile cortex remains at the poles of dividing cells throughout cytokinesis. Whether polar forces influence cytokinetic cell shape is poorly understood. Combining experiments, quantitative imaging and theoretical modeling, we demonstrate that the polar cortex makes cytokinesis inherently unstable and that any imbalance in contractile forces between the poles compromises furrow positioning. We show that limited asymmetric polar contractions occur during normal cytokinesis, and that perturbing the polar cortex leads to cell shape oscillations and division failure. A theoretical model based on a competition between cortex turnover and contraction dynamics accounts for the oscillations and yields predictions on how the mechanical control of cytokinetic shape is achieved. Taken together, our experiments and model show that the physical properties of the entire cell are integrated into a fine-tuned mechanical system ensuring successful cytokinesis.

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Cellular Noise Regulons Underlie Fluctuations in Saccharomyces cerevisiae.

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Stochasticity is a hallmark of cellular processes and different classes of genes show large differences in their noise levels. The roots and consequences of these differences have largely remained unexplored. Here, we systematically dissect the structure of expression noise in S. cerevisiae. On top of local variability unique to a single gene, which is determined by expression level and a low level of global variability shared by all genes, there is strong pathway variability shared across similarly regulated genes. This noise induces quantitative correlations that are strongly predictive of the genes dynamic response to stimulus. Furthermore, analysis of fluctuation induced correlations defines well-structured "noise regulons" spanning distinct functional classes such as the MSN2/4 stress response pathway, amino-acid biosynthesis, and mitochondrial maintenance. Bioinformatic analyses and controlled genetic perturbations reveal PKA and Tor pathway as major contributors to this modular organization. Our results reveal that there is a small number of well-delineated noise regulons operating across a yeast cell, and that pathway noise is a powerful quantitative tool for exploring pathway features and regulatory relationships.

Minisymposium 15: Motors and Microtubule Dynamics

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TPX2 Regulates the Localization and Activity of Eg5 in the Mammalian Mitotic Spindle.

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The assembly and function of the mitotic spindle requires the precise temporal and spatial regulation of numerous motor and non-motor proteins. In mammalian cells, the kinesin-5 family member, Eg5, cross-links and slides apart anti-parallel microtubules *in vitro* and is required for bipolar spindle formation in mitosis. Eg5 localizes to spindle microtubules and is enriched at spindle poles. In live cells, FRAP experiments show that at a population level, Eg5 is highly dynamic in all spindle regions, with a turnover half-time for fluorescence recovery of < 10 sec. Eg5 interacts with the Ran-regulated spindle-assembly factor, TPX2 and the proteins co-localize throughout mitosis. To determine if TPX2 regulates the localization and dynamics of Eg5, we examined spindle formation in cells expressing TPX2 lacking the Eg5 binding domain. In these cells, spindles were disorganized with multiple poles. The TPX2-Eg5 interaction was required for the formation of cold-stable kinetochore fibers and to localize the motor to spindle microtubules, but not spindle poles. FRAP showed that the interaction with Eg5 regulated TPX2 turnover, however Eg5 turnover was not changed in the absence of an interaction with TPX2. Microinjection of the Eg5 binding domain of TPX2 into metaphase cells resulted in spindle elongation, suggesting that TPX2 functions to inhibit Eg5 activity. To test this possibility, *in vitro* motility assays with purified proteins were performed. The results demonstrate that TPX2 reduces the velocity of Eg5-dependent microtubule gliding, inhibits microtubule sliding, and results in the accumulation of motors on microtubules. These results show that the localization and activity of Eg5 are regulated by interactions with the microtubule-associated protein TPX2.

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The depolymerizing kinesins Kip3 (kinesin-8) and MCAK (kinesin-13) are catastrophe factors that destabilize microtubules by different mechanisms.

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Microtubules are dynamic filaments whose plus ends alternate between periods of slow growth and rapid shortening as they explore intracellular space and move intracellular organelles. A key question is how regulatory proteins such as the depolymerizing kinesins modulate catastrophe, the conversion from growth to shortening. To study this process, we reconstituted microtubule dynamics in the absence and presence of the kinesin-8 Kip3 and the kinesin-13 MCAK. Surprisingly, we found that even in the absence of the kinesins, the microtubule catastrophe frequency depends on the length and age of the microtubule, indicating that catastrophe is a multistep process. Kip3 slowed microtubule growth in a length-dependent manner and increased the rate of accumulation of lattice destabilizing features that lead to catastrophe. In contrast, MCAK did not change the feature formation rate, but instead transformed catastrophe into a single step process. Thus, both kinesins are catastrophe factors, but differentially reshape the microtubule cytoskeleton.

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MCAK activity controls microtubule dynamics and directed cell migration.

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Cell polarization during directed cell migration is coordinated by (MT) growth toward the leading edge. Cells can locally control MT growth or shortening through the regulation of depolymerizing kinesins (MCAK), which catalyze the destabilization of MT plus ends, causing MTs to transition from growth to shortening (catastrophe). However, it is not known if MCAK regulation affects polarized MT growth dynamics that mediate cell migration. We hypothesized that MCAK is locally inhibited to establish preferential MT growth toward the leading edge to promote directed migration. To test this hypothesis, we performed high resolution imaging of fluorescently tagged EB3 that dynamically associates with growing MT plus-ends, coupled with automated image-based tracking of MT growth speeds and growth lifetimes (1/catastrophe frequency). We found that knockdown of MCAK (MCAK-KD) promoted increased MT growth lifetimes globally, abolishing the polarized differences in MT growth lifetime in the cell center and edge seen in control cells. As a result, directional cell migration was significantly reduced, suggesting that local regulation of MCAK activity is critical for establishing polarized MT growth and directing cell migration. To determine how local regulation of MCAK might be achieved, we investigated the role of Aurora-A kinase, which when activated by phosphorylation localizes to mitotic centrosomes and behaves as a phospho-inhibitor of MCAK depolymerase activity. Phospho-Aurora-A immunoprecipitation from cell extracts revealed interaction between MCAK and the phospho-active form of Aurora-A. Live-cell measurements of MT growth dynamics showed that overexpression of Aurora-A promotes long-lived MT growth similar to MCAK-KD, suggesting that Aurora-A regulates the depolymerase activity of cytoplasmic MCAK. To identify upstream activators of Aurora-A, we expressed either constitutively active- (CA-Rac1) or dominant negative-Rac1 (DN-Rac1) and measured changes in active Aurora-A via immunolabeling with a phospho-specific Aurora-A antibody. Compared to control, cells expressing CA-Rac1 displayed a 4-fold increase of phospho-active Aurora-A, while in cells expressing DN-Rac1, phospho-active Aurora-A levels were decreased by 3-fold. In addition, live-cell imaging revealed that like GFP-MCAK, GFP-Aurora-A tracks with a subset of growing MT plus-ends in CA-Rac1 cells, but

not in DN-Rac1 cells. Finally, while CA-Rac1 promoted fast and long-lived MT growth, this effect was lost by pharmacologic inhibition of Aurora-A. Together, these results suggest that Aurora-A-mediated regulation of MCAK depolymerase activity is achieved downstream of Rac1 signaling to elicit regional regulation of MT dynamics and thereby promote polarized MT growth and directional cell migration.

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Axon injury triggers a microtubule-based pathway that protects dendrites.

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In order for a single set of neurons to function for the entire lifetime of an animal, these cells need to be able to survive injury and repair damage. Using Drosophila dendritic arborization neurons, we have found that one unexpected response to axon injury is a global increase in the number of growing microtubules.

To understand the pathway that increases microtubule dynamics in response to injury, we performed a candidate screen of microtubule regulators. Reducing levels of microtubule nucleation by RNAi against the core nucleation protein gamma-tubulin 23C, or introducing one copy of a null allele of this gene, reduced the increase in microtubule dynamics in injured neurons but did not affect uninjured cells. Microtubule nucleation is thus required for the increase in number of growing microtubules after axon injury.

We hypothesized that the increased microtubule dynamics might be required for axon regeneration after injury. To test this hypothesis we assayed regeneration in backgrounds with reduced nucleation. No difference from control animals was found when gamma-tubulin 23C was targeted by RNAi. Thus the changes in microtubule dynamics induced by axon injury are likely to be required for an injury-response other than regeneration.

As microtubule dynamics increased in dendrites after a distant axon injury, we hypothesized that rebuilding of dendritic microtubules might act to protect dendrites from retraction or degeneration triggered by losing the axon. To test this hypothesis we made use of the finding that dendrites have an active degeneration program that clears them after they are severed from the cell body. To trigger increased microtubule dynamics we cut the axon 8 hours before dendrite severing. This resulted in a delay in dendrite degeneration. To determine whether the delay in dendrite degeneration was due to the increased microtubule dynamics, we introduced a single mutant copy of gamma-tubulin 23C or targeted the microtubule polymerase msps by RNAi. Both treatments blocked dendrite protection by prior axon injury confirming a role for microtubules in a dendrite protective pathway.

This study identifies a novel program that uses microtubules to protect dendrites after axon injury. This program likely plays an important role in maintaining intact circuits while axons attempt repair.

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Structural Mechanism for Dynein Control by Lis1.

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Cytoplasmic dynein, the large microtubule-based motor protein, is carefully controlled in cells, enabling it to be deployed to specific sites, collect cargoes, and transport them towards the microtubule minus end at set times. Dynein's "engine" is evolved from ring-shaped AAA+ ATPases, and its microtubule-binding domain lies at the tip of a coiled-coil stalk, but how these elements might be acted upon to achieve control remains unknown. Here, using purified proteins from *Saccharomyces cerevisiae*, we dissect how cytoplasmic dynein is controlled by two of its ubiquitous regulators: Lis1/Pac1 and Nudel/Ndl1. By single-molecule microscopy, we find that Lis1 slows dynein velocity, prolongs its encounters with microtubules, and is tethered to dynein by Nudel. High-precision analysis shows that dynein-Lis1 undergoes frequent "anchored" cycles, during which ATP is consumed without the usual microtubule release and forward motion. Unexpectedly, the structural basis for these changes involves Lis1 binding at the interface between dynein's ATPase domain and its microtubule-binding stalk. Lis1 is thus ideally situated to alter allosteric communication in cytoplasmic dynein, which could facilitate its control and function in cells. *J. Huang and A. Roberts contributed equally to this work.*

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The crystal structure of tubulin tyrosine ligase offers insight into tubulin recognition.

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Tubulin tyrosine ligase (TTL) adds a C-terminal Tyr to alpha-tubulin as part of a tyrosination/detyrosination cycle present in most eukaryotic cells. The C-terminal tyrosine in alpha-tubulin serves as an ON/OFF signal for the recruitment of microtubule dynamics regulators. TTL loss causes morphogenic abnormalities and is associated with aggressive cancer progression and poor prognosis. We present the first crystal structure of TTL, defining the structural scaffold upon which the diverse TTL-like (TTLL) family of tubulin-modifying enzymes is built. TTL uses a bipartite strategy to recognize tubulin. It engages the tubulin tail through low-affinity, high-specificity interactions, and co-opts what is otherwise a homo-oligomerization interface in structurally related enzymes to form a tight hetero-oligomeric complex with tubulin. Small-angle X-ray scattering and functional analyses reveal that TTL forms an elongated complex with the tubulin dimer and prevents incorporation of the dimer into microtubules by capping the tubulin longitudinal interface, thereby possibly modulating the partition of tubulin between its monomeric and polymeric forms.

TUESDAY, DECEMBER 6

Symposium 5: Complex Cellular Functions: Linking Networks and Structures

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Virtual movement of a signaling network translated into real movement of a motility network.

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Transient contractile arrays of actin filaments (F-actin) and myosin-2 power diverse biological processes including cytokinesis, wound healing and morphogenesis. Contractile array formation is initiated by the Rho GTPases Rho and Cdc42, which stimulate myosin-2 activation and F-actin assembly in their active (GTP-bound) forms. Upon assembly, contraction itself is thought to direct movement of actomyosin arrays by myosin-2-powered shortening of the array. The relationship of contractile array movement and Rho GTPase turnover was analyzed during single cell wound repair, which entails formation and closure of a ring-like array of F-actin and myosin-2 directed by concentric zones of active Cdc42 and Rho. Remarkably, suppression of contractility prevented physical translocation of F-actin and myosin-2 and active Rho and Cdc42 without preventing closure of the actomyosin array or the Rho GTPase zones. This behavior is explained by an underlying gradient of Rho GTPase activity in which Rho and Cdc42 are preferentially activated at the leading edges of their respective zones and inactivated at their trailing edges. Contraction inhibition partially disrupts the pattern of inactivation while suppression of F-actin turnover disrupts the pattern of both activation and inactivation. The results show that differential nucleotide hydrolysis by Rho GTPases imparts directional information to contractile arrays in a manner analogous to nucleotide hydrolysis by cytoskeletal polymers and reveal unexpected roles for myosin-powered contraction and actin turnover in contractile array function and the spatial regulation of Rho GTPase activity.

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Evolution of Epithelial Organization and the Cadherin-Catenin Complex.

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A tube surrounded by a simple epithelium is the most basic tissue organization in metazoans. Epithelial tubes are formed by actomyosin-based constriction of the apical surface of cells in epithelial sheets. In higher animals, epithelial organization is maintained by cadherin adhesion proteins which bind β-catenin and α-catenin that in turn organizes the actin cytoskeleton. I will discuss recent structure/function studies of the catenin complex in a variety of organisms. The results show that the catenin complex plays remarkably conserved functions in the formation and functional organization of epithelial structures from mammals to slime molds.

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Smell and the Single Neuron: Three-dimensional Reconstructions of Olfactory Neurons Offer Insights into Neuronal Morphologic Diversity and Odor Coding.

K. Baldwin[1]; [1]Department of Cell Biology, Scripps Res Inst, La Jolla, CA

Neurons are among the most morphologically diverse cell types. Neurons have been grouped into thousands of distinct subtypes based on shared functional properties and characteristic somatic morphology and location. Neurons of a particular subtype also exhibit similarities in the

gross scale structure of their axons and dendrites. In sensory neural circuits, differences in the precise connectivity of dendrites determine the information that a neuron carries, while axonal morphology controls the distribution of this information to higher brain centers. In many sensory systems, including vision, hearing and touch, neurons carrying similar sensory information project axons to closely apposed cortical targets producing spatial maps of sensory information that are similar among individuals. To address whether the sense of smell operates using similar principles, we developed long-range viral tracing tools and three dimensional neuronal reconstruction methods to characterize the entire dendritic and axonal arbors of defined olfactory bulb output neurons that carry the "same" sensory information. By reconstructing these neurons and mapping them onto a reference brain we identified unanticipated morphologic diversity among otherwise similar neurons. Neurons responding to the same odor compound project to nearly non-overlapping targets, even within the same animal. These findings have implications for odor perception and suggest a role for stochastic mechanisms in neuronal diversification.

Symposium 6: Mechanism of Multicellular Functions

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The costs of control: Strategies and tradeoffs in robust tissue pattern formation.

A. D. Lander[1,2], W-C. Lo[3], S. Zhou[1], P. Zhang[2], F. Y. Wan[3], Q. Nie[3]; [1]Department of Developmental and Cell Biology, University of Califorina, Irvine, Irvine, CA, [2]Department of Biomedical Engineering, University of Califorina, Irvine, [3]Department of Mathematics, University of Califorina, Irvine, CA

One of the principal ways in which long-range tissue patterns are formed is through the actions of gradients of diffusible morphogens. Conserved, complex regulatory mechanisms are found in most morphogen gradient systems, nearly always including elaborate feedback and feed-forward circuits, with contributions by co-receptors, diffusible inhibitors, multiple morphogens, and regulated uptake. As such circuitry is not essential for creating or interpreting morphogen gradients, it has been hypothesized that it evolved to further "strategic" goals—-e.g. robustness, speed, noise-filtering, flexibility, adaptability, etc. Indeed, the ability of some of these mechanisms to contribute, in principle, to the robustness of patterning has been demonstrated mathematically. Yet scarce attention has been paid to the tradeoffs that such strategies incur. For example, it is straightforward to show that strategies for tolerating uncertainty in the rate of morphogen production generally interfere with strategies for overcoming cell-to-cell variability in morphogen response ("spatial noise"), and *vice versa*. One consequence of such a tradeoff between robustness and noise-tolerance is a limit on the maximum distance over which a morphogen can pattern, a constraint that is indeed observed. Recently, we have begun to systematically identify tradeoffs among strategies for achieving robustness to uncertain production rates of morphogens, receptors and co-receptors. In my talk, I will discuss the general problems of robustness and noise that confront the *decapentaplegic* (BMP) and *wingless* (Wnt) morphogen gradients that pattern the *Drosophila* wing imaginal disc, and introduce the goal of balancing such tradeoffs as a basis for understanding the peculiar regulatory machinery that exists in these gradient systems.

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Shaping the embryo: Cellular dynamics in development.

J. Zallen[1]; [1]Developmental Biology, Sloan-Kettering Institute/HHMI, New York, NY

A major challenge in developmental biology is to understand how large-scale changes in tissue structure are generated on a cellular and molecular level. A conserved structural feature present in many multicellular animals is a body axis that is elongated from head to tail. This elongation is actively generated in the embryo through spatially regulated cell rearrangements, cell shape changes, and cell divisions. Cell rearrangements provide the driving force for axis elongation in frogs, fish, and chicks. In the fruit fly Drosophila, cell rearrangements cause the embryo to double in length from head to tail and narrow in width from back to front, creating the layout of the body plan. This process is amenable to direct live imaging approaches, which reveal a striking directionality in which large populations of cells align their movements along a common axis. To understand how genes encode the forces that drive these polarized cell behaviors and tissue remodeling, we are using cell biological approaches to identify proteins that are asymmetrically localized in intercalating cells, large-scale genetic screens to identify the molecular mechanisms that are required for elongation, and computational methods to analyze cell shape and behavior in three dimensions. We found that proteins involved in cell adhesion and contractile force generation are asymmetrically localized in intercalating cells, where they participate directly in polarized cell behavior. A polarized contractile network provides the global spatial information that guides cell movement, while differential adhesion regulates dynamic interactions between cells. Planar polarized force generation by the contractile actomyosin machinery is regulated by a combination of biochemical signals that establish cell polarity and mechanical feedback systems that coordinate dynamic events between cells. Specifically, we found that nonmuscle myosin II activity not only generates the forces that promote elongation, but myosin localization is also regulated by tension in a mechanism that recruits more myosin to the cortex and propagates contractile behavior from cell to cell. This mechanism triggers a wave of actomyosin contractility that leads to the assembly of multicellular rosette structures that form and resolve directionally, promoting elongation. Rosette behaviors have also been shown to occur in vertebrates and may represent a general mechanism linking single-cell asymmetry to global tissue reorganization.

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Generating multicellular architecture through collective migration.

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The collective migration of cohorts or tissues is a hallmark of organogenesis, wound repair and many invasive cancers. Cells at the leading edge of migrating collectives display many features characteristic of mesenchyme, such as highly dynamic protrusions and reduced apicobasal polarity, whereas the cells that follow become assembled into canonical epithelia. Thus, migrating collectives are generated a process that is highly similar to an epithelial-mesenchymal transition (EMT), with the exception that cell-cell junctions are maintained throughout. It is generally unclear how motility and assembly are balanced across migrating tissues. The zebrafish lateral line primordium is a migrating epithelial tissue that becomes assembled into a series of rosette-like mechanosensory organs en route. Previous genetic studies have shown that an extrinsic stripe of the chemokine SDF1 controls the behaviour of ‘leader’ cells at the tissue edge, whereas internal ‘follower’ cells are assembled into epithelial organs through the activity of an internal FGF-signaling circuit. I will present recent work addressing how cell signaling activities and dynamic cell shape changes are integrated during collective migration.

Minisymposium 17: Cell Biology of Micro-Organisms and the Evolution of the Eukaryotic Cell

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Emergent complexity in myosin V-based organelle inheritance.

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How is adaptability generated in complex biological systems composed of interacting cellular machineries, each with individual, separate and functionally critical jobs to perform? Organelle inheritance is one such system that requires the coordination of several robust and ancient cellular modules, including the cell cycle, cytoskeleton and organelle biogenesis/identity. Budding yeasts have emerged as powerful models to study these processes as organelles compete for access to myosin V motors that travel along polarized actin cables to vectorially deliver bound cargo to the bud. Under the direction of the cell cycle, myosin V motors are recruited to organelles by specific interactions between their carboxyl-terminal globular tail domain and organelle-specific receptors. We used comparative genomics, phylogenetics and secondary structural modeling to characterize the evolutionary history of these organelle-specific receptors. We find that while some receptors are retained widely across the animals and fungi, others are limited primarily to the Saccharomycetaceae family of budding yeast, with the emergent pattern of a conserved biogenic and inheritance factor often paired with an evolutionarily novel inheritance adaptor. We propose an evolutionary model whereby the emergence of myosin V-based organelle inheritance has utilized mechanisms of paralogy, the exploration of sequence space and the appearance of pliable, evolutionarily novel adaptor proteins. Our findings suggest an overarching evolutionary mechanism for how diverse cargoes compete for a single myosin V motor in organelle transport and detail one system's solution to obtaining evolutionary adaptability amongst constrained cellular modules. We are continuing to explore the consequences of the model with functional studies on the regulation of myosin V-based organelle inheritance.

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Comprehensive analysis reveals dynamic and evolutionary plasticity of Rab GTPases and membrane traffic in Tetrahymena thermophila.

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Cellular sophistication is not exclusive to multicellular organisms, and unicellular eukaryotes can resemble differentiated animal cells in their complex network of membrane-bound structures. These comparisons can be illuminated by genome-wide surveys of key gene families. We report a systematic analysis of Rabs in a complex unicellular Ciliate, including gene prediction and phylogenetic clustering, expression profiling based on public data, and Green Fluorescent Protein (GFP) tagging. Rabs are monomeric GTPases that regulate membrane traffic. Because Rabs act as compartment-specific determinants, the number of Rabs in an organism reflects intracellular complexity. The Tetrahymena Rab family is similar in size to that in humans and includes both expansions in conserved Rab clades as well as many divergent Rabs. Importantly, more than 90% of Rabs are expressed concurrently in growing cells, while only a small subset appears specialized for other conditions. By localizing most Rabs in living cells, we could assign the majority to specific compartments. These results validated most phylogenetic assignments, but also indicated that some sequence-conserved Rabs were co-opted for novel functions. Our survey uncovered a rare example of a nuclear Rab and substantiated the

existence of a previously unrecognized core Rab clade in eukaryotes. Strikingly, several functionally conserved pathways or structures were found to be associated entirely with divergent Rabs. These pathways may have permitted rapid evolution of the associated Rabs or may have arisen independently in diverse lineages and then converged. Thus, characterizing entire gene families can provide insight into the evolutionary flexibility of fundamental cellular pathways.

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Functional Genomics of Cell Regeneration in the Giant Ciliate *Stentor coeruleus*.

M. Slabodnick[1], W. Marshall[1]; [1]Univ California-San Francisco, San Francisco, CA

The mechanisms that specify cell shape and organization are not currently understood. Ciliates provide ideal model systems to tackle problems of cell morphology due to their complex cell organization and unique patterning. With current advances in technology along with work done in *Paramecium tetraurelia* and *Tetrahymena thermophila*, it is now possible to apply these tools to studying other organisms. *Stentor coeruleus* is a large, ~1mm long, single cell with a highly patterned cell cortex and the ability to regenerate and reorganize after surgical or chemical manipulations. The ease of surgical manipulations gives *Stentor* significant advantages over other ciliate models. Using the surgical techniques unique to *Stentor* as well as modern RNA interference (RNAi) methods, visualization techniques, and genomic sequencing I will revive *Stentor* as a model for studying cell polarity and organization. With the current state of Next Generation Sequencing it has become feasible for a lab to sequence the genome of a Eukaryotic organism. We have begun our own sequencing effort for *Stentor*'s macronuclear genome in order to facilitate the development of a better experimental toolbox. We have been able to repeat many of the surgical experiments performed by Vance Tartar, Noël de Terra and others. Using data obtained from preliminary sequences I constructed RNAi vectors that target endogenous *Stentor* genes and here I provide evidence that methodology developed for other ciliates can function in *Stentor* as well. Results for RNAi of Alpha-Tubulin and Mob1 result in dramatic changes in cell polarity and organization of the cortex and provide strong evidence that studies in *Stentor* can yield exciting and useful results. Knocking down Alpha-Tubulin, a key structural component in the cortex, results in clear cortical defects and problems with cell regeneration. This is very different from the Mob1 knockdown, which results in the drastic elongation of cells and other cortical aberrations. Using RNAi in conjunction with the unique microsurgical methods available in *Stentor*, it should be possible to restore this classical system to its previous status as a central model for addressing many important questions, including centriole structure, cell polarity, biological pattern formation, and cellular regeneration.

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Systems-level analysis of multicellular microbial community structure.

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Just as the phenotypically different cells that make up multicellular organisms are distributed in tissues with structures that embody specific functions, microbial cells with different metabolic functions form unique spatial structures and coordinate their activities as multicellular units, e.g. biofilms. Fundamentally, microbial communities differ from eukaryotic tissues because their cellular constituents may be genetically distinct; in fact, up to hundreds of different species may be present in a single biofilm. Any number of probes may be designed to identify the different species present in a community; however, the ability to unambiguously distinguish more than a few different labels in a single fluorescence image has been severely hampered by the

excitation cross-talk and signal bleed-through of fluorophores with highly overlapping excitation and emission spectra.
We recently developed a fluorescence labeling, imaging, and analysis method to greatly expand the number of identifiable labels in a single image, which we call Combinatorial Labeling and Spectral Imaging (CLASI). Application of our CLASI technique to human dental plaque using fluorescence *in situ* hybridization (FISH) enabled the first quantitative analysis of the spatial distribution of 15 different taxa of microbes in a biofilm. Proximity analysis was used to determine the frequency of inter- and intrataxon cell-to-cell associations, which revealed statistically significant intertaxon pairings. Cells of the genera *Prevotella* and *Actinomyces* showed the most interspecies associations, suggesting a central role for these genera in establishing and maintaining biofilm complexity. In a proof-of-principle experiment, we further demonstrate that we can distinguish 120 differently labeled *E. coli* in a mixture labeled with binary combinations of 16 fluorophores using a novel linear unmixing algorithm constrained to identify specific combinations of fluorophores. Our results provide an initial systems-level structural analysis of biofilm organization. We believe that the CLASI approach will be useful for a systems level analysis of many complex molecular structures within cells. Supported by the Sloan Foundation and NIH Grants F31DE019576 and RC1DE20630.

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Electrical Spiking in Escherichia coli Probed with a Fluorescent Voltage Indicating Protein.

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Bacteria have many voltage- and ligand-gated ion channels, and population-level measurements indicate that membrane potential is important for bacterial survival. However, it has not been possible to probe voltage dynamics in an intact bacterium. Here we developed a method to measure membrane potential on a single cell which revealed electrical spiking in Escherichia coli. To probe bacterial membrane potential we engineered a voltage-sensitive fluorescent protein based on green-absorbing proteorhodopsin. Expression of the Proteorhodopsin Optical Proton Sensor (PROPS) in E. coli revealed electrical spiking at up to 1 Hz. Within a nominally homogeneous population of bacteria, cells showed a variety of voltage dynamics. Spiking was sensitive to chemical and physical perturbations, and coincided with rapid efflux of a small-molecule fluorophore, suggesting that bacterial efflux machinery may be electrically regulated.

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Control of cell differentiation by the regulatory molecules ppGpp and inorganic polyphosphate.

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The molecules guanosine tetraphosphate (ppGpp) and inorganic polyphosphate (PolyP) function as global regulators of cell physiology in both prokaryotes and eukaryotes. These molecules have the capacity to directly bind and regulate the activity of a range of proteins in the cell including RNA polymerase and proteases. Our recent studies on bacterial cells have focused on understanding the molecular logic underlying regulated synthesis of ppGpp and PolyP, and the downstream control of cell development by these important signaling molecules.

We have defined the input logic and direct transcriptional targets of ppGpp in the oligotrophic bacterium, *Caulobacter crescentus*. The sole ppGpp synthase, $SpoT_{CC}$, binds to and is regulated by the ribosome, and exhibits AND-type control logic. We further demonstrate a regulatory link between the synthesis of ppGpp and PolyP and demonstrate that both molecules function to control of the timing of *Caulobacter* cell differentiation. Specifically, *Caulobacter* differentiates from a motile, foraging swarmer cell into sessile, replication-competent stalked cell during its cell cycle, akin to the G1->S transition in eukaryotic cells. This developmental transition is inhibited by nutrient deprivation to favor the motile swarmer state. Both ppGpp and PolyP inhibit this transition in rich and glucose-depleted medium. Upon exhaustion of available carbon, swarmer cells lacking the ability to synthesize ppGpp or PolyP erroneously initiate chromosome replication, proteolyze the replication inhibitor CtrA, localize the cell-fate determinant DivJ, and develop polar stalks. These results provide evidence that ppGpp and polyP function as cell-type specific developmental regulators.

Minisymposium 18: Cell Migration

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Distinct Roles for Paxillin and Hic-5 in the Regulation of Tumor Cell Plasticity, Invasion and Metastasis.

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Individual metastatic tumor cells can exhibit two interconvertible modes of cell motility during tissue invasion that are classified as either mesenchymal or amoeboid. Indeed, this is thought to be responsible for the ineffectiveness of current invasion-targeted therapeutics. The molecular mechanisms by which invasive breast cancer cells regulate migratory plasticity have yet to be fully elucidated. Herein we show that the focal adhesion adaptor protein Paxillin, a key integrator of Rho family GTPase signaling and the closely related family member Hic-5, regulate MDA-MB-231 breast cancer cell invasion in vitro as well as lung metastasis in vivo. Furthermore, Paxillin and Hic-5 exhibit opposing but potentially integrated functions to coordinate tumor cell morphology and plasticity during 3D extracellular matrix (ECM) migration and invasion. Cells depleted of Paxillin by RNAi exhibited a highly elongated, mesenchymal phenotype, while siRNA-mediated knockdown of Hic-5 induced an amoeboid phenotype. Furthermore, Paxillin and Hic-5 over-expression results in a striking reversal of phenotypes. In evaluating associated signaling pathways, we determined that Rac1 activity was increased in cells devoid of Paxillin while Hic-5 silencing resulted in elevated RhoA activity and associated ROCK-induced non-muscle myosin II contractility. In depth analysis of adhesion dynamics during tumor cell migration through 3D ECM identified Paxillin as a key regulator of 3D adhesion assembly, stabilization and disassembly, while Hic-5 was shown to be essential for the formation of 3D adhesions. Furthermore, analysis of additional tumor cells, including melanoma, fibrosarcoma and pancreatic cancer cell lines, demonstrated a significant correlation between the relative expression of Hic-5 and Paxillin and cancer cell morphology as well as their ability to exhibit spontaneous plasticity during 3D ECM migration. This work was supported by a Susan G. Komen for the Cure Postdoctoral Fellowship (ND) and NIH grants (CT).

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VASP-adhesion-mediated protrusion waves at the motile cell's leading edge.

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Waves of actin received much attention recently. Fish keratocyte cells, which usually exhibit rapid and steady motility, start to exhibit traveling waves of protrusion when plated on highly adhesive surfaces. These waves are the result of oscillations in actin polymerization, not retrograde flow. Protrusion speed correlates strongly with speed of the lateral wave propagation. Inhibition of myosin contraction with blebbistatin abolishes waving. Mature adhesions co-localize with the stalled parts of the leading edge, while F-actin density is elevated at the protruding parts. We hypothesized that some molecular factor limiting actin based protrusion, such that growing actin filaments and maturing adhesions 'compete' for this factor, is responsible for the waving behavior. We further hypothesized that such factor is VASP, protein that associates with growing actin barbed ends as well as with adhesions. We developed a mathematical model of actin protrusion coupled with membrane tension and mediated by adhesion and VASP dynamics. Simulations of this model demonstrated that competition for VASP between adhesions and polymerizing actin network can lead to emergence of the protrusion waves. The model makes a great number of predictions, for example, that the amount of VASP localized to the leading edge should be reduced in waving cells, compared to smooth cells; overexpression of VASP should prevent waving, but depletion of VASP does not increase the fraction of cells that wave; VASP leading edge localization should oscillate, with VASP most enriched at the leading edge prior to initiation of protrusion. Further experiments confirmed these predictions and provided quantitative data to estimate the model parameters. We thus conclude that the oscillations are the result of competition between actin and adhesions for VASP, rather than of existence of a regulatory biochemical oscillator or mechanical tag-of-war. We hypothesize that this waving behavior contributes to adaptation of cell motility mechanisms to perturbed environment.

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Glioblastoma motility occurs in the absence of actin polymer.

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In fibroblasts and keratocytes, motility is actin dependent, while microtubules play a secondary role, providing directional guidance. We demonstrate here that the motility of U87MG glioblastoma cells is exceptional, in that it can occur in cells depleted of assembled actin. Cells display persistent motility in the presence of actin inhibitors at concentrations sufficient to fully disassemble actin and to prevent cell cleavage. Such actin independent motility is characterized by the extension of cell protrusions containing abundant microtubule polymers parallel to the long axis of the protrusion. Strikingly, glioblastoma cells exhibit no motility in the presence of microtubule inhibitors, at concentrations that disassemble labile microtubule polymers and block mitotic progression. Microtubule inhibitors cause the retraction of cell protrusions, and rapid time-lapse shows microtubule inhibited cells blebbing rather than ruffling. In accord with an unconventional mode of motility, glioblastoma cells have unusual requirements for the Rho GTPases. While Rac1 is required for lamellipodial protrusions in fibroblasts, expression of dominant negative Rac1 does not suppress glioblastoma migration. Other GTPase mutants are largely without unique effect, except dominant positive Rac1-Q61L, and constitutively active rapidly cycling Rac1-F28L, which substantially suppress glioblastoma motility. In contrast, overexpression of dynamitin, which suppresses dynein, partially blocks motility. We conclude

that glioblastoma cells can display an unprecedented mode of intrinsic motility that occurs in the absence of actin polymer, and that appears to require polymerized microtubules.

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Phosphorylation of the Arp2/3 complex: conformational changes and regulation in migrating cells.

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Nucleating activity of the Arp2/3 complex drives the assembly of a branched actin filament network at the leading edge of migrating cells for membrane protrusion. Arp2/3 complex binding to nucleation promoting factors (NPFs) of the WASP and WAVE families was previously thought to be sufficient to increase nucleating activity. However, we recently showed that phosphorylation of the Arp2 subunit on either Thr237/238 or Tyr202 is necessary for Arp2/3 complex activation by NPFs (LeClaire et al., JCB 182:647). We now report a structural model for how pArp2 allows reorientation of Arp2/3 subunits to an activation-competent state, and that Arp2 is a substrate for the Nck-interacting kinase NIK. Molecular dynamics simulations predict that phosphorylation of Arp2-Thr237/238 induces a ~10 Å RMSD reorientation of Arp2 relative to Arp3 toward the short pitch dimer orientation, priming the complex for activation by NPFs. Our simulations show an electrostatic network in the vicinity of Arp2-Thr237/238 that includes ARPC4-Arg105/106. The phosphate charge on pThr237/238 disrupts this network to relieve an auto-inhibited conformation. Additionally, a mutant ARPC4-R105/106A renders the complex partially active in the absence of NPFs. We also found that NIK directly phosphorylates Arp2-T237/238 and increases nucleating activity of recombinant wild-type Arp2/3 complex but not a mutant complex containing Arp2-T237A/T238A. In clonal carcinoma cells, GFP-NIK localizes with Arp2/3 at the distal margin of lamellipodia and increases Arp2 phosphorylation and actin polymerization in response to growth factors. In contrast, pArp2, actin polymerization, membrane protrusion, and cell migration are markedly attenuated in cells expressing either a kinase-inactive NIK or Arp2 (T237/238A-Y202A). Our findings suggest a revised model for Arp2/3 complex regulation where electrostatic interactions maintain an auto-inhibited inactive state. Phosphorylation of Arp2 by kinases such as NIK disrupts these interactions and induces conformational changes allowing Arp2/3 complex activation, actin polymerization, and membrane protrusion in migrating cells.

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Arpin, a Novel Protein that Inhibits the Arp2/3 Complex, Controls Lamellipodium Protrusion and Cell Migration.

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The Arp2/3 complex is a major actin nucleator of eukaryotic cells. It generates a branched actin network that generates a pushing force. This force powers the protrusion of the plasma membrane in a structure called the lamellipodium. The Wave complex is the major Arp2/3 activator in the lamellipodium, and the small GTPase Rac activates the Wave complex, when

GTP-bound. Here we present a novel inhibitor of the Arp2/3 complex that regulates dynamics of lamellipodia and cell migration.

We interrogated public databases for proteins containing an acidic C-terminal domain and harboring a W at position -2. This characteristic motif involved in Arp2/3 binding allowed us to retrieve an uncharacterized human protein among known Arp2/3 activators. This protein indeed interacted with the Arp2/3 complex in a GST-pulldown, and inhibited its activation in a dose-dependent manner, as revealed by pyrene-actin assays and by actin filament branching monitored with TIRF microscopy. We call this protein 'Arpin' as a tribute to Monique Arpin, A.G. PhD supervisor, who retired earlier this year, and also because it is a good mnemonic for *Arp*2/3 complex *in*hibitor.

We found that Rac activity induces the interaction of Arpin with the Arp2/3 complex. Consistently, endogenous Arpin localized to the lamellipodium of migrating cells. Upon RNAi-mediated depletion of Arpin in human cell lines, lamellipodia protruded faster, consistent with its inhibitory role on Arp2/3 complex activity. Arpin depleted cells explored a larger territory than the controls, because of increased speed and directionality. A knock-out of the orthologous Arpin gene from *Dictyostelium discoideum* also resulted in faster and more directional amoeba, indicating that Arpin function is conserved from amoeba to man. Fish keratocytes are the best model for persistent migration. Microinjection of purified zebrafish Arpin into keratocytes resulted in cycles of suppression of the existing lamellipodium immediately followed by growth of a new ectopic one.

Together these results argue for an essential inhibitory role of Arpin in modulating Rac signalling toward the Arp2/3 complex. If the WAVE complex is the 'engine' of cell migration, Arpin would be a 'brake' and the 'steering wheel'.

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Cortactin Controls Cell Motility and Lamellipodial Dynamics by Regulating ECM Secretion.

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Branched actin assembly is critical for both cell motility and membrane trafficking. The branched actin regulator, cortactin, is generally considered to promote cell migration by controlling leading edge lamellipodial dynamics. However, recent reports indicate that lamellipodia are not required for cell movement, suggesting an alternate mechanism. Since cortactin also regulates membrane trafficking and adhesion dynamics, we hypothesized that altered secretion of extracellular matrix (ECM) and/or integrin trafficking might underlie motility defects of cortactin-knockdown (KD) cells. Consistent with a primary defect in ECM secretion, both motility and lamellipodial defects of cortactin-KD cells were fully rescued by plating on increasing concentrations of exogenous ECM. Furthermore, cortactin-KD cell speed defects were rescued on cell-free autocrine ECM produced by control cells but not on ECM produced by cortactin-KD cells. Investigation of the mechanism revealed that whereas endocytosed FN is redeposited at the basal cell surface by control cells, cortactin-KD cells exhibit defective FN secretion and abnormal FN retention in a late endocytic/lysosomal compartment. Cortactin-KD motility and FN deposition defects were phenocopied by KD in control cells of the lysosomal fusion regulator Synaptotagmin-7. Rescue of cortactin-KD cells by expression of cortactin binding domain mutants revealed that interaction with Arp2/3 complex and actin filaments is essential for rescue of both cell motility and autocrine ECM secretion phenotypes whereas binding of SH3 domain partners is not required. In conclusion, efficient cell motility, promoted by cortactin regulation of

branched actin networks, involves processing and resecretion of internalized ECM from a late endosomal/lysosomal compartment.

Minisymposium 19: Cellular Mechanisms of Disease and Aging

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Spg20-/- mice reveal multimodal functions for Troyer syndrome protein spartin in lipid droplet maintenance, cytokinesis, and BMP signaling.

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Hereditary spastic paraplegias (HSPs; SPG1-48) are inherited neurological disorders characterized by lower extremity spastic weakness due to a length dependent axonopathy of corticospinal motor neurons. Loss-of-function, frame-shift mutations in the human *SPG20* gene encoding spartin cause autosomal recessive Troyer syndrome (SPG20), an HSP which has additional features of short stature, skeletal defects, cognitive decline, and distal amyotrophy. To identify cellular impairments underlying Troyer syndrome, we generated *Spg20-/-* knockout mice, which develop late-onset hind limb and gait deficits. Though central nervous system pathology appeared largely normal, cerebral cortical neurons cultured from neonatal *Spg20-/-* mice exhibited increased axon branching, a phenotype suppressed by reintroducing spartin and which required its interaction with the ESCRT-III protein IST1. Analysis of the bone morphogenetic protein (BMP) signaling pathway in *Spg20-/-* embryonic fibroblasts indicated that Smad1/5 phosphorylation is elevated, possibly due to observed alterations in BMP receptor trafficking and degradation. Cytokinesis was impaired in embryonic fibroblasts cultured from *Spg20-/-* mice, and binucleated chondrocytes were prominent in epiphyseal growth plates of bones in *Spg20-/-* mice, likely explaining the short stature of patients. Finally, adipose tissue from *Spg20-/-* animals exhibited increased lipid droplet numbers and alterations in perilipin levels, supporting a key role for spartin in lipid droplet maintenance. Taken together, our results support multimodal adaptor functions for spartin that provide important insights into HSP pathogenesis.

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The Oculo-Cerebro-Renal Syndrome of Lowe as a Ciliopathy: Role of Ocrl1 in Primary Cilia Assembly.

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Lowe syndrome is a devastating, X-linked genetic disease characterized by the presence of congenital cataracts, profound learning disabilities and renal dysfunction. Unfortunately, children affected with Lowe Syndrome often die early of health complications including renal failure. Although this syndrome was first described in the early fifties and the affected gene, OCRL1, was identified more than 17 years ago, the mechanism by which Ocrl1 defects lead to Lowe syndrome's symptoms remains unknown.

Here we show that Lowe syndrome display some of the the characteristics of a ciliopathy. Specifically, we found that patients' cells have defects in the assembly of primary cilia and this phenotype was reproduced by knock-down of Ocrl1 in cells in culture. Importantly, this defect could be rescued by re-introduction of WT Ocrl1 in both, patient's and Ocrl1 knock-down cells. In addition, a zebrafish animal model of Lowe syndrome exhibited cilia defects and multiple morphological and anatomical abnormalities typically seen in ciliopathies. Mechanistically, we show that Ocrl1 is involved in protein trafficking to the primary cilia in a Rab8-, Appl1- and IPIP27/Ses-dependent manner via the secretory and endocytic pathways.
Taking into consideration the relevance of the signaling pathways hosted by the primary cilium, our results suggest hitherto unrecognized mechanisms by which Ocrl1 deficiency may contribute to the phenotypic characteristics of Lowe syndrome. This conceptual change in our understanding of the disease etiology may provide an alternative avenue for the development of therapies.

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Microtubule Link between Cell Cycle and Neurotransmitter Receptor Defects in Neurodegenerative Disease.

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Microtubules serve as the highways upon which ATP driven motor proteins move key cellular components, such as proteins, vesicles, chromosomes, and large macromolecules, including microtubules themselves, from one part of the cell to another. Many neurodegenerative diseases show defects in the microtubule transport system, underlining its importance in normal cellular physiology. Our data support a unifying hypothesis stating that defects in microtubule dependent transport are induced by the key neurodegeneration-associated proteins Aβ or Tau and generate neurons with an aneuploid chromosome complement and with reduced neurotransmitter and neurotrophin cell surface expression and function. Previously, we found that mutant amyloid precursor protein and presenilin genes that cause familial AD induce chromosome mis-segregation and aneuploidy in AD patients, transgenic mice, and cultured cells. Confirmatory results from other labs showed that 30% of neurons in early AD cortex are aneuploid/hyperdiploid. We have now found that Aβ added to human cells or *Xenopus* egg extracts impairs the formation and stability of mitotic spindles and directly inhibits three microtubule motor kinesins, Eg5, KIF4A and MCAK, which are essential for the normal structure and function of the mitotic spindle. Very similar motors, and in particular Eg5 itself, are also present in neurons. Eg5 harbors polymorphisms that increase AD risk, is inhibited in neurons treated with Aβ and is almost completely inhibited in brains of the APP/PS transgenic mouse model of Alzheimer's disease. Furthermore, Aβ treatment or chemical inhibition of Eg5 results in mitotic defects and poor localization of the NMDA receptor to the plasma membrane. Like Aβ treatment, inhibition of Eg5 blocks LTP, a brain slice model of memory Most recently, we also detected aneuploid neurons in brains of patients with fronto-temporal dementia caused by mutant Tau as well as in mouse models of FTD, and are currently testing mutant cells expressing FTD mutant Tau genes. Together these data suggest that the memory impairment in AD and related disorders may derive from an inhibition of microtubule function, which can disrupt both neurogenesis and neuroplasticity.

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RNA-binding proteins with prion-like domains in ALS and FTLD-U.

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Amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig's disease) is a debilitating, and universally fatal, neurodegenerative disease that devastates upper and lower motor neurons. The causes of ALS are poorly understood. A central role for RNA-binding proteins and RNA metabolism in ALS has recently emerged. The RNA-binding proteins, TDP-43 and FUS, are principal components of cytoplasmic inclusions found in motor neurons of ALS patients and mutations in TDP-43 and FUS are linked to familial and sporadic ALS. Pathology and genetics also connect TDP-43 and FUS with frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U). It was unknown whether mechanisms of FUS aggregation and toxicity were similar or different to those of TDP-43. To address this issue, we have employed yeast models and pure protein biochemistry to define mechanisms underlying TDP-43 and FUS aggregation and toxicity, and to identify genetic modifiers relevant for human disease. We have identified prion-like domains in FUS and TDP-43 and provide evidence that these domains are required for aggregation. Our studies have defined key similarities as well as important differences between the two proteins. Collectively, however, our findings lead us to suggest that FUS and TDP-43, though similar RNA-binding proteins, likely aggregate and confer disease phenotypes via distinct mechanisms.

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A Novel Role for N-glycans in *Drosophila* Aging.

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Aging is a universal but poorly understood biological process that involves a complex interplay between environmental and genetic factors. Model organisms such as *C. elegans* and *Drosophila* have provided a unique opportunity to search for, and identify, both environmental factors and genes that can regulate the life span of these organisms. We have recently found that *N*-glycans play a novel and unexpected role in regulating *Drosophila* life span. Specifically, we have studied the role of the enzyme GnT1 (encoded by Mgat1), which is required for synthesis of paucimannose N-glycans in *Drosophila*. We have shown that null mutations in *Drosophila* Mgat1 are viable but exhibit defects in locomotion, brain abnormalities and a severely reduced life span. To further define the role of Mgat1 in organismal life span we used a combination of transgenics and RNAi technology to manipulate the expression of Mgat1 in the nervous system. We found that knock down of Mgat1 in the CNS could recapitulate the null mutant phenotype demonstrating that Mgat1 was required in the CNS. Remarkably, we also found that neuronal expression of a wild type Mgat1 transgene not only rescued the shortened life span of Mgat11 null mutants but also resulted in a dramatic increase in life span. While the wildtype control flies had a mean and maximum life span of 54 and 70 days, respectively, our rescued flies had a mean and maximum life span of 127 and 165 days, respectively. Interestingly, targeted expression of Mgat1 in the CNS of Mgat11 null mutants rendered them significantly more resistant to hydrogen peroxide than control flies demonstrating that the increase in longevity is also associated with an increase in oxidative stress resistance. Finally, we showed that while dietary restriction was not absolutely essential for the increased life span, it plays a role in the process. Altogether, these observations suggest that Mgat1-dependent N-glycosylation plays an important role in the control of *Drosophila* life span. Current studies are aimed at determining how Mgat1 and N-glycans regulate life span and whether the ability of Mgat1 to increase life span is conserved across species.

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Molecular Mechanisms distinguishing Reproductive Aging from Somatic Aging.

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Female reproductive cessation is the earliest aging phenotype humans experience, occurring midway through life, and is governed by declining oocyte quality. We have shown previously that C. elegans reproduction, which also persists for only half the worms' lifetime, is similarly limited by oocyte quality decline (Luo, et al. Cell 2010). Moreover, the transcriptional changes that worm oocytes undergo with age are similar to those in aging mammalian oocytes, and these changes are reversed in mutants with extended reproductive spans.
Both the TGF-b Sma/Mab and Insulin/IGF-1 signaling pathways regulate reproductive aging (Luo, et al. PLOS Genetics 2009), but IIS extends life span, as well (Kenyon, et al. 1999). We had previously identified the downstream DAF-16 targets of IIS that are responsible for long life span (Murphy, et al. Nature 2003). We wondered to what degree the mechanisms that extend reproductive span and life span are shared. In fact, very few of the terms overlap, suggesting that post-mitotic, non-proliferating tissues utilize one specific set of genes, while mitotically proliferating and cell-cycle-arrested cells utilize completely different mechanisms to keep cells youthful and functioning.

Minisymposium 20: Cilia and Centrosomes

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Dissecting the molecular difference between MTOC-competent and non-competent centrioles.

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Our recent results revealed that a Plk1-dependnet modification, which occurs in early mitosis, is required to convert centrioles to centrosomes/MTOCs at late mitosis. Without such modifications, centrioles can neither organize MTOC nor duplicate regardless of their age, configuration (engaged/disengaged), or how they are formed (canonical or *de novo*). Only modified (or MTOC-competent) centrioles can duplicate in S phase, and segregate in the following mitosis through association with spindle poles. This leads to a tight coupling between duplication and segregation, i.e., only centrioles that can segregate themselves are allowed to duplicate. This mechanism, which critically maintains the homeostasis of centrioles in cycling cells, may highlight why centrioles have emerged as centrosomes/MTOCs in higher eukaryotes.

In such a regulatory scheme, centriole duplication in cycling cells inevitably generates hybrid centriole pairs, each consisting of one modified and one unmodified centriole. We propose that unmodified centrioles carry unknown inhibitory activities that locally suppress duplication. This "short-range feedback inhibition" model could nicely explain how centriole engagement blocks re-duplication. Our proteomic study in searching for molecules that associate with unmodified centrioles has generated supporting evidence for this model.

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GCP6 is a substrate of Plk4 and required for centriole duplication.

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Centrioles are microtubule-based structures that organize the centrosome and nucleate cilia in most eukaryotes. Centriole duplication occurs once per cell cycle and is thought to be initiated at the G1/S boundary with the formation of procentrioles at each parental centriole. A central role in the control of centriole biogenesis and duplication has been attributed to Plk4, a member of the Polo-like kinase family. Microtubule nucleation requires γ-tubulin which exists in two major protein complexes: the γ-tubulin small complex (γ-TuSC) and the γ-tubulin ring complex (γ-TuRC). GCP6 is a member of the γ-TuRC but its role in human cells and the regulation of its functions remain unclear. We find that depletion of human GCP6 prevents the assembly of the γ-TuRC and induces a high percentage of monopolar spindles. These spindles are characterized by a loss of centrosomal γ-tubulin and reduced centriole numbers. GCP6 is localized in the pericentriolar material (PCM) but also at distal portions of centrioles. In addition, GCP6 is required for centriole duplication and Plk4-induced centriole overduplication. GCP6 interacts with and is phosphorylated by Plk4. Moreover, we find that Plk4-dependent phosphorylation of GCP6 regulates centriole duplication. Our data suggest that GCP6 is a critical subunit of the the γ-TuRC and a novel target of Plk4 in centriole biogenesis.

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The BBSome at the crossroad of signaling, trafficking and tubulin acetylation.

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The primary cilium exposes signaling receptors and concentrates signaling cascades to send specific molecular responses into the cell. Such remarkable signaling properties rely on the physical separation between the cilium and the rest of the cell and on molecular machines that move proteins through diffusion barriers. Our overarching goal is to reconstitute these transport activities in cell-free systems and as a first step, we have established an in vitro system that faithfully recapitulates macromolecular diffusion between ciliary lumen and cytoplasm.
Independently, our biochemical dissection of the archetypical ciliopathy Bardet-Biedl Syndrome (BBS) uncovered a complex of seven BBS proteins that we named the BBSome. We have now demonstrated that the BBSome sorts signaling receptors –such as the somatostatin receptor 3 (SSTR3)– to cilia. While initial results indicated that the BBSome functions as a coat complex akin to clathrin, COPI and COPII, we will present recent structural insights indicating that the BBSome may resemble more closely the coat adaptors that bridge membrane proteins to the lattice elements of coats. These results suggest the existence of currently unknown components the organizes the BBSome into a latticed coat.
Additionally, we discovered the long-sought α-tubulin K40 acetyltransferase TAT1 as a BBSome-associated protein. We have now uncovered an essential role for TAT1 in the maintenance of long-lived microtubules and for cell migration, thus supporting a role for stable microtubules in cell migration. Fascinatingly, acetylation takes place inside the lumen of microtubules and, utilizing TAT1 as a probe for lumen accessibility, we have found that transient openings along the microtubule sidewall allow for entry of proteins into the microtubule lumen thus suggesting the existence of a new facet of microtubule dynamicity inside the cell.

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The importance of a single primary cilium: extra centrosomes and cilia compromise ciliary signaling.

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Primary cilia perform important sensory functions, and are associated with a range of human diseases, termed ciliopathies. We show that cells with extra centrioles, a phenotype frequently observed in cancers, also form extra cilia. Such super-ciliated cells have a reduced ciliary concentration of the Sonic hedgehog pathway protein Smoothened, leading to decreased Shh pathway activation. A similar ciliary dilution phenotype was observed with the serotonin receptor Htr6, fibrocystin gene PKHD1, and GTPase Arl13b. The presence of extra cilia disrupted epithelial organization in 3-D spheroid culture. Finally, mutation of the tuberous sclerosis disease gene Tsc2 also results in super-ciliated cells and in dilution of ciliary protein. Thus, the presence of extra cilia disrupts ciliary signaling, and may contribute to ciliopathy and cancer disease phenotypes.

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IFT25 Couples Movement of the Hedgehog Signaling Components to Intraflagellar Transport.

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The intraflagellar transport (IFT) system is required for assembling primary cilia, sensory organelles that cells use to respond to their environment. IFT particles are composed of about 20 proteins and these proteins are highly conserved across ciliated species. However, IFT25 and its binding partner, IFT27, are unusual in that they are found in Chlamydomonas and mammals but not in Drosophila or Caenorhabditis even though these organisms assemble cilia. To elucidate the function of the IFT25/IFT27 module, we generated an Ift25 mutant mouse. Unlike all previously studied mice with null mutations in IFT genes, which do not assemble cilia and die at mid term, the homozygous Ift25 null mutants form cilia normally and survive through gestation. Mutant mice die at birth with multiple phenotypes indicative of Hedgehog signaling dysfunction. Loss of IFT25 leads to the depletion of IFT27 but not other IFT proteins. Cilia lacking IFT25 have defects in the dynamic transport of multiple Hedgehog signaling components, including Patched-1, Smoothened, and Gli2, and fail to activate the pathway upon stimulation. This is the first example of a null IFT mutation that perturbs Hedgehog signaling independent of ciliary architecture. Thus, IFT function is not restricted to assembling cilia where signaling occurs but also plays a direct role in signal transduction events.

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An ARL3-UNC119-RP2 GTPase cycle delivers myristoylated NPHP3 to the primary cilium.

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The membrane of the primary cilium is a highly specialized compartment that organizes proteins to achieve spatially ordered cell signaling. Disrupting ciliary organization leads to diseases called ciliopathies, with phenotypes ranging from retinal degeneration and cystic kidneys to neural tube defects. How proteins are selectively transported to and organized in the primary cilium remains unclear. Using a proteomic approach, we identify the ARL3 effector UNC119 as a binding partner of the myristoylated ciliopathy protein NPHP3. We map UNC119 binding to the N-terminal 200 residues of NPHP3 and find the interaction requires myristoylation. Creating directed mutants predicted from a structural model of the UNC119-myristate complex, we find that UNC119 binds myristoylated cargos through a highly conserved set of phenylalanines within a hydrophobic beta sandwich and identify phenylalanine residues essential for myristate binding. Further, we find ARL3, UNC119b, but not UNC119a, and the ARL3 GAP RP2 are required for NPHP3 ciliary targeting, and that targeting requires UNC119b myristoyl-binding activity. Finally, we discover a new UNC119-binding protein, C5orf30, that may regulate myristoyl-cargo binding. Our results uncover a selective, membrane-targeting GTPase cycle that delivers myristoylated proteins to the ciliary membrane, and suggest that other myristoylated proteins may be similarly targeted to specialized membrane domains.

Minisymposium 21: Intracellular Sorting and Trafficking

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Morphological and functional identity of organelles of the early plant secretory pathway.

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A fundamental question in eukaryotic cell biology is how cells maintain efficient compartmentalization and control the delivery and integration of biomolecules into specialized organelles. In our lab, we address these questions using the plant secretory pathway as a model. This pathway is vital to the inner workings of the cell and for communicating with the external environment during growth and in response to stress; it consists of several organelles that synthesize, shuttle, and store a large part of the cell's proteins, lipids, and sugars. In plants, the activities of the endoplasmic reticulum (ER) and Golgi apparatus, the initial organelles of the secretory pathway, are also fundamental for the synthesis and deposition of the building blocks of energy-rich compartments such as the cell wall and storage vacuole. Both organelles have unique architecture and functions, which are maintained despite exchange of membranes and lumenal contents with other organelles. Efforts in our lab focus on identifying the mechanisms governing the morphology and function of the ER and the Golgi and defining the extent to which their architecture influences their function. To address these questions we have carried out forward genetic screens based on confocal microscopy analyses of Arabidopsis seedlings expressing ER and Golgi markers. The screens are designed to identify mutants with aberrant distribution of the ER and Golgi markers compared to non mutagenized seedlings. Through the

characterization of these mutants we are identifying novel genes and mutations that uncover new information on the mechanisms for the integrity of the ER and the Golgi with respect to other organelles, cytoskeleton and flow of biosynthetic cargo. Our most recent findings will be presented in this talk.

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The first 5 seconds in the life of an edocytic clathrin coated pit.

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Formation of endocytic clathrin coated pits and vesicles requires the rapid and highly orchestrated generation of a scaffold made of a very large number of interacting proteins whose assembly results in entrapment of cargoes and membrane deformation. Key for this process is the organized recruitment of clathrin triskelions and AP-2 adaptors. In order to understand the molecular events occurring at the early stages of pit formation we developed a method applicable to living cells and based on total internal reflection fluorescence microscopy that allows visualization and tracking with single-molecule sensitivity and 100 millisecond temporal resolution of clathrin and AP-2 as they are recruited during the first few seconds in the life of a coated pit. By combining this imaging method with newly developed analytical tools to cells expressing fluorescently tagged clathrin and AP-2, we established that coated pits start by the simultaneous recruitment of one clathrin triskelion and two AP-2s. This first recruitment step typically lasts ~2 s, followed by a second step of similar characteristics. We also found that there is no need to pre-cluster AP-2s at the site of coated pit formation and that the presence of the phosphoinositide PIP2 is essential for pit initiation.

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Molecular Architecture of the Transport Channel of the Nuclear Pore Complex.

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The largest and most versatile transport channel of eukaryotes is anchored in the center of the nuclear pore complex and is thought to be composed of three nucleoporins, Nup54, Nup58 and Nup62. Here, we report the crystal structures of the interacting domains between these three nucleoporins. Supported by biochemical, biophysical and mutational data we pieced together the molecular architecture of the mammalian transport channel. Located in the channel mid-plane is a Nup54·Nup58 ring that can undergo large re-arrangements yielding diameter changes from about 20 nm to 40 nm as the channel expands from inactive 'closed' to active 'open' forms, respectively. Triple α-helices of Nup62·Nup54 project alternately up and down from either side of the mid-plane ring and form nucleoplasmic and cytoplasmic entries. The channel consists of as many as 224 copies of the three nucleoporins amounting to a molar mass of 12.3 million daltons and providing 256 unstructured Phenylalanine-Glycine repeat regions for routing captured transport substrates.

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Sedlin binds Sar1p and controls type II procollagen trafficking by modulating the cycle of COPII.

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Genetic defects of sedlin, a conserved component of the TRAPP complex, cause spondyloepiphyseal dysplasia tarda (SEDT), a condition characterised by impaired chondrogenesis that results in short stature, flattening of the vertebrae and premature osteoarthritis. Prompted by the consideration that sedlin is ubiquitously expressed but that sedlin mutations cause cartilaginous-restricted dysfunctions, we hypothesized that sedlin might exert a role in the transport of chondrocyte-specific cargoes, such as type II procollagen (PCII). This hypothesis was reinforced by the fact that certain mutations in PCII give rise to autosomal dominant forms of spondyloepiphyseal dysplasia. We tested this hypothesis by analyzing the involvement of sedlin in the transport of different classes of secretory cargoes and found that sedlin is selectively required for PCII to exit the ER, while it is not essential for the ER exit of small soluble and membrane-associated cargoes. We have also identified the molecular mechanism underlying this role of sedlin in its ability to bind the GTPase Sar1 and to control the membrane-cytosol cycle of Sar1 itself and of the COPII coat complex at the level of the ER exit sites. Sedlin depletion and/or mutation in SEDT patients slows down the Sar1 cycle and prolongs the membrane association of Sar1-GTP at ER exit sites, thus inducing constriction and premature fission of nascent carriers which fail to incorporate the large PC protofibrils but are still competent for smaller secretory cargoes.

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The Creb3l2/Sec23a/Sec24D axis in collagen secretion and cartilage morphogenesis.

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The COPII machinery is the primary mediator of ER-to-Golgi transport and appears to be dynamically regulated according to secretory demand, although the mechanisms of this regulation are only beginning to be elucidated. Using forward genetics coupled with a positional cloning strategy, we have identified zebrafish mutants with defects in the COPII components, *crusher/sec23a*, *bulldog/sec24d*, and *feelgood/creb3l2*, each of which leads to a malformed head skeleton. Although both early cartilage patterning and initial ECM deposition proceed normally, COPII mutant chondrocytes progressively accumulate collagen within the ER, resulting in a failure to maintain cartilage ECM and impaired chondrocyte maturation. Importantly, this trafficking deficiency appears to be collagen-specific, as shown by the normal localization of a variety of other secretory proteins, including glycosaminoglycans, integrin β1, and N-cadherin. We further identified Creb3l2 as a direct transcriptional regulator of select COPII genes, including *sec23a* and *sec24d*. This establishes Creb3l2 as the first identified transcription factor to couple the secretory activity of cells to the progression of developmental programs. Collectively, our work has shown that Creb3l2/Sec23a/Sec24D are critical for collagen secretion during the maturation stage of chondrogenesis and suggests that this secretory axis is central to a broad transcriptional regulatory network that optimizes cellular machinery to facilitate trafficking in highly secretory cell types.

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Genome-wide siRNA screens identify novel networks regulating membrane traffic and organelle biogenesis in the secretory pathway.

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The secretory pathway in mammalian cells has evolved to facilitate protein secretion and the transfer of a huge variety of cargo molecules to internal and cell surface membranes in a specific and timely manner. Extensive genetic and biochemical efforts over many years have identified and characterized much of the core machinery of this pathway. However, it is clear that regulatory components and their inter-dependence on other cellular metabolic events and pathways need to be elucidated to reach a more comprehensive understanding of the secretory pathway.
In order to identify such putative molecules involved in the regulation of the secretory pathway and organelle biogenesis, we have used high throughput/high content microscopy in intact cells to assess on a genome-wide scale the effect of knockdowns by RNAi, on processes such as constitutive protein transport, Golgi integrity and function of vesicular coat complexes. Our data reveal an unexpected relationship between secretory pathway function and genes involved in general metabolic integrity or signal transduction pathways. This provides the basis for an integrative understanding of the global cellular organization and the regulation of the secretory pathway and organelle biogenesis.

Minisymposium 22: Mitosis

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Insights into the micromechanical properties of the metaphase spindle.

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During cell division, the microtubule-based metaphase spindle is subjected to forces that act in diverse orientations and over a wide-range of timescales. Currently, we cannot explain how this dynamic structure generates and responds to forces while maintaining overall stability, as we have a poor understanding of its micromechanical properties. Here we combine the use of force-calibrated needles, high-resolution microscopy, and biochemical perturbations to analyze the vertebrate metaphase spindle's timescale- and orientation-dependent viscoelastic properties. We find that the metaphase spindle is mechanically anisotropic, and exhibits either viscous-like or elastic-like behavior depending on the timescale of applied force. We also find that spindle viscosity depends on the dynamics of microtubule crosslinking and the density of the filament. Spindle elasticity can be linked to kinetochore and non-kinetochore microtubule rigidity, and also to spindle pole organization by kinesin-5 and dynein. These data suggest a quantitative model for the micromechanics of this cytoskeletal architecture and provide insight into how structural and functional stability is maintained in the face of forces, such as those that control spindle size and position, and can result from deformations associated with chromosome movement.

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Spindle assembly sub-reactions without microtubule polymerization dynamics.

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Mitotic and meiotic spindles are thought to self-organize by a combination of microtubule polymerization dynamics and motor protein activity. Rapid microtubule turnover by dynamic instability is an essential part of normal spindle assembly, but it is interesting to ask, to what extent can spindles assemble in the absence of such dynamics? We addressed this question using Xenopus egg extracts that recapitulate meiosis-II spindle assembly, and high-speed supernatant (HSS) fractions derived from them. Use of HSS also addresses possible requirements for membranes and other particulate components. We used taxol and DMSO to force rapid microtubule polymerization and block subsequent polymerization dynamics. Randomly assembled microtubules were able to re-organize over time into asters, parallel bundles and, under some conditions, spindle-shaped assemblies. These arrays recruited known spindle assembly factors, such as NuMA and TPX2 to appropriate sites, and their assembly depended on dynein and Eg5. Their length scales seem to arise mostly from the initial polymerization conditions, and to a lesser extent from subsequent slow length changes. We conclude that important sub-reactions in spindle morphogenesis can occur in the absence of polymerization dynamics. These data help explain the finding that spindle-like assemblies are sometimes able to form in extracts and living cells even when key dynamics regulators are perturbed.

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Nuclear divisions under space constraints studied in single embryo extract.

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Early development in the *Drosophila* embryo is governed by rapid nuclear divisions in a large syncytium. Initially at the pre-blastoderm stage nuclei divide in the interior of the embryo and later the majority migrate towards the cortex where they are tightly packed. Proper nuclear positioning is crucial at this stage as the embryo is prepared for cellularization of the blastoderm. An open question is what governs nuclear spreading through the entire embryo and the spatial relationship between the nuclei in the pre-blastoderm stage in contrast to the blastoderm stage when nuclei are anchored at the cortex. Using a novel *ex vivo* assay in which cytoplasm extracted from individual syncytial embryos is encapsulated in microchambers, we monitor nuclear divisions by confocal fluorescence microscopy. We first demonstrate that the cytoplasmic extract maintains its functionality: nuclear size, spindle morphology, cycle timing and synchrony are preserved. We find that in the absence of spatial constraints the separation of chromosomes and nuclei in pre-blastoderm extract is biphasic; fast chromosome segregation during anaphase is followed by slow separation of nuclei at telophase and early interphase, leading to even spacing of nuclei. Separation is accompanied by the formation of large centrosomal asters. Confining the extract in microchambers allowed us to test how the dynamics of nuclear divisions respond to spatial constraints. Large spindle deformations in confined pre-blastoderm extract indicate strong pushing forces generated by both the spindle midzone and the astral microtubules. The second, slow phase of separation is abolished, and instead we observe frequent spindle fusion during the next mitosis. Our results suggest that in pre-blastoderm divisions individual nuclei occupy a volume with characteristic size (radius ~15 µm). Remarkably, at this stage this length does not scale with the available space, but is an intrinsic property of the dividing nucleus, defining a domain which it occupies in the absence of membranes. This domain appears critical as two nuclei forced into the same domain collapse

and fuse. Our findings provide a conceptual framework for the rapid spreading of nuclei inside a large syncytium. This spreading of dividing nuclei within domains of constant size is distinct from the tightly packed nuclear divisions occurring at the embryo cortex, where nuclear domain size oscillates and spindle length is progressively reduced. Our novel *ex vivo* assay will allow further insight into what defines the identity of spindle domains and their biomechanical properties in a syncytium.

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Centromere protein Q self-assembles into toroidal complexes and promotes microtubule catastrophe.

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Human kinetochores are multi-protein mechanochemical engines that form attachments to the plus-end of spindle microtubules1. By tightly controlling plus-end dynamics the kinetochore is able to coordinate the set of movements that align and segregate chromosomes during mitosis. The conserved constitutive centromere associated network (CCAN)is emerging as a key part of the machinery that regulates tubulin exchange at microtubule plus-ends with one subunit, the CENP-Q octomer, being able to bind microtubules in vitro. Here we show that purified CENP-Q octomers can self-assemble in a nucleotide-independent manner into toroidal complexes (Q-rings) that can bind to the microtubule lattice. Strikingly, CENP-Q octomers, and to a greater extent Q-rings, are able to disassemble microtubules in vitro by reducing the rate of tubulin assembly at microtubule plus-ends, thereby promoting catastrophe. Q-ring disassembly is promoted by free tubulin so that ring driven microtubule disassembly is self-limiting. Using total internal reflection microscopy we reveal that CENP-Q molecules preferentially target microtubule ends. In human cells, depletion of CENP-Q causes a reduction in the turnover of kinetochore microtubules and defects in chromosome alignment. This work identifies CENP-Q as the founding member of an entirely new class of microtubule dynamics regulator that caps and slows growth of microtubule ends. Within the kinetochore this activity is essential to control microtubule plus-end dynamics and allow accurate chromosome segregation.

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Recruitment of the human Cdt1 replication licensing protein by the loop domain of Hec1 is required for proper kinetochore microtubule attachment.

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The Cdt1 protein is critical for DNA replication origin licensing during G1 phase and is degraded during S phase followed by re-accumulation in G2 phase. We now provide evidence that Cdt1 has an essential function in mitosis entirely independent of its function in replication. Cdt1 localizes to kinetochores during mitosis through interaction with Hec1 (hNdc80), a member of the highly conserved Ndc80 complex that forms the core microtubule (MT) attachment site at kinetochores. G2-specific depletion of Cdt1 or injection of a function blocking anti-Cdt1 antibody into mitotic vertebrate cultured cells arrests them in late prometaphase with high levels of Mad1 at aligned kinetochores and abnormally unstable, cold-sensitive kMTs. Biochemical and cell biological analysis indicate that Cdt1 binds to a unique, flexible loop extending from the middle of the rod domain of Hec1 that we find is also required for forming robust kinetochore microtubule attachments. Mutations in the loop domain prevent Cdt1 localization to kinetochores

and arrest cells in prometaphase with high levels of Mad1 at aligned kinetochores, as observed after Cdt1 depletion. Analysis of kinetochores in control and Cdt1-depleted cells using super-resolution fluorescence microscopy indicates that Cdt1 binding to Hec1 supports a conformational change in the Ndc80 complex that is compatible with proper spindle microtubule attachment. These results show that Cdt1 is a novel regulator of Hec1 function and that Cdt1 bound to the loop domain of Hec1 has important roles in kinetochore microtubule attachment and the spindle assembly checkpoint. Because Cdt1 is actively degraded until the end of S phase to ensure once-and-only once genome duplication, we suggest that this newly recognized, essential and direct mitotic role reinforces the dependence of mitotic events on the completion of S phase.

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System-level feedbacks during mitotic progression.

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Progression through mitosis is characterized by irreversible transitions at mitotic entry (G2/M), at metaphase-to-anaphase (M/A) and at mitotic exit (M/G1). I will argue that the irreversibility of these transitions is based on system-level feedbacks in the molecular regulatory mechanisms. In particular, positive (or double-negative) feedback circuits in these regulatory networks create one-way toggle (bistable) switches with two alternative stable steady states. The irreversibility of the G2/M transition is caused by switch-like activation of Cdk1:CycB. The M/A transition is made irreversible by the mitotic checkpoint which blocks anaphase until all chromosomes are bioriented on the mitotic spindle. The mitotic checkpoint is dependent on Cdk1:CycB activity which is down-regulated in mammalian cells before progression into anaphase, thereby blocking checkpoint re-activation during anaphase. The bistable switch created by the mitotic checkpoint is important to suppress the negative feedback loop caused by Cdk1:CycB dependent activation of APC/C (Anaphase Promoting Complex/Cyclosome) and CycB degradation. I will discuss experimental data with computational modeling in order to illustrate the sequential engagement of the bistable switches during mitotic progression.

References:

He, E., Kapuy, O., Oliveira, R.A., Uhlmann, F., Tyson, J.J. & Novák, B. (2011): System-level feedbacks make the anaphase switch irreversible.
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Minisymposium 23: Nuclear Organization and Control of Gene Expression

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The dynamics of splicing factor interactions with active genes in living cells.

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Splicing factors are co-transcriptionally recruited to the nascent transcript as it emerges from the active polymerase. Using tandem gene arrays that contain genes with introns and exons we could show that spliceosome assembly occurs at the active site of transcription. To obtain information about the recruitment kinetics of splicing factors to active genes in vivo we used live-cell microscopy in which both the splicing factors and the produced mRNAs could be fluorescently visualized. Typically, studies that have followed splicing factor diffusion dynamics

in the nucleus of living cells have utilized fluorescently-tagged splicing factors that were expressed from viral promoter-driven constructs (e.g. CMV or SV2). Overexpression of splicing factors might shift the endogenous balance of splicing factors and could influence the outcome of splicing decisions. We therefore used recombineered BACs containing GFP-splicing factor genes, i.e. these splicing factors were expressed from their endogenous promoters, leading to physiological levels of expression of the fluorescently tagged splicing factors. Each of these BACs was separately integrated into U2OS Tet-On cells already harboring the gene array. Using a combination of techniques for measuring intra-cellular kinetics, namely, fluorescence recovery after photobleaching (FRAP), fluorescence loss after photobleaching (FLIP), and fluorescence correlation spectroscopy (FCS), we measured the kinetics of a variety of splicing factors in the nucleoplasm, nuclear speckles and when engaged with the actively transcribing gene. Using computer simulations we provide measurements of the engagement times of splicing factors with active genes, and show that splicing factors exhibit different dynamics depending on their nuclear location.

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High-Resolution Mapping Reveals Role for Three-Dimensional Chromatin Architecture During Stem Cell Differentiation.

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Chromatin structure, and the epigenetic modifications that alter this structure, are emerging as critical factors in the establishment of cellular state. High-throughput sequencing studies suggest that ES cells accumulate a multitude of epigenetic marks in response to developmental cues. Because hundreds of genome-wide epigenetic data sets now exist across multiple cell types, the challenge lies in finding combinatorial patterns that indicate how epigenetic marks are integrated into functional signals that regulate gene expression.

Genomes of higher eukaryotes are intricately packaged into several hierarchical levels of organization. Therefore, consideration of the epigenome in its native 3-D context will be critical for the discovery of patterns that may be obscured when these structural features are not taken into account. The objective of this project was to elucidate how 3-D chromatin architecture changes in a cell type-specific manner during development.

Here we report a kb-resolution analysis of higher-order chromatin structure during neural lineage commitment. We leveraged Chromosome-Conformation-Capture-Carbon-Copy (5C) technology in combination with high-throughput sequencing to generate structure maps for pluripotent ES cells, multipotent ES-derived neural progenitor cells, and mouse embryonic fibroblasts. An alternating primer design was used to query six 1-2 Mb-sized genomic regions around key developmentally regulated genes (i.e. Oct4, Sox2, Nanog, Klf4, Nestin, Olig1-Olig2) in a massively parallel and unbiased manner. These data reveal a complex chromatin interaction network in ES cells that undergoes extensive reorganization during neural lineage commitment. Furthermore, new regulatory elements and combinatorial patterns of epigenetic marks unique to looping structures were identified by the integration of structure maps with recent genome-wide ChIP-Seq and RNA-Seq data sets. Finally, an iterative process between computational and experimental approaches was used to assemble a catalogue of novel active and poised regulatory elements involved in neural differentiation. These results are significant toward understanding how the genome and the epigenome act in concert to regulate the formation of a diverse array of tissue-types during development.

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Visualization of Eukaryotic DNA Mismatch Repair Reveals Distinct Recognition and Repair Associated Intermediates.

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DNA Mismatch Repair (MMR) increases the fidelity of DNA replication by eliminating mispaired bases resulting from replication errors. MMR proteins are conserved from bacteria to humans and people with defective mismatch repair develop cancers at much higher rates due to an increased accumulation of mutations. In eukaryotes, Mispaired bases are recognized by two partially redundant heterodimer complexes, Msh2-Msh6 or Msh2-Msh3. After the mismatch recognition factors bind a mispaired base, accessory factors including the Mlh1-Pms1 complex are recruited, ultimately targeting repair to the daughter DNA strand. We used high-resolution microscopy to visualize functional fluorescent-tagged versions of the Msh2-Msh6 and Mlh1-Pms1 MMR complexes in living S. cerevisiae cells. Msh2-Msh6 forms foci in S-phase that colocalize with nuclear foci composed of the DNA replication machinery, often called replication factories. This localization was independent of mispair recognition. The association of Msh2-Msh6 with replication factories is completely dependent on the interaction of Msh6 with the DNA polymerase clamp PCNA (Pol30). Replication factory-association accounts for 10 to 15% of MMR in wild-type cells but is essential for MMR in the absence of the exonuclease Exo1. Mlh1-Pms1 forms nuclear foci that are dependent on mispair recognition by Msh2-Msh6 or Msh2-Msh3. Surprisingly, these foci rarely colocalize with Msh2-Msh6 foci. Mlh1-Pms1 foci increase in frequency in response to increased levels of mispaired bases. These foci also increase in response to the disruption of processes downstream of mispair recognition, indicating an accumulation of foci that cannot be efficiently processed. Based on these data, we conclude that Mlh1-Pms1 foci are sites of active mismatch repair; this is the first time mismatch repair intermediates have been observed in eukaryotes. These results suggest that (I) mispair recognition involves either the concentration of Msh2-Msh6 at replication factories or a second distinct pathway that depends on both Msh2-Msh6 and Exo1, and that (II) accumulation of Mlh1-Pms1 triggered by Msh2-Msh6-dependent mispair recognition defines sites of active MMR.

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The budding yeast nuclear envelope adjacent to the nucleolus serves as a “membrane sink” during mitotic delay.

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To identify proteins and processes that affect nuclear structure, we conducted an automated microscopy screen of the entire budding yeast deletion collection for mutants that exhibit an abnormal nuclear shape. Prominent among those identified were mutants in DNA recombination and repair genes. The aberrant nuclei in these mutant strains had a single extension that always coincided with the nucleolus, while the morphology of the DNA mass was unchanged. We show that the alteration in nuclear shape in these mutants is an indirect consequence of a mitotic delay, as inhibiting mitotic progression by other means (e.g. nodocazole treatment, which depolymerizes microtubules and activates the spindle checkpoint, or expression of non-degradable Pds1p, which blocks anaphase initiation) also resulted in nuclei with a nucleolus-associated extension. Moreover, inactivating the DNA damage checkpoint in a DNA repair mutant reduced the occurrence of nuclei with extensions, further indicating that nuclear extensions are a consequence of a mitotic delay. In contrast, arresting cells in S phase or in G2 did not alter nuclear morphology. We also found that phospholipids continued to accumulate

during the mitotic delay, and that inhibiting phospholipid synthesis prevented the appearance of nuclear extensions during a mitotic arrest. Our data suggest that accumulation of phospholipids during a mitotic arrest leads to the accumulation of nuclear membrane that is not added evenly throughout the nuclear envelope, but is sequestered to a membrane compartment that is associated with the nucleolus. We hypothesize that in this way, cells avoid a disruption to intra-nuclear organization in the nuclear compartment containing the bulk of the DNA mass. Our findings also show that membrane addition to the nucleus during mitosis is independent of spindle-driven nuclear elongation, and suggest the existence of a mechanism that directs nuclear envelope expansion specifically in mitosis.

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Phosphoinositide levels modulate nuclear morphogenesis.

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Nuclear morphogenesis during sperm head formation is critical for creating functional sperm and for male fertility. During late stages of sperm development, round spermatid nuclei undergo dramatic changes in shape and volume. These changes are associated with chromatin condensation and with microtubule-dependent nuclear elongation. Chromatin condensation and remodeling involve a switch from a histone-based chromatin configuration, present in early round spermatid nuclei, to a protamine-based configuration, present in mature sperm nuclei. Concomitantly, the nucleus elongates by reorganizing the perinuclear microtubule cytoskeleton and its association with the basal body and the nuclear membrane. Here, we show that levels of phosphatidylinositol phosphates (PIPs) are critical for shaping the sperm head and for chromatin condensation during Drosophila spermiogenesis. Spermatids in which levels of phosphatidylinositol 4,5-bisphosphate (PIP2) have been reduced show profound defects in nuclear shaping; the nuclei do not mature and the males are sterile. Posttranslational modifications of histones are impaired. Protamines get incorporated into nuclei despite histones not being completely removed. Transition proteins are missing in these spermatids and repair of double-stranded DNA breaks is incomplete. We previously showed that reducing PIP2 levels in testes leads to defects in basal body docking at the nucleus and to nuclear scattering (Wei et al., J Cell Sci 2008; Fabian et al., MBoC 2010). Our present data suggest these defects could be due to absence of Klaroid, a SUN domain protein that requires normal levels of PIP2 for its localization on the inner nuclear membrane. We show that PIP2 localizes to the nucleus and along microtubules during late stages of spermatid development. Therefore, our data strongly suggest that spatially restricted PIPs control sperm head development by regulating cellular processes involved in chromatin remodeling and interactions between the nuclear membrane and nucleo-cytoskeleton.

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Regulation of the Dbp5 nucleotide cycle by Nup159 and Gle1/IP$_6$.

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Eukaryotic gene expression is a highly regulated process that requires proper processing and transport of messenger (m) RNA-protein complexes known as messenger ribonucleoproteins (mRNPs). mRNPs are synthesized co-transcriptionally as RNA binding proteins load onto the nascent pre-mRNA transcript. The specific complement of mRNP proteins exchange throughout the mRNA's life cycle, directing downstream events such as splicing, polyadenylation, export from the nucleus, translation and degradation. One class of proteins, the DEAD-box proteins (Dbp) that function as RNA dependent ATPases, plays an integral role in the regulation of each

step in the mRNP life cycle. In *S. cerevisiae*, DEAD-box protein 5 (Dbp5) is an essential protein implicated in mRNP biosynthesis and required for mRNP export. Terminal steps in mRNP release from the nuclear pore complex (NPC) are controlled by Dbp5 interactions with another essential NPC-associated protein Gle1 and the small molecule inositol hexakisphosphate (IP_6). Both Dbp5 and Gle1 localize to the cytoplasmic side of the nuclear envelope through interactions with NPC components Nup159 and Nup42, respectively. At these cytoplasmic NPC filaments, the RNA-dependent ATPase activity of Dbp5 is stimulated by IP_6-bound Gle1 to mediate remodeling and release of proteins from mRNP complexes. To date, the model of mRNP export has assigned the role of the Dbp5-Nup159 interaction as anchoring Dbp5 at the NPC to position it close to Gle1 for activation. As such, Dbp5 is thought to be spatially controlled for remodeling mRNPs for cytoplasmic release thereby preventing re-entry into the nucleus. Utilizing *dbp5* mutants that result in dbp5 proteins that do not bind to Nup159 *in vivo* or *in vitro*, we recently showed that this binding event is not strictly required for efficient RNA export, suggesting another function for the Dbp5-Nup159 interaction. Using *in vitro* reconstitution assays to test the effect of Nup159 on other known functions of Dbp5, we determined that Nup159 promotes the release of ADP from Dbp5. Furthermore, Gle1-IP_6 promoted ATP loading onto Dbp5, thereby completing the nucleotide cycle. We propose a new model for the Dbp5 cycle during mRNA export at the NPC wherein Nup159 and Gle1-IP_6 act to promote the nucleotide cycling of Dbp5. Additionally, we suspect that along with Gle1-IP_6 regulation of Dbp5 in translation termination, a yet unidentified cytoplasmic ADP release factor works to regulate Dbp5 in translation. Overall, our work provides the first evidence for a Dbp regulated by an ADP release factor and makes light of the possibility for other Dbps to require similar co-factors.

Minisymposium 24: Stem Cells and Pluripotency

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Mechanisms regulating maintenance of stem cells and the stem cell niche.

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Adult stem cells support tissue homeostasis and repair throughout the life of an individual. Many stem cells lose the capacity for self-renewal when removed from their local microenvironment (“niche”), indicating that the niche plays a major role in controlling stem cell fate. Numerous intrinsic changes occur with age that result in altered stem cell behavior and reduced tissue maintenance and regeneration. However, changes to the local and systemic environments also occur that result in decreased stem cell activity or alterations in lineage commitment.
In the *Drosophila* testis, stem cells depend upon signals from the apical hub, a cluster of somatic cells that express the self-renewal factor Unpaired (Upd). Upd secretion activates the JAK-STAT pathway in adjacent stem cells to promote stem cell maintenance in young males; however, aging results in a dramatic decrease in *upd* expression, with a concomitant loss of germline stem cells (GSCs). Here we present genetic and biochemical data to demonstrate that IGF-II mRNA binding protein (Imp) counteracts endogenous small interfering RNAs to stabilize *upd* RNA and contribute to maintenance of the niche. However, Imp expression decreases in hub cells of older males, similar to *upd*, which is due to targeting of *Imp* by the heterochronic microRNA *let-7*. Therefore, in older males *upd* mRNA is unprotected and degraded. Our data reveal how small RNAs can act at multiple levels to target key self-renewal pathways, resulting in loss of stem cell niche function and decreased stem cell maintenance with age.

Understanding the mechanistic basis for aging-related changes in stem cell behavior will lead to the development of strategies to treat age-onset diseases and facilitate stem cell based therapies in older individuals.

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The regulation of asymmetric stem cell division by centrosome orientation checkpoint.

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Adult stem cells are critical for tissue homeostasis, especially for short-lived differentiated cell populations, such as blood, skin, and sperm cells. A hallmark of stem cells is the ability to give rise to daughter cells committed to differentiation, while maintaining themselves the potential of self-renewal. Asymmetric cell division is a common strategy for stem cells to achieve the balance between self-renewal and differentiation. Perturbation of this balance can lead to either tumorigenesis by overgrowth of stem/stem-like cells, or tissue degeneration by depletion of stem cells.

Drosophila germline stem cells (GSCs) provide a useful model system in which to study the function and regulation of adult stem cells. *Drosophila* GSCs and the niche in which they reside have been identified and can be studied at single-cell resolution. *Drosophila* male germline stem cells (GSCs) always divide asymmetrically to give rise to a daughter cell that remain within the niche, maintaining stem cell identity, and the other daughter cell that is displaced away from the niche, committing to differentiation.

We have shown that the centrosome orientation in *Drosophila* male GSCs is tightly linked to the asymmetric division of the GSCs. GSCs in interphase maintain stereotypical centrosome orientation with respect to the niche component, hub cells, leading to perpendicularly oriented spindle in mitosis. Interestingly, the mother centrosome is always located close to the hub, whereas the daughter centrosome migrates to the opposite pole to set up the perpendicular orientation of mitotic spindle. However, how the precise centrosome orientation is ensured has not been investigated.

Here we show that *Drosophila* male GSCs possess a checkpoint that monitors the centrosome orientation before entering mitosis. This checkpoint delays/ arrests the cell cycle of GSCs when centrosomes are misoriented, preventing mitosis with misoriented spindle that may lead to symmetric stem cell division. Our data demonstrate that Par-1 kinase is one of the critical components of this novel centrosome orientation checkpoint. We also identified Cyclin A as the downstream target of Par-1. Cyclin A is sequestered on spectrosome, a germline specific ER-like organelle, by Par-1 till the centrosomes are correctly oriented. Interestingly, we found that the C terminal fragment of Cyclin A is required for its localization on spectrosome. We are embarking a biochemical pull-down experiment followed with mass spectrometry to identify the interacting partners with this spectrosome targeting motif/domain. This may also open new avenue for identifying other components of the centrosome orientation checkpoint. Taken together, we propose that centrosome orientation is tightly regulated and monitored in male GSCs to ensure asymmetric outcome of the cell division.

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Measuring electrical dynamics in human iPS-derived cardiomyocytes with a fluorescent voltage-indicating protein.

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Human iPS-derived cardiomyocytes are a promising platform for drug screens and studies of cardiac electrophysiology in healthy and diseased states. However, conventional patch clamp measurements are laborious, multi-electrode arrays do not probe membrane potential directly, and voltage-sensitive dyes are highly phototoxic. Here we describe a fluorescent voltage-indicating protein (VIP) based on a modified microbial rhodopsin protein, whose fluorescence sensitively and accurately reflects membrane potential. Expression of this VIP in iPS-derived cardiomyocytes yielded optical measurements of membrane potential in quantitative agreement with electrophysiological data. We used high-speed imaging to observe activation fronts propagating across and between cells with sub-millisecond temporal resolution. Co-expression of VIPs with channelrhodopsin enabled fully non-contact electrophysiology in which stimulus and response were both mediated by light. These results open the way for high-throughput and high-content optical studies of electrophysiology in human iPS-derived cardiomyocytes.

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Characterization of blastema formation and pattern reformation during lower jaw regeneration in zebrafish.

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Zebrafish possesses the remarkable ability to regenerate the complicated structures by formation of the characteristic blastema. It is disputed how the blastemal cells arise. Little is known about changes in gene activities that occur during the course of regeneration. To decipher the cellular and molecular processes that underlie restoration of the amputated lower jaw, we have investigated cellular transitions and transcriptional profile of the deregulated genes during the blastema formation and pattern reformation.

In the first two days, the wound epithelium is reconstituted by a stratified epidermal layer. Following complete re-epitheliation, fibroblasts, fragmented muscle cells, and cells from blood and damaged endothelium are activeated. These cells aggregate under the new formed wound epidermis, and contribute to the growing mass of mesenchymal tissue (blastema). In the following days, a large part of the blastema undergoes a transitional chondrogenesis while the rest of blastema cells are respecified to restore the original tissues including bone, muscle, pigment and connective tissue. Cell identity and selected cell marker analyses reveal that these blastemal cells possess a complex property of neural crest cell and muscle progenitor. Our observations indicate that epithelial- mesenchymal interaction and local microenvironmental cues induce transdifferentiation and dedifferentiation of pre-blastemal cells, and subsequent redifferentiation. Combing our time course-based whole transcriptome profiling, we have identified the signaling induction in relation to tissue memory of blastemal cells in the lower jaw regeneration process.

Key words: zebrafish, jaw, regeneration, blastema, cellular memory

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Insights into Nuclear Reprogramming via Heterokaryon RNA Sequencing.

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Induced pluripotent stem cells (iPS) can be produced from virtually any somatic cell by the overexpression of a few transcription factors, a process termed “nuclear reprogramming”. At present, the molecular mechanisms underlying reprogramming are not well understood, in large part due to an inability to analyze early stages of reprogramming at the molecular level in iPS.
We hypothesized that there are as yet unidentified molecular regulators critical to the early onset of reprogramming. To identify key regulators of reprogramming we developed a synchronous, high efficiency, rapid reprogramming approach consisting of heterokaryons (interspecies multinucleate fused cells) in which activation of human pluripotency genes occurs rapidly (24hrs) and efficiently (70% of single heterokaryons), enabling early mechanistic studies.
We conducted a transcriptome-wide investigation of heterokaryon reprogramming using high throughput RNA sequencing. Heterokaryons were isolated over a three day time-course and subject to RNA sequencing to characterize the early gene expression dynamics of the mouse and human transcriptome during the early stages of nuclear reprogramming. Our results show significant changes in transcriptional output of both the mouse and human nucleus, including the induction of key human pluripotency genes and chromatin remodelers. Our results also validate using heterokaryon RNA sequencing as a discovery tool via the identification of previously published molecules known to have a role in reprogramming towards pluripotency. In conclusion, the speed and efficiency of reprogramming in the heterokaryon system provides a means to identify critical transcription factors and epigenetic regulators via RNAseq, providing temporal information and transcriptional insights into mechanisms of reprogramming and cell fate.

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Hippo/YAP signaling in somatic stem cells.

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During development and regeneration, proliferation of tissue-specific stem cells is tightly controlled and monitored to produce organs of a predetermined size. The molecular determinants of this process remain poorly understood. We are particularly interested in studying the function of the emerging highly-conserved Hippo/YAP signaling cascade, and its effects on tissue size, homeostasis and cancer. Our previous studies have demonstrated that Hippo signaling can be a very potent regulator of organ size in mice and have also provided a conceptual link between organ size regulation and stem cell activity through Hippo signals. Our work is now aimed at fully dissecting the components and the role of this cascade in somatic stem cells utilizing a variety of genetic, biochemical, and high throughput technologies. Work describing the function of the pathway in the epithelial stem cell compartments will be discussed. Insight into these processes will shed light on fundamental aspects of tissue regeneration and facilitate the development of therapeutic approaches based on cellular transplantation

E.B. Wilson Medal Presentation and Address

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EB Wilson, MBL and the Physical Properties of Protoplasm.

G. G. Borisy[1]; [1]Marine Biological Laboratory, Woods Hole, MA

No text submitted.

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Mitosis Futures: the past is prologue.

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The mechanisms by which spindles organize and segregate chromosomes have fascinated and frustrated many generations of cell biologists. Since the discovery of spindle microtubules, progress has been comparatively rapid, and our understanding of spindle mechanism has grown with increasing speed as the techniques of modern cell and molecular biology have been brought to bear on the problem. The discovery of spindle motor enzymes has motivated a wide range of studies on the ways these nanomachines might contribute to chromosome organization and segregation over distances as big as half a cell diameter. Modern methods for imaging, both by fluorescence microscopy of live cells and by electron microscopy of cryo-immobilized samples, have revealed the movements of spindle microtubules that underlie chromosome motion itself. Analogous methods have, however, shown that chromosome-to-pole motion in yeast cells does not require pole-directed motors enzymes, suggesting that tubulin depolymerization too can contribute to mitotic chromosome motility. To make things even more interesting, some motor enzymes can regulate tubulin dynamics, so an understanding of chromosome segregation will certainly require the evaluation of multiple contributing factors. Genetics will help, but many spindle components can be mutated without blocking mitosis, so alternative modes of experimentation are likely to be valuable. Some recent work has used the strategy of assembling isolated spindle proteins into extra-cellular devices that can duplicate aspects of spindle function under well-controlled conditions. This talk will review some recent history of mitosis research and examine current work in that context, looking forward to where the field may go in the near future.

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Molecular motors: Where do we go from here?

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A mere forty years ago it was unclear what motor molecules exist in cells that could be responsible for the variety of non-muscle cell movements, including the "saltatory cytoplasmic particle movements" apparent by light microscopy. One wondered whether such cells might have a myosin-like molecule, well known to investigators of muscle. Now we know there are more than a hundred different molecular motors in eukarotic cells that drive a wealth of cell biological processes and help organize the cell’s dynamic city plan. Furthermore, *in vitro* motility assays, taken to the single molecule level using techniques of physics, have allowed detailed characterization of how these motors transduce the chemical energy of ATP hydrolysis into mechanical movement. Still, there is much to do for the eager student interested in molecular motor research.

WEDNESDAY, DECEMBER 7

Minisymposium 25: Cancer Cell Biology

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DNA secondary structures and epigenetic determinants of cancer genome evolution.

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An unstable genome is a hallmark of many cancers. It is unclear, however, whether some mutagenic features driving somatic alterations in cancer are encoded in the genome sequence and whether they can operate in a tissue-specific manner. We performed a genome-wide analysis of 663,446 DNA breakpoints associated with somatic copy-number alterations (SCNAs) from 2,792 cancer samples classified into 26 cancer types. Many SCNA breakpoints are spatially clustered in cancer genomes. We observed a significant enrichment for G-quadruplex sequences (G4s) in the vicinity of SCNA breakpoints and established that SCNAs show a strand bias consistent with G4-mediated structural alterations. Notably, abnormal hypomethylation near G4s-rich regions is a common signature for many SCNA breakpoint hotspots. We propose a mechanistic hypothesis that abnormal hypomethylation in genomic regions enriched for G4s acts as a mutagenic factor driving tissue-specific mutational landscapes in cancer.

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The transcription factor ZNF217 is an oncogene that promotes an increase in progenitor cells, increases metastasis, and acts via the AKT pathway.

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Amplification of 20q13 occurs in many cancers including 20-30% of primary human breast cancers and correlates with poor breast cancer prognosis. The transcription factor ZNF217 is a candidate oncogene within this region. In patient cohorts we found that patients expressing high ZNF217 in breast tumors had reduced survival and increased chemoresistance. To study the consequences of Znf217 overexpression we generated a transgenic mouse model with inducible Znf217 expression. Znf217 overexpression in the mammary epithelium induced premalignant and invasive lesions. In addition, Znf217 overexpression in a mouse breast cancer model generated primary tumors with increased numbers of progenitor cells that expressed markers of both the luminal and myoepithelial cells. In addition, Znf217 overexpression promoted increased motility in mouse mammary epithelial cell (MEC) lines and metastasis in vivo.

To identify an inhibitor of ZNF217 function we correlated the IC50s for drugs from the Developmental Therapeutics database with ZNF217 expression levels in NCI60 tumor cell lines and identified the AKT inhibitor triciribine. Treatment of our mouse mammary tumor models with triciribine decreased the Znf217-induced tumor burden, inhibited AKT and MAPK activation in

the tumors and inhibited progenitor cell self-renewal by mammosphere assays. ZNF217 overexpression in MECs made them resistant to the chemotherapy doxorubicin, while treatment with triciribine generated synthetic lethality with doxorubicin. Taken together, we find that ZNF217 overexpression induces multiple phenotypes required for tumor progression including increased progenitor cell phenotype, increased motility and increased resistance to cytotoxic drugs, indicating that it is an oncogene and potential drug target.

Supported by grants from the National Cancer Institute, Stand Up to Cancer-AACR Dream Team Translational Cancer Research Grant, and the American Cancer Society.

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Tumor Suppressor Gene p16/INK4A Dependent Regulation of Cell Cycle Exit in a Spontaneous Canine Model of Breast Cancer.

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p16/INK4A is an important tumor suppressor gene which arrests cell cycle in early G1 phase inhibiting binding of CDK4/6 with cyclin D1 leaving the Rb tumor suppressor protein unphosphorylated and E2F bound and inactive. We have previously shown that p16 is frequently mutated in canine mammary cancer comparable to human breast cancer. Because p16 expression persists following cell cycle exit, we hypothesize that p16 has a role in exit from cell cycle that becomes defective in cancer cells and that p16 has alternate binding partners other than CDK4/CDK6 in quiescent or differentiated cells. Well characterized p16-defective canine mammary cancer cell lines (CMT28, CMT27, and CMT12), normal canine fibroblasts (NCF), and p16-transfected CMT cell clones (CMT27A, CMT27H, CMT28A, and CMT28F) exhibiting a rescued phenotype, have been used to investigate expression of p16 after serum starvation into quiescence followed by re-feeding to induce cell cycle re-entry. CMT cells lack p16 expression either at mRNA or protein expression levels, while p27, CDK4, CDK6, cyclin D1, and Rb, appear to be expressed at normal levels. We have successfully demonstrated cell cycle arrest and synchronous cell cycle re-entry in parental CMT12, CMT28 and NCF cells as well as p16 transfected CMT27A, CMT27H, CMT28A, and CMT28F cells and confirmed this by 3H-thymidine incorporation and flow cytometric analysis of cell cycle phase distribution. p16 transfected CMT27A and CMT27H exited cell cycle post serum-starvation in contrast to parental CMT27 cells. NCF, CMT27A, and CMT28F cells expressed up-regulated levels of p27 mRNA coincidently with elevated expression of p16 mRNA as cells exited cell cycle and entered quiescence. To find alternating binding partners of p16, co-immunoprecipitation was performed in quiescent CMT27A cells which resulted in unique co-immunoprecipitation of the p53-associated and putative tumor suppressor 14-3-3σ protein only in quiescent CMT27A cells in comparison to exponential cells. Levels of 14-3-3σ mRNA expression also rose along with p16 in quiescent NCF cells. Because quiescence and differentiation are associated with decreased levels of cyclin D1 and/or CDK6 our data demonstrating that p16 is up-regulated during quiescence suggests the presence of alternative binding partners for p16, such as 14-3-3σ, in promoting and maintaining the quiescent and/or differentiated phenotype (funded by NCI/NIH).

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Mad1 upregulation causes chromosomal instability and resistance to microtubule poisons.

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The mitotic checkpoint ensures accurate chromosome segregation during mitosis. Reduced expression of mitotic checkpoint components causes aneuploidy, an abnormal chromosome number, and can promote tumorigenesis. Mice heterozygous for the mitotic checkpoint component Mad1 develop increased levels of spontaneous and carcinogen-induced tumors. However, at the genomic and mRNA level, Mad1 is as likely to be upregulated as downregulated in human cancers. The effects of Mad1 upregulation on mitosis, aneuploidy and transformation are currently unclear. We have found that Mad1 protein is frequently overexpressed in human breast cancers and that upregulation of Mad1 is a marker of poor prognosis. Upregulation of Mad1 causes aneuploidy and promotes transformation. Increased expression of Mad1 weakens mitotic checkpoint signaling and results in mislocalization of the Mad1 binding partner Mad2 from kinetochores, producing a mitotic checkpoint phenotype similar to that caused by reduction of Mad2. Cells overexpressing Mad1 are resistant to microtubule poisons, including those currently used to treat human cancers, and express reduced levels of the p53 tumor suppressor. These results suggest that levels of Mad1 must be tightly regulated to prevent aneuploidy and transformation.

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Efficient Elimination of Cancer Cells by Deoxyglucose-ABT-263/737 Combination Therapy.

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Genotoxin-based chemotherapies are most effective in treating tumors at early stages of development, while recently developed targeted therapies can be very effective on certain types of cancer at later stages. For most of the common cancer types, however, these targeted therapies are not available because potential targets are not even identified in most of cancer types, let alone have targeting drugs in development. Furthermore, the recent data from the Cancer Genome Atlas Project also suggested that targeting and inactivating just one protein would not be enough to kill cells in most cancer types, implying that multiple targeted drugs may be required to kill most cancer cells. Here we described a new therapy that is safe and effective in eliminating many cancer cells, including checkpoint defective, genotoxin-resistant, PTEN-deleted, late stage cancer cells.

The new therapy is based on combining 2-deoxyglucose (2DG), a glycolysis inhibitor, with ABT-263/737 (ABT), molecules targeting the anti-apoptotic Bcl-2 family of proteins. As single agents, ABT-263 and ABT-737 (ABT), molecular antagonists of the Bcl-2 family, bind tightly to Bcl-2, Bcl-xL and Bcl-w, but not to Mcl-1, and induce apoptosis only in limited cell types. The compound 2-deoxyglucose (2DG), in contrast, partially blocks glycolysis, slowing cell growth but rarely causing cell death. Injected into an animal, 2DG accumulates predominantly in tumors but does not harm other tissues. However, when cells that were highly resistant to ABT were pre-treated with 2DG for 3 hours, ABT became a potent inducer of apoptosis, rapidly releasing cytochrome c from the mitochondria and activating caspases at submicromolar concentrations in a Bak/Bax-dependent manner. Bak is normally sequestered in complexes with

Mcl-1 and Bcl-xL. 2DG primes cells by interfering with Bak-Mcl-1 association, making it easier for ABT to dissociate Bak from Bcl-xL, freeing Bak to induce apoptosis. A highly active glucose transporter and Bid, as an agent of the mitochondrial apoptotic signal amplification loop, are necessary for efficient apoptosis induction in this system. This combination treatment of cancer-bearing mice was very effective against tumor xenograft from hormone-independent highly metastasized chemo-resistant human prostate cancer cells, suggesting that the combination treatment may provide a safe and effective alternative to genotoxin-based cancer therapies.

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Integration of Growth Factor, MAP Kinase, and DNA Damage Signaling Networks in Cancer and Cancer Treatment.

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Protein kinases and phosphoserine/threonine-binding domains such as 14-3-3 proteins, FHA domains, Polo-box domains, and BRCT domains function together within signaling networks to control growth factor responses, cell cycle progression, and the response to DNA damage. How signals from these pathways are integrated and processed to control cell phenotypic responses is unclear. To address this, we have been developing systems biology-based models of DNA damage signaling where kinase activities, protein phosphorylation, and phosphoprotein-binding events for multiple signaling pathways are quantitatively measured at densely sampled points in time, along with cellular responses such as cell cycle arrest, autophagy, and apoptosis. The resulting large dataset of signals and responses are then related to each other mathematically using partial least squares regression, principal components analysis, and time-interval stepwise regression. We have used this approach to examine the response of breast cancer and osteosarcoma cells to DNA damaging chemotherapy and gamma radiation in the presence or absence of small molecule inhibitors of growth factor signaling pathways. The resulting models, built from thousands of signaling measurements and hundreds of cellular response assays, reveals surprisingly paradoxical context-dependent roles for several well-established protein kinase signaling pathways in controlling cell cycle arrest, apoptosis and senescence after DNA damage. Furthermore, the analysis has identified novel therapeutic approaches that can therapeutically re-wire signaling pathways within tumor cells to dramatically improve tumor cell killing by conventional clinical agents.

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Minisymposium 26: Cell Biology of RNA

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Transcriptional control of mRNA export and low expressing genes: New clues from single molecules.

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Some of the most important genes controlling key processes in cellular regulation are expressed in low amounts virtually undetectable; yet their function is absolutely necessary for normal cell function. Here we report new methods to study gene expression at the single molecule level devoted to quantify mRNAs expressed in populations at levels far below one molecule per cell. We will also show how these approaches bring new clues on the link in between Transcription and mRNA physiology.
We found that different promoter architectures enabling equal loading and initiation rates of polymerases on a gene yielded notably different elongation rates. Moreover, we found that properly spliced mRNAs transcribed from a same gene under different activating conditions can have drastically different fates, one being exported and translated while the other is retained in the nucleus where it accumulates in nuclear SC35 domains (speckles).

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Characterization of the Enlargement of Speckles of SF2/ASF due to Loss of Function of Smu1 in the Mammalian Temperature-Sensitive Mutant.

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A temperature-sensitive (ts) CHO-K1 mutant cell line, tsTM18, exhibits chromosomal instability with decreased DNA synthesis at the nonpermissive temperature, 39°C. An amino acid substitution in Smu1 underlying the ts phenotypes of tsTM18 cells was identified previously. We also found a ts defect in splicing of the *unc52/perlecan* gene. In the present study, we have generated cell lines expressing Smu1 tagged with green fluorescent protein (GFP) to study the dynamics of Smu1 in living cells. The hybrids complement deficiencies in tsTM18 cells and allow them to grow normally at 39°C. GFP-tagged Smu1 is found in speckles in many discrete nucleoplasmic sites, and most of these also contained SF2/ASF. SF2/ASF is a member of the serine/arginine (SR)-rich splicing group of factors that are necessary for spliceosome assembly and can influence alternative splicing. SF2/ASF is also involved in the integrity of genome maintenance. In tsTM18 cells cultured at 39°C, the Smu1 ts defect appears to alter SF2/ASF localization, suggesting a physiological association of Smu1 with SF2/ASF. The significant decrease of Smu1 may lead the enlargement of speckles of SF2/ASF. These data show the importance of Smu1 as a regulator of splicing and genome maintenance.

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β-actin mRNA compartmentalization by ZBP1 controls focal adhesion stability and directed cell motility.

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Localization of β-actin mRNA is facilitated by the zipcode binding protein, ZBP1. Binding to the cognate zipcode within the 3'UTR of β-actin mRNA enables ZBP1 to asymmetrically distribute mRNA and control translation of its target, a process known to be necessary for directed cell motility. This study utilized a ZBP1 KO fibroblast cell line to compare β-actin mRNA dynamics to wild-type cells. TIRF microscopy and mRNA particle tracking in live cells enabled us to discover a specific compartment near focal adhesions where β-actin mRNA dwells for periods greater than one minute. In ZBP1 KO fibroblasts, the probability of tracking β-actin mRNA within the adhesion environment is significantly reduced. Consequently, adhesion lifetimes are reduced in ZBP1 KO cells. This supports the hypothesis that ZBP1 facilitates mRNA localization to strengthen focal adhesions and therefore direct cell migration. To test directly whether mRNA localization to adhesions can alter adhesion dynamics and cell motility we utilized a novel mRNA tethering technique. We tethered β-actin mRNA to focal adhesion complexes through MS2 stem loops in the 3'UTR of β-actin mRNA and the MS2 coat protein fused to vinculin. This produced a significant increase in adhesion lifetime and adhesion size in cells expressing MS2-β-actin mRNA. Additionally, cell velocity was significantly reduced in cells with tethered β-actin mRNA. These experimental results lead us to conclude that β-actin mRNA compartmentalization to adhesions, mediated by ZBP1, strengthen adhesions and therefore produce an asymmetric force distribution within the cell to control its directionality.
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Test driving the 60S subunit during ribosome biogenesis.

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In eukaryotic cells, ribosomes are largely preassembled in the nucleus. When the large (60S) ribosomal subunit enters the cytoplasm, it is functionally inactive and requires the release of export and assembly factors and the addition of ribosomal proteins to generate functionally active subunits. We recently delineated the order of these maturation events and found that assembly of the ribosome stalk was a prerequisite for the release of the subunit anti-association factor Tif6 followed by release of the nuclear export adapter Nmd3. Because we had previously found that the ribosomal protein Rpl10 was required for the release of Nmd3, we asked if Rpl10 was also required for the release of Tif6. Rpl10 is located in a cleft between the central protuberance and the GTPase stalk and contains an internal loop that embraces the P-site tRNA. Deletion of the P-site loop of Rpl10 trapped Tif6 in the cytoplasm, indicating a novel role for Rpl10 in release of Tif6, prior to its role in Nmd3 release. Mutagenesis of the P-site loop yielded mutants that specifically affect 60S biogenesis. The release of Tif6 is known to require the GTPase Efl1, a paralog of the translation translocation factor eEF2. Surprisingly, these rpl10 mutants could be suppressed by a mutant allele of Tif6 that has weakened affinity for the ribosome and can bypass the need for Efl1. Because Efl1 is normally required for the release of Tif6, this result implicates Efl1 in signaling between Rpl10 and Tif6. Indeed, we identified mutations in Efl1 that bypass P-site loop mutations in Rpl10. Based on the crystal structure for eEF2, the suppressing mutations in Efl1 map to domain interfaces of eEF2 that are important for the conformational changes in eEF2 that drive translocation. Molecular dynamics simulations of

these mutants show that one cluster of suppressing mutations disrupt the hydrophobic core of a critical subunit interface, suggesting that they facilitate a conformational change in ribosome biogenesis that mimics the changes eEF2 undergoes during translocation. These results suggest that during maturation, the 60S subunit undergoes a "test drive" that assesses the correct assembly and ability of the subunit to support the conformational dynamics required during translation. We suggest that this assessment of 60S assembly by Efl1 is multi-faceted, monitoring assembly of the stalk, correct folding of the sarcin-ricin loop and the correct assembly of the P-site of the ribosome. We propose that interrogation of the P-site of the ribosome represents a quasi-functional quality control check of the large subunit, prior to its first round of *bona fide* translation.

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The human enhancer of mRNA decapping, Hedls, regulates the decapping enzyme, Dcp2, protein levels and activity.

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mRNA decay is a key step in the regulation of gene expression. A critical step in several mRNA decay pathways is decapping. A major decapping complex consists of the decapping enzyme Dcp2 as well as several decapping enhancers. An outstanding question is what role does each of these enhancers play in regulating the activity of Dcp2. We have identified a role for the metazoan specific decapping enhancer Hedls (also called Ge-1/Edc4) in the regulation of Dcp2 protein stability and activity. We observed that knockdown of Hedls results in the accumulation of deadenylated mRNA using a beta globin reporter containing the AU-rich element from the 3'UTR of granulocyte-macrophage colony-stimulating factor mRNA. This suggests that Hedls is required for the efficient 5'-3' decay of the message following deadenylation. We also observed that endogenous Dcp2 levels were reduced dramatically upon Hedls depletion. In addition exogenously expressed Dcp2 protein is highly unstable but can be stabilized by Hedls co-expression or by deletion of or five point mutations within the C-terminal Hedls interaction domain of Dcp2. Thus Hedls promotes Dcp2 activity at least in part by interacting with and preventing the activity of a C-terminal Dcp2 instability domain. These results provide novel insights into the regulation of mRNA decay mediated by the Dcp2 decapping complex.

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The Chp1-Tas3 core is a multifunctional platform critical for gene silencing by RITS.

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In the fission yeast *S. pombe*, the RNAi machinery regulates heterochromatin in coordination with chromatin regulators. The RNA-induced Initiation of Transcriptional gene Silencing (RITS) complex is at the center of this process, integrating the two pathways and directing heterochromatin formation and maintenance. Within the RITS complex the GW protein Tas3 links the large chromodomain protein Chp1 to the RNAi effector protein Ago1. Current evidence suggests that small RNAs loaded into Ago1 act cooperatively with the high affinity for histone H3K9 methyl marks of Chp1's chromodomain to target the RITS complex to specific genomic regions where recruitment of the histone methyltransferase Clr4 and the RNA dependent RNA polymerase Rdp1 result in a self enforcing process of heterochromatin formation and gene silencing.

We have solved the crystal structure of the complex between Chp1 and Tas3, forming the core of the RITS complex. The structure reveals a very intimate and unusual interaction between the two proteins with the Tas3 N-terminal fragment being engulfed by two domains of Chp1. We furthermore identified a third domain in Chp1 that shares strong similarity with PIN domains of proteins involved in RNA decay and telomere silencing. Deletion of the Chp1 PIN domain in *S. pombe* compromises specifically the silencing of subtelomeric transcripts, thereby revealing that Chp1 and Tas3 provide a heterochromatin bound platform with roles that extend beyond recruitment of Ago1.

Minisymposium 27: Cell Cycle Dynamics and Checkpoints

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A forward genetic screen for cell cycle control genes in the plant kingdom.

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Current evolutionary analysis, predominantly based on molecular systematics, indicates that fungi and animals diverged more recently from each other than from all other eukaryotic branches, including plants. This has the implication that intense research over the past decades into cell cycle control in fungi and animals may mistake fungal/animal (‘opisthokont’) control principles for features of a ‘universal’ eukaryotic cell cycle control system. A forward, unbiased exploration of cell cycle control in other branches of the eukaryotic family is necessary both for direct understanding of cell cycle control in that branch, and for allowing the possibility of deducing truly universal features of eukaryotic cell cycle control. For these reasons, and because of the fundamental importance of plants for life on earth, we have initiated a forward genetic screen for cell cycle control genes in the green alga Chlamydomonas reinhardtii, a member of the plant kingdom (Viridiplantae). Our approach integrates classical genetic methodologies with resequencing of mutant genomes, with the aim of obtaining both a comprehensive ‘parts list’ and initial functional understanding of cell cycle control in Viridiplantae.

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Entry into mitosis is linked to membrane growth in budding yeast.

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Addition of new membrane to the cell surface by vesicle transport is a key mechanism of cell growth. Here, we report that blocking transport of vesicles to the site of cell growth causes a checkpoint arrest before entry into mitosis in budding yeast. The arrest is enforced via coordinated control of the budding yeast homologs of Wee1 and Cdc25. We discovered a signaling pathway that acts upstream of Wee1 and Cdc25 to relay signals regarding the status of vesicle transport. Signaling via this pathway is dependent upon vesicle transport and appears to be proportional to membrane growth. We hypothesize that transport of vesicles to the site of cell growth generates a signal that is proportional to membrane growth, and that the strength of the signal is read by downstream components to determine when sufficient growth has occurred to allow entry into mitosis. Thus, growth-dependent signaling could represent a new mechanism by which growth is integrated with cell cycle progression. In addition, a growth-dependent signaling model for mitotic checkpoint function could reconcile different views of the checkpoint as functioning to control cell size or morphogenesis.

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A quantitative approach to exploring spindle assembly checkpoint signaling.

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The spindle assembly checkpoint (SAC) is a crucial surveillance mechanism within the eukaryotic cell cycle that ensures proper chromosome segregation. Despite a wealth of cell biological and biochemical data about the SAC, its complex in vivo dynamics are still only fragmentarily understood. To obtain insight into this intricate signaling mechanism, we have combined mathematical modeling with quantitative experiments in fission yeast. As an accurate foundation for our mathematical models, we have quantified the abundance of SAC proteins in vivo in single cells. Furthermore, we have modulated the protein abundance of single SAC proteins in steps between 0 and about 200 % of the endogenous level. This enabled us to determine the range of SAC protein abundance that allows checkpoint activity, and enabled us to test models for the signaling mechanism. We find that the range of expression that allows SAC activity varies greatly between different SAC proteins. Interestingly, subtle reduction of some SAC proteins leads to a bimodal distribution of the SAC response. This implies the presence of an ultrasensitive step in the signaling mechanism, which could explain how the SAC can robustly respond to even a single misattached chromosome.

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A novel cyclin dependent kinase couples cell size to cell division.

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Cell size is coupled to the cell division cycle in diverse eukaryotes, but size checkpoint control is relatively unexplored. The unicellular green alga *Chlamydomonas reinhardtii* divides by multiple fission where mother cells undergo repeated rounds of S and M phase in rapid succession. A sizer mechanism couples mother cell size to division number such that larger mother cells undergo more S/M cycles than smaller mother cells, thus ensuring a uniform daughter size distribution. We identified a sizer protein, CDKG1, that is a D cyclin dependent kinase which phosphorylates the retinoblastoma-related protein MAT3 as its key substrate. Loss of CDKG1 causes inadequate division and results in large daughters, while misexpression of CDKG1 causes supernumary divisions and results in small daughters. CDKG1 is nuclear-localized and is produced in mother cells just prior to division. It is then diluted during successive rounds of S/M and eliminated upon reentry into G0 or G1 phase. The concentration of CDKG1 in pre-mitotic cells is set by mother cell size, and provides a link between cell size and the extent of cell division. Cell-size-dependent accumulation of rate-limiting cell cycle regulators such as CDKG1 is a potentially general mechanism for size control.

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Mps1/Mph1 kinase recruits SAC components to kinetohores through phosphorylation of KNL1/Spc7.

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The spindle assembly checkpoint (SAC) delays anaphase onset in the presence of unattached kinetochores. Bub1 is a well conserved SAC protein and temporarily recruited to kinetochores in mitosis. In addition to SAC function, kinetochore associated Bub1 has a role in proper chromosome segregation in part through recruiting shugoshin proteins to centromeres.The

kinetochore localization of Bub1 requires its binding partner Bub3 as well as another SAC protein kinase Mps1/Mph1. But how Mps1/Mph1 regulates the kinetohore localization of Bub1 is unknown. Here, we show that Mph1 promotes the interaction of the Bub1-Bub3 complex with the kinetochore protein KNL1/Spc7 through phosphorylation of Spc7 in fission yeast. Mph1 phosphorylates Spc7 at multiple sites containing conserved MELT repeats in vitro. In cells expressing the non-phosphorylatable spc7-12A mutant, the kinetochore localization of Bub1 and Bub3 are abolished, resulting in impaired chromosome segregation and SAC. Moreover, artificial targeting of Mph1 to kinetochores or expression of the phospho-mimetic mutant spc7-12E causes ectopic localization of Bub1 and Bub3 at kinetohores in interphase. Our results suggest that Spc7 is a hitherto unknown critical target of Mph1 in both chromosome segregation and SAC.

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Regulation of mitosis in Giardia, a divergent eukaryote, is independent of the Anaphase-Promoting Complex or Ubiquitination.

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Giardia intestinalis, a human parasite responsible for diarrheal disease, is a member of the Diplomonads, potentially one of the most highly divergent lineages in the eukaryotic kingdom. We investigated the role of *Giardia* cell cycle proteins including cyclin B, aurora kinase and polo-like kinase to determine whether their regulation differs from those in other eukaryotes. In every eukaryote studied to date, cyclin B is targeted by the Anaphase Promoting Complex (APC) for proteasome-mediated degradation prior to the onset of anaphase by ubiquitin conjugation. There are no APC subunits in *Giardia*, and the *Gardia* cyclin B (*Gi* cyclin B) lacks the conserved sequences required for recognition by the APC in other eukaryotes. Overexpression of *Gi* cyclin B in fission yeast leads to mitotic arrest, a phenotype similar to that observed when N-terminal mutants of yeast mitotic cyclins are expressed. This phenotype can be suppressed by fusing the yeast degradation sequence to *Gi* cyclin B. One of the endogenous gene copies of cyclin B in *Giardia* was tagged with a 3HA epitope and experiments using morpholino-mediated gene knockdown show that *Gi* cyclin B is essential for mitotic entry in *Giardia*. Using immunofluorescence and quantification assays we showed that cyclin B is degraded before the cells return to G1. Both *in vivo* experiments using proteasomal inhibitors and *in vitro* ubiquitination experiments using recombinant ubiquitin show that, though *Giardia* ubiquitin can be effectively conjugated to substrates, *Gi* cyclin B and other APC substrates such as aurora kinase and polo-like kinase are not ubiquitinated. In addition cells treated with proteasomal inhibitors do not arrest in the cell cycle. This is the first example of a mitosis that is not regulated by the APC, or ubiquinitination by an E3 ligase and it may reflect an evolutionary ancient form of cell cycle regulation. To further improve our understanding of cell cycle regulation in *Giardia* we have isolated by affinity purification proteins interacting with aurora and polo-like kinase from *Giardia*. Work is in progress to identify these proteins and their contribution to cell cycle regulation.

Minisymposium 28: Cell-Pathogen Interactions (Viruses and Bacteria)

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The Chlamydial Toxin CPAF Promotes Early Exit from Mitosis in Infected Cells.

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Chlamydia trachomatis has been epidemiologically linked to cervical cancer in patients with a prior human papilomavirus (HPV) infection. *Chlamydia* causes multinucleation of the host cell, a potential pathway for chromosomal instability. Two mechanisms that are known to initiate multinucleation are cell fusion and cytokinesis failure. Our studies ruled out cell fusion and demonstrated that multinucleation is caused by cytokinesis failure. From this data, we aimed to determine the mechanism of cytokinesis failure due to *Chlamydia* infection. We have previously reported that chlamydial infection of the host cell overrides the Spindle Assembly Checkpoint (SAC) causing mitosis to be shortened. Our findings demonstrate that the Chlamydial Protease-like Activity Factor (CPAF) functionally inactivates cyclin B1 and cyclin dependent kinase 1 (Cdk1) through cleavage, effectively bypassing the SAC. This checkpoint is necessary for an organized cell division. In this study we demonstrate that premature entry into anaphase causes a increased rate of DNA tangles that results in cytokinesis failure of chlamydial infected cells.

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Brush border microvilli-derived vesicles detoxify bacterial products and regulate epithelial-microbial interactions.

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The continuous monolayer of intestinal epithelial cells (IECs) that lines the gut lumen functions as the site of nutrient absorption and as a physical barrier that prevents the translocation of microbes and associated toxic compounds into the peripheral vasculature. IECs also express host defense proteins such as intestinal alkaline phosphatase (IAP), which detoxify bacterial products and prevent intestinal inflammation. Our laboratory recently showed that IAP is enriched on vesicles that are released from the tips of IEC microvilli and accumulate in the intestinal lumen (McConnell et al, 2007; McConnell et al, 2009). Here, we show that these native ‘lumenal vesicles’ (LVs): (i) contain catalytically active IAP that can dephosphorylate lipopolysaccharide (LPS), (ii) cluster on the surface of native lumenal bacteria, (iii) prevent the adherence of enteropathogenic E. coli (EPEC) to epithelial monolayers, and (iv) limit bacterial growth. We also find that IECs upregulate LV production in response to EPEC and defects in LV production are associated with significant changes in the intestinal microbiota. Together these results suggest that microvillar vesicle shedding represents a novel mechanism for distributing host defense machinery into the intestinal lumen, and that microvillus-derived LVs modulate epithelial-microbial interactions and exert a direct effect on the lumenal microbiome.

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Mitochondrial dynamics in *Listeria monocytogenes* infection.

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Mitochondria are dynamic organelles central to energy production and several other cellular processes, such as calcium buffering and apoptosis. As such, they are targeted by pathogens to subvert cellular function. We studied the effects of infection by the intracellular pathogen *Listeria monocytogenes* on mitochondrial dynamics and function and could recently show that it profoundly alters mitochondrial dynamics, causing transient fragmentation of the mitochondrial network. Such mitochondrial fragmentation occurs early during infection, is specific to pathogenic *L. monocytogenes*, and is not observed upon infection with several other intracellular pathogens. The relevance of mitochondrial dynamics for *L. monocytogenes* infection is highlighted by the finding that siRNA-mediated inhibition of mitochondrial fusion or fission alters infection efficiency. We identified the secreted pore-forming toxin listeriolysin O as the main bacterial factor responsible for disruption of the mitochondrial network and for mitochondrial function modulation. Our work suggests that transient disruption of mitochondrial dynamics and function represents a novel strategy used by pathogenic bacteria to interfere with cellular physiology at the onset of infection, which opens new avenues to study mitochondrial dynamics and function.

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Dynamics of ESCRT protein recruitment during retroviral assembly.

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The assembly of retroviruses is the dependent upon the interaction of various components encoded both by the virus and the host cell. The separation of retroviral particles from host membranes is promoted by the cellular ESCRT (Endosomal Sorting Complex Required for Transport) complexes and associated proteins. The ESCRT complexes and associated proteins mediate other membrane scission reactions, such as multi-vesicular body formation and the terminal stages of cytokinesis. These proteins are believed to be sequentially recruited to the site of membrane scission, and then complexes are disassembled by the ATPase Vps4A. However these events have never been observed in living cells and their dynamics are unknown. Characterizing the relative time course of these molecules is complicated in cells where each virion is in a different state of assembly. To characterize the dynamics of these molecules, we studied the assembly of individual virions. By quantifying the recruitment of several ESCRT and associated proteins during the assembly of two retroviruses, EIAV and HIV-1, we show that Alix progressively accumulated at viral assembly sites, coincident with the accumulation of the major viral structural protein, Gag, and was not recycled after assembly. In contrast, ESCRT-III and Vps4A were only transiently recruited when the accumulation of Gag was complete. These data suggest that the rapid and transient recruitment of proteins that act late in the ESCRT pathway and carry out membrane fission is triggered by prior and progressive accumulation of proteins that bridge viral proteins and the late-acting ESCRT proteins.

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Salmonella enterica and epithelial cells: life in a vacuole or the cytosol?

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Salmonella enterica is an intracellular bacterial pathogen that resides and proliferates within a membrane-bound vacuole in phagocytic and non-phagocytic cells of its mammalian hosts. Although essential to disease, how Salmonella escapes from its intracellular niche and spreads to secondary cells within the same host, or to a new host, is not known. We recently described that a subpopulation of Salmonella are released from the Salmonella-containing vacuole and hyper-replicate in the cytosol of epithelial cells. These bacteria are transcriptionally distinct from intravacuolar Salmonella. They are induced for the invasion-associated type III secretion system and possess flagella; hence, they are primed for invasion. Epithelial cells laden with these cytosolic bacteria undergo inflammatory programmed cell death and are extruded out of the monolayer, releasing invasion-primed and -competent Salmonella into the lumen, allowing for completion of the infectious cycle. To distinguish cytosolic from vacuolar Salmonella, we have developed a drug-based assay that we are using to define bacterial factors that contribute to vacuole lysis and cytosolic replication in epithelial cells.

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Viral interior design: Rewiring the host to generate organelle platforms for replication.

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RNA viruses manipulate multiple host components of the secretory pathway to generate organelles that are specialized for replication and are distinct in protein and lipid composition from the host cell. We show that specific picornaviral proteins promote recruitment of host phosphatidylinositol-4-kinases to membranes, while suppressing recruitment of host membrane coat proteins. This results in the biogenesis of uncoated membrane platforms that are highly enriched in phosphatidylinositol-4-phosphate (PI4P) lipids and contain a specific subset of host endocytic and secretory proteins. We find that the PI4P-rich lipid microenvironment is essential for viral RNA replication in both picorna- and flavivirus families; and that this unique lipid microenvironment may regulate the recruitment and assembly of viral replication machinery including viral RNA polymerases which we show have a specific novel binding site for PI4P lipids. Our findings reveal how RNA viruses can selectively exploit specific elements of the host to form specialized organelles, and identify host phosphatidylinositol-4-kinases and PI4P lipids as key panviral regulators of viral RNA replication and hence potential targets for the development of novel panviral therapeutics.

Minisymposium 29: Collective Cell Behavior and Morphogenesis in Development

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Regulation of collective cell movements and morphogenetic diversity *in vivo*.

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Morphogenetic cell movements are very diverse and many cells travel in groups. We study the similarities and differences between the mechanisms of collective cell migrations and those of

single cells. We would also like to understand how cells self-organize into tissues. We use ovarian development in the fruit fly as our major experimental model. Border cell migration serves as a genetically tractable, in vivo model for collective cell migration. We have identified signals that regulate when, where and which cells migrate and invade. Recent studies suggest that E-cadherin plays diverse and central roles both in organizing the cluster as well as in guidance and movement of the cells, in contrast to the general notion that E-cadherin inhibits cell motility. We have also discovered a novel role for Src in cell motility in vivo. On the other hand, using a photo-activatable form of the small GTPase Rac, we have shown that local activation of Rac in one cell can guide the whole cluster in a new direction. This is very similar to the function of Rac in single cells. In addition to promoting protrusion locally, focal Rac activation inhibits protrusion of other cells in the cluster. We are exploring the mechanism by which activation or inactivation of Rac in one cell affects the behavior of other cells in the cluster. Taken together our results support the hypothesis that morphogenetic diversity arises from combinatorial use of modular mechanical properties including cell-cell and cell-matrix adhesion, myosin-mediated contractility, and Rac-mediated protrusion.

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Actin turnover balances forces between cells during epithelial invagination.

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Embryonic development requires that coordinated cell shape changes collectively deform tissues to generate organs with diverse forms and functions. Cell shape changes result from forces generated by actin networks that are coupled to adhesive complexes. During Drosophila gastrulation, pulsed contraction of an apical actin and myosin II network coupled to adherens junctions drives apical constriction of mesoderm cells, which is important for epithelial invagination. While the role of actin turnover is well established for individual cell migration, the importance of actin turnover for the coordinated movement of an epithelial sheet is not known. To examine the importance of actin turnover, we titrated the rate of actin polymerization by injecting different concentrations of cytochalasin D, which blocks barbed end growth, and imaged cell and cytoskeletal dynamics in live embryos. We observed a transition from a general disruption of contractility in all cells with high doses of cytochalasin D to a mesoderm specific disruption in cell-cell connections at low doses. At low cytochalasin D neighboring actomyosin networks continually lost and reformed connections, resulting in an unbalanced ‘tug-of-war’ between cells of the mesoderm. A similar phenotype was observed in mutants for the formin, dia, suggesting that formin mediated actin polymerization is required to maintain cell-cell connections. Live imaging of F-actin in wild-type embryos demonstrates that pulsed contractions generate transient holes in the actin meshwork across the apical surface. We propose that rapid actin turnover is required to fill holes generated by network contraction in order to balance forces across adhesive contacts.

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Angular morphomechanics in the establishment of multicellular architecture.

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We report a novel type of human cell motility in which cells undergo coherent angular motion to assemble into polarized multicellular spherical structures (acini) when placed in a 3D basement membrane surrogate gel. We visualize the complete evolution from the single cell to an acinus. We address a fundamental question: what are the physical laws that govern the geometry of acinar structures? We link the functional relevance of this distinct coherent angular motion (CAMo) to the ability to attain the resultant architecture, and determine the relevance of adhesion and tissue polarity to the outcome. CAMo is conserved from primary human cells to

established breast cell lines where the final realized geometry is spherical. Cancer cells do not display CAMo motility but random motility. Upon 'phenotypic reversion' of malignant cells, both CAMo motility and correct architecture are restored.

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Self-organizing and stochastic behaviors during the regeneration of a large population of hair stem cells.

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The hair follicle undergoes cyclic degeneration and regeneration cycle throughout life. The length of growing (anagen) and resting phase (telogen) can determine the hair length. Long resting phase mean shorter or no hair, i.e., alopecia. We have developed ways to visualize hair stem cell activation over entire skin in living mice. With this, we found cyclic BMP signaling from subcutaneous adipose layer regulates stem cell activation during hair regeneration (Plikus et al. 2008, Nature. 451:340-344. Cyclic dermal BMP signaling regulates stem cell activation during hair regeneration). More molecular analyses showed wnt serves as activators and hair growth patterns are governed by simple rules based on a pair of activator/inhibitor signaling. Regeneration in a population of hair follicles spreads like chain reaction, forming diverse wave patterns (Plikus et al. 2011, Science 332:586-589. Self organizing and stochastic behaviors during the regeneration of hair stem cells). Mathematical modeling reveals unexpected self-organizing and stochastic nature of this novel stem cell behavior, which emerge only at higher organ population level, allowing hair regeneration to become a very adaptable trait. These variations are seen among different animal species with different needs for hairs: robust spreading in rabbits, gradual wave spreading in mice, and random growth with loss of coupling among follicles in human skin. The hair wave can also vary under different physiological conditions of a same individual, such as in puberty, pregnancy and aging. We show that change in macro-environment is one major factor that contributes to alopecia in aging mice. We have developed ways to modify macro-environment for hair follicles, and hence can stimulate many more resting phase hair follicles to re-enter growing phase.

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Par-1 Controls Non-Muscle Myosin II Activity and Dynamics to Regulate Collective Cell Migration.

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Many cells migrate in collective groups during tissue morphogenesis, wound healing and tumor invasion and metastasis. In single migrating cells, localized actomyosin contraction couples with actin polymerization and cell-matrix adhesion to regulate cell protrusions and retract trailing cell edges. In contrast, we have only a limited understanding of mechanisms that coordinate actomyosin dynamics in collective cell migration. We study the migration of *Drosophila* border cells, which move as a cohesive group of 6-10 epithelial-derived cells during late oogenesis. We previously observed that mutants of the cell polarity protein Par-1 (MARK), a serine-threonine kinase, result in defective border cell delamination and migration. We now show that these defects are caused by perturbations in cytoskeletal dynamics due to disruption of a previously unknown signaling pathway between Par-1 and non-muscle myosin-II (Myo-II). Using live time-lapse imaging, we show that Myo-II is required for two critical features of border cell migration: initial detachment of the border cells from the surrounding epithelium, and extension of cell

protrusions of normal length and direction. We identified a robust genetic interaction between the myosin regulatory light chain (MRLC) homolog *spaghetti squash* (*sqh*) and *par-1* and, remarkably, found that active Sqh/MRLC strongly suppresses par-1-dependent mutant phenotypes. We show that Par-1 regulates dynamic subcellular localization of Sqh/MRLC in live border cells, and, furthermore, that Par-1 regulates the activity of Myo-II. We have discovered that Par-1 phosphorylates and inactivates myosin phosphatase, thus promoting phosphorylation of Sqh/MRLC and increasing Myo-II activity. Finally, while myosin phosphatase is distributed uniformly in the border cell cluster, we find that Par-1 localizes to the cluster rear along with active Myo-II; in the absence of Par-1, spatially distinct active Myo-II is lost. Our study reveals that Par-1 kinase is a principal regulator of polarized Myo-II activity within the border cell cluster through localized inhibition of myosin phosphatase. Polarity proteins such as Par-1, which intrinsically localize, can thus directly modulate the spatiotemporal actomyosin dynamics required for proper collective cell migration.

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Spatiotemporal regulation of somitogenesis by the oscillator networks of the segmentation clock.

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A bilateral pair of somites forms periodically by segmentation of the anterior ends of the presomitic mesoderm (PSM). This periodic event in time and space is regulated by a biological clock called the segmentation clock, which involves cyclic gene expression. In mice, Hes7 expression oscillates by negative feedback, and mathematical models have been used to generate and test hypotheses to aide elucidation of the role of negative feedback in regulating oscillatory expression. Hes7 induces coupled oscillations of Notch and Fgf signaling in the posterior PSM, while Notch and Fgf signaling cooperatively regulate Hes7 oscillation, indicating that Hes7 and Notch and Fgf signaling form the oscillator networks. Oscillations in Notch signaling generate traveling waves, thereby periodically segregating a group of synchronized cells. Notch signaling activates, but Fgf signaling represses, expression of the master regulator for somitogenesis Mesp2, and coupled oscillations in Notch and Fgf signaling dissociate in the anterior PSM, which allows Notch signaling-induced synchronized cells to express Mesp2 after these cells are freed from Fgf signaling. These results indicate that Notch signaling periodically defines the prospective somite region (spatial periodicity), while Fgf signaling regulates the pace of segmentation (temporal periodicity).

Minisymposium 30: Organelle Biogenesis and Autophagy

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Molecular organization and function of the Golgi stacks.

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The unique structure of the Golgi in almost all eukaryotic cells is a stack of flattened cisternal membranes, but how this structure is formed at the molecular level and why its formation is important for cellular functions remain elusive. We have developed an in vitro system to reconstitute the process of mitotic Golgi disassembly and post-mitotic reassembly in mammalian cells, which allowed us to reveal the molecular mechanism of the Golgi biogenesis during cell division. Mitotic Golgi fragmentation involves membrane vesiculation coupled with cisternal unstacking; post-mitotic Golgi reassembly is mediated by membrane fusion to form single cisternae and stack formation. Stack formation directly involves the Golgi stacking protein

GRASP65 and GRASP55, which play complementary and essential roles in Golgi cisternal stacking by forming mitotically regulated trans-oligomers. By depletion of GRASP65/55 we are able to manipulate Golgi stack formation and thus determine the biological significance of stacking. We demonstrate that Golgi cisternal unstacking stimulates COPI vesicle budding and thus enhances protein transport. Golgi fragmentation, however, impairs protein sorting and alters the glycosylation of cell surface proteins and reduces cell adhesion. Subsequently, cell adhesion and migration were reduced when the Golgi was unstacked, which was probably the result of reduced ¦Á5/¦Â1 integrin expression. Furthermore, total protein synthesis and the proliferation of cells with unstacked Golgi were enhanced. Inhibition of Golgi disassembly at the onset of mitosis also affects cell cycle progression. We propose that Golgi stack formation is a flux regulator for protein trafficking and thereby functions as a quality control mechanism for protein sorting and modification. Structural and functional Golgi defects in disease models are explored in this study.

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ER tubules mark sites of mitochondrial division.

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Mitochondrial structure and distribution are regulated by division and fusion events. Mitochondrial division is regulated by Dnm1/Drp1, a dynamin-related protein that forms helices around mitochondria to mediate fission. Little is known about what determines sites of mitochondrial fission within the mitochondrial network. Given that ER and mitochondria exhibit tightly coupled dynamics and have extensive contacts, we tested whether ER plays a role in mitochondrial division. We show that mitochondrial division occurs at positions where ER tubules contact mitochondria and mediate constriction prior to Drp1 recruitment. These data demonstrate that ER tubules play an active role in defining the position of mitochondrial division sites.

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Coordinating Mitochondrial Biogenesis and Redistribution to Achieve Proper Inheritance of Mitochondrial Content in Budding Yeast.

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Mitochondria must grow with the growing cell to ensure proper cellular physiology and inheritance upon division. But it is unknown how mitochondrial content scales with cell size during asymmetric cell growth and division. Is the same mitochondrial to cell size ratio maintained uniformly throughout the growing cell to produce identical mother and daughter cells? Or is there an asymmetry in the inheritance of mitochondrial content? We previously developed a novel computational method that quantifies mitochondrial networks as 3D mathematical graphs to measure the physical size of the mitochondrial network. Using this method, we found mitochondrial inheritance to be very asymmetric. Regardless of mother size or mitochondrial content, all buds attained the same average mitochondrial to cell size ratio with the same kinetics during budding. In contrast, aging mothers experienced a continued decrease in their mitochondrial to cell size ratio over successive generations. The proper mitochondrial volume ratio in the bud must is generated by a combination of net biogenesis (sum of new synthesis and turnover) in each, and net redistribution (sum of all movement and retention of tubules) between, mother and bud compartments. We calculated the relative contributions of net biogenesis and redistribution to the mitochondrial content in the bud. We found that redistribution dominated during the first half of budding and biogenesis in the bud took over

during the second half. A final 60-75% of the mitochondria inherited by the buds originated in their mothers. Mitochondrial biogenesis in the mothers could not keep up with the required redistribution into their buds. In the youngest, first generation mothers decreased redistribution was compensated for by increased biogenesis in their buds to achieve the same volume ratio as the rest of the population. We delayed or sped up mitochondrial inheritance with deletion or overexpression of Ypt11p and Mmr1p, representing two of the three mitochondrial inheritance pathways. In both cases the kinetics of mitochondrial inheritance were significantly altered but buds still reached the proper mitochondrial volume ratio in time for division. We conclude that cells actively regulate the mitochondrial volume ratio in their buds by regulating both redistribution and biogenesis to ensure proper inheritance of mitochondrial content.

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mTORC1 Senses Amino Acids Through a Lysosomal Inside-Out Mechanism that Requires the Vacuolar H+-ATPase.

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The mTOR Complex 1 (mTORC1) kinase is a master growth regulator that senses amino acids through a poorly understood mechanism. A key event is the amino acid-induced activation of the Rag GTPases, which promotes the translocation of mTORC1 to the lysosomal surface, the site of mTORC1 activation. A central question is where in the cell amino acids are sensed and how their sensing leads to Rag activation. Here, we identify the vacuolar H+-ATPase (v-ATPase) as necessary for amino acid signaling to mTORC1. The v-ATPase functions upstream of the Rag GTPases and promotes amino acid-mediated recruitment of mTORC1 to the lysosomal surface. Using an assay that recapitulates amino acid signaling to mTORC1 in a cell-free system, we show that the catalytic movement of the v-ATPase, but not the lysosomal pH gradient, is necessary for amino acids to regulate the Rag GTPases and promote mTORC1 translocation. Moreover, using this system we implicate the lysosomal lumen as the site where amino acid signaling initiates. These results identify the v-ATPase as a new component of the mTOR pathway and delineate a lysosome-associated machinery for amino acid sensing.

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The interaction of MiT/TFE transcription factors with lysosomes contributes to regulation of lysosomal homeostasis.

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The MiT/TFE family of transcription factors (MITF, TFEB, TFE3) regulates expression of many genes encoding proteins involved in autophagy and the biogenesis of lysosomes and lysosome-related organelles. In this study, we show that the regulation of MiT/TFE transcription factor abundance in the nucleus is tightly linked to autophagy-inducing stimuli and overall lysosomal status and that this regulation depends on a physical interaction of these transcription factors with lysosomes via a conserved region at their amino terminus. This lysosomal recruitment promotes MiT/TFE phosphorylation within a motif that confers a phosphorylation-dependent interaction with 14-3-3 proteins. This interaction retains MiT/TFE proteins mostly in the cytoplasm under basal conditions. In response to specific autophagy-inducing stimuli and/or perturbation of lysosomal function, phosphorylation of the14-3-3 binding site is attenuated, the interaction with 14-3-3 proteins is lost and the MiT/TFE proteins accumulate in the nucleus. Building on the previously established ability of these transcription factors to up-regulate the expression of genes within the autophagy-lysosomal pathway, our data suggests a novel homeostatic feedback mechanism that couples demand for lysosomal activity to the

transcriptional control of the expression of genes encoding proteins critical for the function of this organelle.

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The PX-BAR protein SNX18 is required for autophagy.

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Autophagy is a catabolic pathway targeting cytoplasmic material for lysosomal degradation, thereby protecting cells from accumulation of toxic components and enabling cells to survive scarce nutrient supplies. Macroautophagy is characterized by the formation of double-membraned vesicles, but the membrane remodeling events required for formation of autophagic vesicles are still not completely understood. However, the class III PI3K/Vps34 complex and phosphoinositol-3-phosphate are of core importance to induction of autophagy. Since PX domain proteins are known to bind PI3P and other phosphoinositides and mediate membrane remodeling and trafficking events, we performed an imaging-based siRNA screen targeting all human PX domain proteins using GFP-LC3 autophagosome formation as a read-out and found depletion of the PX-BAR protein SNX18 to strongly inhibit autophagosome formation. Consequently, overexpression of SNX18 increases LC3 lipidation and GFP-LC3 spot formation and we demonstrate that membrane binding of SNX18 is required for efficient autophagosome formation. Moreover, SNX18 colocalizes with and interacts with the autophagy-associated proteins LC3 and TBK1. In conclusion, our study identified the PX-BAR protein SNX18 to be involved in membrane events required for autophagosome formation.

Minisymposium 31: Signal Transduction Networks

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Exploring the design principles of signaling circuits in space and time.

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Living cells are able to carry out diverse information processing tasks and to control complex spatiotemporal behaviors using molecular signal transduction networks. To understand the design principles of such networks, we are complementing traditional analytical approaches with synthetic approaches of trying to build novel or systematically modified networks that can carry out target functional behaviors. Using modular components that control phosphorylation and GTPase signaling, we are exploring the construction of synthetic spatial self-organizing circuits, as well as circuits with complex dynamical responses. We are also using parallel enumeration based computational approaches to explore the space of networks that can robustly execute such tasks. We hope to gain a deeper understanding between network structure and function through such studies. We also hope to gain a better understanding of the evolutionary landscape through which such behaviors emerge.

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Spatio-temporal control of intracellular ROS concentration in PDGF signaling revealed by single Eu^{3+}-doped nanoparticle imaging.

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For many cell functions, notably those requiring an asymmetric response, such as directed migration, spatio-temporal organization of signaling pathways is important for the cell response regulation. PDGF (Platelet Derived Growth Factor) induces migration in numerous contexts, such as reparation of vascular lesions or metastasis formation, inducing ROS (Reactive Oxygen Species) production as second messenger. The potential lethality of concentrated ROS and the importance of intracellular organization for migration require a tight control of their concentration. However, the dynamics of ROS production and organization is so far mostly unknown. By imaging single Eu^{3+}-doped nanoparticles[1], we probed the intracellular ROS response with high temporal and spatial resolution. We thus measured the absolute ROS concentration in normal or tumoral cells and revealed specific temporal patterns of ROS production under PDGF stimulation. We measured an integration time of several minutes required for the ROS response formation. This response formation is shorter for tumoral cells. We satisfyingly explained this fact by modeling the diffusion-limited dimerization of PDGFRs. This may constitute a temporal filtering mechanism for preventing cell responses to transient signals and its impairment in tumoral cells could be of great physiological relevance for the metastatic transition. We moreover quantitatively measured a transactivation of EGFR by PDGF stimulation and revealed its role in the dynamics of the cell response. By using a microfluidic system, we furthermore apply spatially controlled PDGF stimulation and displayed the maintenance of asymmetric ROS concentration in the cell under a PDGF gradient. This likely relies on a balance between ROS diffusion and degradation. This balance controls the local ROS concentration and thus the cell response. Altogether, our results reveal the tight regulation of the ROS spatio-temporal organization by the cell, which illustrates how the spatio-temporal control of transduction pathways is crucial for the buildup of the cell response.

[1]Casanova, Bouzigues et al. Nat. Nanotech. **4**, 581 (2009)

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Dynamics: SH2 domain binding/unbinding on EGFR.

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Epidermal growth factor (EGF) stimulation triggers downstream signaling cascades through binding of Src homology 2 (SH2) domain containing proteins to the phosphorylated tyrosine (p-Tyr) residues of the EGF receptor (EGFR). This signaling plays a crucial role in cell proliferation, metastasis, survival, tumorigenesis, etc. However the dynamics of bindings of SH2 domain to p-Tyr on the EGFR are not well quantified. Applying novel single-particle-tracking photoactivated localization microscopy (spt-PALM) techniques combined with the total internal reflection (TIR) optics, we quantified kinetic parameters for following tyrosine phosphorylation rate and dissociation rate between EGFR and tdEos- fused SH2 domains of several signaling molecules (Grb2, Plc□1, Shp2, Src, Nck1, Crk, Jak2, ShcA, CblA) in live cell. We mapped the progression of the EGFR-SH2 complexes over the EGF induction time and measured binding kinetics. Our data shows each SH2 domain produced a different binding curve as a function of time after EGF stimulation, suggesting phosphorylation rates are different for different tyrosine residues on EGFR and each SH2 domain has binding specificity. Analyzing multiple exponential distributions of an individual binding time of SH2 domain, we obtained the dissociation rate from

0.05 to 10 S^{-1}. This finding implies that there exist multiple states of EGFR-SH2 complex such as transient, monomer or clustering binding states. The clustering of EGFR-SH2 complex developed as a function of EGF stimulation time was confirmed by tracking approximately 1-5 $x10^4$ individual complexes with the time-course PALM imaging. Interestingly, a negative correlation between binding time of SH2 domain on EGFR and their mobility was observed. This result, along with PALM analysis, suggests that downstream signaling may require stable clustering of EGFR-SH2 complexes. In conclusion, our analyses not only confirmed binding specificity of SH2 domains, but also suggested that each specific p-Tyr residue on the receptor has a specific phosphorylation rate.

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Targeted Proteomics Analysis of Signalling Dynamics: a View of the Adaptor Protein GRB2 mediated events.

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Signalling pathways are commonly organized through inducible protein-protein interactions, mediated by adaptor proteins that link activated receptors to cytoplasmic effectors. However, we have little quantitative data regarding the kinetics with which such networks assemble and dissolve to generate a specific cellular response. We have identified 90 proteins and 36 phosphorylation sites associated with the GRB2 adaptor protein in human cells. We have found that GRB2 nucleates a remarkably diverse set of protein complexes, involved in multiple aspects of cellular function. To comprehensively and quantitatively investigate changes in GRB2-based protein interactions in growth factor stimulated cells, we have designed a targeted mass spectrometry method, AP-SRM (affinity purification-selected reaction monitoring). The data define context-specific and time-dependent networks that form around GRB2 following stimulation, and reveal core and growth factor-selective interaction subsets. These results illustrate the reliability of AP-SRM in the quantitative analysis of dynamic signalling networks. They also suggest that capturing a key hub protein and dissecting its interactions by SRM is an approach that can be broadly applied to quantify signalling dynamics.

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KLF5 acetylation, which is induced by TGFbeta but interrupted by oncogenic signaling, underlies the dual functions of TGFbeta in gene regulation and cell proliferation control.

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During epithelial homeostasis, stem cells divide to produce progenitor cells, which not only proliferate to generate the cell mass but also respond to cellular signaling to transition from a proliferative state to a differentiation state. Such a transition involves functional alterations of transcriptional factors, yet the underlying molecular mechanisms are poorly understood. In addressing this question, we found that the pro-proliferative factor KLF5 becomes anti-proliferative upon TGFbeta-mediated acetylation in an in vitro model of epithelial homeostasis. KLF5 is not only essential for cell proliferation, it is also indispensable for TGFbeta-induced anti-proliferation in epithelial cells. Without TGFbeta, KLF5 inhibits p15 expression and induces Myc expression, but when TGF-¦Â is present, KLF5 becomes a cofactor of TGFbeta to induce p15 and suppress Myc expression in the same cells. Mechanistically, TGFbeta recruits acetylase p300 to acetylate KLF5, and acetylation in turn induces the assembly of KLF5 with other TGFbeta transcriptinoal effectors including Smad2-4 and Miz-1 and their binding to p15 and Myc promoters, resulting in the reversal of KLF5 function. Failure in KLF5 acetylation prevents TGFbeta-assemblled p300-KLF5-Smads complex on gene promoters, reversing the function of

TGFbeta in gene regulation and proliferation control. It was reported that TGFbeta and KLF5 have dual roles in tumoirigenesis, and that Ras oncogenic signaling converts TGFbeta from a tumor suppressor to a tumor promoter. We therefore also examined whether Ras signaling modulates TGFbeta function by interrupting TGFbeta-induced KLF5 acetylation and the assembly of the p300-KLF5-Smads transcriptional complex. We found that Ras inhibited TGFbeta-induced KLF5 acetylation and interfered with TGFbeta in p15 induction and Myc repression. In addition, TGFbeta-induced Smad3 phosphorylation at the C-terminal region was necessary for TGFbeta to induce KLF5 acetylation, and Ras interrupted this phosphorylation. Ras signaling further interrupted the interactions among p300, KLF5 and Smads, as well as the binding of the p300-KLF5-Smads complex onto the TGFbeta-responsive promoter elements for both p15 and Myc. Our findings suggest that 1) TGFbeta-induced KLF5 acetylation is a "switch" that that turns on differentiation in proliferating progenitor cells; 2) deacetylation of KLF5 underlies the reversal of TGFbeta function in gene regulation and cell proliferation control; and 3) oncogenic signaling reverses TGFbeta function by interrupting KLF5 acetylation.

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Cross-talk and information transfer in mammalian and bacterial signaling.

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In both mammalian cells and bacteria, simple phosphorylation circuits play a very important role in cellular function. Bacteria have hundreds of two-component signaling systems that involve phosphotransfer between a receptor kinase and a response regulator. In mammalian cells a similar pathway is the crucial TGF-beta signaling pathway, where extracellular levels of TGF-beta family ligands lead to activation of cell surface receptors that phosphorylate Smad proteins, which in turn activate many genes. In TGF-beta signaling the multiplicity of external ligands begs the question as to how cells are able to distinguish signals coming from different extra-cellular ligands, but transduced through a small set of Smads. Here we use information theory with stochastic simulations of simple networks to address this question. We find that when signals are transduced through the same Smad, the cell cannot distinguish between different levels of the external ligands. Increasing the number of Smads from one to two significantly improves information transmission as well as the ability to discriminate between different external ligands. Surprisingly, both total information transmitted through the channel and the capacity to discriminate between the external ligands are quite insensitive to the cross-talk between the two Smads as long as they are not nearly identical. In sharp contrast, we find that two-component systems in bacteria show a significantly sharper decline in information transfer in the presence of cross-talk. This suggests that mammalian signal transduction can tolerate a high amount of cross-talk. This may have played a role in the evolution of new functionalities from small mutations in signaling pathways and allowed for the development of cross-regulation. Insensitivity to cross-talk also could increase robustness due to redundancy in signaling pathways. On the other hand, bacterial two component systems are much less robust against cross-talk which may provide an explanation for the lack of cross-regulation in most two component systems.

Symposium 7: Design Principles of Cells and Tissues

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Inside of the cell, meet the extracellular universe: merging tissue engineering and systems biology.

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Tissue engineering is the process of creating functional 3D tissues using cells combined with scaffolds or devices that facilitate cell growth, organization and differentiation. At its inception, the major vision of the field was on building clinical therapies to replace human heart, liver, cartilage and other tissues, by combining cells with polymer scaffolds. The vision for clinical therapies has arguably evolved to include more emphasis on regenerative medicine approaches that harness endogenous pathways. Further, an important new direction has emerged – creation of models of human tissues that capture 3D physiology for use in applications ranging from drug development to studies of disease pathophysiology. For both application areas, an integration of design principles from molecular to macroscopic length scales is required, using (or developing) mathematical models of cell behavior. For example, at the molecular level, presenting adhesion ligands in a clustered compared to random format enhances cell adhesion and influences migration in a manner predicted by a mathematical model of migration; at a macroscopic level, the density of cells in an implanted scaffold is limited by nutrient diffusion, and this constraint influences the strategies for cell transplantation therapies for tissue defects such as bone. An emerging frontier is in linking the networks of extracellular cues that stimulate cells from the microenvironment to the intracellular signaling networks that dictate downstream phenotypic responses. Comprehensive analysis of intracellular protein kinase signaling networks, coupled with mathematical analysis, have begun to yield rules for cell fate decisions as a function of cell state and the temporal evolution of cell state. These systems biology approaches offer new insight into design of extracellular microenvironments to influence cell fate. At the same time, incorporation of macroscopic design principles through design of 3D environments enhances investigation of cell fate, as exemplified by use of tissue-engineered systems for predicting liver toxicology in drug development.

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The Flagellar Length Control System.

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One of the most fundamental engineering problems faced by a cell is how to control the size of its organelles. Cilia and flagella of eukaryotic cells provide an excellent model system in which to explore the design principles of size control systems, due to their simple one-dimensional geometry and easy visibility. Moreover, since flagella are motile structures, their size can be directly related to their function and to the evolutionary fitness of the organism. We will present a simple model for length control based on quantitative measurements of transport within flagella, and show tests of this model using length-altering mutations and analysis of intraflagellar transport and length fluctuations in living cells of the unicellular green alga Chlamydomonas reinhardtii.

SUNDAY, DECEMBER 4

Actin and Actin-Associated Proteins I

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Distributed deconvolution analysis of fluorescence images reveals a regulatory mechanism for thin filament lengths in human skeletal muscle.

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Skeletal muscle is striated and consists of arrays of repeating sarcomere units, which contain interdigitating thin (actin) filaments and thick (myosin) filaments. The lengths and regulation of each of these filament systems are important since the amount of overlap between the thin and thick filaments dictates the force that can be produced by the muscle. This study aims to determine if human thin filament lengths are regulated by nebulin via a ruler mechanism, as is commonly thought, or by some other mechanism, potentially involving tropomodulin (Tmod). Pectoralis major and deltoid muscle biopsies were obtained from human patients, and cryosections were immunostained using Tmod, nebulin, and α-actinin antibodies as well as phalloidin and imaged by confocal fluorescence microscopy. Line scans of myofibrils from images of the samples were analyzed using distributed deconvolution to accurately determine lengths associated with each thin filament component. Across all patients and muscle biopsies, it was found that Tmod and phallodin lengths were comparable (Tmod: 1.27±0.09 μm, n=1091 myofibrils; phalloidin: 1.24±0.06 μm, n=1146 myofibrils), confirming that Tmod localizes to the pointed ends of the thin filaments. However, Tmod lengths were found to be significantly greater than the lengths associated with the nebulin N-terminus (0.90±0.07 μm, n=1214 myofibrils) for all samples, with an average difference from the Tmod lengths of 0.37±0.11 μm, suggesting the existence of a nebulin-free pointed-end extension. Based on these findings, nebulin cannot be a ruler that dictates human thin filament lengths. Rather, human thin filaments extend past the nebulin N-terminus and up to, but not past, Tmod. These data complement previous studies showing that thin filament lengths are not regulated via a nebulin ruler mechanism in mouse and rabbit muscles. The existence of nebulin-free pointed-end extensions in human thin filaments suggests a unifying mechanism for thin filament length regulation across mammalian species.

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Heterogeneity of thin filament lengths in human skeletal muscles: implications for biomechanical function.

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During skeletal muscle contraction, force is produced when actin (thin) filaments slide past myosin (thick) filaments, resulting in sarcomere shortening. The degree of force production is proportional to the amount of overlap between the thin and thick filaments, which, in turn, depends on their lengths. Both thin and thick filament lengths are precisely regulated and uniform within a muscle fiber. While thick filament lengths are essentially constant across muscles and species (~1.65 μm), thin filament lengths are highly variable both across species and across muscles of a single species, depending on the dynamic activities of specific combinations of tropomodulin (Tmod), tropomyosin, and nebulin isoforms. However, thin filament length heterogeneity has not been assessed in humans. Here, we used a high-resolution immunofluorescence and image analysis technique (distributed deconvolution) to

directly test the hypothesis that human thin filament lengths vary across muscles and individuals. Using deltoid and pectoralis major muscle biopsies from patients undergoing shoulder replacement surgery, we identified substantial intra- and inter-patient thin filament length variability. Thin filament lengths ranged from 1.19±0.08 μm to 1.37±0.04 μm, based on Tmod localization with respect to the Z-line, corresponding to optimum sarcomere lengths of 2.38-2.58 μm to 2.74-2.94 μm, respectively. Tmod localized 0.28 μm to 0.47 μm further from the Z-line than the N-terminus of nebulin, confirming that human thin filaments have nebulin-free pointed-end extensions whose lengths are modulated by Tmod and comprise up to 34% of total thin filament length. Thin filament length was negatively correlated with the percentage of type-2X myosin heavy chain within the biopsy, establishing the existence of a relationship between thin filament lengths and fiber types in human muscle. Together, these data challenge the widely held assumption that human thin filament lengths are constant. Our results also have broad relevance to musculoskeletal modeling, surgical reattachment of muscles, and orthopaedic rehabilitation.

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The Limch1, a novel microfilament associated protein, associated with to the sarcomeric type stress fiber.

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Limch1 as a novel protein engaged in cell migration was identified in systematic screening of misexpressing proteins in border cell migration in Drosophila oogenesis. This novel protein comprises an N-terminal calponin homologous (CH) domain, and a C-terminal LIM domain flanking its central undefined coiled coil stretch. Immunofluorescence microscopy investigation in subcellular localization indicated Limch1 mainly distributes on stress fibers. The CH domain and LIM domain of Limch1 both are capable of localizing the Limch1 to stress fibers by domain characterization with overexpression of GFP tagged CH domain or LIM domain either in the U2OS cells, in contrast, the GFP fused central coiled coil stretch randomly mislocalized in cytoplasmics. Indeed, the Limch1 is associated with the sarcomeric type stress fiber (SSF). The association of Limch1 with SSF was dissected by a costaining with α-actinin as a Z-disc marker as in the sarcomere of skeletal muscle myofibrils and the SSF in non-muscle cells. Subsequently, the ROCK inhibitor administration was performed to examine the biological role of Limch1 implicated in physiological function of SSF. While the inhabiting ROCK activity leads to Limch1 disassociated from the SSF, it suggested Limch1 might be a downstream effector of ROCK signaling. The ROCK is, however, a crucial regulator involved in SSF assembly, and mediated actomyosin contraction. The treatment of ROCK inhibitor caused Limch1 detached from SSF may imply that Limch1 likely plays an important role in the contractile SSF assembly and function. Furthermore, SSF contraction is an essential force generator in migratory cell. Take together, the misexpression of Limch1 in Drosophila delayed the border cell migration during oogenesis, it hinted that association of Limch1 with SSF may manage contractile SSF assembly, and operate SSF dependent force generation for cell migration.

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A novel actin filament class that regulates glucose uptake and GLUT4 trafficking.

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A primary defect in Type 2 diabetes is alterations to glucose uptake in skeletal muscle and adipose tissue. Insulin-stimulated glucose uptake requires the trafficking of GLUT4-containing vesicles from intracellular stores to the cell surface. We have identified a novel population of actin filaments defined by the cytoskeletal tropomyosin (Tm) isoform Tm5NM1. Analysis of Tm5NM1 transgenic (Tg) mice suggests these filaments play a role in glucose uptake. In Tm5NM1 Tg mice, whole body glucose clearance and insulin-stimulated glucose uptake into white adipose tissue (WAT), skeletal muscle and heart was increased. This was specific to Tm5NM1, as glucose clearance was unaltered in mice expressing an alternative Tm, Tm3. Gene expression profiling (Illumina microarray), quantitative RT-PCR and Western blot analysis of WAT from the Tm5NM1 Tg mice detected an increase in genes involved in actin filament turnover and GLUT4 trafficking, including myosin motors (Myolc, $P<0.05$) and components of the exocyst complex (Sec8, $P<0.005$). In keeping with Tm5NM1's reported role in stabilising actin filaments, there was a 30% increase ($P=0.019$) in filamentous actin (detected by phalloidin staining) in Tg WAT. In 3T3L1 adipocytes, insulin-stimulation (30 min.) resulted in a shift in Tm5NM1 localisation to the plasma membrane, consistent with a role in GLUT4 trafficking. This effect was specific to Tm5NM1, as insulin had little impact on the localisation of other Tm isoforms. Finally, in 3T3-L1 adipocytes stably expressing Tm5NM1, there was an increase in insulin-stimulated GLUT4 movement to the plasma membrane compared to vector control cells. We propose that Tm5NM1 actin filaments promoterecruitment of GLUT4 trafficking machinery that in turn results in enhanced insulin-stimulated GLUT4 translocation to the plasma membrane and glucose uptake.

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Progression of hepatitis C virus infection to hepatocellular carcinoma is associated with increased expression and co-localization of filamin A and angiogenic markers.

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Background: Around 80% of hepatitis C virus (HCV) infected patients develop chronic HCV infection. HCV-infected patients have a high risk for developing HCC but mechanisms leading to the increased risk for liver cancer are still unclear. Angiogenesis, a physiological process involving the formation of new capillaries from pre-existing vessels, plays a pivotal role in majority of malignancies favoring growth and metastasis of several types of cancer including HCC. Our recent study showed an increase in expression of filamin A (fila), an actin-binding protein in HCV expressing cell lines compared to parental cell line. Fila is a regulator of R-ras which is a precursor of RAF/MEK/ERK pathway, is one of the main mediators of angiogenesis. The objective of this study was to investigate whether increased liver angiogenesis could be a predictor of HCC occurrence in patients with HCV-related cirrhosis. Furthermore we determined the association between fila and angiogenic markers in HCV induced cirrhosis and progression

to HCC. Methods: Expression profile and co-localization of fila and angiogenic markers in livers of human subjects with HCV infection, HCV with HCC, and HCC VS. control was determined. HCV replicon cell (Con1) and parental cell (Huh7.5) lines were treated with either IFN-a or sorafenib (soraf), an antitumor drug targeting towards VEGF receptors (R2 and R3) and c-Raf or in combination for 24 hours and 48 hours to asses the effect on fila and HCV proteins. Results: The result showed higher expression of fila, VEGF, R2 and R3 with the progression of the disease from HCV to HCC. Cells treated with either IFN-α or soraf showed a dose and time-dependent decrease of fila and HCV proteins. The combination treatment showed synergistic effects on fila. Conclusions: A positive association and co-localization was found between higher expression of fila and angiogenic markers with progression of HCV infection to HCC in both human subjects and cell lines. This study suggests that the interaction between fila and angiogenic markers in hepatocytes may contribute to the progression of the HCV to HCC and can be used as a potential target for development of new therapy in the treatment of HCV with HCC.

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The Microtubule Plus End Tracking Protein EB1 Binds to Actin and Promotes Actin Polymerization.

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Coordination between the actin and microtubule cytoskeletons is vital for complex processes such as cell division and multicellular development, but the mechanisms of this cross-talk remain mysterious. We show that the microtubule plus-end tracking protein (+TIP) EB1 binds to actin filaments directly, and it promotes actin polymerization both in vitro and in vivo. EB1 also colocalizes with actin under certain conditions in a variety of cell types. EB1 plays a pivotal role in the MT cytoskeleton because it the structural core of the +TIP network, a web of interacting proteins that are master regulators of MT dynamics. The observation that EB1 binds to actin suggests that EB1 plays a direct and perhaps central role in the process of actin-MT coordination.

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Filamin is required for maintenance of F-actin and calcium signaling in *C. elegans.*

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We are using *C. elegans* as a model system to study how cells respond to mechanical forces *in vivo*. The spermatheca—a simple myoepithelial tube—experiences dramatic stretching forces caused by oocytes during ovulation. We hypothesize that the stretching of the spermatheca initiates a signaling cascade to release the oocyte from the spermatheca into the uterus. We identified filamin, a mechanosensitive scaffold protein, as being required for the release of oocytes from the spermatheca. The *C. elegans* filamin ortholog, FLN-1, has a well-conserved overall structure, including an actin-binding domain, and a series of 20 immunoglobulin-like repeats. FLN-1 is expressed in the somatic gonad, and colocalizes with F-actin. Analysis of a deletion allele indicates filamin is required to maintain the actin cytoskeleton in the spermatheca and uterus. Filamin-deficient animals accumulate embryos in the spermatheca, and consequently lay damaged eggs and exhibit reduced brood sizes. PLC-1/phospholipase C is also required for the exit of embryos from the spermatheca, and analysis of double mutant animals suggests that PLC-1 and FLN-1 act in the same pathway. Because PLC-1 is thought to

be upstream of intracellular calcium release, we used GCaMP—a genetically encoded calcium indicator—to image calcium during ovulation and spermathecal transit. Using worms expressing GCaMP we show that entry of an oocyte into the spermatheca initiates a distinctive series of autonomous calcium transients, which likely result in spermathecal constriction that propels the fertilized oocyte into the uterus. Loss of filamin results in drastically delayed onset of calcium signaling, followed by abnormal calcium oscillations. Importantly, mutations that enhance IP_3 signaling increased Ca^{2+} signaling and partially rescued the filamin defect. As expected, loss of PLC-1 abolished the calcium transients. The calcium pulses likely modulate actomyosin contraction to constrict the spermatheca, pushing the oocyte into the uterus. Given the effect on calcium signaling and the known mechanosensory role of filamin, we hypothesize that filamin is required in the spermatheca to respond to increased tissue tension, and to initiate calcium signaling. Understanding how cells sense and respond to mechanical forces has implications for development, cancer metastasis, and normal organ function.

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The distinct roles of nucleo-cytoskeleton connections in three-dimensional cell motility.

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The role of actin filament organization in motility on conventional flat substrates has been studied extensively. However, cells often migrate in vivo in an extracellular matrix milieu that is intrinsically three-dimensional (3D) and the role of actin filament architecture in 3D cell motility is less well understood. Here we use quantitative functional live-cell assays to show that, while recently identified linkers of nucleoskeleton to cytoskeleton (LINC) complexes play a minimal role in conventional 2D migration, they play a critical role in regulating actin architecture in cells in 3D matrix and mediate protrusion dynamics, which in turn drive matrix remodeling and migration. LINC complex molecules Nesprin 2 giant and Nesprin 3 differentially modulate 3D motility by directly regulating the organization of a subset of actin filament bundles connected to the nucleus. A simple mechanical model explains why nucleo-cytoskeleton connections play no significant role in 2D motility, but are essential in 3D motility.

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Architecture and dynamics of F-actin in somatic nuclei.

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Actin filaments have been suggested to participate in a number of processes in eukaryotic nucleus, including transcription, chromatin remodeling and active gene transport. Studies in oocyte systems have revealed an extensive nuclear F-actin network that plays crucial roles in nuclear integrity and chromosome segregation, but the architecture and precise functions of actin in somatic nuclei are unknown. We have developed a novel utrophin-based reporter construct for the detection of actin filaments in somatic nuclei. Expression of this reporter in HeLa and U2OS cells reveals a pool of small (<0.5 um diameter) discrete nuclear F-actin puncta. These nuclear F-actin puncta are highly mobile and are resistant to latrunculin B, suggesting a low turnover rate. Surprisingly, nuclear F-actin puncta are excluded from chromatin and do not localize to known nuclear landmarks. These results indicate that nuclear F-actin puncta are unlikely to participate directly in chromatin-based processes, and may instead serve as scaffolds in the interchromatin space.

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Interaction of Telomeres and the Nucleoskeleton.

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The mammalian telomere consists of several kilobases of ‘TTAGGG’ repeats bound by the 6-protein ‘shelterin’ complex. Telomere maintenance is very important for long-term cell replenishment and genomic stability; more than 90% of human cancers have high telomerase levels, while haploisufficiency for the telomerase enzyme or mutations in telomere-associated proteins cause crippling premature aging syndromes in humans. We set out to examine the interaction between telomeres and nucleoskeletal components to determine if their interaction was modulated by telomere damage. After introducing fluorescently tagged versions of a telomere binding protein, an actin binding domain, and a DNA-damage sensing section of 53BP1 into cancer cells, we induced telomere damage by overexpressing a mutant telomerase template and quantified changes in signal and relative localization of all of the components. In addition to the expected increase in colocalization between telomeres and 53BP1, we saw an increased association between telomeres and nuclear actin foci after induction of telomere damage. These associations were modulated by inhibition of the DNA damage response protein ATM, which is known to be important in sensing telomere damage. We therefore hypothesize that a functional interaction between telomeres and the nucleoskeleton represents one of several control systems important to recognition and/or repair of damaged telomeres.

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Mechanical Characterization of the Actomyosin Cortex in Single Cell C. elegans Embryos.

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Polarization of the one-cell C. elegans embryo (zygote) is essential for proper development. Polarity is established in response to a transient symmetry-breaking cue and then maintained for many minutes prior to the first asymmetric division. During maintenance, the anterior cortex is highly enriched in ARP2/3 mediated branched actin and non-muscle Myosin II. While underlying genetic and biochemical mechanisms have been much explored, little is understood about the mechanics of polarity maintenance – in particular about how the zygote maintains a stably positioned anterior-posterior (AP) boundary despite the asymmetric distribution of force generating elements. More generally, the zygote cortex offers an attractive platform for developing microrheological techniques to probe mechanisms underlying actomyosin-based contraction and force transmission in animal cells.

Here, we combine video rate fluorescence microscopy and particle tracking to characterize spatiotemporal dynamics of actin filaments. We use phalloidin-conjugated quantum dots to report local motions of filamentous actin (F-actin). We mapped the trajectories of quantum dots at 30 fames per second at the zygote cortex during maintenance phase. In wild type (WT) embryos, the mean squared displacement (MSD) of actin bound quantum dots appears diffusive at long times with an apparent diffusion coefficient of 0.02 um^2/s and 0.03 um^2/s in the anterior and posterior halves respectively. In embryos that lack cortical myosin during maintenance, the MSD is again diffusive at long times with apparent diffusion coefficients reduced by a factor of four compared to WT. We find that the distribution of actin bound quantum dot displacements at 30 Hz is not Gaussian indicating that cortical actin filaments experience heterogeneous microenvironments that may result from variations in actin density,

actin turnover, actin architecture, or myosin force generation. Significantly, posterior bound quantum dots, which experience lower densities of both branched actin and Myosin II, show a higher occurrence of large and rapid (5 – 11 um/s) displacements compared to anterior. These differences are abrogated in embryos lacking cortical myosin, suggesting that myosin drives the large fluctuations. Moreover, reduced fluctuations in the anterior, where Myosin II is highly enriched, suggest that myosin-driven fluctuations are suppressed in the presence of ARP2/3-dependent (branched) actin, perhaps because branched actin networks are an unsuitable substrate for myosin force generation. Displacement distributions in both anterior and posterior halves become Gaussian at longer times indicating that inhomogeneities in cortex microenvironments and/or driving forces have randomized.

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Cyclase-associated Protein 1 (CAS-1) Promotes Actin Filament Turnover in Cooperation with ADF/Cofilin And Is Essential for Muscle Actin Organization in C. elegans.

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Cyclase associated protein (CAP) is a conserved multi-domain protein that binds to monomeric actin. At high concentrations, CAP inhibits actin polymerization, while it also catalytically promotes actin filament turnover in cooperation with ADF/cofilin and regulates actin dynamics in yeast, fruit fly, Dictyostelium, and mammalian cultured cells. However, little is known about the role of CAP in a morphogenetic process of highly differentiated cytoskeletal structures. In the nematode C. elegans, the cyclase-associated protein-1 (cas-1) gene encodes a CAP protein. Here, we report that CAS-1 promotes actin filament turnover in cooperation with UNC-60B (a muscle-specific ADF/cofilin) and is essential for actin filament organization in the body wall muscle. We generated recombinant full-length CAS-1, an N-terminal half (CAS-1N) containing the helical-folded domain, and a C-terminal half (CAS-1C) containing the proline-rich motif, the WASP-homology 2 domain, and the ß-sheet domain for in vitro studies. In the presence of UNC-60B, full-length CAS-1 enhanced the rate of actin filament turnover as determined by the rate of phosphate release. While CAS-1N and CAS-1C independently bound to G-actin, either fragment failed to enhance actin filament turnover in the presence of UNC-60B, indicating that both N- and C-terminal domains are required for this activity. Full-length CAS-1 strongly enhanced exchange of G-actin-bound ATP even in the presence of UNC-60B that inhibits exchange of ATP. However, CAS-1N and CAS-1C did not enhance exchange of G-actin-bound ATP, indicating again that both N- and C-terminal domains are required for this activity. We generated a specific antibody against CAS-1 and found that CAS-1 was specifically expressed in the body wall muscle and localized to the M-line and a part of the thin filaments within sarcomeres. A cas-1-null mutant was homozygous lethal, and most of the mutant worms die in larval stages. The cas-1-null mutants showed disorganized actin filaments in the body wall muscle. In wild-type, UNC-60B localized to the thin filaments, while it mislocalized to the center of actin aggregates in the cas-1-null mutants. On the other hand, localization of CAS-1 was largely unaffected in unc-60B mutants, indicating that CAS-1 localizes to sarcomeres independently of UNC-60B. These results suggest that cooperative effects of CAS-1 and UNC-60B on actin filament turnover are essential for sarcomeric actin organization in striated muscle.

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Remodeling of Actin Filaments by Cofilin and Phalloidin.

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Cofilin/ADF proteins play key roles in the dynamics of actin, one of the most abundant and highly conserved eukaryotic proteins. We used cryo-electron microscopy to show that the cofilin induced change in the filament twist is due to a unique conformation of the actin molecule unrelated to any previously observed state. The changes between the actin protomer in naked F-actin and in the actin-cofilin filament are greater than the conformational changes between G- and F-actin. This illustrates the structural plasticity of actin and suggests that other actin-binding molecules may also induce large but different conformational changes. We used cryo electron microscopy to reveal the structure of F-actin decorated with phalloidin - a small polypeptide widely used to stabilize actin filaments in in vitro experiments including the motility assay. We show that phalloidin and cofilin impose very different structural states on F-actin. In contrast to the previously accepted notion that phalloidin stabilizes a particular structural state of the actin filament we show that multiple structural states of F-actin exist in the filaments saturated with phalloidin. Our results suggest that the plasticity of actin-phalloidin filaments may explain their ability to support acto-myosin motility.

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Dynamic modes of F-actin and its D-loop and their modulation by cofilin.

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Conformational dynamics of filamentous actin (F-actin) is essential for its regulation by actin binding proteins that modulate the actin cytoskeleton. Previous solution studies showed that the plasticity of the DNase I binding loop (D-loop; residues 40–50) on subdomain 2 of actin, and its contacts with the hydrophobic loop (H-loop), the C-terminus, and the W-loop at the interprotomer space create multiple filament states, including the destabilized ones. Here, we explored the nucleotide dependence of D-loop states via site directed fluorescence or electron paramagnetic resonance (EPR) spin probing of cysteine mutants of a.a. 40-50. Neither the reactivity towards fluorescent label acrylodan nor the spectral analysis of spin labeled D-loop mutants show any residue dependent helical periodicity, suggesting a highly mobile and disordered D-loop irrespective of the bound nucleotide. EPR spectra of spin-labeled mutants indicate the presence of multiple D-loop conformers in F-actin. These conformers provide greater versatility of actin binding surfaces and can be selectively stabilized by the binding partners. Focusing on cofilin, we show by site directed fluorescence and spin labeling that cofilin changes the emission properties of acrylodan and the mobility of spin labels attached to D-loop cysteine mutants. Furthermore, the kinetics of copper-catalyzed disulfide cross-linking in equimolar copolymers of D-loop cysteine mutants with either wildtype (C374) actin or mutant S265C/C374A (on the H-loop) or mutant F169C/C374A (on the W-loop) is altered in the presence of cofilin, favoring the unstable conformers of F-actin. These polymerization impaired oligomers of actin, created by such disulfide traps, can be rescued by cofilin, as detected by light scattering and electron microscopy imaging. Overall, our results emphasize the importance of F-actin dynamics and structural modes and their functional role in actin interactions with its binding proteins.

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Investigating actin binding sites and bundling behavior of TRIOBP-4.

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TRIOBP is an actin-bundling protein that helps form resilient rootlets of hair cell stereocilia in the inner ear. TRIOBP binds to actin filaments and forms highly dense F-actin bundles. The formation mechanisms of the actin bundles are very unique, because TRIOBP may be able to wrap around actin filaments, instead of connecting between the actin filaments. TRIOBP has three isoforms: TRIOBP-1, -4, and -5. In TRIOBP-4/5 knockout mice, stereocilia rootlets failed to develop and hair cells subsequently degenerated resulting in profound deafness. We have been investigating the structural basis of TRIOBP function. We are determining the number and sequence of the actin binding sites in TRIOBP-4, and their interaction with actin filaments using in vitro biochemical assays and cell biological methods. TRIOBP-4 has two repeated domains, R1 and R2 and we hypothesized that these would be the binding sites to F-actin. To test this, we purified TRIOBP-4 mutants with either the R1 or R2 domain removed. These deletion mutants of TRIOBP-4 were still able to form F-actin bundles. F-actin bundle dynamics were visualized by TIRF microscopy in real time. Localization and actin dynamics in the cells were observed. We found that the repeat sequences, R1 and R2, have different mechanisms for actin bundling formations.

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Optimising models to examine the specificity of anti-tropomyosin compounds for Tm5NM1.

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Disrupting the actin cytoskeleton of cancer cells represents an attractive chemotherapeutic strategy. However given the critical role of actin in all cellular functions, a more refined approach is necessary to prevent excessive toxicity. The actin cytoskeleton is organised into functionally distinct compartments by a repertoire of low and high molecular weight (LMW/HMW) tropomyosin isoforms. Malignant cells demonstrate an increased reliance on a subset of LMW tropomyosins. Importantly, these isoforms are distinct from muscle tropomyosins regulating sarcomeric contraction. Using a novel class of anti-tropomyosin compounds we have demonstrated preferential disruption of endogenous LMW tropomyosin-containing actin filaments in SHEP neuroblastoma and SK-MEL-28 melanoma cells. Ascertaining the specificity of the compounds for Tm5NM1 has proven more challenging given that Tm5NM1 is a structural protein with no simple functional readout by which to assess activity. OBJECTIVE: We aim to optimise assays which will allow us to investigate the nature and specificity of the Tm5NM1-compound interaction. SUMMARY OF RESULTS: Drug-induced changes to the alpha-helical structure of LMW isoform Tm5NM1 were measured by circular dichroism (CD). Preliminary CD findings revealed a more stable secondary structure of Tm5NM1 model peptides in the presence of drug. We next sought to investigate the effect of drug binding on the function of Tm5NM1 as a regulator of the actin filament. While the compounds tested did not alter the affinity of Tm5NM1 for filamentous actin, Tm5NM1 bound with greater cooperativity in the presence of the drug. Further to this, the slowed rate of actin filament elongation seen with Tm5NM1 saturation was reversed in the presence of compound. Therefore, we postulate that

Tm5NM1 targeting compounds may act to alter actin filament dynamics. Tm5NM1 has also been shown to enhance actin-activated myosin II ATPase activity. The effect of Tm5NM1 targeting compounds on the actomyosin II ATPase activity is the focus of further investigation. CONCLUSIONS: We have described a novel class of compounds which modulate the activity of Tm5NM1 and may serve as a valuable tool for dissecting the role of Tm5NM1-containing actin filaments in cellular models. These findings have significant clinical implications for targeting distinct actin filament populations involved in tumour cell function.

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Correlation between tropomyosin-binding and actin-capping abilities of tropomodulin.

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Tropomodulin (Tmod) is an actin-capping protein that binds two tropomyosins (TM) at the pointed end of the actin filament in order to prevent further actin polymerization and depolymerization. Capping ability is highly influenced by TM and is 1000 fold greater in its presence. There are four Tmod isoforms (Tmod1-4), three of which, Tmod1, Tmod3 and Tmod4, are expressed in skeletal muscles. Earlier studies revealed that Tmod/TM interactions are isoform-specific. It was found that the affinity of Tmod1 to skeletal striated αTM (stTM) is higher than that of Tmod3 and Tmod4. In this study, we used synthetic peptides corresponding to the first and second TM-binding sites of Tmod1 to measure affinities by circular dichroism. We showed that mutations R11K, D12N and Q144K in TM-binding sites of Tmod1 decreased the affinity of Tmod1 to stTM and made it similar to that of Tmod3 and Tmod4. To find out if these mutations affect actin-capping properties, they were introduced into full length Tmod1. TM-dependent actin-capping ability of Tmod1, wild type and mutants, was tested using a pyrene-actin polymerization assay. We showed a significant decrease of inhibition of actin pointed-end polymerization in the presence of Tmod1 [R11K/D12N/Q144K] as compared with wild type Tmod1. This indicates a direct correlation between TM-binding and actin-capping abilities of Tmod. We suggest that actin-capping ability of Tmods depends on their TM-binding affinity and therefore preferential binding of Tmod isoform to actin filament should depend on TM isoform, associated with these filaments.

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Identification and Functional Analysis of Conserved Residues in Fission Yeast Tropomyosin.

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Tropomyosin (Tm) is a global regulator of actin filament function. We have taken a bioinformatics approach in a structure-function analysis of this protein in Schizosaccharomyces pombe. S. pombe has a single, essential Tm gene (cdc8) that is required for required for growth, formation of the contractile ring and actin cables, for normal transport of actin-containing dots on the cables, and for normal conjugation. S. pombe Tm, like its vertebrate counterparts, regulates formin function, inhibits actin crosslinking and severing proteins, and can inhibit or activate myosin activity, depending on the myosin isoform. Our objective is to identify the regions and residues required for these universal functions. We posit that that mutation of specific conserved residues in tropomyosin will affect these, and have unanticipated effects in this model system. First, we constructed a phylogenetic tree of 30 fungal protein sequences from 27 species using maximum-likelihood and Bayesian analyses. Then, the substitution rates

at individual codon sites was determined using PAML to identify the most conserved residues. Many of the conserved codons are also conserved in animal Tm sequences, even though the fungal sequences are shorter, spanning the length of four or five actin subunits, compared to six or seven for animal Tms. We have screened a series of mutants of 22 conserved residues (amino acid residues 6, 16, 23, 30, 41, 58, 82, 86, 93, 96, 103, 104, 107, 110, 114, 117, 118, 121, 131, 138, 139, 153) in 16 mutants for the ability to rescue growth of a cdc8-ts mutant (cdc8-27) at the restrictive temperature (35 oC) when overexpressed in a pREP41 vector. Most of the mutated residues are in the surface positions of the Tm coiled coil, those most likely to be available for interacting with other proteins. While all except two of the mutants rescue growth at 35 oC, we have observed subtle differences when monitoring the nuclear number (DAPI staining), actin cytoskeleton (phalloidin staining) and septum formation (Calcofluor staining) with time after transfer to the restrictive temperature. These differences include the position and number of nuclei, cellular shape and length distribution, position and organization of the septum and contractile rings, and the frequency of abnormal cell divisions. The results will give insights into Tm-dependent cellular functions that can be corroborated by genetic experiments as well as in vitro analysis of purified proteins. Supported by MDA, NIH, Aresty Research Center for Undergraduates at Rutgers University.

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Multiple nonmuscle tropomyosin isoforms perform overlapping but distinct functions in *Drosophila.*

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Nonmuscle tropomyosin (Tm) is an actin-binding protein, which is implicated in many cellular processes, including locomotion, intracellular trafficking, and cytokinesis. Metazoans typically express multiple non-muscle isoforms of Tm (up to 40 isoforms from four different genes in humans). Although previous studies indicated that *Drosophila melanogaster* expresses only one nonmuscle isoform (Tm1PA) from two different genes, we have discovered that *Drosophila* S2 cells express at least two additional nonmuscle Tm isoforms, using a combination of immunoblotting, RT-PCR, and RNAi. Using double-stranded RNAs targeting specific isoforms, we found that different isoforms may perform overlapping but distinct cellular functions. Knockdown of a subset of Tm isoforms results in aberrant cell spreading and increased adhesion, while knockdown of a different subset of Tm isoforms alters the structure and dynamics of the actin cytoskeleton at the leading edge of interphase cells. Knockdown of all known isoforms from both Tm genes perturbs cytokinesis in S2 cells. Given the diversity of phenotypes, we are currently exploring how these Tm isoforms play different roles in the actin cytoskeleton. We have found that fluorescently tagged Tm isoforms exhibit different localization patterns and retrograde flow rates in S2 cells, which may indicate the presence of two distinct Tm bound actin networks. Our preliminary results suggest that the isoforms are differentially regulated by acetylation, and that this may affect their affinity for filamentous actin. We speculate that differences in their affinities for filamentous actin and their effects on actin regulatory factors (such as cofilin and myosin II) underlie the functional differences between the different isoforms.

Higher-Order Actin-Based Structures

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Entrapment of Intracytosolic Bacteria by Septin Cage-Like Structures.

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Actin-based motility is used by various pathogens for dissemination within and between cells. Yet host factors restricting this process have not been identified. Septins are GTP-binding proteins that assemble as filaments and are essential for cell division. However their role during interphase has remained elusive. We have discovered that septin assemblies are recruited to different bacteria that polymerize actin. We observed that intracytosolic *Shigella* either become compartmentalized in septin cage-like structures or form actin tails. Inactivation of septin caging increases the number of *Shigella* with actin tails and enhances cell-to-cell spread. TNF-α, a host cytokine produced upon *Shigella* infection, stimulates septin caging and restricts actin-tail formation and cell-to-cell spread. Finally we show that septin cages entrap bacteria targeted to autophagy. Together these results reveal an unsuspected mechanism of host defense that restricts dissemination of invasive pathogens, and highlight the septins as a novel and unconventional component of the cytoskeleton.

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The Shs1p C-terminus is required for septin ring geometry and cell morphology.

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Septins are a class of conserved GTP-binding proteins that function in diverse cellular processes. Individual septin proteins assemble into hetero-oligomeric complexes, which can further polymerize into nonpolar filaments. These filaments complex to form higher-order structures, including rings and bundled filaments, that function in cytokinesis and act as membrane barriers. Although *in vitro* analyses have unveiled the composition and arrangement of individual septin filaments, the mechanisms of septin assembly into the higher-order structures seen *in vivo* are not well understood. The filamentous fungus *Ashbya gossypii* expresses five septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Sep7/Shs1p) that localize to hyphal tips and form cortically-attached rings throughout the hyphae. These rings maintain a persistent but dynamic association with the plasma membrane over many hours of growth. We have shown that the kinases Elm1p and Gin4p regulate the stable association of septin structures with the cell cortex. A possible kinase target is the C-terminus of Shs1p, a region containing nine phosphorylation sites that we have identified by mass spectrometry. Additionally, the Shs1p C-terminus harbors a predicted coiled-coil domain, which may play a role in septin-septin interactions. Expression of Shs1p lacking the C-terminus as the only Shs1p in *Ashbya* results in aberrant formation of a specific subset of septin rings that arise at hyphal branch points. Branch rings containing Shs1ΔC-GFP assemble extra cortical filamentous extensions adjacent to otherwise normal-appearing rings. Shs1ΔC-GFP immunoprecipitates with the other four septins, indicating that a loss of septin complex association is not the cause of the aberrant branch ring phenotype. Time-lapse microscopy reveals that the extensions appear concurrently with the septin ring formation, suggesting that loss of the C-terminus of Shs1p leads to misregulation of the septin assembly process, specifically an inability to scale the ring properly at assembly. In addition to septin abnormalities, these cells display increased hyphal branching frequency, suggesting that either the C-terminus of Shs1p is required for normal cell morphology and/or that aberrantly scaled septin rings can alter sites of polarity. We hypothesize that the phosphorylation sites and/or the coiled-coil domain of Shs1p may be

responsible for the inability of Shs1ΔC-GFP to grow and form septin rings properly and are currently analyzing alleles containing point mutations in the phosphorylation sites. This work illuminates the role of specific septin domains in higher-order septin structure regulation.

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Self-Organization of Cytoplasmic Actin-Myosin-Formin Network.

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In eukaryotic cells, specialized actomyosin structures such as stress fibers and cytokinetic contractile ring are well known, but there is very limited understanding of the cytoplasmic actomyosin network. To elucidate this actomyosin network organization, we perturb it using Latrunculin A (LA). This treatment led to emergence of asters with diameter of 0.5-1.2μm, visualized by fluorescently tagged Lifeact or beta-actin in regions of cytoplasm not associated with remaining stress fiber or focal adhesions. These asters were very dynamic moving vigorously, often fusing with each other. Using dual color TIRF microscopy live imaging, myosin clusters were found either between or co-localized with actin aster centers, while filamin A and formin protein DAAM1 co-localized with the centers. Notably, PALM microscopy revealed that even in non-treated cells DAAM1 was localized in patches spaced similarly to the asters that emerged after the LA treatment. Actin aster movements induced by LA can be stopped by adding blebbistatin, a myosin II inhibitor, or largely reduced by treatment with formin inhibitor SMIFH2. On the other hand, in filamin A-/- cells, LA treatment resulted in formation of asters moving more rapidly and fusing and splitting more frequently than in control cells. Re-introduction of filamin A into the knockout cells decreased the asters' dynamics significantly. Increasing the level of filamin A in the control cells caused the reduced velocity or complete block of the aster motility. We developed a computational model which, in agreement with these data, suggests that myosin motors slide actin filaments into the multiple asters with centers stabilized by cross-linkers and reinforced by formin-mediated nucleation, while myosin is stabilized at the edges between the asters where its contractile action causes aster movements. Modeling suggests that the dynamic-aster mode of self-organization is optimal for mechanical connectedness of the cytoplasm in perturbed actin networks.

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Actin Assembly Kinetics Determine the Architecture of α-Actinin Crosslinked F-actin Networks.

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The spatial and temporal regulation of the actin cytoskeleton is required for numerous aspects of eukaryotic cell physiology including adhesion, polarity, migration, division, endocytosis, and intracellular trafficking. In concert with actin regulatory proteins, actin filaments (F-actin) are organized into bundles of axially aligned filaments or meshworks cross-linked at high angles.

Understanding the biochemical and physical processes regulating the assembly of actin filaments into meshworks or bundles is central to developing a quantitative and predictive understanding of cytoskeletal organization. Here we show that the kinetics of actin polymerization play a crucial role in controlling the morphology of *in vitro* filament networks assembled with α-actinin by a combination of quantitative imaging, microrheology and computational modeling approaches. Cross-link mediated bundle formation only occurs in dilute solutions of actin filaments with a predominately viscous microenvironment facilitating rotational and translational diffusion of actin filaments. During the time period of high filament mobility, α-actinin concentration and filament density controls the rate of bundle formation. Furthermore, we show that the network morphology can be modulated from a highly bundled network to an isotropic meshwork that by altering only nucleation kinetics. Thus, the morphology of cross-linked F-actin networks reflects a metastable state determined by the kinetics of filament nucleation, elongation and α-actinin mediated aggregation. These results underscore the importance of physical constraints in regulation of actin cytoskeletal organization into higher ordered structures in a dense and crowded cytoplasm.

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In-vivo measurement of stress fiber contraction dynamics with high resolution and dynamics range.

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Force generation is critical to the regulation of many fundamental cellular processes including stem cell differentiation, tissue development, and tumor progression. Stress fibers, the principle generators of contractile forces, are linear chains of bundles of actin filaments of opposing polarity that are pulled together by bipolar myosin filaments in a manner similar, though less ordered, to muscle myofibrils. Though force was not directly measured, recent experiments suggest a surprising richness in how stress fiber dynamics regulate force and mechanotransduction.

We present a high resolution, high dynamic range Nano-Electro-Mechanical Systems (NEMS) based force measurement tool with wide applicability to measure forces generated by individual adherent cells. Which we demonstrate here with measurement of the force from a single stress fiber within a cell as the cell is perturbed by Cytochalasin D (CD) and recovers after CD removal. We measure a contraction upon initial exposure to CD of ~4nN, force steps during both CD induced force collapse and post-CD force recovery of ~1nN, and small force oscillations, ~400pN peak-peak, with frequency linearly dependent upon the cell generated force throughout.

The data validate a two part model – steady state and kinetic – of stress fiber force generation. The steady state model, adapted from recent work that features actin treadmilling and polymerization forces in addition to actomyosin contraction, describes the initial contraction and force steps. The kinetic model is based upon stochastic, abrupt failure and activation of sarcomere like units within the stress fiber and describes the overall force-time response.

This work demonstrates the ability of high performance NEMS to connect quantitative biophysical modeling with specific cell level function. With these capabilities we confirm the importance of actin polymerization dynamics in understanding stress fiber response and present a detailed mechanism by which cytochalasins reversibly disrupt cellular force generation.

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In silico myosin motors and contractile networks.

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Motor proteins are critical to cells in processes such as cellular contractility, motility, cell division, and intracellular transportation and compartmentalization. Thus detailed simulations of these processes require motor representations that can be "benchmarked" to encapsulate key features of the biochemical and mechanical motor characteristics.

A biological myosin molecule is continuously flexible (with distinct regions of differing rigidity) and mechanical models might vary from molecular dynamics approaches (i.e. representation of each molecule and the various force fields of interaction) to multiple rigid bodies connected by springs, to simple elastic representations. Ever more detailed and physically realistic models of a motor protein have the potential to capture finer facets of motor behavior, but at a computationally cost.

Our eventual goal with this model is to simulate very large networks of actin filaments, myosin motors, and crosslinkers. Thus a very simple and computationally inexpensive motor representation is appropriate. We propose a two-spring myosin motor for 2-dimensional simulations. One spring is co-linear with the actin filament —this spring has the stiffness measured for a myosin crossbridge and a bi-modal rest length to enact a powerstroke— while an orthogonal spring keeps the filament and motor from drifting apart.

Each single motor is encoded through Monte Carlo methods to exhibit the measured transition rates between biochemical states (i.e. the state of the bound nucleotide). The ADP-release rate is modulated by force-state, as indicated by experiment. The model prescribes a 5.5nm step-size and a measured value for crossbridge stiffness, which guarantees appropriate force interaction between filament and myosin with each powerstroke.

We show that groups of motors exhibit the expected average duty-ratios. In *in silico* gliding filament assays filaments demonstrate appropriate speed dependence and eventual saturation with increasing myosin density. And under resisting loads these model motors can reproduce the expected concave force-speed curve.

As an element in networks with filaments and crosslinkers we demonstrate how these motors can lead to filament buckling and different contractile topologies, dependent on motor heterogeneity and density.

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Actin pattern formation in reconstituted actomyosin cortices.

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In eukaryotic systems the actomyosin cortex is involved in fundamental processes such as cytokinesis, cell migration and cell polarization. Actomyosin contractility is involved in the generation of cortical flows helping cells to polarize, which is essential for asymmetric cell division and hence for the development of multicellular organisms. In *Caenorhabditis elegans* zygotes cortical flows are accompanied by the formation of dynamic actomyosin networks. However, the physical relation between actomyosin contractility, network formation and cortical flows are not well understood.

We therefore set out to build minimal *in vitro* systems, namely consisting of membrane bound actin filaments and myosin (filaments) motors in order to reconstitute and mimic general features of contractile actomyosin cortices present in model organisms. In particular, we aim to understand the behavior of motor filaments and the role of the membrane during actomyosin network formation.
By imaging fluorescently labeled actin and myosin filaments with TIRF microscopy we show that the addition of myosin filaments to the membrane bound actin layer leads to the formation of interconnected actin clusters. By varying the density of the membrane bound actin layer we obtain different morphologies of the actin pattern after addition of the motors. We demonstrate that similar to active gels in bulk solution, the process of actin pattern formation depends on the concentration of ATP. At saturating ATP concentration actin pattern formation is inhibited, while lowering the ATP concentration to a critical level leads to the formation of actin clusters. This process is reversible by the addition of ATP to actomyosin clusters thereby resuming the original morphology of the actin meshwork. However, addition of the crosslinker Fascin at ATP saturating conditions leads to pattern formation, although more slowly. This indicates that crosslinking is necessary for the contraction of the actin filament layer. Visualization and tracking the motion of individual fluorescently labeled myosin filaments revealed that the processivity of the motor filaments increases when the ATP concentration is lowered. We propose that upon ATP depletion some myosin filaments start to function as crosslinkers inducing the actin meshwork contraction. We hypothesize that the concomitant overall increase of the processivity of myosin filaments further accelerates the contraction of the actin filaments into actomyosin clusters.

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Thickness and dynamics of the actomyosin cortex.

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Cellular shape changes are, in essence, mechanical processes that are governed by the physical properties of the cell. One physical property in particular that has been implicated in cell shape change is tension, which is controlled by the actomyosin cortex, a thin layer of proteins that directly underlies the plasma membrane. Cell tension arises from two major properties of the cortex: (1) the amount of stress generated by myosin motors within the cortex and (2) cortex thickness. In order to further understand the regulation of cortical tension, we have developed an assay to measure cortex thickness in living cells that relies on a precise, quantitative analysis of fluorescence images of the cortex and the plasma membrane. In this assay, we measure cortex thickness by determining the spatial separation between the cortex and the plasma membrane, and we have used this technique to track changes in thickness during dynamic cell shape changes, including bleb retraction and cell division. Furthermore, we have used a similar image analysis approach, combined with the use of photoactivatable probes, to investigate the dynamics and spatial regulation of actin assembly in the steady-state cortical network. By interpreting these photoactivation data through a simple geometrical description of the cortex, we are not only able to more precisely extract information about the spatial dynamics of actin assembly, but we also attain an independent measurement of cortex thickness that is consistent with our thickness measurements using cortex and membrane peak separation. Comparing the results of these experiments with photobleaching data, we are further trying to understand the regulation of actin dynamics and thickness at different stages of the cell cycle, both in order to uncover the underlying mechanisms involved in previously observed changes in cells' physical properties and shape throughout the cell cycle and also to more generally determine the relationship between cortical actin dynamics and cortex thickness.

The understanding of such relationships and of the basic principles of cortical actin dynamics are essential in understanding how the coordinated actions of these proteins contribute to larger-scale physical properties.

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Actin network architecture determines myosin motor activity.

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The organization of actin filaments into higher-ordered networks governs overall eukaryotic cell shape, mechanical integrity and directed movement. The global architecture of the actin cytoskeleton is determined by coordinated actions of a large number of actin regulatory proteins that modulate filament assembly and disassembly dynamics. Myosin motors also plays a critical role in these processes and reorganize filament structures through sliding (e.g. contractility) and/ or depolymerization. Understanding the molecular mechanism of such complex spatiotemporal orchestration is extremely challenging in cells, where hundreds of different proteins act simultaneously on overlapping actin sub-structures. Here, we use geometrically controlled and polarized in vitro actin networks to evaluate how myosin motors influences filament architecture. Direct visualization of filaments indicates that myosins selectively disassemble randomly-oriented and anti-parallel actin filament structures while parallel actin filament bundles are unaffected by myosin contractility. This “orientation selection” reveals how the overall organization and dynamics of the actin cytoskeleton is controlled by actomyosin contractility. General principles governing the spatial organization of actin filaments in cells emerge from this work.

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Spatial structure of actin cytoskeletons associated with nuclear membrane.

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Current study aims to elucidate the spatial organization of cytoskeletal actin filaments in the cytoplasm with special reference to the association with nuclear membrane and its alternations during cell cycle. Cytoskeletal actin filaments in living cell have been investigated so far exclusively with fluorescent light microscopy in conjunction with GFP tag method. However, fine structural changes with time were not detected well. Observation was restricted on the stress fiber with relatively strong fluorescence and at ventral side of the cell. We compared live cell images with electron microscopic images in a strict sense. In order to detect real spatial structure of cytoskeleton, high voltage TEM (1000 KV), high resolution SEM and immune-freeze etching technique were applied to unroofed whole cells in addition to light microscopic live cell imaging . Our innovative methods arrested incredibly more abundant actin filaments besides the stress fibers than in fluorescent microscopy. Remarkable amount of fine actin filaments occupying the whole cytoplasm extended in all directions with aggregation and dispersion to form meshwork, and eventually divided cytoplasmic space into several domains. Furthermore many stress fibers as well as non-bundled actin filaments attached onto the nuclear membrane while associating with intermediate filaments. On closing to the M phase, stress fibers were disappeared together with the nuclear membrane for a short time (within one minute). Therefore, stress fibers seem to play an important role to disperse the nuclear envelope, though mesh work formed by fine actin filaments was not changed significantly. Then, restoration of

nuclear envelope took a long time more than 20 minutes after mitosis, when stress fibers were formed again gradually.

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Retinoic acid controls cultured and harvested epithelial cell sheet's shrinkage via the regulation of actomyosin contraction.

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Primary oral mucosal epithelial cells show a self-stratification similarly to those in vivo under optimum culture condition supplemented with serum. After being incubated in 20 ºC for 30 min, confluent cells on a poly(*N*-isopropylacrylamide)-grafted temperature-responsive cell culture insert spontaneously detached themselves as a contiguous cell sheet. Interestingly, the harvested cell sheets shrank to approximately 30% of their initial size. By utilizing the culture insert, stratified epithelium-like cell sheets were also harvested even without serum, and the harvested cell sheets failed to show any shrinkage. In addition, the cell adhesion ability to culture substratum was significantly weak under serum-free culture condition. Therefore, we hypothesized that these differences depend on cell cytoskeleton and attempted to investigate cytoskeletal organization of cell sheets.
Several cytoskeletal inhibitors were added in a serum-containing cell sheet culture system. Shrinkage of each harvested cell sheet was inhibited when actin polymerization, Rho kinase, myosin light chain kinase, and myosin ATPase were inhibited for 24 h before the cell sheet harvest, while intermediate filament or microtubule inhibition showed no effect. In addition, ATP synthesis inhibitor NaN_3 also inhibited the shrinkage. These results strongly indicate that cell sheet shrinkage was controlled by an actomyosin contraction. HE staining showed the thickness of harvested cell sheet in serum-free condition was thinner than that of serum-containing culture. Lysophosphatidic acid and thrombin, which are known as Rho activating factors, showed no effect on the cell sheet shrinkage. Furthermore, the receptor antagonists of these receptors were added in serum-containing culture, but no shrinkage was inhibited. Retinoic acid, an epidermal proliferation and differentiation regulator contained in serum, was added to oral mucosal epithelial cell culture, resulting in shrinkage similar to the serum-containing condition. Moreover, the shrinkage was suppressed by the actomyosin specific inhibition.
In conclusion, the cell sheet shrinkage was found to depend on cytoskeleton organization, especially on actomyosin contraction, and the cell sheet shrinkage via actomyosin contraction was regulated by retinoic acid.

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Supervillin couples myosin-dependent contractility to podosomes and enables their turnover.

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Podosomes are actin-rich adhesion and invasion structures. Especially in macrophages, podosomes exist in two subpopulations, large precursors at the cell periphery and smaller podosomes (successors) in the inner cell regions. To date, the mechanisms that differentially regulate these subpopulations are largely unknown. Here, we show that the membrane-

associated protein supervillin localizes preferentially to successor podosomes and becomes enriched at precursors immediately prior to their dissolution. Consistently, podosome numbers are inversely regulated by supervillin protein levels. Using deletion constructs, we find that the myosin II-regulatory N-terminus of supervillin (SV 1-174) is crucial for these effects. Moreover, phosphorylated myosin light chain (MLC) localizes at supervillin-positive podosomes, and time-lapse analyses show that enrichment of EGFP-supervillin at podosomes coincides with their coupling to contractile myosin IIA-positive cables. Because supervillin binds only to activated myosin IIA, and a dysregulated N-terminal construct (SV 1-830) enhances phospho-MLC levels at podosomes, preferential recruitment of supervillin to podosome subpopulations may both require and induce actomyosin contractility. Importantly, using siRNA-treated cells, we demonstrate that supervillin and myosin IIA cooperate to regulate podosome life time, podosomal matrix degradation and cell polarization. In sum, we show here that podosome subpopulations differ in their molecular composition and identify supervillin, in cooperation with myosin IIA, as a critical factor in the regulation of podosome turnover and function.

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The scaffolding protein EPB50 is a highly dynamic and essential component of microvilli regulated by its PDZ interactions.

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Most cells are or have the potential to be polarized. Polarity requires the ability to control the protein composition of the plasma membrane in time, in location, and in response to signaling pathways. A well-studied example is epithelial cells that have distinct apical and basolateral domains. Microvilli, which define the apical surface of epithelial cells, are dynamic finger-like protrusions of the apical membrane supported by bundled filamentous-actin (F-actin) cores. Despite their abundance on many cell types, the regulation of microvilli remains poorly understood. One regulator is Ezrin Binding Phosphoprotein of 50 kD (EBP50), a microvillar scaffolding protein with two PDZ domains followed by a C-terminal ezrin binding domain. We have shown that both the first PDZ and ezrin binding domains of EBP50 are required for microvillar formation, and that phosphorylation regulates its ability to engage multiple PDZ ligands simultaneously. Because of the dynamic nature of microvilli, we are currently investigating the dynamics of microvillar components using both FRAP and photo-activation in live epithelial cells. We were surprised to find that EBP50 is extremely dynamic compared to other microvillar proteins. Of particular interest is how EBP50 can exhibit such high turnover compared to ezrin, which binds to EBP50 with 1-3nM affinity in vitro. Surprisingly, we also find that mutation of either of EBP50's PDZ domains results in reduced turnover in microvilli and that this is due to an increased association with ezrin, a much less dynamic regulator of microvilli. These unexpected results require dramatic revision to current models of microvillar regulation and rethinking as to how a cytoskeletal scaffolding protein is able carry out its structural function amid such rapid molecular turmoil.

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PKC phosphorylation of cortactin is implicated in the regulation of actin dynamics.

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Actin bundle formation is essential for variety of cellular events including the formation of neuronal growth cones. Recently we have reported that dynamin 1-cortactin ring complexes bundle actin filaments in growth cone filopodia (Takei et al., 2010, 50th ASCB meeting). In the present study, we show that PKC phosphorylation of cortactin is implicated in the actin

dynamics in the growth cone. Cortactin was rapidly phosphorylated in metabolically ^{32}P-labeled NG108-15, a human neuroblastoma cell line, during growth cone collapse by LPA. The phosphorylation was inhibited by Ro-31-8220, a PKC inhibitor, indicating that cortactin is a substrate for phosphorylation by PKC. By double immunofluorescence of SH-SY5Y, cortactin colocalized with PKCα, an isoform enriched in SH-SY5Y, along the growth cone filopodia. Cortactin and PKCƒ¿ interacted as confirmed by immuneprecipitation. Treatment of SH-SY5Y with phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, increased cortactin-PKCα interaction simultaneously with PMA-induced growth cone collapse. Consistently, cortactin-dependent actin bundle formation in vitro was reduced by cortactin phosphorylation via PKCα. MALDI-MS analysis revealed multiple PKC phosphorylation sites of cortactin. Taken together these results strongly suggest that PKC phosphorylation of cortactin represents a mechanism involved in the regulation of actin dynamics in growth cones.

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The Role of Protocadherin 24 in Enterocyte Brush Border Assembly.

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Intestinal epithelial cells (IECs) possess a highly ordered apical array of actin-based, membrane protrusions known as microvilli. Collectively, these microvilli form the brush border, which is known to play a key role in both nutrient absorption and host-cell defense. A mature, functional brush border is characterized by ~1000 tightly packed microvilli, which extend into the gut lumen to a near identical length. Despite playing a critical role in gut function, little is known about the molecular mechanisms underlying brush border assembly during enterocyte differentiation. Using the CACO-2_{BBE} cell culture model system, we observed that microvilli cluster together during enterocyte differentiation, interacting with each other at their distal tips to form ‘tepee’-shaped structures. High magnification images of these clusters revealed that microvilli are physically connected to one another by thread-like links. Similarly, quick-freeze deep-etch electron microscopy of native intestinal tissue revealed an extensive network of inter-microvillar links that exists within a mature brush border. Towards the goal of identifying the constituent(s) of these links, we have identified and characterized the functional role of a cadherin superfamily member, protocadherin-24 (PCDH24), which exhibits striking enrichment at the tips of microvilli of both CACO-2_{BBE} cells and native intestinal tissue. PCDH24 is composed of 8 extracellular cadherin domains, a single-pass transmembrane spanning region and a short cytoplasmic tail. Over-expression of PCDH24 in CACO-2_{BBE} cells was correlated with microvillar clustering, while knockdown resulted in an abnormal brush border assembly, in which cells lost the ability to form a tightly packed brush border, with microvilli of uniform length. Pull-down analysis using a tagged PCDH24 cytoplasmic domain incubated in CACO-2_{BBE} cell lysates demonstrated an interaction with the molecular scaffold protein harmonin-a. Harmonin-a colocalized with PCDH24 at the tips of microvilli of CACO-2_{BBE} cells and native tissue. A yeast-two-hybrid screen identified an interaction of harmonin-a with the motor protein, Myo7b. Similar to PCDH24 and harmonin-a, Myo7b was found to localize to the distal tips of microvilli in CACO-2_{BBE} cells and native tissue. We propose that PCDH24, harmonin-a and Myo7b form a complex, which localizes to the distal tips of microvilli and functions to promote the tight packing and uniform length of microvilli during brush border assembly.

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Regulation of Macrophage Podosome Patterning by Proline-Serine-Threonine Phosphatase Interacting Protein 1 (PSTPIP1).

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The autoinflammatory disease, pyogenic sterile arthritis, pyoderma gangrenosum and acne (PAPA) syndrome, results from mutations that appear to activate PSTPIP1. PAPA syndrome patients suffer from uncontrolled, phagocyte-mediated inflammation in the skin and joints, but the reason for this accumulation has remained unclear. PSTPIP1 is a member of the F-bar family of proteins and has important interactions with the actin nucleating protein, WASp, features which suggested that it could play a role in cytoskeletal regulation. We therefore tested the hypothesis that PSTPIP1 would regulate the function of macrophage podosomes, which are actin-based structures that mediate adhesion, chemotaxis, and ECM degradation in myeloid-lineage cells. Lentiviral transduction of shRNA targeting PSTPIP1 resulted in an approximately 90% decrease in PSTPIP1 expression in the human monocyte cell line, THP-1. Differentiation of the THP-1 cells on fibrinogen-coated cover slips and staining for actin and vinculin revealed that PSTPIP1 deficiency caused increased formation of highly-organized podosome rosettes. One of the unique functions of podosomes, as compared to other adhesion types, is the ability to degrade extracellular matrix. We found that PSTPIP1 deficient THP-1 macrophages had an increased ability to degrade a fluorescent-gelatin extracellular matrix as compared to controls. Preliminary data suggest that rescuing the PSTPIP1 knockdown with a form mutated in PAPA syndrome results in reduced podosome number, organization, and degradative capacity as compared to controls. These results suggest that the normal function of PSTPIP1 is to inhibit podosome organization, and that this inhibitory feature is amplified in PSTPIP1 mutants. The correlation of increased podosome organization and degradative capacity is consistent with studies in osteoclasts that demonstrate the necessity of podosome rosette formation prior to degrading bone matrix. Preliminary data also show that PSTPIP1 deficiency also results in decreased chemotaxis of THP-1 monocytes to CCL2. These findings imply that PSTPIP1 is important for podosome patterning and chemotaxis in macrophages. Additionally, they suggest that a potential mechanism underlying phagocyte accumulation in the skin and joints of PAPA syndrome patients is the altered interaction of macrophages with the ECM via dysregulation of podosomes.

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Extraction and Analysis of 3D Cytoskeletal Networks Based on Open Active Contour Models.

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Network structures formed by actin and microtubule filaments are present in many kinds of fluorescence microscopy images. To quantify their conformations and dynamics, we propose a fully automated method to extract these networks and analyze their topology. This method handles well intersecting filaments, and can also detect relatively faint structures from noisy microscopic images in both 2D and 3D. First we automatically initialize a large number of Stretching Open Active Contours (SOACs) from ridge points in a multi-scale fashion. These SOACs then elongate along the bright centerlines of laments by minimizing an energy function. During their evolution, they merge and stop growing at junctions, forming a network that represents the topology of the filament ensemble. We further dissect converged SOACs at junctions and reassemble these SOAC segments by a graph-cut spectral clustering method,

respecting continuity at junctions. The proposed approach is generally applicable to extracting network and intersecting curvilinear structures in noisy 2D and 3D images. We demonstrate its potential using two kinds of data: (1) actin filaments imaged by Total Internal Reflection Fluorescence Microscopy (TIRFM) in vitro in 2D; (2) 3D actin cytoskeleton networks formed by actin filaments in fission yeast imaged by spinning disk confocal microscopy in live cells. Results have shown that the method can not only extract the skeleton of the networks but also reveal quantitative information of their topology.

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Time fluctuation of single cell rheology by atomic force microscopy.

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Living cell is a soft viscoelastic material, which is strongly related to the highly dynamic and continuous remodeling of cytoskeleton. Previous studies have consistently revealed that the ensemble-averaged rheological properties, e.g. the complex shear modulus, G*, of various types of cells forced in an external stress followed a power-law rheology. However, it is little known how G* fluctuates in time and the time distribution is related to the ensemble one. Here we investigated the time distributions of G* of single mouse fibroblast cells, which were arranged on microarray, for t = 2.5 h by atomic force microscopy (AFM) as a function of frequency, f. It was observed that (1) the frequency dependence of time-averaged G* followed a power-law rheology, (2) the time distribution of G* exhibited a log-normal distribution, and (3) the variation of the time distribution, σ, decreased with increasing f. These results were similar to those observed in the ensemble (cell-to-cell) measurements. Moreover, we found that σ varied in each cells and was small compared with the ensemble variation and that σ(t) of time distribution attained a constant value at ~ 2 h. The results indicate that single cells are weekly trapped in a partial region in their possible states of single cell rheology.

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Alterations of Cell Edge Dynamics and Actin Cytoskeleton Define Migration Properties of Transformed Fibroblasts.

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Leading edge protrusion plays an important role in cell migration. It is well known that neoplastic transformation is accompanied by significant changes in the cytoskeletal organization and leads to enhanced invasion and metastasis of malignant cells. However, specific behavioral and cytoskeletal changes accompanying the invasive phenotype are poorly understood. In this study, we investigated distribution, dynamics, and the cytoskeletal architecture of leading edge protrusions in control fibroblast cell lines and their transformed derivatives, and compared these parameters to the cell migratory phenotypes. The most significant differences between transformed and non-transformed cell types were found their protrusive behavior. Two studied control cell lines, human skin fibroblasts (1036) and human embryo lung fibroblasts (MRC5), had an elongated polarized cell shape and a dominant leading edge. More rarely, a few protrusive areas were observed. In contrast, the corresponding transformed cell lines, human fibrosarcoma HT-1080 and SV-40-transformed MRC5 cells (MRC5-V1 and MRC5-V2), were poorly polarized and had multiple protrusion, frequently in the form of ruffles. However, the percentage of cell perimeter occupied by protrusions was significantly greater in transformed cells (up to 95% in HT-1080 cells) than in control fibroblasts, where protrusion-containing cell edges occupied ~50% of cell perimeter. Kymograph analysis revealed that lamellipodia of transformed cells advanced a shorter distance in each cycle than control cells, but the frequency of protrusions was increased compared to control cells. Importantly, although

transformed cells moved slower and more randomly than control fibroblasts in 2D cultures, they displayed an increased transmigration in Boyden chambers and greater invasion of Matrigel. Additional differences revealed in transformed fibroblasts included smaller and more randomly organized stress fibers and focal adhesions, a decreased cell area, and an increased thickness of lamellae, as compared to control cells. We propose that observed redistribution of the protrusive activity, together with changes in the actin cytoskeleton and the leading edge dynamics, leads to altered motile behavior of transformed fibroblasts suitable for invasive migration.

Microtubule Dynamics and Its Regulation I

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An imaging based survey of CLASP-interacting protein functions in regulating microtubule dynamics.

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The microtubule (MT) cytoskeleton is a key effector downstream of various signaling pathways and is subject to tight regulation in response to intrinsic and extrinsic cues, often through transient interactions with a variety of microtubule-associated proteins (MAPs). Current research in our laboratory focuses on Drosophila CLASP, a microtubule (MT) plus-end tracking protein, and its interactors. CLASP contains binding domains for both MT and actin, suggesting it may be important in coordinating MT behavior with other cellular components. In this study, we have performed a dynamic imaging-based screen to define the microtubule-regulatory functions of recently identified candidate CLASP interactors. Double-stranded RNA (dsRNA) was used to knock down expression of 45 candidates in Drosophila S2 cells. Using EB1-GFP as a marker for the growing MT plus-end, we captured time-lapse movies of cells treated with dsRNA for 72 hours and plated on Concanavalin A to facilitate their adhesion and spreading. Imaging was followed by systematic, multi-parametric quantification of microtubule dynamics using plusTipTracker, a Matlab-based open-source software. PlusTipTracker allows for a fully automatic unbiased detection, classification and analysis of changes in MT behavior. Our results suggest that we are able to detect subtle but significant changes in MT dynamics at time points where loss of protein expression has no obvious impact on gross MT morphology and/or tubulin stability as assessed using immunofluorescence. Overall, approximately 50% of the CLASP-interacting proteins significantly affected EB1 localization at the MT plus-end and/or had significant and reproducible effects on MT dynamics. As predicted from their MT assembly-promoting activities, knockdown of APC, Clip-190 and Shortstop, among other genes, cause a significant alterations in growth dynamics such as increased growth speed or decreased growth lifetimes. However, although genes thought to play similar roles in MT regulation, such as MT plus-end interacting proteins (+TIPs), showed similar phenotypes, subtle but mutually exclusive functional profiles (“fingerprints”) emerged. This suggests that these proteins act in comparable but distinct ways to regulate the cytoskeleton. For example, knockdown of p150glued and Clip190 increased MT dynamics; however, while both exhibit an increased lifetime of MT growth events, p150glued appears to affect primarily MT catastrophe and rescue while Clip190 affects the rate of transition between MT growth and pause. In addition, despite their identification as CLASP interactors, candidate actin-binding proteins showed little influence on MT dynamics in these highly adherent cells. Finally, we have demonstrated a novel role in regulating MT dynamics for proteins involved in signal transduction, protein translation, and GTP hydrolysis.

This study shows that automated quantitative analysis of MT dynamics provides a powerful tool to define and compare the specific actions of proteins that populate broad functional classes.

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p150^{Glued} is a multifunctional microtubule modulator.

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Dynactin subunit p150^{Glued} binds to and activates cytoplasmic dynein. However, p150^{Glued} may also have important dynein-independent effects on microtubules. Here, we determine the mechanisms by which p150^{Glued} both catalyzes microtubule nucleation and promotes microtubule polymerization. Full length native p150^{Glued} is a dimer that encodes two tandem N-terminal microtubule-binding sites: a CAP-Gly domain followed by a basic region. We find that an N-terminal monomeric construct which lacks the dimerization domain cannot nucleate microtubules. An equivalent construct dimerized by the canonical GCN4 coiled coil promotes microtubule nucleation in a robust concentration-dependent manner, significantly reducing the lag time associated with microtubule nucleation. Both the CAP-Gly and basic domains are necessary for nucleation; dimeric constructs representing physiological splice forms that lack either domain cannot nucleate microtubules. Further, a single point mutation in the CAP-Gly domain, linked to inherited Parkinsonism, is also sufficient to block nucleating activity. Using TIRF microscopy to directly observe microtubule polymerization, we also see that monomeric p150^{Glued} only weakly promotes polymerization, while dimeric p150^{Glued} containing both domains act as an anticatastrophe factor and markedly enhances polymerization, more than doubling the rate of assembly. Finally, we show by size exclusion chromatography and FRET that, while the monomeric construct binds only a single soluble tubulin subunit, dimeric p150^{Glued} forms a ternary complex with two tubulin subunits, potentially coordinating them in close lateral register. We propose that, when dimerized, the tandem CAP-Gly and basic domains of p150^{Glued} contribute to nucleation by lowering the energy of an unfavorable substep in tubulin oligomerization, and promote polymerization by locally increasing the concentration of tubulin subunits near the microtubule. These functions may be dynamically regulated in the cell by the differential expression of p150^{Glued} splice forms. Importantly, these novel functions of dynactin may be particularly relevant in the extended axonal processes of mammalian neurons, consistent with our observation that a Parkinson's disease-associated mutation abrogates function.

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Dissecting biological function of EB1 family and Dis1/TOG/XMAP215 family proteins in interphase HeLa cells.

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Recently, two classes of microtubule-end-binding proteins, EB1 (end-binding 1) family and Dis1/TOG/XMAP215 family proteins, have been proven to autonomously associate with growing microtubule plus end and positively regulate microtubule growth using in vitro reconstitution systems (Bieling et al., Nature, 2007, 45, 1100-5; Brouhard et al., Cell, 2008, 132, 79-88). Although the in vitro systems have revealed their mechanistic properties for plus end recognition and microtubule growth promoting activities, functional redundancy or synergy between them in biological processes is obscure.

In this study, we compared the roles of EB1 family and ch-TOG, a *Xenopus* XMAP215 homologue, in microtubule organization using HeLa cells. In interphase HeLa cells, many

microtubule plus ends are attached and stabilized at the peripheral regions of cells. CLASP proteins are potent microtubule-anchoring factors in HeLa cells: CLASPs bind to EB1 at their middle portion and this interaction is involved in the stable attachment of microtubule ends to the basal cell cortex (Mimori-Kiyosue et al. JCB, 2005, 168, 141-153). Consistent with this previous observation, siRNA-mediated nockdown of EB1 reduced microtubule density at the cell periphery, while ch-TOG was remained at the microtubule ends. On the contrary, knockdown of ch-TOG increased CLASP accumulation and microtubule density at the cell periphery. High-resolution SIM (structured-illumination microscopy) technique revealed that ch-TOG localizes to distinct portion of EB1/CLASP-accumulating microtubule plus end, where is the more distal of the EB1 comet. These observations suggest that ch-TOG and EB1/CLASP complex can act independently at microtubule plus ends and exert opposite effects on microtubule stability at the cell periphery.

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Quantitative Live Imaging and Function of Microtubule Plus-End-Tracking Proteins in the Neuronal Growth Cone.

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Proper neural connections, essential to nervous system function, depend upon precise navigation by the neuronal growth cone. A fundamental problem in growth cone cell biology is how guidance pathways are integrated to coordinate cytoskeletal dynamics, thus driving accurate steering. To address this question, we focus on the plus-ends of microtubules (MTs), which explore the growth cone periphery and play a role in growth cone steering. MT plus-end dynamics are regulated by a conserved family of proteins called “plus-end-tracking proteins” (+TIPs). Yet, it is unclear how +TIPs interact with each other and with plus-ends to control MT behavior. Additionally, it is largely a mystery how signaling mechanisms downstream of extracellular cues coordinate +TIPs to guide the growth cone in the right direction.

One conserved node of integration for multiple guidance pathways is the Abelson kinase (Abl). Our work identified the +TIP XMAP215 and its co-factor Maskin as genetic antagonists of the +TIP CLASP, which is a direct Abl substrate. We also found that accurate axon guidance requires XMAP215 and Maskin *in vivo*, and that they antagonize Abl function.

In order to determine how +TIPs directly affect MTs inside the growth cone, we analyzed and quantified parameters of +TIP dynamics using PlusTipTracker, a Matlab-based open-source software, following acquisition of high-resolution live-imaging data of tagged +TIPs within cultured *Xenopus* growth cones. Simultaneous dual channel imaging of +TIPs, including EB1, CLASP, and XMAP215, demonstrate that while all of them localize to the plus-ends, they do so with different dynamics of binding. We are particularly interested to investigate how manipulations in Abl signaling lead to changes in +TIP binding patterns and subsequent MT dynamics. Furthermore, we also examine +TIP function by manipulating their expression levels in *Xenopus* growth cones. For example, partial knock-down of XMAP215 leads to a 40% increase in EB1-GFP velocity, which is unexpected given that XMAP215 is known to have MT polymerase activity. We are currently investigating the mechanism underlying this surprising phenotype. These combined results will tell us how +TIPs differentially behave within the growth cone, thus bringing us one step closer to illuminating how the growth cone MT+TIP complex may respond to changes in guidance cue signaling to affect MT dynamics and growth cone behavior.

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A novel method using dual color-coded display (dCCD) of EB3-GFP to examine spatial and temporal regulation of microtubule dynamics in live cells.

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Investigating the spatial and temporal control of microtubule assembly in live cells is critical to understanding many cellular processes in development and disease. Traditionally, fluorescent tagged tubulin molecules were used to follow microtubule dynamics, but recently, GFP-tagged plus end binding proteins (+TIPs) became the tool of choice, because they allow the visualization of the plus or growing ends in dense microtubule networks. However, the current methodology relies heavily on tracking microtubules over a period of several minutes and requires frequent imaging to obtain the dynamic information for each microtubule. To complement the tracking method and to minimize the damage of live cells that are sensitive to fluorescence excitation, we developed a dual color-coded display (dCCD) method that uses a two-color coding scheme to visualize EB3-GFP labeled microtubule ends and to rapidly detect dynamic changes of microtubule assembly in real time. Using digital image processing algorithms, we developed computational tools to identify and separate the distinctive color-coded microtubules ends that correspond to different events of microtubule assembly, including growth, rescue, and catastrophe/pause. By constructing a space map of these events, we can obtain a global view of microtubule dynamics in the cell, and compare it in different regions at different times. This method requires minimal imaging and has high sensitivity and accuracy to capture the change in microtubule dynamics. It has been tested in several cell types, including fibroblasts and epithelial cells, as well as motile structures such as nerve growth cones, and provided a fresh new look of microtubule regulation in live cells. Thus, the dCCD method offers a novel approach to studying microtubule dynamics in complex cellular processes.

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Synergistic Modulation of Microtubule Dynamics by EB1 and CLIP170 Involves Displacement of the Stably Bound Tubulin-GDP-P_i at Microtubule Ends.

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Microtubule plus end-tracking proteins (+TIPs) target to growing microtubule plus ends and are precisely positioned to modulate microtubule dynamics. The objective of this study was to elucidate the molecular mechanisms by which the +TIP proteins EB1 and CLIP 170 cooperatively regulate microtubule dynamics. Because CLIP170 can auto-inhibit itself through an intramolecular interaction between the CAP-Gly domains and its zinc-knuckle motifs, we used a truncated CLIP170 version devoid of the zinc-knuckle motifs (CL12, 27.175 kDa, S48–M300). Using small angle X-ray scattering, we found that CL12 adopts a largely extended conformation with two non-interacting CAP-Gly domains. Similar analysis of EB1 indicated that, in solution, EB1 is an elongated dimer. Analytical ultracentrifugation of EB1 and CL12 mixtures indicated that they form a 1:1 molar complex. By video microscopy, we found that EB1 and CL12 individually modulated steady-state microtubule dynamics- mainly by increasing the growth rate and decreasing the shortening rate. When EB1 and CL12 were present together, their effects on dynamics were several fold stronger than when present separately. For example, a 1:20 molar ratio of EB1 to tubulin increased the growth rate 30% and decreased the shortening rate 21%. The same molar ratio of CL12 to tubulin increased the growth rate 19% and the shortening rate 18%. However, at a highly dilute 1:80 molar ratio to tubulin, the mixture

of EB1 and CL12 still increased the growth rate ~30% and much more strongly decreased the shortening rate (by ~50%). Using [γ32P]GTP, we further found that the +TIPs partially depleted the microtubule ends of stably-bound tubulin-GDP-P_i, indicating that EB1 and CLIP170 may regulate microtubule dynamics in association with affecting the stabilizing cap at microtubule ends. Consistent with their effects on dynamics, depletion of tubulin-GDP-P_i was considerably higher with reconstituted EB1-CL12-microtubules than with microtubules assembled with EB1 or CL12 alone. The data support the hypothesis that +TIP proteins act synergistically to regulate microtubule dynamics by modulating the cap mechanism.

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TON2/FASS promotes microtubule nucleation in interphase plant cells.

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Arabidopsis *TON2* gene encodes the B'' regulatory subunit of the protein phosphatase PP2A. *ton2* mutants display severe defects in the cortical microtubule arrays: the density of the interphase cortical microtubules is reduced and the preprophase band is completely absent. To address the role *TON2* in the formation of cortical microtubule arrays we created and analyzed transgenic plants co-expressing microtubule nucleation marker GCP2:GFP and the microtubule marker Cherry:TUB5. These lines allowed us to compare the frequency of microtubule nucleation events the epidermal cells of wild-type and the *ton2* mutant. In the *ton2* mutant total nucleation frequency was 6.7 events per 100 um area in 1 hour, compared with 18.6 events in wild type. This corresponds to 2.8 fold reduction in microtubule nucleation activity in the *ton2*. We also found that cortical microtubules required the *TON2* function for the reorientation in response to the light signal. Our data indicate that *TON2* function is required for efficient nucleation of cortical microtubules and their reorganization. We hypothesize that the *TON2*-regulated MT nucleation plays a critical role in the organization of the cortical microtubule cytoskeleton during PPB formation, in cell morphogenesis and in response to environmental cues.

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DRB perturbed the interaction between casein kinase-2 and microtubules, depolymerized microtubules and induced apoptosis in MCF-7 cells.

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Casein Kinase 2 (CK2) has various roles in cell functioning including signaling, transcription and apoptosis. CK2 has been shown to interact with microtubules and to increase microtubule assembly, though its significance remains unknown. In the present study, we have explored the role of CK2 in regulating the assembly and stability of microtubules. Double immunofluorescence and co-immunoprecipitation experiments suggested that CK2 interacts with microtubules in cells. The pharmacological inhibition of CK2 by DRB, (5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside), a small molecule inhibitor of CK2, induced apoptosis in MCF-7 cells. The cells exposed to DRB showed increased nuclear localization of p53 indicating the activation of p53 dependent apoptotic pathway. It inhibited the proliferation of MCF-7 cells with half maximal inhibitory concentration of 5.8 ± 0.2 μM. DRB enhanced the antiproliferative effect of microtubule depolymerizing agents including colchicine, nocodazole and vinblastine, whereas it reduced the effect of taxol, a microtubule stabilizing agent. Further,

CK2/tubulin ratio in MCF-7 cells was found to decrease by 31% and 54% in the presence of 20 and 40 μM DRB, respectively, indicating that DRB perturbed the interaction of CK2 and microtubules in MCF-7 cells. DRB treatment induced disassembly of cellular microtubules in MCF-7 cells. The ratio of soluble to polymeric tubulin increased from 0.42 in control cells to 1.3 in the presence of 36 μM DRB suggesting that DRB-treatment decreased the polymeric mass of tubulin in cells. However, DRB did not affect the assembly of purified tubulin *in vitro* indicating that it did not directly inhibit microtubule polymerization. In addition, DRB inhibited the growth of nocodazole-depolymerized microtubules after nocodazole washout suggesting that it suppresses the reassembly of microtubules in cells. The results together suggested that CK2 may have a role in regulating microtubule assembly dynamics.

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Spatial Partitioning of a Model Cytoplasm by Microtubule Asters.

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The cytoplasms of large egg and embryo cells are organized primarily by radial arrays of microtubules called “asters.” Asters center pronuclei after fertilization and paired sister aster position the cleavage furrow in telophase. In polyspermic fertilization and natural syncytial embryos, asters space themselves and nuclei out regularly in cytoplasmic domains. Using interphase *Xenopus* egg extract to recapitulate aster behavior, we asked three questions: how do asters grow, how do neighboring asters interact, and how do asters move? Permeabilized sperm nuclei, with their associated centrosome, and Ca^{++} were added at time zero to mimic polyspermic fertilization. In some experiments, sperm nuclei were replaced by centrosomes, or beads coated with Aurora A antibody which mimic centrosomes (Tsai & Zheng, 2005, *Curr Biol 15*, 2156-63). Aster growth and interaction were scored by live imaging of thin squashes between passivated coverslips. Asters expanded by a combination of classic radial polymerization and an additional mechanism that adds new microtubules near the periphery. When growing asters contacted each other they formed interaction zones of low microtubule density, as seen in whole embryos. Asters tended to move away from these zones, and thus space themselves regularly. Aster movement, but not aster growth or aster-aster interactions, was blocked by dynein inhibitors. Normal aster growth and formation of interaction zones depended on the kinesin Kif4, as assayed by immunodepletion. Interaction zone formation, but not aster growth, depended on Aurora B kinase activity, as assayed by drug inhibition. Both Kif4 and Aurora B localized to microtubule bundles in interaction zones by live imaging, though they may be recruited by different mechanisms. We propose a mechanistic model for microtubule depletion at aster-aster interaction zones that involves Kif4, Aurora B, and a hypothetical microtubule crosslinker. This limits microtubule growth, leading to aster movement away from the zone by dynein-dependent pulling. Our model begins to explain positioning of centrosomes, nuclei, and cleavage furrows in early embryos.

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Cytoplasmic self-organization of internal membranes, microtubule- and actin-cytoskeleton inside microfluidics generated droplets.

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Rodionov and Borisy suggested that internal membranes are critical for the self-organization of cytoplasmic melanophore fragments [1]. On the other hand, Nedelec et al. reported that purified tubulin and motors alone result in microtubule aster formation in vitro [2]. Since then, the role of intracellular membranes in cytoplasmic self-organization has been discussed. Using microfluidic systems to generate droplets, we describe the self-organization of cytoplasmic frog egg extract in a spatially confined compartment surrounded by a pegylated, inert scaffolding oil. We characterized the temporal and spatial interplay of internal membranes and cytoskeletal components with directly labeled Tubulin and DiI staining. In the absence of centrosome, microtubules emerge from multiple nucleation sites which eventually coalesce. In parallel, initially dispersed internal membranes accumulate in the center of the droplet. Finally, microtubules form a cage around the internal membranes. This self-centering of microtubules and internal membranes can be prevented by inhibition of dynein motors with vanadate and by disruption of microtubules with nocodazole. In the late stage of cytoplasmic self-organization inside a droplet, the microtubule cage gets laterally constricted. Since the final lateral constriction can be prevented by latrunculin, but not by blebbistatin or nocodazole, it is probably due to curvature-induced cortical actin in a Myosin II independent manner.
To define determinants of three-dimensional arrangement, we addressed aspects of stiffness and gravity. The positioning of the microtubule cage and internal membranes along the z-axis is apparently due to both factors, while the lateral arrangement is clearly associated with differential stiffness of the scaffolding compartment.

Tang S., Renz M., and Driscoll M. contributed equally.
Field C., and Lippincott-Schwartz J. are corresponding authors.

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Induced transverse organization of Arabidopsis cortical microtubule arrays.

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Cortical microtubule arrays are critical for cell morphogenesis in plants and provide a unique model for investigating microtubule interaction and regulation. Microtubules form complex and dynamic patterns at the plant cell cortex in the absence of a centrosome or a known microtubule-organizing center. In rapidly elongating Arabidopsis epidermal cells, the cortical array organizes into a highly co-aligned network transverse to the cell axis and patterns extra-cellular matrix deposition. We hypothesize that plant cells create transverse microtubule array patterns by regulating the localization of nucleation sites and subsequent trajectories of new microtubules. To address our hypothesis, we have developed a treatment regimen using

exogenously applied plant hormones to induce a transverse microtubule array organization. We are examining the kinetics of array organization by imaging GFP:Tubulin or GFP:Eb1 expressing cells that are transitioning to the transverse alignment. Using the hormone induction as an assay, we show that organization into a transverse array does not require an intact actin cytoskeleton and is insensitive to Brefeldin A treatment, which blocks polarized secretion of several cell polarity markers. Quantitative tracking of the GFP:Eb1 probe revealed a strong bias for microtubule plus ends moving out of the outer periclinal cell face into the anticlinal cell faces. Hormone treatment changes the microtubule plus end distribution and promotes a gradual influx of new transverse microtubules from the lateral anticlinal faces of the cell that progressively organize the array into a transverse alignment. We propose from these data that transverse microtubule array organization is actively driven by the redistribution of microtubule nucleation sites into positions that favor transverse microtubule trajectories.

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Rho-kinase controls asymmetric patterning of microtubules essential for anterior-posterior axis formation in epithelial cells.

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Microtubules (MTs) have been found to play a central role in cell shape determination and polarized cell movements. In migrating cells, through acquiring an asymmetric configuration, the MT cytoskeleton contributes to the polarization of cellular activities that allow the cell to migrate in a directional fashion. Despite the importance of MTs for migratory cell polarity, relatively little is known about the molecular mechanisms by which MTs acquire a specific arrangement that enables asymmetric distribution of cellular activities.

In the present study we focus on the question how the organization of MT networks may be signaled in the cytoplasm by the Rho-kinase/ROCK-mediated pathway. Inhibition of ROCK activity induces symmetric discoid-shaped epithelial cells plated on a planar substrate to polarize along the anterior-posterior axis. Based on this observation, we decided to check whether this cell shape polarity reflects polarity in MT organization. In contrast to non-treated (NT) cells, dynamic MTs in ROCK-inhibited cells show an overall gradient of decreasing density from anterior to posterior. Close inspection of MT tracks collected over time in living cells revealed that cells with compromised ROCK activity switch the patterns of MT organization from a meshwork of randomly oriented MTs to an array of MTs with a strong bias toward the anterior. Therefore, we then set out to measure parameters of MT dynamic instability in these cells, since changes in such parameters are often used to explain distinct patterns in MT organization. By doing so, we showed that in ROCK-inhibited cells the growth of anterior MTs is often interspersed with episodes of pauses and shortenings that decrease the time MTs spent processively growing. The ratio of MT growth lifetimes between anterior and posterior MTs (lifetime A/P ratio) in ROCK-inhibited cells is ~0.5 compared to ~1 in NT cells. Moreover, unlike NT-cells, ROCK-inhibited cells show asymmetric distribution of MT growth, pause, and shortening densities between anterior and posterior. A/P ratio for these parameters in ROCK-inhibited cells is ~3 vs. ~0.8 in NT cells. Comparison of posterior MT dynamics in NT cells vs. ROCK-inhibited cells show absence of significant differences. Thus, we conclude that dramatic changes in MT arrangement seen upon ROCK suppression are explained by the local modulation of dynamics of MTs growing toward the cell anterior pole. This modulation presumably favors the accumulation of growing MTs in the vicinity of anterior cortical regions. Interestingly, treatment of ROCK-inhibited cells with Taxol, an MT-stabilizing drug that disorganizes polarized MT arrays, resulted in the loss of the anterior-posterior axis. In such conditions, the cells undergo dramatic spreading to the point where they formed round cells that looked like fried eggs.

Taken together, these findings suggest that ROCK-mediated signaling in epithelial cells attenuates anterior-posterior axis formation by organizing dynamic MTs into symmetric meshworks. Delineating the molecular mechanism underlying this phenomenon as well as determining its physiological role in morphogenetic cell behaviors is an ongoing part of our research program.

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Role of tubilins in structural and functional organization of energy metabolism in the muscle cells.

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It is becoming clear that the structure of the cell is important for the organization and energy metabolism of cells. Nowadays, it is known that tubulins are important for cell's energy metabolism; previous studies in our lab showed how βII-tubulin is the isotype attached to the outer mitochondrial membrane giving to VDAC its selective permeability in cardiac cells. Furthermore, this restricted permeability for ADP and ATP, favours their recycling in the coupled MtCK-ATP synthasome reactions in mitochondria, connecting oxidative phosphorylation to PCr synthesis within a supercomplex.
Based on these previous studies in cardiac cells, the present study shows the intracellular distribution of the different tubulin β isotypes in skeletal muscles (gastrocnemius white, gastrocnemius red, soleus and extensor digitorum longus), which are composed of fast-twitch, slow-twitch and mix-twitch fibres; finding out how this structural organization is playing a main role in the energy metabolism of cells, controlling the energy fluxes of ADP and ATP through the outer mitochondrial membrane and controlling VDAC's affinity.
Immunofluorescent staining and western blot analysis were carried out in adult skeletal muscle cells, revealing a different tubulin isotypes distribution in each skeletal fibre analysed, which may suggest us how the intracellular distribution of structural proteins are playing a role in the compartmentalisation and use of substrates.

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In vivo imaging of microtubule dynamics throughout tissue biogenesis and cell differentiation.

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Microtubules (MTs) are dynamic polymers composed of alpha/beta-tubulin heterodimers. The alternating polymerization and depolymerization of MTs, called dynamic instability, is important for interphase microtubule functions and spindle assembly and dynamics during cell division. Since many agents that block mitotic progression directly affect MT dynamics, this phenomenon is of great interest to develop and improve anticancer drugs. Measurements of dynamic instability in vitro and in cultured cells and early embryos have revealed differences in the speeds of growth and shortening and the frequencies of catastrophe and rescue in vitro and in vivo and among cell types. However, it is not known how these data relate to MT behavior in

intact animals. Specific unknowns include how the geometry (2D versus 3D) of the cell environment, and the degree of cellular differentiation contribute to dynamics. These are all important considerations for understanding MT biology in development and pathologies, and to manipulate MTs with pharmaceuticals.
We have developed methods to measure MT dynamics in an intact animal. We created a C. elegans strain in which GFP-tagged tubulin is expressed specifically in the vulval epithelial cells, several uterine muscle cells and their respective precursors. This system allows us to measure and compare MT dynamics in cells of the same lineage at different developmental stages within the same organism. Time-lapse acquisitions at one second intervals are made using real-time confocal imaging, and image processing and analysis are done with ImageJ and custom Matlab programs. MT dynamics can be observed in 2 dimensions with high spatial and temporal resolution. Protein depletion by lineage-specific RNAi and small molecules are used to dissect the role of known and candidate MT-associated proteins at each developmental stage.
Interestingly, MTs from differentiated cells have a higher growth rate than those of precursor cells (8.39 +/- 2.50 vs. 6.13 +/- 1.60 μm/min), and display long biphasic growth excursions, slowing as they near the cell periphery. Precursors cells' MTs have shorter growing phases and higher catastrophe and rescue frequencies. We screened 90 candidate MT regulators and found that MTs in differentiated cells and precursors cells respond distinctly to protein depletions. In the future, we will use this model to study mitotic MT dynamics in vivo. Ultimately our novel method will facilitate identification of drug targets, and eventually drugs, that can specifically disrupt MTs in dividing cells, sparing post-mitotic neurons unlike current anticancer drugs.

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Microtubule Dynamics and Nucleation in Live Muscle Fibers.

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The organization of microtubules in proliferating cells is understood well enough that even static images convey dynamic information about microtubule origin and growth direction. But in cells with a non-conventional microtubule network, as is the case for skeletal muscle fibers, this is impossible. Muscle fibers are large, multinucleated cells. Their microtubules form complicated 3D lattices which include the nuclei and the numerous small Golgi complexes. We know nothing of muscle microtubule dynamics, polarity, or origin and therefore can neither understand the role of microtubules in muscle function, nor predict the consequences of the microtubule disruptions observed in many pathological conditions. In order to bring to light microtubule dynamics in muscle fibers, we first sought a suitable fluorescent microtubule marker. Several were tested, first by transfection of the C2 muscle cell line, and then by injection and electroporation in the mouse Flexor Digitorum Brevis (FDB) muscle. It became clear however that different markers best suit each system. EB3-GFP and GFP-tubulin are well incorporated in the microtubules of FDB fibers and can be followed for extended periods of live cell confocal imaging, allowing us to visualize entire microtubules and their plus-ends. We find that muscle microtubules are highly dynamic and grow at rates comparable to those of proliferating cells (about 16 micrometers/min). They move longitudinally and transversely, and can switch from one to the other. The plus-end of the microtubules are all along the lattice, suggesting that this scaffold is made of microtubule bundles. To resolve the question of microtubule origin we have been following recovery from nocodazole-induced depolymerization. Numerous asters are seen, originating from the Golgi complex elements, which must be associated with or directly involved in the nucleation process. In support for such a role, we find that microtubule recovery is affected by Brefeldin A. We are also using shRNA to knock down specific Golgi proteins such as GM130 in order to assess their function in muscle microtubule nucleation. Nucleation from the

many Golgi elements gives the muscle microtubule network hundreds of origins and minus ends, and the potential for local regulation of trafficking and organelle positioning. The tools developed have allowed us to see for the first time how microtubules form and move in live muscle fibers and provide us with a platform for exploring the role of microtubules in muscle and the consequences of their defects in muscle diseases.

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Quantitating Microtubule Dynamics in Intact Mammalian Epidermis.

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Microtubules, integral components of a cell's cytoskeletal network, influence cell shape and motility, function in organelle transport and organize into the mitotic spindle that facilitates chromosome organization and segregation during cell division. In stratified mouse epidermis, microtubule organization varies between cell layers: microtubules within the proliferative basal cells focus around centrosomes, whereas microtubules within the differentiated layers lose association with the centrosome and reorganize to the cell cortex. It remains unclear, however, whether proliferative and differentiating epidermal cells exhibit distinct microtubule nucleation and growth rates. In this study, we engineer mice to express a fluorescently tagged microtubule end-binding protein EB1 to facilitate microtubule tracking during nucleation from interphase centrosomes *in vitro* and *in vivo*. In addition, this tool will assist in characterizing *in vivo* microtubule dynamics during asymmetric cell divisions in epidermal basal cells. Previous research has defined a complex of proteins that localize to the apical cortex of basal cells to drive spindle reorientation and asymmetric division. One of these proteins, NuMA, has binding domains in its N- and C-terminus that facilitates its interactions with dynein and microtubules, respectively. We are particularly interested in determining whether these NuMA/microtubule interactions play a role in the regulation of both microtubule dynamics and spindle orientation during epidermal stratification. Taken together, these studies will lend further insight into the function of interphase and mitotic microtubule dynamics during mammalian epidermal morphogenesis.

Cilia and Flagella I

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3-D architecture of growing flagella.

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Many cells have a cilium or flagellum, organelles that generate cell motility and important sensory and signaling organelles. Two of the most studied flagella with a canonical 9+2 structure are those of the green-algae Clamydomonas reinhardtii and the parasitic protozoa Trypanosoma brucei. C. reinhardtii has two flagella that are reabsorbed and shed prior to mitosis. T. brucei has a single flagellum for about half its cell cycle when a new flagellum starts growing along the pre-existing flagellum in preparation for mitosis. Thus, growing flagella can be found in recently divided C. reinhardtii and in T. brucei cells with two flagella. The large protein complexes containing the flagellar building blocks are delivered to the site of axonemal elongation in an evolutionary conserved process called intra flagellar transport (IFT). IFT transport is well studied, yet we know little about the order by which these structural components of the axoneme assemble into the growing flagellum. To examine the process of axoneme elongation, we studied the tips of growing flagella in C. reinhardtii and T. brucei using

electron tomography. The axoneme is disorganized during growth in long T. brucei flagella since associated proteins are not added at the same rate as MTs grow. We also show that membrane extension is not dependent on axonemal pushing.

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A novel distal appendage protein required for primary cilium formation.
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Primary cilia (PC) are microtubule-based organelles that play important mechano- and chemosensory roles in eukaryotic cells, and whose function is compromised in a number of diseases including polycystic kidney disease, obesity, and certain forms of mental retardation. The basal body that functions in PC assembly derives from the older mother centriole, which can be distinguished from the younger mother by the acquisition of subdistal and distal appendages, which are required for cilia formation. Here we have identified CCDC41 as a component of the mother centriole. We found that CCDC41 is recruited to mother centrioles between late S and the beginning of G2 phases of the cell cycle, coincident with the recruitment of two other previously characterized appendage proteins, Cep164 and Odf2, but prior to the recruitment of a third appendage protein, centriolin. Furthermore, CCDC41 colocalizes with Cep164, a distal appendage protein, but not with Odf2 or Centriolin, both subdistal appendage proteins. Knockdown of CCDC41 resulted in a defect in primary cilia formation and length, and a failure to recruit Cep164 and Odf2, but had no effect on centriolin recruitment. We hypothesize that CCDC41 is recruited to mother centrioles during appendage formation in a complex with other appendage proteins that likely includes Cep164.

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The pf27 mutant is defective in radial spoke transport in the cilium.
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Ciliary proteins are generated in the cell body and subsequently targeted to precise locations in the cilium. To address the mechanisms of assembly and transport, we focused on assembly of the radial spoke structure. The radial spoke first assembles as 12S precursor complexes in the cytoplasm. This is followed by IFT mediated transport in the ciliary compartment and docking of a fully assembled 20S radial spoke structure (Diener et al., 2011; Yang et al., 2005; Qin et al. 2004). We took advantage of the Chlamydomonas reinhardtii mutant pf27, which is deficient in ciliary radial spokes (Huang et al., 1981). Comparison of cytoplasmic extracts from pf27 and wild-type show no difference in composition of the 12S radial spoke precursor complex. Interestingly, the spokes that assemble in pf27 are localized to the proximal third of the axoneme, but otherwise are fully assembled into the mature 20S radial spoke complexes. Furthermore, 20S spoke complexes derived from wild-type axonemes are competent to bind pf27 axonemes in vitro. Thus, pf27 is apparently not defective in radial spoke assembly in the cytoplasm or docking of radial spokes to the axoneme. Rather, our results suggest that pf27 is defective in the transport of spoke complexes. To further test this idea, complementation in temporary dikaryons of wild-type and pf27 reveal rescue of radial spoke assembly from tip to base as previously described for other radial spoke mutants (Johnson and Rosenbaum, 1992). Additionally, Next-Generation high-throughput sequencing of pf27 reveals a mutation in a candidate flagellar associated protein (FAP) predicted to be a kinase. Consistent with this prediction, radial spoke proteins exhibit reduced phosphorylation in pf27 axonemes (Huang et al., 1981). Based on these results, we hypothesize that PF27 encodes a protein kinase required for facilitating interaction between the radial spoke precursor complex and the ciliary IFT transport machinery.

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A Novel Cytoplasmic Protein Necessary for Proper Assembly of Eukaryotic Flagella.

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Nearly all organisms in the eukaryotic branch of life are dependent on motile (9+2) cilia. Proper function of motile cilia is essential for normal embryonic development, mucous clearance from the respiratory tract and reproduction. A breakdown of normal ciliary function can lead to Primary Ciliary Dyskinesia (PCD), which is characterized by respiratory disorders, male infertility and female subfertility as well as body plan defects. As cilia have multiple regulatory structures, including the central pair, radial spokes and the dynein regulatory complex, a defect with even a portion of any of these structures may result in PCD. Little is currently understood about how these structures are assembled, this is especially the case with the central pair. Using the model organism *Chlamydomonas reinhardtii*, we have isolated a new central pair mutant called *uncoordinated 1(unc1)*. *Unc1* was originally identified as nearly immotile, resulting from asymmetrically activating flagella that often stall mid-bend. Electron microscopy of unc1 axonemes shows that the C2b projection is missing in 95% of observed cross-sections, and of these about half are missing part or all of the C2 microtubule and its associated projections. Using available antibodies for central pair proteins, western blotting of unc1 axonemes reveals reductions in Klp1 and Hydin, while Cpc1 and PF6 levels are equal to that of wild-type, confirming that unc1 results in C2 specific defects. The *UNC1* locus maps along LG V, and is part of a tandem duplication. Both UNC1 and its tandem duplicate are predicted subtilisin-like serine proteases. Knockdown of the tandem duplicate with an amiRNA construct results in a similar motility phenotype to unc1. Transformation of a epitope-tagged UNC1 construct restores motility with a normal waveform. Isolation of cell bodies, cytoplasmic extracts and flagella from rescued cells shows that UNC1-HA localizes to the cell body, supporting a potential cytoplasmic role for UNC1p in central pair pre-assembly processing events. Supported by NIGMS 44228 to DRM.

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Regulation of ciliary assembly through intron retention.

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The microspore of Marsilea vestita is a meiotic product that produces a male gametophyte. A series of nine divisions in precise planes generates a gametophyte comprising 7 sterile cells and 32 spermatozoids. Each spermatozoid possesses ~140 cilia. Gametophyte development is rapid and temporally precise (reaching completion in 11 h), spatially precise (cells and division planes are fixed within the microspore), and transcriptionally quiescent (the microspore stockpiles proteins and RNA, which are used to carry out spermatogenesis with no new RNA production). We employed deep sequencing (Solexa Illumina) and de novo transcriptome assembly (Trinity) to gain insights into how the gametophyte regulates the storage, processing and translation of stored RNA.

In silico analysis was used to generate a list of annotated ‘early’ transcripts predicted to contain retained introns. Many of these intron-containing transcripts encode proteins whose biological functions are required during late stages of development. A subset of these transcripts (e.g., PF16/SPAG6, PFK, IFT88/OSM-6, FAP234, FAP71, Shaggy/GSK3) has previously been found to be essential for ciliary/flagellar axoneme formation or are associated with axonemal structures. Early in gametophyte development, ~30% of all identified ciliary/flagellar axonemal transcripts contain retained introns. Comparisons of genomic and RNA sequences of ‘early’ intron-retaining ciliary transcripts reveals that these transcripts possess at least 1 intron embedded in the coding sequence both in the desiccated microspore and during early

gametophyte development, while other introns within and beyond the ORFs had been previously removed from the pre-mRNAs. In all cases examined, these retained introns are within the coding sequence of the transcript, code for a premature stop codon, and usually disrupt conserved encoded protein domains. Isoforms of these 'early' intron-containing transcripts were found at later stages of development to lack any introns, and this loss of intron retention results in an mRNA encoding for a full length, functional protein. We performed RNAi knockdowns of intron containing transcripts at the onset of development. Detectable anomalies in phenocopies were not observed until after the developmental time point when these transcripts are spliced, suggesting that the later spliced isoforms of these transcripts are developmentally essential, while the intron-containing transcripts might serve only as translationally/functionally incompetent precursors. While the proportion of ciliary/flagella axonemal transcripts found in our study to retain introns (8) constitutes only a small portion of the ciliome (i.e., 124 components in C. reinhardtii) it is important to note that several of these intron-retaining transcripts encode proteins essential for ciliary/axoneme formation and/or stability (e.g., SPAG6 and OSM-6). Our data suggest that the retention of introns may be a mechanism involved in temporally regulating the onset of translation of essential ciliary axonemal proteins and thus, forestall the assembly of cilia in the rapidly developing gametophyte until late in spermatid maturation. Supported by NSF grant 0842525 to S.M.W.

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Whole genome transcriptome analysis identifies new cilia genes.

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Cilia are microtubule based organelles that project from a cell. Cilia are found on almost every cell type of the human body and numerous diseases are associated with defects in cilia including respiratory infections, male infertility, situs inversus, polycystic kidney disease, retinal degeneration, and Bardet-Biedl Syndrome. Mass spectrometry analysis has identified over 600 proteins that compose cilia. At the base of cilia lie basal bodies that template cilia and recruit proteins for ciliary assembly. To understand how cilia are formed, we used Illumina-based whole genome transcriptome analysis during flagellar growth in the biflagellate green alga *Chlamydomonas reinhardtii*. *Chlamydomonas* cells were deflagellated by pH shock and then sampled at 3, 10, 30, and 60 min during flagellar growth for whole transcriptome analysis. We identified over 1400 genes that were upregulated during flagellar growth. We also used phylogenetic profiling to identify genes that are conserved in organisms with basal bodies, triplet microtubules and motile cilia; we found 65 proteins that have coevolved in *Chlamydomonas*, humans, zebrafish and moss and are missing from land plants, fungi, and nematodes. We focus on those genes that are conserved among this set of ciliated organisms and a set of genes that are up-regulated during flagellar growth. To validate these genes, we used 4-5 different shRNA lentiviral constructs to knockdown each candidate gene in human retinal pigment epithelial cells (hTERT-RPE) stably expressing centrin-GFP, a basal body/centriole marker. We focused our analysis on five phenotypes: the percent ciliated cells, cilia length, numbers of basal bodies/centrioles, distance between basal bodies/centrioles, and cell cycle defects as determined by EdU staining. We show that knockdown of GLOD4, a glyoxylase implicated in retinal degeneration, is upregulated 7-fold following deflagellation in *Chlamydomonas*. Knockdown in RPE cells leads to decreased ciliation of cells and the severity of the ciliary phenotype is correlated to the GLOD4 mRNA levels. ZMYND10, a zinc finger protein containing an MYND domain, is upregulated 71 fold following deflagellation in *Chlamydomonas*. Knockdown in RPE cells leads to cell cycle defects without affecting cilia or basal body/centriole numbers. In total, we validated 22 of our predicted genes and a large proportion of these show

defects in at least one of the five phenotypic categories. Characterization of these genes helped us to gain insight into the molecular mechanism by which centrosomes and cilia are assembled. Research is funded by grants from the National Institutes of Health.

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A Forward Enhancer Screen in C. elegans to Identify Novel Ciliopathy Genes.

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Cilia are signaling organelles and defects in cilia signaling or structure lead to diseases referred to as ciliopathies. Many ciliopathies including, Nephronopthisis (NPHP) and Meckel-Gruber Syndrome (MKS), are autosomal recessive disorders and can involve a series of mutations in multiple, overlapping genetic loci, most of which have yet to be identified. It has been hypothesized that the complex genetic program of ciliopathies leads to a spectrum of related phenotypes determined by the nature of the mutation and/or the combination of different mutations (mutational load) in the patient's background. Genetic screens in *C. elegans* can be utilized to identify candidate genes that contribute to cilia dysfunction. The ciliated sensory neurons (CSNs) of *C. elegans* can be used to assess the mutational load of ciliopathy defects based on quantifiable assays including CSN regulated behaviors and the ability of CSNs to uptake lipophilic dye. Worms unable to take up dye are designated *Dyf,* for dye-filling defective. We previously demonstrated this phenomenon by crossing worms mutant for the homolog of the human ciliopathy gene NPHP4 (*nphp4*$^{-/-}$) with worms mutant for a homolog of a human MKS gene (*mks(x)*$^{-/-}$). Alone, *nphp4*$^{-/-}$ or *mks(x)*$^{-/-}$ have very mild or no *Dyf* phenotypes but when crossed, the resultant *nphp4*$^{-/-}$*;mks(x)*$^{-/-}$ F_2 generation display enhanced *Dyf* phenotypes as well as behavioral phenotypes indicative of CSN defects. Using this *nphp4*$^{-/-}$ *Dyf*-based enhancer phenotype paradigm we performed chemical mutagenesis on *nphp4*$^{-/-}$ mutant worms to identify genes that enhance *Dyf* phenotypes and contribute to mutational load. After outcrossing, we identified nine independent worm strains with synergistic *Dyf* phenotypes on the nphp4$^{-/-}$ mutant background that were more severe than those found in *nphp4*$^{-/-}$ mutants alone. The enhanced *Dyf* phenotype in these strains segregated in ratios indicative of double recessive mutations. Bulk chromosomal analysis and non-complementation tests were used to map mutations and check for allelism. After mapping, we employed Illumina deep sequencing to identify causative genes/alleles. We have begun to analyze the function of new candidates in an effort to understand how mutations in these genes cause synergistic phenotypes with the NPHP4 mutation. Genes from this screen are likely to be strong candidates for novel human ciliopathy genes.

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Structural and functional transition of choroid plexus epithelial cilia revealed by proteomic analysis.

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Mammalian cilia and flagella have been classified into three major subtypes depending on the function and axonemal structure, namely, motile 9+2 cilia, non-motile 9+0 primary cilia and motile 9+0 nodal cilia. In addition, whereas 9+2 cilia are expressed as many as hundreds, 9+0 primary and nodal cilia are usually solitary. One of the rare exceptions is the multiple 9+0

primary cilia expressed in choroid plexus epithelial cells (CPECs). We have previously reported that CPEC cilia modulate the production of cerebrospinal fluid (Narita et al., Traffic, 11:287-301, 2010). In the present study, we performed a proteomic analysis of CPEC cilia to elucidate their exceptional features in molecular basis. By LC-MS analysis of proteins from purified swine CPEC cilia, 868 proteins were identified as a CPEC ciliome, of which 419 were not shared with other ciliome datasets. Unexpectedly, we found several molecules implicated in ciliary motility in the CPEC ciliome. Real-time PCR also validated the expression of these molecules in mouse CPECs. Immunostaining for one of them, radial spoke head 9 homolog (Rsph9), demonstrated that the molecule localized to a subpopulation of CPEC cilia. Live imaging of choroid plexus tissue exhibited that some CPEC cilia could beat vigorously at the neonatal stage, whereas adult CPEC cilia were non-motile. When observed with high-speed video microscopy, the beating pattern appeared to be similar to that of typical 9+2 cilia of ependyma, though several parameters were different. The motility of CPEC cilia did not produce any directional planar flow, as assessed by the movement of fluorescent microbeads added to the medium. Transmission electron microscopy of P1 mouse choroid plexus revealed that the coexistence of 9+0 and 9+2 cilia. In conclusion, our proteomic analysis revealed unique structural and functional changes of CPEC cilia, which highlight the diversities of mammalian cilia. Based on the present study, we propose a new concept on ciliary subtype classification to deal with various atypical cilia systematically, with consideration to possible molecular mechanisms underlying the diversity.

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Epsilon tubulin is essential for the formation and maintenance of basal bodies in Tetrahymena thermophila.

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Basal bodies and centrioles are conserved microtubule based organelles whose improper assembly leads to a number of diseases, including ciliopathies and cancer. Tubulin family members play integral roles in the function of these structures. I have identified the ε-tubulin gene in Tetrahymena thermophila and localized it through immunofluorescence to basal bodies. Immunoelectron microscopy has revealed that ε-tubulin localizes to the three main domains of the basal body: the proximal end, the distal end, and the microtubule scaffold. A complete knockout of ε-tubulin reveals that it is an essential gene required for the assembly and maintenance of the core triplet microtubule structure of basal bodies. Most recently we have identified possible novel binding partners of ε-tubulin that may shed insight into the mechanism of ε-tubulin's function. Concurrently, a mutational analysis of ε-tubulin is underway, with the hope of establishing a structure function relationship for this protein. The results of this research have enhanced our current understanding of the function of ε-tubulin and will further shed light on its role at basal bodies.

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The two human centrin homologues in Tetrahymena have similar but distinct functions at basal bodies.

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Centrins are a ubiquitous family of small Ca2+ binding proteins at centrioles and basal bodies. Structurally, they consist of two domains tethered by a short linker with each domain containing a pair of EF hands, a Ca2+ binding motif. Centrins are grouped into two groups based on sequence similarity to the human centrins, Centrin 2 and Centrin 3, and analyses of components that make up centrioles or basal bodies in different species suggest that they

contain a centrin isoform from each group. The study of centrins has largely focused on those belonging to the human Centrin 2 group, while neglecting those in the human Centrin 3 group. We used the ciliate protist Tetrahymena thermophila to gain a better understanding of the functions of the two centrin groups and to determine their redundancy. We have previously shown that the Tetrahymena Centrin 1 (Cen1), the human Centrin 2 homologue, is required for basal body stability, assembly, orientation of assembly, and separation. Here, we show that the Tetrahymena Centrin 2 (Cen2), the human Centrin 3 homologue, shares similar functions as Cen1. However, an examination of Cen2's molecular mechanisms shows that it behaves differently than Cen1, suggesting that the two proteins are distinct. In all, our data suggest that centrioles and basal bodies require a centrin from both groups in order to function correctly.

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Modular organization of known and novel proteins at the ciliary transition zone.

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Objective: We recently discovered that multiple proteins concentrated within the proximal region of the cilium, termed transition zone, are required for at least two inter-related events in an early ciliogenic pathway: attachment of the basal body to the plasma membrane and formation of a so-called ciliary gate. Our goal has been to identify additional components of the transition zone and understand how they fit within different modules localising to this ciliary region, whose role is critical for maintaining the functional compartmentalisation of the sensory organelle.

Methods: We employ C. elegans as a model system to study the localisation, function, and interactions of known and novel components of the ciliary transition zone. Novel transition zone and other ciliary proteins are uncovered using a combination of comparative genomics, functional genomics, expression profiles, and in vivo cell biology approaches.

Results: Our analyses indicate that MKS-5 (RPGRIP1L/NPHP8), implicated in several ciliopathies, including Meckel syndrome, Joubert syndrome, and nephronopthisis, serves as a major scaffolding protein within the transition zone. Specifically, MKS-5 is required for the proper localisation of all known transition zone proteins tested (three B9 domain proteins, MKS-2, MKS-3, MKS-6, NPHP-1 and NPHP-4), as well as three novel membrane-associated proteins. All proteins can be ascribed to one of two modules, termed "MKS" or "NPHP", with the latter containing NPHP-1 and NPHP-4. These results are largely based on robust genetic interaction data whereby disruption of two or more proteins within the MKS module, or the NPHP module, does not result in prominent ciliogenesis defects—but disruption of any combination of MKS and NPHP module proteins causes major ciliogenic phenotypes. Our findings also provide insights into the hierarchical assembly of MKS module proteins; in particular, we find that MKS-2 and B9 proteins represent 'core' components of the MKS module.

Conclusion: The transition zone, a previously unappreciated region of the cilium that is entirely conserved in evolution, is now implicated in ciliogenesis and gating of the cilium. Our findings provide insights into the organisation and functions of many known transition proteins. In addition, we have uncovered novel components of this ciliary region, revealing its surprising complexity. Furthermore, the novel proteins represent strong ciliopathy candidates for being implicated in ciliopathies such as Meckel/Joubert syndrome and/or nephronophthisis—which collectively affect development and various physiological functions (kidney, eye, etc.).

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Cdc14 association with basal bodies in the oomycete Phytophthora infestans indicates potential new role for this protein phosphatase.

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The dual-specificity phosphatase Cdc14 is best known as a regulator of cell cycle events such as mitosis and cytokinesis in yeast and animal cells. However, the diversity of processes affected by Cdc14 raises the question of whether its cell cycle functions are truly conserved between species. Analyzing Cdc14 in the lower eukaryote Phytophthora infestans, a member of the oomycete group of the Stramenopile kingdom, should provide further insight into the role of Cdc14 since this organism does not exhibit a classical cell cycle. Prior study in this organism already revealed differences in Cdc14. For example, instead of being post-translationally regulated like its fungal and metazoan relatives, PiCdc14 appears to be mainly under transcriptional control. It is absent in vegetative hyphae where mitosis occurs and expressed only during the spore stages of the life cycle which are mitotically quiescent. Since transformants overexpressing PiCdc14 exhibit normal nuclear behavior, the protein likely does not play a critical role in mitotic progression although PiCdc14 is known to complement a yeast Cdc14 mutation that normally arrests mitosis. Further investigation into the role of PiCdc14 uncovered a novel role. Subcellular localization studies based on fusions with fluorescent tags showed that PiCdc14 first appeared in nuclei during early sporulation. During the development of biflagellated zoospores from sporangia, PiCdc14 transits to basal bodies, which are the sites from which flagella develop. PiCdc14 interacted with microtubules in vitro, and abnormal cleavage of sporangial cytoplasm during zoosporogenesis occurred in transformants overexpressing PiCdc14. A connection between Cdc14 and flagella is also supported by their phylogenetic distribution (gain or loss), suggesting an ancestral role of Cdc14 in the regulation of basal bodies or development of flagellated cells.

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Kinesin-13 regulates microtubule dynamicity and ciliary motility in *Tetrahymena thermophila.*

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Previous research localized kinesin-13, a microtubule end depolymerase, to the tip of axonemes, and implicated this protein in negative regulation of ciliary length in diverse ciliated species (Blaineau et al., Curr. Biol., 2007,17:778-82; Dawson et al., Euk. Cell, 2007, 6: 2354-2364; Piao et al., Proc. Natl. Acad. Sci. USA. 2009, 106:4713-8). *Tetrahymena thermophila* has three kinesin-13 encoding genes: *KIN13A*, *KIN13B* and *KIN13C*. When tagged with GFP at the native locus, Kin13Ap-GFP was detected inside the micronucleus and the dividing macronucleus. Kin13Cp-GFP tagged at the native locus localized to the contractile vacuole pore. Kin13Bp tagged with GFP at the native locus did not produce a detectable fluorescence signal, but when overproduced, localized near basal bodies and inside cilia (including tips), and caused rapid disassembly of axonemes. Knocking out either *KIN13B* or *KIN13C* genes alone did not change the gross phenotype. However, cells lacking both *KIN13B* and *KIN13C* grow and move extremely slowly. Cilia of double knockout cells beat more slowly and have decreased curvature when viewed after fixation. Surprisingly, in KIN13B and KIN13C double knockout cells, the number, length and ultrastructure of axonemes and cortical microtubule bundles appear unaffected. However, the double knockout cells are hypersensitive to paclitaxel, indicating a change in microtubule dynamicity. A *GFP-KIN13B* transgene rescued taxol hypersensitivity of double knockout cells and GFP-Kin13Bp localized primarily to the basal

bodies. Based on immunofluorescence, the double knockout cells have increased levels of acetyl-K40 α-tubulin in cilia and cell cortex, consistent with decreased dynamicity of microtubules. We hypothesize that in *Tetrahymena*, kinesin-13 promotes tubulin subunit turnover in cortical and axonemal microtubules possibly by depolymerizing both the plus and minus ends, and that this activity is important for ciliary motility.

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CrKinesin13, a microtubule depolymerizer, depolymerizes cytoplasmic microtubules to control tubulin pool for flagellar assembly in Chlamydomonas.

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Cilia and flagella are microtubule based cellular organelle. They may be reassembled upon completion of cell division, or upon chemically or mechanically induced loss. How the molecular mechanisms underlying cytoplasmic microtubule dynamics to control assembly of this organelle is not well understood. Previously we have found that CrKinesin13, a microtubule depolymerizer, functions in both flagellar assembly and disassembly in Chlamydomonas reinhardtti. CrKinesin13 is phosphorylated in the cell body during flagellar assembly while it is targeted to the flagella during flagellar shortening. RNAi experiment demonstrates that CrKinesin13 is required for both processes. Here, we show that CrKinesin13 regulates the pool of cytoplasmic tubulins by depolymerizing cytoplasmic microtubule to regulate flagellar assembly. Conditions that interfere with cytoplasmic microtubule dynamics affect flagellar assembly. After flagella are removed chemically and the cells are allowed to reassemble flagella, control cells assemble around half length flagella in the absence of protein synthesis while CrKinesin13 RNAi cells assemble no flagella at the same condition. Similarly, adding Taxol to control cells completely blocks flagellar assembly in the absence of protein synthesis. At onset of flagellar assembly, cell body pool of free tubulin increased and cytoplasmic microtubules were shorter and sparse. In contrast, CrKinesin13 RNAi cells showed little or no changes in the amount of free tubulin or morphology of microtubule networks. The phosphorylated residues of CrKineisn13 were determined to be S100 and S522 by mass spectrometry. However, in vitro assay showed that protein phosphorylation did not affect microtubule disassembly activity of CrKinesin13. We conclude that CrKinesin13 controls cytoplasmic microtubule disassembly to regulate flagellar assembly and protein phosphorylation may facilitate CrKinesin targeting to microtubules by interacting with unknown factors. This work was supported by NSFC (Grant 30830057).

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The primary cilium regulates the expression and subcellular localization of kinesin-3 family member Kif13B.

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Intraflagellar Transport (IFT) motors of the kinesin-2 family are known to participate in building of the ciliary axoneme, but additional kinesins may contribute to certain aspects of cilia assembly or function. In *Caenorhabditis elegans* the kinesin-3 family protein Klp6 is required for localization of polycystin-2 to the membrane of sensory cilia (Peden and Barr, Curr Biol, 2005) and for regulation of IFT (Morsci and Barr, Curr Biol, 2011). A kinesin-3-encoding gene from *Drosophila* was found to contain a consensus binding site for cilia-specific RFX-type transcription factors, suggesting a potential ciliary function for this kinesin (Laurencon et al., Genome Biol, 2007). Furthermore, it was demonstrated that the kinesin-3 Kif13A localizes to

centrosomes in HeLa cells (Sagona et al., Nat Cell Biol, 2010), but if any mammalian kinesin-3 family proteins play a role in ciliary assembly or function is unknown.
We recently showed that kinesin-3 Kif13B is strongly up regulated during growth arrest in mouse NIH3T3 cells, suggesting potential cilia-related functions for this kinesin (Thorsteinsson et al., Methods Cell Biol, 2009). Further, we showed that the primary cilium plays a critical role in regulating directional cell migration in mouse fibroblasts (Schneider et al., Cell Physiol Biochem, 2010). Here we report that both endogenous and GFP-fused Kif13B are localized to the base of the primary cilium, as well as to cell-cell adhesion sites, in growth-arrested mouse fibroblasts and other cultured mammalian cell types. This localization is abolished in *Tg737*^{orpk} mouse embryonic fibroblasts (MEFs), which lack primary cilia, and in *inv*$^{-/-}$ MEFs, which are defective in cilia-mediated Wingless/Int signaling, cell polarity, and directional cell migration. Using RT-qPCR we show that the expression level of Kif13B is dramatically reduced in ciliated *inv*$^{-/-}$ MEFs compared to wild type MEFs, indicating that ciliary signaling controls the cellular level and localization of Kif13B. We hypothesize that KIF13B functions downstream of the cilium to promote cell polarity and directional cell migration.

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Phosphorylation of the Kinesin II Motor KIF17 Impacts OS Turnover in Cone Photoreceptors.

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Photoreceptor outer segments (OSs) are highly specialized cellular compartments, which are essential for vision. Various proteins are targeted to the OS where they function in the vision pathway, however, the molecular details of protein targeting to the OS are not well understood. Recent studies have focused on intraflagellar transport (IFT) which is a highly conserved bidirectional transport pathway which occurs along the MT-based ciliary axoneme. The canonical IFT pathway requires several components; the multiprotein IFT particle, the heterotrimeric kinesin II or KIF3 complex (anterograde), and cytoplasmic dynein 2 (retrograde). Previous work from our lab has demonstrated that photoreceptor IFT requires an additional kinesin 2 family motor, the homodimeric KIF17. Inhibition of KIF17 leads to a significant disruption of OS formation without impacting photoreceptor inner segments (ISs), a defect which is not phenocopied by KIF3 complex inhibition. These findings suggest an OS specific, non-redundant role for photoreceptor KIF17-based IFT. However, the mechanism used to coordinate KIF17 and the KIF3 complex is not clear. We have identified a role for phosphorylation, at a highly conserved serine residue in KIF17's tail domain, in regulating KIF17-based photoreceptor IFT. Analysis utilizing non-phosphorylatable and phosphomimetic KIF17 mutants revealed that phosphorylation modulates KIF17's translocation to the distal tip of zebrafish cone photoreceptor cilia. Consistent with this, phosphorylation impacts KIF17 distal tip accumulation in cultured kidney epithelial cells suggesting a conserved mechanism of KIF17 regulation. Because the c-terminus of KIF17 links the motor to the IFT complex we tested if this phosphorylation event, through kinase inhibition assays and overexpression of our mutant constructs, regulates the motor-IFT interaction necessary for OS formation. The aforementioned assays, coupled with biochemical analysis revealed that phosphorylation of the KIF17 tail domain regulates the interaction between the motor and the IFT complex. Next we investigated the role of KIF17 phosphorylation in OS development and maintenance. Compared to WT KIF17 and non-phosphorylatable KIF17, expression of the phosphomimetic KIF17 in zebrafish cones induced a significant increase in OS disc shedding. These findings reveal a new mechanism used to coordinate KIF17 activity in photoreceptor OSs, which is essential for regulating OS turnover.

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Differential Calcium-dependent Regulation of Embryonic Cilia.

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Zebrafish embryos have multiple populations of cilia that emerge from cells in tissues such as the KV, the pronephric kidney, the developing ear and in neuromasts. Each of these ciliated tissues exhibits Ca^{2+} elevations and activated CaMK-II. CaMK-II autophosphorylates (T^{287}) upon Ca^{2+}/CaM stimulation and can be immunodetected in zebrafish embryos, thus identifying subcellular compartments of prolonged and relevant Ca^{2+} signaling. The KV (Kupffer's vesicle) is a transient ciliated organ where cilia-driven fluid flow leads to left-sided elevations of Ca^{2+} and activation of the "nodal" cascade to establish left-right organ asymmetry. CaMK-II is activated in ~4 ciliated cells on the left side of the KV. The pronephric kidney has ductal and cloacal cilia, but only cloacal cilia exhibit activated CaMK-II. Ca^{2+} elevations in the KV and kidney are mediated by polycystin-2 (PKD2), a "TRP" family Ca^{2+} channel. Hair cells in the ear exhibit kinocilia that nucleate otoliths, the bony particles that respond to sound or gravity. P-CaMK-II is enriched at the base of the kinocilium, just above the basal body, in the region corresponding to the transition zone. Activated CaMK-II is also enriched in ciliated neuromast clusters, which are sensory cells that migrate and differentiate along the lateral line. Suppression of CaMK-II causes a loss of left-right asymmetry, and incomplete morphogenesis of the kidney, ear and lateral line. KV cilia are shorter, cloacal cilia disassemble, ear kinocilia beat improperly, while ductal and spinal cord cilia are unaffected. Downstream from CaMK-II, potential targets that could explain these roles include members of the histone deacetylase (HDAC) and kinesin families. These are promising targets of CaMK-II since a) mutants also show defects in left-right asymmetry and kidney development, b) family members have been implicated in Ca^{2+}-dependent ciliary resorption and c) HDAC's and kinesins are known substrates or binding partners of CaMK-II. HDAC inhibitors preferentially elongate cloacal cilia, while ectopic KIF2C interferes with left-right asymmetry. Our results suggest that activated CaMK-II sequesters KIF2C and HDAC family members, such as HDAC6. Upon CaMK-II inactivation, KIF2C and/or HDAC6 become active and cilia are resorbed. These findings are relevant to development and the dynamics of primary cilia.

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The Effect of Calcium on IC138 Phosphorylation.

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Ciliary/flagellar motility, which is driven by the microtubule motor dynein, has been shown to be regulated by changes in intraflagellar calcium. One of the key regulatory dyneins is IC138, an intermediate chain subunit of the inner arm dynein complex, I1. A network of axonemal kinases and phosphatases controls IC138 phosphorylation; when IC138 is phosphorylated the dynein is inactive. Many of the axonemal kinases and phosphatases are associated with the central pair apparatus and the radial spokes. Chlamydomonas mutants lacking these structures are paralyzed and their flagella contain hyperphosphorylated levels of IC138. Moreover, axonemes isolated from these mutants have low microtubule sliding velocities. These phenotypes can be rescued by treatment with phosphatases and kinase inhibitors. Additionally, treatment of wild-type axonemes with a high calcium buffer results in increased microtubule sliding velocities. However, the role of calcium as it relates to the localized phosphorylation of specific dynein subunits is not known. Based on these data, we hypothesize that calcium regulates IC138 phosphorylation. Preliminary data indicate that IC138 phosphorylation levels are altered in response to a high calcium treatment, as seen through western blotting techniques. These results provide additional evidence for a signaling pathway initiated by calcium and transduced

through the central pair apparatus and radial spokes, resulting in the activity/inactivity of the dynein motors. Future studies will determine the exact mechanism through which calcium influences IC138 phosphorylation, and thus dynein activity

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Functional balance between ciliary kinase Mak and a microtubule-associated protein RP1 regulates ciliary length and survival of retinal photoreceptor cells.

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Ciliary dysfunction is associated with various diseases that can be broadly classified as “ciliopathies” in humans. While ciliary components and transport machinery have been well studied, regulatory mechanisms of ciliary formation and maintenance are poorly understood. Here we show that male germ cell-associated kinase, Mak, regulates retinal photoreceptor ciliary length and subcompartmentalization. Mak was localized both in the connecting cilia and outer segment axonemes of photoreceptor cells. In the *Mak*-null retina, photoreceptors exhibit elongated cilia and progressive degeneration. We observed accumulation of IFT88 and IFT57, expansion of Kif3a and acetylated α-tubulin signals in the *Mak*-null photoreceptor cilia. We found abnormal rhodopsin accumulation in the *Mak*-null photoreceptor cell bodies at P14. Electron microscopy analysis revealed aberrant outer-segment disk formation in Mak deficient photoreceptors. In addition, overexpression of RP1, a microtubule-associated protein localized in outer segment axonemes, induced ciliary elongation, and Mak coexpression rescued excessive ciliary elongation by RP1. The RP1 N-terminal portion induces both ciliary elongation and microtubule acetylation, and is phosphorylated by Mak. We identified a phosphorylation site of RP1 by Mak. Furthermore, we investigated whether Mak phosphorylates Kif3a or not. We found that Mak directly phosphorylates Kif3a. These results suggest that Mak is essential for ciliary protein transport, regulation of ciliary length, acetylation of ciliary microtubules, and is required for the long-term survival of photoreceptors.

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A Small Molecule-based Approach for Identifying Molecular Pathways Regulating Ciliary Length.

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Cilia or flagella are organelles that protrude from the surface of most mammalian cells and play diverse roles in sensing light, mechanical, and chemical signals as well as in generating flow of extracellular fluid. These nearly ubiquitous structures are so critical for normal cellular function that even minor alterations in ciliary length, assembly, or function can result in a variety of severe maladies including situs inversus, polycystic kidney disease, retinitis pigmentosa, and many pleiotropic disorders including Bardet-Biedl Syndrome, Joubert, Syndrome, and Meckel Syndrome. The ciliary core is a cylindrical arrangement of microtubule doublets and therefore regulation of ciliary structure and length has rightfully been addressed by studying the intraflagellar transport machinery and motor proteins required for ciliary assembly and disassembly. However, identification of long and short flagella mutants in the unicellular green algae, *Chlamydomonas reinhardtii*, has also highlighted the importance of signaling proteins not restricted to the flagellum. In this study, an unbiased chemical screen was performed to uncover pathways that are critical for ciliary formation and length regulation as well as identify potential targets for therapeutic intervention of ciliary malformation and malfunction. The annotated Sigma LOPAC1280 chemical library was utilized and flagellar phenotype was determined by assays for cell viability as well as flagellar length, motility and severing. Data from all assays were utilized to cluster potential targets for identification of novel pathways regulating flagellar

length and motility. The most frequently targeted protein family found to be involved in flagellar length regulation were the dopamine binding G-protein coupled receptors (GPCRs). *Chlamydomonas* is known to express GPCRs and have catecholamine biosynthesis and metabolism pathways. Further support for the role of this pathway in ciliary regulation comes from other ciliates in which biogenic amines are known to alter ciliary beat frequency and, in some cases, stimulate ciliary regeneration. Additionally, in mammals, dopamine receptors have been localized to neuronal cilia. Clustering analysis also predicted dopaminergic, opioid, and adrenergic receptors as potential regulators of flagellar motility, which was subsequently tested by high speed imaging. Comparison of data from a variety of assays using the same chemical library has successfully identified many novel signaling pathways that are potentially critical for ciliary formation and length maintenance. These include a variety of protein kinase pathways that may be ideal therapeutic targets for rescue of ciliary phenotypes.

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NDR is required for ciliogenesis through the phosphorylation of Rabin8.

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The primary cilium is a microtubule-based membrane projection localized on the surface of many vertebrate cells. Defects of cilium formation (ciliogenesis) and/or function cause an array of genetic disorders, such as Bardet-Biedl syndrome and polycystic kidney disease. Accumulating evidence indicated that vesicular transport mechanism involving Rab8 and its specific guanine nucleotide-exchange factor (GEF) Rabin8 (also known Rab3IP) is required for cilium formation. However, little is known about the regulation mechanisms of Rabin8 activity and localization during ciliogenesis. Nuclear Dbf2-related protein kinase (NDR) is an evolutionarily conserved Ser/Thr protein kinase. Although NDR family kinases are known to have a critical role in polarized growth, morphogenesis and cell cycle progression in yeast and invertebrates, the physiological function of mammalian NDR remains poorly understood. Here, we identified Rabin8 as a physiological substrate of NDR. In vitro kinase assay and western blot analysis revealed that Rabin8 is phosphorylated by NDR in vitro and in cultured cells. Furthermore, we show that NDR-mediated Rabin8 phosphorylation is required for primary cilia formation in hTERT-RPE cells. siRNA-mediated depletion of NDR caused the defect in cilium formation. The defects of ciliogenesis in Rabin8 knockdown cells were rescued by the expression of siRNA-resistant (sr)-Rabin8, but not by expression of a non-phosphorylatable or phospho-mimetic mutant of sr-Rabin8. Intriguingly, these Rabin8 mutants exhibited aberrations in proper accumulation of Rabin8 at the centrosome after serum withdrawal. These results indicate that phosphorylation is required for the Rabin8 function during ciliogenesis. We also provided the evidence that the phosphorylation of Rabin8 does not affect its GEF activity toward Rab8 in cell-free assays. A proteomic approach revealed that 14-3-3 proteins were bound to Rabin8, dependent on the phosphorylation of Rabin8. Together our results suggest that NDR-mediated Rabin8 phosphorylation and subsequent association of 14-3-3 are involved in Rabin8 localization and function during ciliogenesis.

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TSGA14 is mutated in the ciliopathy Joubert syndrome and is required for tubulin glutamylation at the cilium.

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Tubulin glutamylation is a post-translational modification (PTM) occurring predominantly on ciliary axonemal tubulin and has been suggested to be important for ciliary function. However, its relationship to disorders of the primary cilium, termed 'ciliopathies', has not been explored. Here, in Joubert syndrome (JBTS), we identify the JBTS13 locus and the responsible gene as TSGA14, encoding a centrosomal protein of 41 KDa (CEP41). We show that CEP41 is localized to the basal body/primary cilium, and regulates ciliary entry of TTLL6, an evolutionarily conserved polyglutamylase enzyme. Depletion of TSGA14 causes ciliopathy-related phenotypes in zebrafish and mouse, and induces cilia axonemal glutamylation defects. Our data identify loss of TSGA14 as a cause of JBTS ciliopathy and highlights the involvement of tubulin PTMs in the pathogenesis of the ciliopathy spectrum.

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Tubulin polyglutamylation regulates flagellar motility by modulating the function of inner arm dynein "e".

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Tubulin polyglutamylation is a post-translational modification that affects various microtubule-dependent cellular functions. The *tpg1* mutant of *Chlamydomonas* lacks a homologue of mouse TTLL9 (a tubulin polyglutamylase) and has a reduced level of polyglutamylated α-tubulin in the outer-doublet B-tubule. It displays lowered flagellar motility because of deficient interaction between inner-arm dynein and doublet microtubules (Kubo et al. Curr. Biol. 20, 441-445, 2010). *Chlamydomonas* has seven major species of inner-arm dyneins, designated dynein a~g. Dynein f (dynein I1) is a two-headed dynein containing two heavy chains, while all others are one-headed. In the present study, we produced double mutants of *tpg1* and various inner-arm-deficient mutants, and found that the *tpg1* mutation did not affect the lowered motility of mutants lacking inner-arm dynein e, while it greatly reduced the motility of mutants lacking dyneins other than dynein e. This observation suggests that tubulin glutamylation most sensitively affects the function of dynein e. The exceptionally basic microtubule binding site (the stalk tip) of dynein e may account for its high sensitivity to the *tpg1* mutation, which should greatly change the microtubule surface charge. Because dynein e is associated with the dynein regulatory complex (DRC; a multi-protein complex involved in dynein regulation and inter-doublet linkage) in the axoneme, and also because most mutants lacking dynein e examined in this study also lacked part of the DRC, it is possible that the *tpg1* mutation lowered the flagellar motility by affecting the function of the DRC as well as dynein e.

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Identification of a flagellar-specific adenylyl cyclase as a candidate for the enzyme regulated by flagellar adhesion and cilium-generated signaling in *Chlamydomonas* gametes.

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The cilium/flagellum increasingly is being recognized as a key organelle in activation of developmental pathways. During fertilization in the biflagellated green alga *Chlamydomonas*, flagellar adhesion between plus and minus gametes activates a signaling pathway in flagella, leading to an ~15-fold increase in cAMP. Gamete activation triggered by the increase in cAMP elevation prepares the gametes for cell-cell fusion. Previously we described a flagellar adenylyl cyclase activity with regulatory properties specific to gamete flagella. Here we report studies on a gamete-specific adenylyl cyclase, ACgf. The protein has several putative transmembrane domains, one putative TPR domain, and one cyclase homology domain. RT-PCR analysis demonstrated that ACgf transcripts are upregulated during flagellar regeneration. RNA-Seq analysis showed that transcripts are barely detectable in asexual, vegetative cells, 20-fold higher in gametes, and are upregulated another 5-fold during gamete activation. Fractionation of cells expressing an epitope-tagged form of ACgf indicates that the protein is highly enriched in flagella. Thus, ACgf is a candidate for the gamete specific, flagellar adenylyl cyclase whose activity is regulated by cilium-generated signaling in *Chlamydomonas*. Supported by NIH grant GM25661 to W. J. S. and a Wellcome Trust grant to O. B.

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Differential phosphorylation of the Chlamydomonas flagellar length marking protein CALK during flagellar assembly and disassembly.

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The cellular and molecular mechanisms that underlie the assembly and disassembly of cilia and flagella as well as control of organelle length are poorly understood. Previously we showed that the phosphorylation state of the aurora-like protein kinase CALK in Chlamydomonas is a marker of the presence of flagella. Compared to CALK in cells with flagella of steady state length (CALK78), the CALK in deflagellated cells and in cells mutationally or physiologically rendered aflagellate became phosphoryated (CALK80) based on a phosphatase-sensitive electrophoretic shift in SDS-PAGE. We do not yet know the residues(s) whose phosphorylation is responsible for the electrophoretic shift in apparent molecular mass from 78kDa to 80kDa. CALK phosphorylation state also is a marker of flagellar length. During flagellar assembly only CALK80 was present in cells with flagella equal to or less than � length, and CALK80 was dephosphorylated to CALK78 when the flagella became greater than � length. Here, we show that CALK activity and phosphorylation state are differentially regulated during flagellar assembly and disassembly. In vitro protein kinase assays (with mutated forms of CALK) showed that phosphorylation of threonine residue 193 (T193) in the activation loop was essential for CALK to phosphorylate an exogenous substrate, but was not essential for autophosphorylation of CALK. Using an anti-phospho-CALK antibody (pT193), we made the unexpected observation that in cells with flagella of steady state length, even though the unknown residue responsible for the CALK 78-80 shift was not phosphorylated, T193 on CALK was phosphorylated. Upon flagellar loss, however, the unknown residue became phosphorylated concomitantly with dephosphorylation of T193. It was not obligatory for one residue to be phosphorylated and the other dephosphorylated, because when flagellar shortening was triggered (either by NaPPi treatment or during zygote development) the

unknown residue(s) and T193 both were phosphorylated. GST pull-down assays using truncated forms of CALK indicated that the C-terminal, non-kinase domain interacted with N-terminal protein kinase domain. We conclude that CALK interacts both with itself and with other regulatory proteins, and that this protein kinase is at the nexus of multiple cellular pathways that sense and regulate flagellar assembly, flagellar disassembly, and flagellar length. This work is partly supported by the National Natural Science Foundation of China (30830057) and Tsinghua university (2010THZ0).

Intermediate Filaments

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Novel Interaction and Reciprocal Regulation between Annexin A2 and Keratin 17.

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Keratins are cytoplasmic intermediate filament proteins providing crucial structural support in epithelial cells. Keratin expression has diagnostic and even prognostic value in disease settings, and recent studies have uncovered novel non-mechanical functions of keratins in cell signaling. Elevated keratin expression in select cancers is correlated with higher expression of epidermal growth factor receptor (EGFR), whose overexpression and/or mutation give rise to cancer. In order to explore the role of keratins in oncogenic signaling pathways, we examined the regulation of epithelial growth-associated keratin 17 (K17) in response to EGFR activation. K17 is specifically upregulated in detergent-soluble fraction upon EGFR activation, and immunofluorescence analysis revealed alterations in K17-containing filaments. Interestingly, we identified AnxA2 as a novel interacting partner of K17, and this interaction is antagonized by EGFR activation. K17 and AnxA2 proteins show reciprocal regulation. AnxA2 overexpression delays EGFR-mediated change in K17 detergent solubility, and downregulation of K17 expression results in decreased AnxA2 phosphorylation at Y23. These findings uncover a novel interaction involving K17 and AnxA2, and identify AnxA2 as a potential regulator of keratin filaments.

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The Role of Tubulin Polyglutamylation on The Regulation of Neuronal Cytoskeleton.

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Microtubules, a major cytoskeletal component, form the structural basis of neuronal morphology and partake in directing intracellular transport. Tubulins are known to be highly polyglutamylated in neurons, and we, along with another group, have recently shown that a part of tubulin tyrosine ligase-like (Ttll) proteins catalyzes polyglutamylation of tubulins though the subsequent function of this polyglutamylation remains unclear. Present study demonstrates that Ttll1 and Ttll7 play major roles in adding glutamate chain to alpha and beta tubulin respectively in the brain. Ttll1 or Ttll7 single-knockout mice exhibited only a slight decrease in total polyglutamylation. To reveal the polyglutamylation function in the brain, we generated Ttll1-Ttll7 double-knockout mice which showed significant decrease in neuronal polyglutamylation. Immunohistochemical and electron microscopic analyses showed fewer neurofilaments in dendrites of cortical neurons in the double-knockout mice compared to the wild type, while the neurofilament distribution in Ttll1 or Ttll7 single-knockout mice was similar to that of the wild

type. This aberrant arrangement was specific to dendritic neurofitaments of double-knockout mice. The distributions of neurofilaments in axons and of microtubules in both dendrites and axons were comparable to that of the wild type. These results suggest that polyglutamilation regulates dendritic neurofilament distribution in the brain.

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Monitoring the desmin-nebulin assembly kinetics by single molecule approaches.

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Recent mathematical models support the idea that desmin intermediate filaments act as mechanical tethers to stabilize Z-discs between adjacent myofibrils and the extracellular matrix. Desmin association to nebulin is a key element in the maintenance of sarcomere connectivity, which is thought to be responsible for the lateral alignment of myofibrils. While the localization of the major integral proteins of striated muscle sarcomeres has been well characterized, the mechanisms that regulate the spatial organization with associated intermediate filaments are not yet well understood, particularly in regards to their role in muscle disease. To gain insight into the molecular mechanisms responsible for desminopathy, our study aims to understand structurally how desmin physically binds to nebulin fragments in vitro. We evaluated the early assembly kinetics using stopped flow experiments of two desminopathy-associated desmin mutants mapping to desmin binding peptides localized in the head (S46Y) and coil 1b (E245D) regions of desmin. Our data showed comparable light scattering at the tetramer level for desmin E245D and wild-type, while increased signals were obtained for desmin S46Y. However, during filament elongation no major differences were observed. Our sedimentation velocity analysis shows that the binding profiles of the nebulin fragment M160-164 shifted to higher s-values when mixed with either of these mutant desmin as compared to when mixed with wild-type desmin, suggesting that the interaction of these proteins responds to changes in desmin filament architecture. Using solid-phase binding assays, we detected that mutant desmin E245D bound with an increased binding capacity to nebulin M160-164 as compared to wild-type desmin, while no changes were detected for the interaction with mutant desmin S46Y. Using a combination of atomic force and total internal reflection microscopies ongoing single molecule experiments are evaluating in real-time the effect of nebulin fragment binding to fluorescently labeled desmin during late stages of filament elongation. All together our approach offers a powerful strategy to determine how complex cellular networks in muscle cells -intermediate filaments and sarcomeres- function in space and time.

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Desmin exhibits versatile strategies to connect with sarcomere thin filaments.

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Desmin intermediate filaments (IF) form an intricate three-dimensional meshwork that organize and integrate myofibers inside muscle cells. Highly regular arrays of interdigitating actin and myosin filaments compose the sarcomeres, muscle's minimal contractile units demarcated by Z-discs structures within myocytes. Limited data is available regarding the mechanisms that regulate the spatial organization of intermediate filaments and sarcomeres. It is not completely understood why the relative abundance of nebulin and actin filament lengths distribution differs in skeletal and cardiac muscles. In this study, we hypothesize that desmin IFs attach to the actin thin filaments in skeletal muscle via nebulin, while in cardiac muscle this connection is mediated

by nebulette. Moreover, a deeper understanding on the linkage between intermediate filament and sarcomeres may be relevant to deciphering molecular causes for muscle diseases such a desminopathy caused by inherited mutations in desmin. To unravel the nature of the association of desmin IFs and sarcomeres, we compared the binding affinity of desmin to nebulette M1-5 to that of nebulin M160-164 by solid-phase binding assays. Our results show that both fragments bound strongly to desmin with comparable disassociation constants (Kd ~17nM). Furthermore, we show that desminopathy-associated mutant desmin E245D (coil 1B) and T453I (tail), localized within nebulin-binding desmin peptides, reveal a tight specific association with either nebulette or nebulin fragments (Kd ~5nM). Using negatively stained electron microscopy we detected nebulette binding to desmin filaments in a similar fashion as that observed for nebulin. We also observed areas of co-localization for desmin and nebulette in cardiomyocytes near the Z-band region of the sarcomere. Our finding that a marked decrease of nebulette and actin protein levels in heart tissues from desmin null mice supports a strong functional relationship between nebulette and desmin. Ongoing studies are using siRNA to silence nebulette in cardiomyocytes to determine the effects its downregulation has on sarcomeric markers (F-actin and tropomodulin) and Z-disc associated IFs (plectin and synemin) distribution. We predict that most likely desmin and actin transcript and protein levels will be significantly decreased in myocytes depleted of nebulette. We present a working model in which desmin association with nebulette tailors specific functions to satisfy physiological demands of involuntary cardiac muscle, which in voluntary skeletal muscles are met instead by desmin association with nebulin.

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Regulation of Vimentin Expression Modulates p53 Levels and Apoptosis in Cancer Cells.

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Vimentin, a mesenchymal-specific intermediate filament (IF) protein, is often express in epithelial cancers exhibiting a de-differentiated phenotype. Vimentin is associated with increased chemoresistance and metastatic potential. Chemoresistance is a multifactorial phenomenon which is a major cause of treatment failure in human cancer. Some IF proteins are known to promote resistance to stress-induced apoptosis, but the role of vimentin in this process is still unknown. In the present study, we investigate the role of vimentin in response to apoptosis-induced chemotherapeutic drugs in different epithelial carcinoma cell lines. Surprisingly, using siRNA, our results show that vimentin downregulation decreases caspase-3 and PARP cleavage induced by cisplatin, doxorubicin and TNFalpha. This unexpected result could be explained by the decrease of p53 protein levels in vimentin-downregulated cells. Indeed, we observed that vimentin knockdown decreases p53 protein levels in a proteasome-independent manner. PI3K/Akt pathway is well known to promote chemoresistant phenotype in different cancers and Akt can also regulate p53 function. Our results show that vimentin-induced p53 decrease is associated with an increase of Akt activation/phosphorylation and XIAP protein levels. We propose that vimentin knockdown results in an increase of Akt activity which in turn regulates p53 levels. Previous studies already shown that vimentin can sequester p53 in cytoplasm. However, to our knowledge, these results represent the first indication that vimentin could regulate p53 levels and Akt activity in response to apoptosis-induced chemotherapeutic drugs. We have recently showed that Akt isoforms regulates vimentin expression in epithelial cancer cells, suggesting that it is directly involved in PI3K/Akt signaling pathway. These results support the hypothesis that modulation of vimentin expression plays an active role in cancer progression. Supported by NSERC to MC and IRSC to EA.

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Vimentin's Effect on Stiffness Depends on Cell Type.

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The mechanical characteristics of intermediate filaments (IF), including vimentin, support previous experimental results that showed roles for IF in the maintenance of cell mechanical properties and force resistance. Vimentin's involvement in cell motility was also recently demonstrated, and the formation of lamellipodia in regions devoid of vimentin provided further evidence that vimentin may locally stabilize the plasma membrane. We are investigating the effects of vimentin expression and distribution on cell stiffness, as well as how substrate stiffness affects vimentin dynamics. In order to compare the local stiffnesses of regions containing or not containing vimentin filaments, we generated micron-resolution stiffness maps of live cells expressing a fluorescently-tagged vimentin and correlated the stiffness and fluorescence intensity at each pixel. Our results demonstrate that in mouse embryo fibroblasts (mEF) on glass coverslips, regions containing vimentin filaments are less stiff than regions in which no vimentin is evident. Similar results are obtained when cells are immunostained for their endogenous vimentin. Vimentin-containing regions of mEF or epithelial MCF-10A cells, both of which endogenously express vimentin, are less stiff than regions where vimentin filaments are not present. These studies suggests that when cells express all three cytoskeletal filament types, cytoskeletal regions formed by relatively rigid F-actin and microtubules alone are stiffer than regions in which the more flexible vimentin filaments are present. Intriguingly, transient transfection with vimentin in MCF-7 epithelial cells, which do not express vimentin endogenously, increases their stiffness relative to non-transfected cells, possibly because in this case the added vimentin compounds the stiffness of the existing keratin IF network. We compared the average stiffnesses of clusters of single point measurements made over the nuclear region, the perinuclear region or near the cell periphery. In all three regions, MCF-7 cells expressing vimentin are stiffer than non-transfected cells of similar morphology on the same coverslip. We are also investigating how substrate stiffness affects the distribution and dynamics of the vimentin network. FRAP experiments in cells plated on substrates of varying stiffnesses demonstrate that the rate of vimentin subunit turnover is greater in cells grown on soft (5-10 kPa) than on very stiff (glass) substrates. These results suggest that one effect of vimentin expression is to 'tune' a cell for existence on a substrate of certain stiffness. Supported by NIH-GM083272 and GM096971.

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Electron Microscopy Structure Studies of Keratin Filaments.

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A disorganization of the keratin cytoskeleton in the outer skin layers physically creates a skin barrier dysfunction causing increased water permeability. This is a central feature in many skin diseases such as atopic dermatitis as well as for genetic disorders that are based on specific keratin mutations. We aim at understanding how cellular keratin filaments achieve this assignment and how keratin filaments are spatially organized and regulated.

We use negative staining, cryo-electron microscopy and cryo-electron tomography to characterize the structure, assembly and organization of keratin filaments in vitro. Purified recombinant keratin polymerizes into structurally variable filaments depending on dialysis conditions. Filaments in physiological buffer were vitrified on electron microscopy grids by

plunge-freezing. By this method macromolecular assemblies can be imaged in their native states without fixative or staining artifacts. These in vitro studies can be compared to electron micrographs of vitreous sections of native human skin. The advantages with cryo-electron microscopy are that intramolecular as well as intermolecular organization and the 3D properties are maintained during preparation and data collection. This allows us to study at molecular detail the 3D structure of keratin filaments and reconstruct a model for assembly and skin keratinocyte maturation.

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Mechanical Modeling of Keratinocyte Cell Spreading.

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Keratin intermediate filaments (IFs) are known to form crosslinked arrays so as to fulfill their structural support function in epithelial cells and tissues subjected to external stress. How the crosslinking of keratin IFs impacts the morphology and differentiation of keratinocytes in the epidermis and related surface epithelia remains an open question. Experimental measurements established that keratinocyte spreading area is inversely correlated to the extent of keratin IF bundling in two-dimensional culture. In an effort to quantitatively explain this relationship, we developed a simple continuum mechanical model in which isotropic cell spreading is considered as a first approximation. Relevant physical properties such as actin protrusion and adhesion events have been included in this model. The corresponding response of lamella formation at the cell periphery was included as a mathematical formula related to Bell's equation, along with experimental data relating time-dependent changes in keratinocyte surface area during spreading. Simulation results confirm the notion that the mechanical properties of crosslinked keratin filaments impact cell spreading, and detail the kinetics of this effect. The proposed model also provides insight into the distribution of stress within the cell and the impact of adhesion events during spreading. These *in silico* findings provide further support to the notion that differentiation-related changes in the density and intracellular organization of keratin IFs impact the morphological characteristics in epidermis and related stratified epithelia.

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A wound-induced keratin regulates Src activity during keratinocyte migration and tissue repair.

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The overall objective of this study is to mechanistically characterize the migratory enhancement of keratin 6 null mouse keratinocytes. Injury to the epidermis triggers an elaborate homeostatic response resulting in tissue repair and recovery of the vital barrier function. The type II keratins 6a and 6b (K6a, K6b) are among the genes induced early on in wound-proximal keratinocytes and maintained during re-epithelialization. Paradoxically, genetic ablation of K6a and K6b in mouse results in enhanced keratinocyte migration. Here we show, primarily by analyzing keratinocytes in primary culture settings, that this trait results from activation of Src kinase and key Src substrates that promote cell migration. Endogenous Src physically associates with keratin proteins in keratinocytes in a K6-dependent fashion, as seen in co-immunoprecipitation and co-localization studies done in primary culture. Purified Src binds K6-containing filaments in vitro in co-sedimentation and far western overlay experiments via its SH2 domain in a novel, phosphorylation-independent manner, resulting in kinase inhibition. Detergent-resistant membrane preparations show that K6 occurs in lipid rafts, a key site of Src regulation, and rafts

from K6 null keratinocytes are depleted in both keratin and Src. We conclude that K6 negatively regulates Src kinase activity and the migratory potential of skin keratinocytes during wound repair, and possibly in related contexts such as cancer.

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Vimentin's Role in Lipid Droplet Biology.

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Adipoctyes are specialized cells containing numerous lipid droplets (LDs) that are surrounded by vimentin intermediate filament (VIF) cages. Using the 3T3-L1 system as a model for adipocyte differentiation we have investigated the interaction between LDs and VIF. In pre-adipocytes the VIF network extends throughout the cytoplasm; as differentiation progresses VIF wrap around medium and large LDs as reported by others. Electron micrographs of platinum replicas of adipocytes clearly show that VIF wrap around and terminate on the LD surface. To determine if there is a physical link between VIF and LDs, cells were treated with nocodazole to depolymerize microtubules; in pre-adipocytes this causes the VIF to re-organize into a perinuclear cap. In adipocytes, however, VIF remain wrapped around the lipid droplets after nocodazole treatment, suggesting a physical link between VIF and LDs. To extend our microscopic findings we isolated LDs from adipocytes using sucrose density gradient centrifugation and found that VIF co-purified with the LDs, in agreement with previously published proteomic studies. Since the major function of LDs is to store fatty acids in the form of triacylglycerides we have initiated studies to determine if VIF modulate lipolysis, the process of release of fatty acids from adipocytes. We have found that stimulation of adipocytes with isoproterenol (an adrenergic receptor agonist) results in phosphorylation of VIF by PKA. PKA is the major activator of lipolytic processes in the adipocytes. In order to determine whether there is a specific role for VIF in lipolysis, vimentin expression was reduced using shRNA. The loss of vimentin resulted in a 42% reduction in the rate of lypolysis as measured by glycerol release, suggesting that VIF play an important role in lipolysis.

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Assembly properties and cellular distribution of human lamin A progeria mutants.

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We have analysed the assembly properties of eight point-mutated variants of lamin A causing premature ageing: T10I, Y45L, A57P, R133L, L140R, S143F, E145K, Y376L, as well as the “classical” lamin A mutant progerin. We followed a robust assembly protocol for paracrystal formation, employing high concentrations of CaCl2, and screened the assembly products by transmission electron microscopy. With wt-lamin, regular paracrystals of different size are abundant from 30 min on. By one hour, extensive paracrystalline arrays are observed at low magnification (3,000x) that upon closer inspection exhibit the well known regular transverse banding pattern with a 24.5-nm axial repeat. Surprisingly, the majority of the mutant variants assembled into structures seemingly identical to those exhibited by wt-lamin A, except for the E145K and the A57P mutant. Whereas the former produced long fibers with some kind of ordered substructure – but no regular transverse banding pattern – the latter formed exclusively non-structured aggregates. Similarly, under standard filament forming conditions, the A57P-lamin A did not assemble into extended filaments but only into aggregates like under paracrystal conditions.

When the mCherry-tagged A57P-lamin A was transfected into human U2OS cells, the protein did not integrate into the nuclear envelope but was distributed, different from mCherry-wt lamin A, in extensive fibrillar arrays throughout the cytoplasm. Hence, the mutation of alanine to proline at position 57 of lamin A compromises its assembly properties strongly, both in vitro and in vivo. Despite the fact that this alanine is in a b- rather than an a-position of the heptad repeat pattern, the proline at this position appears to have a dramatic effect on the structure of the coiled-coil dimer and thereby impedes all types of regular higher-order assembly. Furthermore, alanine 57 is not conserved in the various intermediate filament proteins and therefore most probably mediates a lamin A-specific molecular interaction.

Myosins

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Regulating motor force in skeletal muscle contraction.

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In active biological contractile processes, multiple motors can perform coordinated and synchronous actions while individual myosin motors appear to randomly attach to and detach from actin filaments. Recent experiment has demonstrated that, during skeletal muscle shortening at a wide range of velocities, individual myosin motors maintain a force about a fixed value during a working stroke. To understand how such force “homeostasis” can be so precisely regulated in an apparently chaotic system, here we develop a molecular model within a coupled stochastic-elastic theoretical framework. The model reveals that the unique force-stretch relation of myosin motor and the stochastic behavior of actin-myosin binding cause the average number of working motors to increase in linear proportion to the filament load, so that the force on each working motor is rather precisely regulated, in excellent agreement with experiment.

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Nonmuscle Myosin II Drives Contractile Ring Constriction by Exerting Tension on but not Translocating Actin-Filaments.

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During vertebrate cytokinesis it is thought that contractile ring constriction is driven by nonmuscle myosin II (NM II) translocation of antiparallel actin-filaments, similar to muscle contraction. Here we report in vivo, in situ and in vitro observations that challenge this hypothesis. NM II-B is essential for normal cardiac myocyte development. Ablation of NM II-B in mice resulted in defects in cardiac myocyte cytokinesis. Surprisingly, expression of mutant NM II-B R709C that cannot translocate actin filaments and has a substantially diminished MgATPase activity, in place of wild-type NM II-B, successfully rescues multinucleation in NM II-B ablated cardiomyocytes in mouse hearts. Graded siRNA knockdown of NM II-B in cultured COS-7 cells reveals that the amount of NM II limits contractile ring constriction. Time-lapse analyses show that both the rate and extent of ring constriction depends on the level of NM II expression. In addition expression of motor-impaired mutant NM IIs (NM II-B R709C, NM II-A N93K and NM II-A R234A) restores contractile ring constriction in COS-7 cells depleted of NM II-B, even though these mutant NM IIs are incapable of translocating actin-filaments. However contractile ring constriction is blocked by blebbistatin inhibition of myosin II strongly bound to actin in cells expressing either wild-type or mutant NM IIs. These results support a role for NM II

in generating tension but not translocating actin-filaments during contractile ring constriction. This role is substantiated by in vitro transient kinetic experiments with baculovirus-expressed NM II proteins using stopped-flow analyses. The mechanochemical properties of mutant NM II-B R709C show extremely high affinity for actin, despite loss of actin translocation. Under loaded conditions, mutant NM II exhibits prolonged strong actin attachment during which a single mechanoenzymatic cycle spans most of the time of cytokinesis. This prolonged attachment promotes simultaneous binding of essentially all NM II heads to actin, thereby increasing tension generation and resisting expansion of the ring and cell cortex, but further preventing translocation of actin-filaments.

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Defining the Properties of Nonmuscle Myosin IIs Using Full Length Proteins.

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Nonmuscle myosin IIs (NMIIs) are filament-forming myosins which are involved in a wide variety of cellular processes including cell adhesion, migration and cytokinesis. There are three paralogs of NMII in humans and mice, based on heavy chain expression by three different genes, located on different chromosomes. Owing to the difficulty in expressing the full length proteins (composed of dimers of a 230 kDa heavy chain and two pairs of light chains), previous studies on NMII have used tissue purified protein or expressed fragments of the molecule; both cases present potential drawbacks for in vitro protein characterization. To approach their native properties, we have successfully expressed full length wild type and mutated NMII proteins using the Sf9-baculovirus system. We also expressed two chimeric NMII proteins [Wang A et al. PNAS 2010, 107(33):14645-50] and GFP-NM II fusion proteins. We find that: I) although full length NMIIA, IIB and IIC exhibit biochemical differences, the morphology of the filaments determined by negative-staining electron microscopy (EM) is essentially indistinguishable among the three paralogs. In the presence of ATP all three paralogs display a similar ability to adopt the 10S compact conformation. II) EM images of chimeric molecules show that the tail domains of the paralogs are interchangeable in terms of filament formation and formation of the 10S compact conformation; III) In contrast to a previous report, the presence of point mutations in full length NM IIA proteins (N93K, D1424N, E1841K) causing human diseases has little or no obvious effects on filament formation; IV) GFP fused to NM II allows us to directly analyze in vitro motility (IVM) by TIRF microscopy.

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Mutations in the Motor and Rod Domains of Murine Nonmuscle Myosin II-A Cause Similar Defects and Mimic Human *MYH9*-Related Disease.

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Point mutations in *MYH9*, the gene encoding nonmuscle myosin heavy chain IIA (NMHC II-A), underlie autosomal dominant syndromes in humans (incidence 1 in 500,000). The abnormalities can manifest as macrothrombocytopenia, granulocyte inclusions, progressive proteinuric renal

disease, cataracts, and sensorineural deafness. To gain insight into the pathological mechanism of *MYH9*-related disease in humans, we generated three mouse lines, each with a different mutation in *Myh9* (R702C, D1424N and E1841K). Each line develops *MYH9*-related disease similar to that found in human patients. R702C mutant *human* cDNA fused with GFP was introduced into the first coding exon of *Myh9*, and D1424N and E1841K mutations were introduced directly into the corresponding exons. Homozygous R702C mice die at embryonic day 10.5-11.5 while homozygous D1424N and E1841K mice are viable. All heterozygous and homozygous mutant mice show macrothrombocytopenia with prolonged bleeding times, a defect in clot retraction and increased extramedullary megakaryocytes. Studies of cultured megakaryocytes and live cell imaging of megakaryocytes in the bone marrow show that heterozygous R702C megakaryocytes form fewer and shorter proplatelets with less branching and larger buds. The results indicate that disrupted proplatelet formation contributes to the macrothrombocytopenia in mice and most likely in humans. We also observed premature cataract formation, kidney abnormalities including albuminuria, focal segmental glomerulosclerosis with progressive kidney disease and mild hearing loss. Our results show that heterozygous mice with mutations in the myosin motor or filament-forming domain manifest similar phenotypes to humans with *MYH9*-related disease.

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HCM and DCM causing mutations affect the velocity and force producing capacity of human beta-cardiac myosin.

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Over 250 mutations in the β-cardiac myosin heavy chain can cause either hypertrophic or dilated cardiomyopathy (HCM or DCM), but the underlying molecular effects on the myosin molecule remain elusive. The primary reason the effect of HCM and DCM mutations on purified human β-cardiac myosin has not been elucidated is due to difficulties in expressing this protein in a functional and highly purified form. However, recent advances have resulted in the ability to express functional human α– and β-cardiac myosin in a mouse myoblast cell line. Using this expression system, we have purified wildtype human α– and β-cardiac S1 to which human cardiac essential light chain (MYL3) is bound. Analysis of these proteins in an in vitro motility assay reveals a velocity of ~ 0.5u/s for the β-cardiac S1 and ~1 u/s for the α–isoform at room temperature. Single molecule analysis using a dual beam optical trap assay to measure the stroke size under low load and the maximum force that single molecules of myosin can produce also show significant differences between the isoforms. Expression and purification of mutated forms of human β-cardiac S1 corresponding to mutations observed in patients with HCM (R403Q, R453C) and DCM (S532P) has been carried out, and ongoing analysis suggests these mutations alter both velocity and force production of the myosin motor.

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Structure of Human β-Cardiac Myosin Motor Domain at 3.2 Å.

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Point mutations in the human ß-cardiac myosin heavy chain are linked to devastating human cardiac diseases such as familial hypertrophic and dilated cardiomyopathies. Over 192 individual disease-causing mutations have been identified in the ß-cardiac myosin heavy chain alone. A systematic analysis of the mutations in the human myosin has been hindered by the

instability and heterogeneity of the protein when obtained from human specimens and by the lack of an adequate expression system to produce human ß-cardiac myosin. We showed that the principle obstacle for the expression of vertebrate striated muscle myosin is motor domain folding which requires a regulated folding pathway unique to striated muscle. As a proof of principle we constructed a human ß-cardiac myosin Motor Domain (MD)::GFP chimeric protein with a GFP domain substituting for the light chain binding region and expressed it in muscle cells using adenovirus vectors. The human ß-cardiac myosin MD::GFP chimera expressed in this manner is a functionally active motor that has been purified, crystallized, and its structure determined at 3.2 Å resolution. The human cardiac myosin MD is structurally homologous with the other two striated myosin motor domains in the PDB: the chicken skeletal myosin S1 and the scallop striated myosin S1. The protein was crystallized without nucleotide and has a sulfate ion in the ß-phosphate binding loop similar to the earlier structures. There are two non-identical cardiac MD's in the crystallographic asymmetric unit and both MD's are in a near-rigor conformation with an open 50 KDa cleft and extended switch 2 loop. The two distinct cardiac MD::GFP molecules are tightly packed against one another and are essentially identical up to the converter domain-lever arm-GFP extension, that projects at different angles from the two molecules. The structure determination of the human ß-cardiac myosin MD provides a new opportunity to investigate the mechanics of this motor, analyze the structural changes that lead to disease, study cardiac myosin specific drug interactions, and explore new drug designs. (Supported by a grant from the AHA, NIH and HHMI.)

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Influence of the Activity of Myosin II on the Stiffness of Suspended Cells.

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Mechanical properties of cells, e.g. stiffness, are closely related to cell function. For example, we could show that cancerous cells are less stiff than normal cells (Guck et al. 2005) or that the deformability of a haematopoietic precursor cell line (NB4) changed significantly when cells were ATRA differentiated. Cellular stiffness is mainly defined by the cytoskeleton, specifically the amount and cross-linking of the different filaments. Here, we investigated the influence of the activity of the molecular motor myosin II on the stiffness of different cell types. Adherent cells often form actin stress fibers which have the potential to change the stiffness of the cells by simple physical reinforcement of the cytoskeleton but also play a role for myosin II binding to actin. Therefore, we chose to measure cells in suspension without any physical contact to avoid stress fibers. This was possible using a dual beam laser trap (optical stretcher), which enabled us to hold and stretch single cells at a high throughput rate. We compared the deformability of three different cell types (NB4 cells, 3T3 fibroblasts and TNGA cells) before and after the treatment with 50 ÂµM blebbistatin and found a significant stiffening of blebbistatin treated cells, a result which has been reported with the opposite trend measured on adherent cells by AFM before. Further experiments with Y-27632 and 12,13-dibutyrate have been performed. These experiments sheet new light on the influence of molecular motor activity on cellular stiffness, but also raise awareness of the adherence status of cells when investigating their mechanical properties.

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Differential Evolutionary Constraints On The Coiled-Coil Proteins Tropomyosin, Paramyosin, and Myosin II In Invertebrate Striated Muscle.

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The myofilaments of invertebrate striated muscle contain three coiled-coil proteins: tropomyosin (Tm) in the thin filament, and paramyosin (Pm) and myosin II (Myo) in the thick filament. The coiled-coil domains are important for both the assembly of the filaments and the packing of the proteins in the filaments. Using protein sequences from GenBank, the PCOILS coiled-coil prediction program and BLAST, we analyzed percent amino acid sequence (AA seq) divergence for identical AAs only in the coiled-coil domains of these three proteins for 62 invertebrates representing four major Phyla: Nematoda, Arthropoda, Plathyhelminthes, and Mollusca. Taken as a whole, our results demonstrate that the AA seq of these coiled-coil proteins are very highly constrained, with Tm being the most constrained in the majority of organisms examined (varying 0% – 1% within most Orders). Given actin's high conservation throughout Eukaryota, it is consistent that Tm's coiled-coil domain is so highly constrained. Phyla members were also subdivided to make comparisons between more closely related organisms. Our results also demonstrate differential constraints for the coiled-coil domains of Tm, Pm, and Myo, both between Phyla and within a Phylum. In Nematoda, in the genus *Caenorhabditis*, the order of constraint is: Tm > Myo B > Pm > Myo A (including the 322 AA region essential for stability). In *Caenorhabditis*, where there are two isoforms of myosin in the thick filament, it was unexpected that Myo B has the highest constraint and Myo A has the lowest constraint given that Myo A, the filagenins and Pm constitute the thick filament core, with Myo B added to complete the assembly. However, for plant and animal parasitic nematodes, the general order of constraint is: Pm > Tm > Myo B > Myo A with the Tm sequences diverging 2% - 6% within an Order. Parasitic nematodes are the only animals studied where Pm is the most highly constrained coiled coil. In Arthropoda, in the genus *Drosophila*, the order of constraint is: Myo > Tm > Pm. In the three most closely related *Drosophila* species (time to the last common ancestor (TLCA) < 10 MYA), the Tm's are identical, but for six more distantly related species (TLCA ≈ 10 - 35 MYA), the Tm coiled coils diverge ≈ 6%. For the other Orders of arthropods, however, the order of constraint is: Tm > Myo > Pm. Of special interest in insects is the Tm of indirect flight muscle. In those insects where the coiled-coil domain starts near the N-terminus and is then split by a long polyproline rich region, their coiled coils are slightly less constrained than Tm-2, while for those where the coiled-coil domain is centrally located, those are significantly less constrained than Tm-2. In Plathyhelminthes, the order of constraint is: Tm > Myo > Pm. In Mollusca, with a limited data set, Pm and Tm are equally constrained. With paramyosin located in the core of the thick filament, it seems counterintuitive (with the exception of the parasitic nematodes), that its coiled-coil domain is less constrained than myosin's, as one might assume that the assembly of the thick filament core would necessitate a closely packed, and hence, more highly constrained protein structure. However, it appears that the packing of the myosin onto the outside of the thick filament requires the greater constraint. The differential selection pressures on the maintenance of the coiled coils of tropomyosin, paramyosin, and myosin suggests that the exact role of each coiled-coil domain in the assembly of the thick and thin filaments may vary considerably within invertebrate striated muscle.

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Structural characterization of thick filaments from *Drosophila* flight muscles.

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Flightin is a novel myosin rod binding protein that is essential for flight muscle function in *Drosophila*. The absence of flightin compromises the structural integrity and functional rigidity of thick filaments. We have initiated an electron microscopy analysis of negatively stained thick filaments with the goal of identifying the structural basis for the effect of flightin mutations on thick filament and muscle properties. Previous attempts at defining the structure of *Drosophila* IFM thick filaments were hampered by the lack of helical order. Here we investigated experimental conditions required to order myosin heads along the thick filament backbone. We show that thick filaments isolated in near physiological relaxing solution with the addition of blebbistatin, a small-molecule noncompetitive inhibitor of myosin II shown to stabilize the helical order of tarantula thick filaments, allow the collection of two dimensional images of *Drosophila* thick filaments with well ordered myosin heads. The diffraction pattern of filaments in relaxing solution produced clear layer lines 1 (43.5nm), 3 (14.5nm) and 6 (7.2nm). The addition of blebbistatin produced more layer lines and the subsidiary maxima of the Bessel functions are more persistently clear. Tomographic reconstruction provided strong evidence of a hollow core. A detailed three dimensional model of the thick filament from the fruit fly flight muscle will be generated by 3D electron microscopy and image processing. A combination of molecular labeling techniques and comparison of filament structures with and without flightin will result in the identification and localization of flightin in the thick filament. These studies will provide the first higher resolution three dimensional structure of thick filaments from a model genetic organism and will provide the foundation for the structural analysis of *Drosophila* flight muscle mutants with known genetic, biochemical, and mechanical defects. In addition, these studies will serve as a model for understanding how myosin binding proteins contribute to the structural and biomechanical properties of thick filaments.

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The E706K IBM3 myosin mutation depresses the chemomechanical properties and the stability of the molecular motor.

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Hereditary myosin myopathies are muscle diseases with variable clinical features and age of onset. Inclusion body myopathy 3 (IBM3) is an autosomal dominant myopathy associated with a missense mutation (E706K) in the myosin heavy chain IIa gene (MYH2). The disease is mild in childhood but appears progressive in adulthood, with proximal muscle weakness affecting movement. Biopsies from adult patients reveal dystrophic alterations and rimmed vacuoles consistent with an increased expression of the mutant motor with advanced age. Here we developed the first Drosophila melanogaster model system to study IBM-3. We constructed a transgene encoding E706K myosin and expressed it in Drosophila indirect flight and jump muscles. Flight and jump abilities were nearly absent in homozygotes. Homozygosity permits acquisition of highly pure samples of mutant myosin to investigate the unambiguous molecular consequences of the E706K mutation. The mutant myosin displayed 80% lower actin sliding velocity and 90 and 88% reductions in basal and actin stimulated ATPase activities compared to wild-type myosin. Heat-induced aggregation kinetics of myosin proteolytic fragments suggest that E706K S1 is more susceptible to aggregation relative to control S1. This observation was

directly verified via electron microscopy (EM), which revealed E706K myosin heads bear a dramatic propensity to unfold and to aggregate relative to wildtype heads. At 23ºC, 77.5% of control molecules exhibited two well-resolved independent heads compared to only 22.5% of mutant myosin molecules. A five minute, 37ºC incubation induced 80.9% of the control molecules' heads to form intra- or intermolecular aggregates versus 95.3% of the mutant myosin heads. This test suggests E706K myosin is far more labile than wildtype myosin. Furthermore, EM reveals E706K myofibrils exhibit Z-disk streaming, a loss of thick filaments and M-lines, sarcolemmal membrane invaginations and protrusions and they lack round shape and hexagonal packing of myofilaments. Cylindrical spiral membranes that resemble sarcolemmal inclusions of human skeletal myopathy are also found. Therefore, the ultrastructural hallmarks seen in adult patients also appear in our fly model. The depressed motor properties and the propensity for the mutant myosin to collapse and aggregate likely contribute to the muscle weakness observed in our fly model and possibly in senescent patients.

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Myosin 1s Move Rapidly on Dorsal Surfaces of Lamellipodia.

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Because the actin network in active lamellipodia is continuously assembling at the edge, moving inward at speeds up to 200 nm/s and disassembling, there is a question about how actin binding proteins and edge receptors are transported to the leading edge. There has been some indirect evidence of an active transport system but the basis of that transport is unknown. We show here that several myosin 1 isoforms (1G/1B/1C/1F) rapidly move to the leading edges of lamellipodia at different rates up to 5 μm/s. Photoactivation of PAmCherry-myosin 1G shows rapid outward movement on the upper surfaces of lamellipodia. Movement depends upon PH domains for lipid binding and upon ATPase activity. Surprisingly, at membranes bound to fibronectin-coated surfaces (ventral surface of lamellipodia), activated myosin 1G particles move in all directions; although at rapid rates (average velocity 3.4 ± 1.23 μm/s). Thus, it appears that myosin 1s actively move on dorsal actin to the leading edge and are not carried by fluid flow or some other biophysical mechanism. We suggest that myosin 1s transport actin, actin-binding proteins and some membrane receptors to the leading edges of lamellipodia to enable treadmilling. Further, myosin 1s can concentrate proteins at sites of actin polymerization.

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Effect of Myosin1e Knockdown on Cell Adhesion, Spreading, and Random Cell Migration.

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Class I myosins are actin-dependent molecular motors consisting of a head, neck, and tail; the tail domain contains TH1 (lipid binding), TH2 (proline-rich) and SH3 domains. Myosin1e is one of two "long-tailed" class I myosins, and has been implicated in both endocytic and exocytic membrane trafficking. In previous studies, knock out of another long-tailed myosin I, myosin1f, resulted in an increase in cell adhesion and spreading, and decreased cell motility, due to increased exocytosis of integrin-containing secretory vesicles (Kim *et al.*, 2006, Science **314**:136). The objective of the present research is to investigate whether myosin1e, like myosin1f, affects cell adhesion, spreading, and random cell migration in B16F1 mouse melanoma cells. The effect of myosin1e knockdown on both cell adhesion and cell spreading was examined on an integrin-dependent substrate, laminin, and an integrin-independent substrate, poly-L-lysine. Myosin1e knockdown resulted in significantly less adhesion and cell spreading on laminin compared to controls after 30 minutes. However, no significant difference

in adhesion and spreading was observed in myo1e knockdown cells plated on poly-L-lysine. Time-lapse videomicroscopy was used to track single cell migration in myo1e knockdown and non-targeting control cells. Preliminary random cell migration results demonstrate that myo1e knockdown cells exhibit less migratory behavior after two hours compared to controls. These findings are in contrast to results with myosin 1f-deficient cells, which exhibited increased adhesion and decreased cell motility. Nevertheless, these results suggest that myosin 1e has a role in cell adhesion, spreading, and random cell migration in B16F1 cells, possibly via regulation of integrin presentation at the cell surface.

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Myosin-1a tail homology 1 (TH1) domain targets to phosphatidylserine enriched microvilli using multiple membrane binding motifs.

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One of the most abundant components of the enterocyte brush border is the actin-based monomeric motor, myosin-1a (Myo1a). Within brush border microvilli, Myo1a forms lateral cross-bridges between the central actin bundle and the apical membrane. Proper physiological function of Myo1a depends on its ability to bind microvillar membrane, an interaction mediated by a C-terminal tail homology 1 (TH1) domain. As little is known about mechanistic details of the Myo1a-TH1/membrane interaction, our goal in this study was to characterize this binding event. In contrast to previously characterized class 1 myosins, Myo1a did not preferentially interact with phosphoinositol (4,5) bisphosphate (PIP2) in lipid binding assays. Additionally, mutating the conserved Myo1-pleckstrin homology (Myo1-PH) domain did not significantly impact microvillar enrichment of Myo1a-TH1 in cellular targeting assays. Using PIP strips to assay for other putative lipid binding partners in vitro revealed that Myo1a-TH1 might preferentially bind phosphatidylserine (PS). Myo1a-TH1 displayed striking colocalization with PS in microvilli of polarized epithelial cells. When co-expressed with the well characterized, bona fide PS binding domain from lactadherin-C2 (Yeung et al., Science; 2008), quantification of microvillar enrichment showed Myo1a-TH1 targeting was reduced, suggesting lactadherin-C2 can compete with Myo1a-TH1 for microvillar membrane binding sites. We sought to use our microvillar targeting assay to identify the minimal regions of Myo1a-TH1 required for microvillar enrichment. Structure/function analysis revealed that an N-terminal basic region, which is conserved across class 1 myosins, and a cluster of basic residues in the distal C-terminus of Myo1a-TH1, are both required for proper Myo1a-TH1 targeting to microvilli. Single molecule TIRF microscopy revealed that, of these two identified motifs, the C-terminal basic motif makes a greater contribution to the membrane bound lifetime of Myo1a-TH1. Taken together, our results suggest Myo1a membrane binding potential is distributed throughout TH1 rather than localized to a single motif and that the detailed mechanism of membrane targeting varies across vertebrate class 1 myosins. This variation may underlie differences in subcellular localization and function observed for distinct myosin-1 isoforms.

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Myosin IIIA kinase activity is regulated by phosphorylation of the kinase domain activation loop.

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Myosin IIIA (MYO3A) is the only member of the myosin superfamily that contains both a motor and kinase domain. We have found that the motor activity is decreased by autophosphorylation while little is known about the regulation of the kinase domain. We confirmed from sequence

analysis that MYO3A is a member of the STE kinase family and that threonine 184 (T184) is a highly conserved phosphorylation site among STE family members. We demonstrate by mass spectrometry that T184 is phosphorylated and identify several other potential phosphorylation sites in the kinase and motor domains. We present the probability of phopshorytion at each site and an examination of the regulatory role of T184. The kinase activity of MYO3A 2IQ with the phosphomimic (T184E) or phosphoblock (T184A) mutations demonstrates that kinase activity is reduced 30-fold as a result of the T184A mutation. Interestingly, the actin-activated ATPase activity of MYO3A 2IQ is impacted by the T184A/E mutations suggesting coupling between the motor and kinase domains. Furthermore, abolishing motor activity with a mutation in the motor active site reduces kinase activity 5-fold. To determine how altered kinase activity modulates the cellular functions of MYO3A, we plan to transfect GFP tagged MYO3A with the point mutations T184A/E into COS-7 cells. In addition, these experiments will be performed in the presence and absence of Espin 1, a known cargo for MYO3A in the stereocilia of inner ear hair cells. Our results suggest that phosphorylation of the kinase domain enhances MYO3A kinase activity, while phosphorylation of the motor domain deceases motor activity. We present a model for how the unique regulation of MYO3A allows it to function as an actin-bundle based transporter and in the formation of actin-bundle based protrusions.

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Overlap of Cargo Adaptor Binding Sites on Myosin V Coordinates the Movement of Cargoes.

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Myosin V motors move multiple cargoes within each cell type. These cargoes move to distinct locations at different times, which raises the question of how myosin V motors select individual cargoes. Specificity is achieved in part through organelle specific adaptor proteins that link the motor to individual cargoes. Here we show that the cargo-binding domain of myosin V also plays a critical role in the selection of cargoes. We show that the cargo binding domain of yeast Myo2 contains at least two distinct binding regions comprised of overlapping binding sites for nine known cargo-specific adaptor proteins. The simpler region interacts with two of these adaptors, Mmr1 and Vac17. Time-lapse analysis of organelle inheritance reveals a direct role for Myo2 interaction with Mmr1 and Vac17 in the transport of mitochondria and vacuoles, respectively. Moreover, we demonstrate that Vac17 and Mmr1 compete for access to Myo2 in vitro and in vivo. Importantly, point mutations that disrupt the interaction of Vac17 with Myo2 cause an increase in the amount of mitochondria inherited. Conversely, mutations that disrupt Mmr1 interaction with Myo2 result in an increase in the amount of vacuoles inherited. Our data suggest that the overlap in cargo adaptor binding sites modulates the total amount of mitochondria and vacuoles that are transmitted from the mother cell to the bud, and thereby plays a role in the distribution of organelles.

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Tropomyosin is Essential for Processive Movement of a Class V Myosin from Budding Yeast.

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In the budding yeast *S. cerevisiae*, the class V myosin Myo2p transports cargo along actin cables from the mother cell to the growing bud. Most class V myosins are processive, able to walk for a micron or more along actin filaments without dissociating, reflecting their role as cargo

transporters. Thus it was surprising when *in vitro* experiments characterized Myo2p as non-processive. However, these experiments were performed under non-physiologic conditions in that they used a track of bare skeletal muscle actin filaments. Here we show that Myo2p is processive on a more native actin track. Myo2p walks for several microns as a single motor along actin filaments containing tropomyosin, but shows no movement on bare actin. Different tropomyosin isoforms have varying effects on Myo2p behavior, but the native yeast tropomyosins Tpm1p and Tpm2p result in the strongest processivity. Smooth muscle tropomyosin also supports processive movement, but to a lesser extent. In contrast, nearly identical results were obtained with yeast and skeletal muscle actin. While moving processively, single quantum-dot labeled Myo2p motors moved at a speed of 5.5 µm/s with a step size of 34 nm. We are currently determine the mechanism by which tropomyosin affects Myo2p function. Initial results indicate that tropomyosin increases the fraction of each ATP hydrolysis cycle that Myo2p is strongly bound to actin (the duty ratio), and recruits more motors to actin filaments. Our results show that tropomyosin is crucial for the proper function of yeast myosin V. We speculate that several class V myosins in higher organisms that are non-processive *in vitro*, including human myosin Vc, may be processive on actin-tropomyosin tracks. In addition, there is growing evidence that myosin isoforms are differentially regulated by tropomyosin, and that tropomyosin may target the correct myosin to the proper actin structure in the cell.

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A novel isoform of spire1 encodes a mitochondrial actin nucleator.

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Highly dynamic structures, mitochondria play an essential role in cellular energy production, calcium signaling and buffering, and apoptosis regulation. To efficiently carry out these processes, mitochondrial transport and positioning must be tightly regulated. Mitochondria move bi-directionally along microtubule tracks, frequently pausing and reversing direction. It is often necessary, however, for mitochondria to reach microtubule-deficient areas of the cell, such as synapses, growth cones, and microvilli. Myosins have been implicated in the transport of mitochondria to such microtubule-deficient regions, as well as in the frequent pausing events, yet the mechanisms of such events remain elusive. Myosin Va is a molecular motor that transports a diverse array of organelles, secretory vesicles, membranous cargo, mRNA, and vesicles along actin tracks. Identification of the proteins and complexes involved in myosin-dependent transport is paramount for obtaining a clear understanding of these transport processes. To this effect, we performed a biochemical screen using affinity chromatography to identify proteins that bind the globular tail domain of myosin Va. We identified spire1 as a protein that binds myosin Va with high affinity and specificity, and performed subsequent assays to verify direct binding of spire1 to the myosin Va tail *in vitro*. Amplification of the spire1 gene from mouse brain cDNA yielded a novel splice variant that localizes to the mitochondria in numerous cell types. Further analyses revealed that an alternate exon of 58 residues is both necessary and sufficient for mitochondrial localization. Spire1 is a multi-domain protein that nucleates actin through its WH2 domains. It also interacts with formin2 through its KIND domain, yet its role in mitochondrial dynamics remains unexplored. Further analyses using purified proteins, affinity chromatography, mouse models, and live-cell imaging, are being utilized to elucidate the mechanism by which Spire1 may mediate mitochondrial dynamics and localization.

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Myosin VIIa and sans localization at inner ear hair cell stereocilia upper tip-link density (UTLD) implicates these Usher syndrome proteins in mechanotransduction.

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Inner ear hair cell stereocilia convert mechanical vibrations into electrical signals via the coordinated interactions of multiple proteins precisely positioned within the mechanotransduction (MET) complex. The MET complex is present at both ends of a “tip-link”, a bridge comprising cadherin-23 and protocadherin-15, which connects two adjacent stereocilia. Electron microscopy has revealed a protein-dense plaque underlying the stereocilia membrane at each end of the tip-link. The upper tip-link insertion density (UTLD) is presumed to contain the scaffolding protein harmonin, and a cluster of motor proteins that continually pulls on cadherin-23, creating a resting tension on the tip-link that is essential for optimal mechanosensitivity. While early immunolocalization and functional studies suggested that myosin 1c was the tensing or adaptation motor, later localization attempts failed to detect myosin 1c concentrated at the predicted UTLD site. More recent electrophysiological studies with myosin VIIa showed that this Usher syndrome protein was essential for maintaining a resting tension on the tip-link. However, there have been no reports of localization of myosin VIIa at the UTLD. Therefore, the identity of the tip-link tensing motor and the localization of myosin VIIa have both remained unresolved. Using immunofluorescence we show that myosin VIIa and sans, another Usher syndrome protein known to interact with myosin VIIa, cluster together with harmonin-b at the UTLD. We confirm myosin VIIa and sans localization at the UTLD by transfecting hair cells with GFP-tagged constructs for these proteins. Based on the median value of the distribution of immunofluorescence reactivity for each protein, the estimated copy number for myosin VIIa, sans, and harmonin-b is shown to be 8:9:23 respectively. The high number of myosin VIIa molecules that cluster at the UTLD is consistent with a direct role for myosin VIIa in maintaining tip-link tension. Co-transfection studies in COS7 cells show that myosin VIIa, sans, and harmonin-b form a tripartite complex, and that each protein is capable of interacting with one another independently. We propose that myosin VIIa, sans, and harmonin-b form the core components of the UTLD complex, and that myosin VIIa is likely the motor element that exerts tension on the tip-link.

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Relative functions of myosin VIIa isoforms 1 and 2.

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Myosin VIIa plays a key role in transport of melanosomes and phagosomes in the retinal pigment epithlelium (RPE), in opsin transport in photoreceptor cells, and in stereocilia formation in the hair cells of the cochlea. Loss of myosin VIIa functions causes progressive retinal degeneration, leading to blindness, and cochlear dysfunction in Usher syndrome type 1B (USH1B). Two transcript variants of myosin VIIa have been reported, isoform (IF) 1 and IF 2. IF 1 has an additional 38 amino acids in the second FERM domain of the tail region.

To assess the relative functions of these two IFs, we first analyzed their association with cellular targets of myosin VIIa. Using confocal microscopy with ARPE 19 cells, we found that the cellular localization of the two isoforms differs. IF 1 is not targeted to phagosomes, which result from uptake of photoreceptor outer segment membranes. In contrast, IF 2 is targeted to these compartments, where it colocalizes with lysosomal-associated membrane protein 2. Real time PCR analysis of the transcription rates of IF 1 and IF 2 in the eyecup, RPE, neural retina and

cochlea of mice showed that IF 2 is transcribed at significantly higher levels than IF 1 in all of these tissues. The same results were obtained with samples of human RPE and neural retina.

Our results suggest that IF 2 accomplishes the major functions of myosin VIIa in the RPE, neural retina, and cochlea. Gene therapy with viral vectors is a promising approach for the treatment of the retinal defects of USH1B. However, the limited capacity of these vectors requires the use of cDNA inserts. Our analysis identifies IF 2 as the most appropriate myosin VIIa transcript variant to use for USH1B gene therapy approaches in the RPE and neural retina.

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Myosin-10 interacts with wee1: implications for mitotic spindle structure and function.

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The unconventional myosin, myosin-10 (myo10), contributes to the structural integrity and/or function of various cellular structures including filopodia, meiotic and mitotic spindles. Previous studies from our lab of intact epithelium of the Xenopus laevis (hereafter Xenopus) embryo revealed that myo10 localizes to the mitotic spindle and that depletion of myo10 protein results in several mitotic spindle-related phenotypes including spindle elongation, failed anchoring, mitotic delay and spindle pole fragmentation [1]. However, the mechanisms by which myo10 contributes to proper spindle structure and function are currently unknown. Here we have characterized the distribution of GFP-tagged, full-length myo10 and several myo10 fragments in the embryo epithelium via 4D microscopy (x, y, z and time). Our data demonstrate that the subcellular distribution of GFP-myo10 is cell cycle dependent and that various interaction domains within the myo10 protein regulate different aspects of myo10 function. In particular, a pool of myo10 is nuclear and this localization, as well as spindle pole localization, both appear to be directed by a domain of the myo10 tail. Further results reveal that expression of a portion of the myo10 tail, the MyTH4 domain, induces some but not all of the spindle phenotypes resulting from myo10 depletion. In particular, GFP-MyTH4 expression induces multipolar spindles and abnormal spindle positioning at mitosis, but unlike myo10-depletion spindle length is normal. Preliminary results suggest that the ability of GFP-MyTH4 to produce phenotypes requires microtubule binding, but loss of microtubule binding enhances the localization of this fragment to the spindle pole, suggesting that the MyTH4 domain coordinates multiple myo10 functions. Interestingly, a yeast 2-hybrid screen identified wee1, a mitotic regulatory kinase, as a myo10 interacting protein. In vitro binding assays confirm a direct interaction between myo10 and wee1, and expression of wee1 and wee1 mutants in Xenopus embryos produce spindle phenotypes similar to GFP-MyTH4 expression. Moreover, the expression pattern of expressed, kinase-dead wee1 is perturbed in myo10-depleted cells compared to controls. These results reveal a novel interaction between myo10 and a cell cycle regulatory kinase with implications for regulation of the mitotic spindle structure and function.

[1] Woolner, S., et al. (2008). J Cell Biol 182, 77-88.

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"Headless" Myo10 is a negative regulator of full-length Myo10 and inhibits cortical axon outgrowth.

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Filopodia have critical functions in many stages of neuronal development, including axon outgrowth and targeting. Myo10 is an unconventional myosin that induces filopodia formation and moves within filopodia by walking along filopodial actin to reach the tip, where it is enriched. The motor (head) domain is required this intrafilopodial motility, while the tail region can bind to putative cargoes. Myo10 binds PIP3 with 1 of its 3 PH domains, permitting interaction with the cell membrane and positioning Myo10 within the crucial PI3 kinase pathway. The MyTH4 domain binds microtubules, allowing Myo10 to mediate actin-microtubule interactions. The tail region ends with a FERM domain that can bind integrins and the netrin receptors DCC and neogenin. The nervous system expresses both the full-length motor and a "headless" isoform that lacks most of the motor domain and thus cannot move on actin. We have hypothesized that this headless isoform may act as an endogenous dominant negative of the full-length motor, but this hypothesis has not been tested and the function of headless Myo10 remains unknown. We used shRNA specific to each isoform to determine their functions in mouse cortical neurons. Knockdown of full-length Myo10 decreased axon outgrowth by 35% and axon branching by 27%, while knockdown of headless Myo10 increased outgrowth by 90% and increased branching by 57%. To test whether these opposing effects reflect antagonism of full-length Myo10 by the headless isoform, we transfected COS-7 cells with each isoform. Expression of full-length Myo10 was sufficient to increase filopodia more than 5-fold, but co-expression of headless Myo10 prevented this filopodia induction. Together, these data indicate that full-length Myo10 is required for normal axon development and that headless Myo10 acts as a negative regulator of the full-length isoform.

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Investigating Bundle Selection of Myosin X *In Vivo*.

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Cells organize their contents and regulate cell shape and mechanics through molecular motors functioning on cytoskeletal filaments. Myosin X, an actin-based motor that concentrates at the distal tips of filopodia of mammalian cells, selects the fascin-actin bundle at the filopodial core for motility. While poorly processive on single actin filaments, it takes processive runs on actin bundled by fascin. Recently we showed that the post-IQ region is the main contributor to myosin X's selectivity. This region contains a charged single alpha-helix (SAH), which may impart unique mechanical or affinity properties to the motor. The structural character of this region was perturbed by insertion of a free swivel (GSGGSG flexible linker) after the SAH domain. The post-SAH swivel mutant showed no preference for bundled actin for motility *in vitro*, thus providing support to a selectivity model where the search space of the forward head for the next binding site is constrained to neighboring filaments in a bundle. Here we investigate if interrupting the "selectivity module" has the described effect in live cells. To research its *in vivo* behavior we overexpressed the post-SAH swivel mutant in the U2OS cell line and compared it to wild type myosin X as well as our GCN4 forced dimer used in *in vitro* assays. We observe that overexpressed GCN4 forced dimer performs in the cell in the same manner as full-length

myosin X. Introducing a free swivel in the SAH domain region affects the selective nature of myosin X in the natural cytoskeleton environment of the cell interior, as co-localization of GFP labeled protein with filopodial tips does not occur for the Swivel mutant.

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Myosin XI chaotic oscillations precede F-actin's during polarized growth of *Physcomitrella patens* cells.

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Tip growth is essential for land colonization by bryophytes, plant sexual reproduction, and water and nutrient uptake. Because this specialized form of polarized cell growth requires both a dynamic actin cytoskeleton and active secretion, it has been proposed that the F-actin-associated motor, myosin XI is essential for this process. Nevertheless, the relationship between myosin XI and F-actin during tip growth is not known. Here, we use the highly polarized cells of the moss Physcomitrella patens to show that myosin XI and F-actin localize, in vivo, at the same apical domain and their signals exhibit an oscillatory pattern. These oscillations may represent an ancestral oscillator to the one observed in pollen tubes and root hairs. We also found that both signals follow chaotic dynamics, and surprisingly, phase analysis shows that myosin XI increase precedes F-actin's. We propose a model where myosin XI coincides with a signal that activates actin polymerization.

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A novel cyclin-related protein interacts with a class XIV myosin and affects elongation of the macronucleus at amitosis in Tetrahymena thermophila.

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In Tetrahymena thermophila, the macronucleus divides amitotically. At the onset of amitosis, intramacronuclear microtubules form an array oriented parallel to the axis of macronuclear elongation (Williams and Williams, JCS 20:61). The parallel array is not known to function as a spindle. Nuclei that fail to form the parallel array also fail to undergo macronuclear elongation resulting in grossly unequal division of the macronucleus, a phenotype observed in a MYO1 (Myosin class XIV) knockout and in the overexpression of GFP-tagged Myo1 MyTH4 domain (Gotesman et al., CSK 235:220; Williams et al., JEM 47:561). In the present study, mass spectrometry of immunoprecipitates containing GFP-Myo1 FERM identified a novel 7.4 kDa (p7) Tetrahymena protein with 34 % homology to cyclin-like proteins in Giardia and Trypanosoma. A peptide antibody against p7 detected a 7.4 kDa polypeptide on immunblots of wild type lysate. In a strain expressing a fusion of GFP and p7, antiGFP affinity pulled down the 35.5 kDa fusion protein and therefore confirmed its stable expression. Mass spectrometry identified Rab1B as a potential binding partner of the fusion protein. The p7 antibody detected the fusion protein and endogenous p7 on an immunoblot of lysate from the fusion-expressing strain. GFP-p7 colocalized with antitubulin to intramacronuclear microtubules. However, colocalization of the two signals was not uniform throughout the macronucleus, and separate GFP-p7 and antitubulin signals were also apparent. In contrast, there was significant colocalization of GFP-p7 and antiMyo1 throughout the macronucleus and the micronucleus. Eight hours after initiation of GFP-p7 over expression, 90% of the cells contained randomly-oriented intramacronuclear microtubules, which did not organize into a parallel array over an additional eight-hour period of observation, and the macronuclei did not achieve full elongation. Subsequent macronuclear division in overexpressing cells was either inhibited or grossly unequal. In wild type and in cells

expressing GFP alone, formation of the parallel array and subsequent amitosis appeared normal. This study indicates a novel cyclin-related protein affects elongation of the macronucleus, possibly through interaction with Myo1 and a subset of intramacronuclear microtubules. [Supported by National Science Foundation]

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Biochemical analysis of the myosin-XIX (MYO19) motor domain.

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Myosin-XIX (MYO19) is a novel unconventional myosin involved in mitochondrial dynamics. Analysis of the MYO19 amino acid sequence reveals that the motor domain contains the sequences required for ATPase and motor activity. Interestingly, sequence alignments of the motor domains of thirty MYO19 orthologs indicate conserved, class-specific differences in the motor domain when compared to well-characterized myosins, suggesting possible class-specific enzymatic properties. A tryptophan residue at the end of the P-loop is well conserved in 29 of 30 the MYO19 sequences analyzed, but not present in any other myosin classes. A class-19-specific cysteine substitution within switch I is conserved in 24 of the 30 sequences analyzed. The interaction between myosin and actin is known to be charge-dependent. The predicted actin-binding regions of MYO19 contain differences in charge when compared to the actin binding regions of other myosins, but were conserved at many of the residues implicated in interactions between actin and myosin. To determine the steady-state kinetics, we expressed and purified an S1-like fragment (MYO19-1IQ) as well as a fragment containing the motor and complete neck (MYO19-3IQ) using an insect cell-baculovirus expression system. The ATPase activity of both constructs was actin-activated. MYO19-1IQ also displayed a relatively strong actin affinity in actin co-sedimentation assays. MYO19-3IQ was also capable of moving actin filaments in the *in vitro* motility assay. As exogenous expression of full length GFP-MYO19 resulted in increased mitochondrial dynamics, we generated a construct with a point mutation predicted to interfere with nucleotide binding and examined the effect of expression of this construct on mitochondrial dynamics. Expression of the GFP-tagged construct containing a putative motor-inactivating mutation did not result in increased mitochondrial dynamics as assayed by displacement index (DI). Further characterization of this motor will allow us to elucidate the cellular function of this novel myosin and may suggest cellular instances in which MYO19 mediates mitochondria-actin interactions, such as in transport or anchorage. Supported by NCI-K01CA160667 to OAQ.

Chemotaxis and Directed Migration

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Open Microfluidics for Passive Chemoattractant Gradient Generation.

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The ability to elicit chemoattractant gradients in 3 dimensional channels has provided investigators with a whole new set of experimental platforms for cell migration studies. However, a major drawback with these devices is that it is difficult to load cells. In addition, chemoattractant gradients are generally provided by laminar flow and expose the cells to shear

forces that influences cell migration. We have developed several platforms in both polydimethylsiloxane (PDMS) and glass to overcome both of these problems. First, we have combined PDMS technology with a micropipette system and can provide a passive gradient to cells traversing 3D microfluidic channels. Using this system allows us to perform highly quantitative experiments on a cell's response to chemical gradients with varying slopes and mean concentrations. We have also developed a device that allows us to break down and re-establish the polarity of cells while migrating through a channel. Lastly, we have developed devices that are made entirely of etched glass. These glass devices are durable, optically superior, easy to unclog and fill, and are reusable. We have developed several devices in both bulk glass and in cover slips that have incorporated 3D microfluidic channels and have used them to image migrating Dictyostelium discoideum cells. Channels were fabricated using a laser ablation system which consists of a diode-pumped frequency doubled Neodymium-doped Yttrium Orthovanadate (Nd:YVO4) laser pump (Verdi), a Ti:Sapphire laser oscillator (Tsunami) and a regenerative amplifier (RegA9000). These on-chip devices produce passive gradients for migration assays and are useful for confocal and epifluorescent microscopes that are outfitted with environmental chambers or bulky equipment that do not permit the use of a micromanipulator and micropipette system as is typically used to create a passive gradient. We have etched 3D channels in a variety of configurations that range in diameter from several hundred nanometers to tens of microns and have elicited passive gradients of cAMP from single and multiple channels that lead to the successful recruitment of D. discoideum cells. Like the PDMS devices, these platforms are created so that the system is "open". An open system provides easy cell loading and allows the careful control of passive chemoattractant gradients for many hours and potentially days.

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Unraveling G-protein signaling mechanisms during chemotaxis.

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Many of the signaling pathways controlling chemotaxis are identical in mammalian cells and in the social amoeba, *Dictyostelium discoideum. D. discoideum* exhibits chemotaxis toward folic acid during vegetative growth and to cAMP during aggregation and multicellular development. Since the majority of signaling components are conserved between folic acid and cAMP chemotaxis, mutants defective for folic acid chemotaxis should also exhibit a defect cAMP chemotaxis. While some mutants can have severe effects on gene regulation during cAMP development we find that we can typically rescue these developmental defects by co-developing mutants with wild-type cells. In this study, polarity and adhesion mutants (rckA and rap1) were assayed for their ability to chemotax under a variety of conditions. RckA is a Regulator of G-protein Signaling (RGS) domain-containing kinase implicated in managing cell polarity. Rap1 is a Ras-like small G protein that is involved in adhesion, chemotaxis, and polarity. The Rap1-CA and Rap1-DN mutations varied in their effects on cAMP chemotaxis depending on whether the mutants were co-developed with wild-type cells or were chemotaxing to folic acid during vegetative growth. RckA null mutants displayed a dramatic gain-of-function phenotype in their ability to chemotax to cAMP when assayed under these conditions. However, rckA mutants did not show a chemotaxis defect towards the chemoattractant folic acid. When the Rap1-CA or Rap1-DN mutations were expressed in a rckA null strain, cAMP chemotaxis was normal when these cells were co-developed with wild-type cells. However, these same cells displayed dramatic defects when tested for their ability to chemotax toward folic acid. These results suggest that RckA is functioning upstream of Rap1 during cAMP chemotaxis. However, during folic acid chemotaxis RckA and Rap1 seem to be regulated in different pathways and only effect

folic acid chemotaxis in the rckA null:Rap1 double mutants. The results also demonstrate that folic acid chemotaxis is not regulated similar to cAMP chemotaxis upstream of the small G-proteins. To our knowledge this is the first evidence of a heterotrimeric G-protein associated protein regulating downstream small-G proteins in *D. discoideum*.

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Inhibition of Glutaminyl Cyclase Attenuates Cell Migration Modulated by Monocyte Chemoattractant Proteins.

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Glutaminyl cyclase (QC) catalyzes the formation of N-terminal pyroglutamate (pGlu) in peptides and proteins. pGlu formation in chemoattractants may participate in the regulation of macrophage activation and migration. However, a clear molecular mechanism for the regulation is lacking. In this study, we explored the role of QC-mediated pGlu formation on monocyte chemoattractant proteins (MCPs) in inflammation. We first demonstrated in vitro the pGlu formation on MCPs by QC using mass spectrometry. With purified N-terminal uncyclized MCPs precursor (preMCPs) and pyroglutamate-containing MCPs (pMCPs), we showed that MCPs-stimulated macrophage migration is dependent on the pGlu formation. A potent QC inhibitor, PBD150, significantly decreased the effect of preMCPs on macrophage migration. QC siRNA revealed a similar inhibitory effect. Lastly, we tested whether inhibiting QC can modulate LPS-stimulated macrophage activation. We demonstrated that, in both U937 cells and human peripheral blood-derived monocytes, QC activity is increased by LPS stimulation. Furthermore, PBD150 significantly decreases the effect of LPS. These results strongly suggest that QC-catalyzed N-terminal pGlu formation of MCPs is required for macrophage migration and provide new insights into the role of QC in the inflammation process. Our results also suggest that QC could be a target for anti-inflammatory drug.

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Polar localization of chemotactic proteins in Vibrio parahaemolyticus.

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Chemotaxis is the process by which bacteria bias their flagella-assisted swimming in response to their environment. Recent work in the rod-shaped bacterium *Vibrio cholerae*, revealed that ParC plays a key role in promoting the polar localization and segregation of chemotaxis proteins. The purpose of our work is to study ParC and chemotaxis protein localization in *Vibrio parahaemolyticus,* another enteric pathogen, which unlike V*. cholerae,* has lateral as well as polar flagellae. To detect the subcellular localization of ParC and chemotaxis proteins within *V. parahaemolyticus*, we fused genes encoding fluorescent proteins to the genes encoding ParC and to genes encoding other chemotaxis proteins, including VP2226 and CheW. In wild-type *V. parahaemolyticus* time-lapse fluorescence microscopy revealed that ParC and VP2226 localize to the old flagellated pole in newborn cells forming a unipolar focus. Later in the cell cycle, each is recruited to the new pole resulting in bipolar foci. Consequently, after cell division, each daughter cell inherits a ParC/VP2226 focus at the old pole. However a new CheW focus does not form at the pre-divisional proximal pole until post-cell division. This shows a hierarchy in the protein recruitment to the new pole suggesting that ParC, as in *V. cholerae,* is important for recruitment of chemotaxis proteins to the developing pole, but that unlike *V. cholerae,* not all proteins are recruited in the same time frame. We have constructed a *V. parahaemolyticus parC* deletion mutant and analyses of this strain will reveal if ParC is required for proper localization

and segregation of chemotaxis proteins. Our *parC* deletion mutant exhibited a swimming defect, suggesting that ParC is essential for optimal chemotaxis. Preliminary swarmer assays using our *parC* deletion mutant suggest that ParC deficiency does not impair the lateral flagellar system. Finally, a bacteria-two-hybrid assay, revealed that there are multiple inter and intra protein-protein interactions among *V. parahaemolyticus* chemotaxis proteins. Future work includes analyses of requirement for ParC in both flagellar systems, understanding the nature of chemotaxis protein-protein interactions, and utilizing an infant rabbit model to test the effect of ParC deficiency on the intestinal colonization ability of *V. parahaemolyticus*.

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Nociceptin is a Chemorepellent in Tetrahymena thermophila.

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Tetrahymena thermophila are free-living, ciliated, eukaryotic organisms that respond to stimuli by moving toward chemoattractants and avoiding chemorepellents. Chemoattractant responses involve faster ciliary beating, which propels the organisms forward more rapidly. Chemorepellent signaling involves ciliary reversal, which disrupts forward swimming, and causes the organism to jerk back and forth, swim in small circles, or spin in an attempt to get away from the repellent. Many food sources, such as proteins, are chemoattractants for *Tetrahymena*, while a variety of compounds are repellents. Repellents in nature are thought to come from the secretions of predators, or from ruptured organisms, which may serve as “danger” signals. Several hormones involved in human pain signaling have been shown to be chemorepellents in *Tetrahymena,* including substance P, ACTH, PACAP, VIP, and nociceptin.
We have been studying the response of *Tetrahymena* to nociceptin, using pharmacological inhibitors in order to elucidate components of the nociceptin signaling pathway. We have found that G-protein inhibitors and a number of mammalian tyrosine kinase inhibitors have no effect on nociceptin avoidance. However, the tyrosine kinase inhibitor, genistein, inhibits avoidance to nociceptin, likely by an unrelated mechanism. Nociceptin avoidance is also inhibited by the calcium chelator, EGTA, and partially inhibited by the ER calcium ATPase inhibitor, thapsigargin. Whole cell electrophysiology experiments in a calcium-containing buffer show that addition of 50 µM nociceptin to the buffer causes a sustained depolarization of approximately 30 mV. This depolarization is nearly eliminated in the presence of EGTA, further supporting the hypothesis that calcium is involved in nociceptin signaling.
J-113397, an inhibitor of the human nociceptin receptor, also inhibits nociceptin avoidance in *Tetrahymena*, though other nociceptin antagonists we tested had no effect on avoidance. Further experimentation on this organism will give a more complete picture of the signaling pathway, as well as allowing greater comparison between nociceptin avoidance in *Tetrahymena* and nociceptin signaling in vertebrates.

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NANIVID: A new technology to analyze shallow gradient chemotaxis *in vitro* and *in vivo.*

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Cancer cells create a unique microenvironment *in vivo* that enables tumor cell migration and dissemination to distant organs. To better understand the role of the tumor microenvironment in dissemination, special tools and devices are required to monitor the interactions between different cell types and the role of chemotaxis in migration and dissemination *in vivo*. Here we

describe the design and optimization of a new, versatile chemotaxis device called the NANIVID (NANo IntraVItal Device). The device is fabricated using BioMEMS techniques and consists of etched and bonded glass substrates, a soluble factor reservoir, fluorescent tracking beads and a microelectrode array for cell quantification. The reservoir contains a customized hydrogel blend that is loaded with epidermal growth factor (EGF). Upon hydration, EGF will diffuse out of the hydrogel to create a well-defined chemotactic gradient that can be sustained for many hours in order to attract tumor cells to the device. Here we show that mammary tumor cells *in vitro* and *in vivo* are chemotactic to the NANIVID. A new insight derived from this study is that tumor cells are capable of following much shallower gradients than previously thought possible. In particular, tumor cells can chemotax up a gradient that is as shallow as 0.7% (Fit R^2 =0.55), indicating that migratory tumor cells found in shallow gradients around co-migratory stromal cells are sufficient for initiating chemotaxis and migration in tumors.

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The Role of CXCR4 and CXCR7-Mediated Chemokine Signaling in Zebrafish Keratocyte Motility.

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The role of CXCR4 in regulating immunity, cell growth, cancer, angiongenesis, and development has been examined in many systems. Although it is known that the cognate, and only known, ligand for CXCR4 is CXCL12 (SDF-1) and that this cytokine is a known promoter of cellular migration, the role of this receptor in regulating non-immune cell migration is a recent development and has not been previously examined in fish epidermal keratocytes. Our data show that both CXCR4 and CXCL12a are differentially expressed during primary keratocyte explant culture. In our cell sheet migration assays, addition of CXCL12a to explant culture media increases keratocyte motility while addition of AMD3100 (a CXCR4b antagonist) appears to inhibit cell migration. The recently discovered chemokine receptor, CXCR7, binds CXCL11 as well as CXCL12a, although no reports to date have linked an effect of CXCR7 to cell motility. Both CXCR7 and CXCL11 appear to be differentially expressed during explant culture and in our migration assays, the addition of CXCL11 increases keratocyte motility. In order to determine the relative contribution of these two receptors on keratocyte motility, we performed the same migration assays on zebrafish homozygous for nonsense mutations in either CXCR4b or CXCR7b. Our data show that CXC receptors may have a more prominent role in regulating non-immune cell motility than previously thought, providing a first look at how cytokine signaling and the acute inflammatory response affect keratocyte cell migration.

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N-3-oxo-dodecanoyl-L-homoserine lactone inhibits epithelial cells migration via Rac1/Cdc42, IQGAP1 and actin remodeling.

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In gram-negative bacteria, cell-to-cell communication based on N-acyl-homoserine lactone (HSL) quorum sensing molecules is known to coordinate the production of virulence factors and biofilms. These bacterial signals can also modulate human immune cell behavior, including cell motility. The aim of this study was to investigate the effect of 3-oxo-C12-HSL from Pseudomonas aeruginosa on the migration of human intestinal epithelial Caco-2 cells. Using wound-healing and migration assays we found that 3-oxo-C12-HSL inhibits Caco-2 cells migration in a dose- and time-dependent manner. The changes in cell migration were paralleled by F-actin cytoskeleton reorganization as evidenced by phalloidin staining and confocal

microscopy. Moreover, for the first time we demonstrated by proteome analysis, Western blot, immunostaining and confocal imaging that the 3-oxo-C12-HSL down-regulates expression levels of the Rho GTPases (Rac1 and Cdc42) as well as their effector, IQGAP1, which binds and stabilizes the active forms of Rho GTPases. Taken together, our findings provide a novel insight on modulation mechanisms between bacterial quorum sensing molecule 3-oxo-C12-HSL and epithelial cells at the sites of bacterial infection.

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Ca^{++} chemotaxis in Dictyostelium discoideum.

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Using a newly developed microfluidic chamber, we have demonstrated *in vitro* that Ca^{++} also functions as a chemoattractant of aggregation-competent *D. discoideum* amoebae. Effective Ca^{++} gradients are extremely steep compared to effective cAMP gradients. Given that Ca^{++} chemotaxis is co-acquired with cAMP chemotaxis during development of this organism, we speculated on the role Ca^{++} chemotaxis might play, notably the possibility that steep, transient Ca^{++} gradients may be generated during natural aggregation in the interstitial region between neighboring cells. In searching for a potential Ca^{++} chemotaxis receptor, we discovered that deletion of IplA (inositol triphosphate receptor-like protein), a putative Ca^{++} channel, resulted in loss of Ca^{++}, but not loss of cAMP chemotaxis. We also found that *iplA*$^{-}$ cells could not accurately orient towards the aggregation center at the onset of each normal cAMP wave generated and relayed by a majority population of wild-type cells. In support of the independence of two chemotactic systems, we found that of the four myosin II heavy chain kinases, deletion of either myosin heavy chain kinase A (MHCKA) or myosin heavy chain kinase C (MHCKC), blocked Ca^{++}, but not cAMP, chemotaxis. These mutant phenotypes were similar to that of the *iplA*$^{-}$ mutant. Furthermore, we show that neither Ca^{++} nor Ca^{++}/calmodulin directly regulates these kinases intracellularly, a further indication that Ca^{++} chemotaxis is mediated through a surface receptor, potentially IplA. These discoveries increase the complexity of the contextual framework, a paradigm in place for forty years, for interpreting how chemotaxis functions in the natural aggregation of the model organism *D. discoideum*. Finally, we are investigating the role Ca^{++} chemotaxis may play in mammalian cell migration, especially during cancer cell metastasis.

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HS1-dependent Rac activation is necessary for neutrophil chemotaxis.

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HS1 is a 75-kDa protein that is primarily expressed in hematopoietic cells and is heavily phosphorylated in response to B- and T-cell receptor signaling. HS1 is important in antigen-induced proliferation of lymphocytes and like its homologue cortactin, is thought to stabilize F-actin branching through an N-terminal domain that binds Arp2/3 and tandem repeat domains that bind F-actin. HS1 contains a C-terminal proline-rich region and an SH3 domain that are important for a variety of protein-protein interactions including Src family kinases and Vav1. Despite progress in understanding HS1 function in lymphocytes, the function of HS1 during neutrophil chemotaxis remains unknown. Neutrophil chemotaxis is dependent on dynamic actin turnover at the leading edge. Here we investigate the function of HS1 during neutrophil chemotaxis using the neutrophil-like cell line, PLB-985 cells. We show that HS1 co-localizes

with F-actin at the leading edge of PLB-985 cells and primary neutrophils during chemotaxis. HS1 is tyrosine phosphorylated in response to fMLP stimulation and this is dependent on both adhesion and the activation of Src family kinases. Moreover, HS1-deficient cells show impaired activation of Rac GTPases in response to fMLP, suggesting that HS1-mediated Rac activity is necessary for neutrophil chemotaxis. Finally, we also show that phosphorylation of the RhoGEF Vav1 is impaired in HS1-deficient cells providing a link between HS1 and Rac activation. Taken together, our findings suggest that HS1 is necessary for Rac activation through the modulation of Vav1 GEF activity to allow for efficient neutrophil chemotaxis to fMLP.

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Actin Crosslinking Proteins, Cortexillin I and II, are Required for cAMP-signaling During Dictyostelium Chemotaxis.

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Dictyostelium has long been an excellent model for understanding the molecular basis of chemotaxis and intracellular and extracellular cAMP signaling. Binding of cAMP to G-protein-coupled cAMP receptors (cAR1) on the cell surface of Dictyostelium amoebae initiates a series of molecular responses. Expression of cAR1 and adenylyl cyclase (ACA) is markedly increased. Gß? released from the heterotrimeric G-protein coupled to cAR1 activates myosin II (via guanylyl cyclase and cGMP), and also activates RasC and RasG which initiates synergistic and partially redundant pathways. One pathway activates target of rapomycin 2 (TORC2) and protein kinase B stimulating actin polymerization at the cell front which, together with actomyosin II contraction at the rear of the cell, drives chemotaxis. The second Ras pathway activates phosphatidylinositol 3-kinase (PI3K), CRAC, ACA and cAMP synthesis. Binding of cAMP to cAR1 also leads to phosphorylation and activation of extracellular signal regulated kinase 2 (ERK2), which also contributes to ACA activation, as does TORC2. Synthesis and secretion of cAMP relays the cAMP signal to neighboring cells. Thus, chemotaxing amoebae form head-to-tail cell streams migrating to aggregation centers where multicellular mounds form and differentiate and develop into mature fruiting bodies. Both cortexillin I and II (ctxI and ctxII), 444 and 441 residues respectively, form parallel dimers with two globular heads and a coiled-coil helical tail. Both cortexillins accumulate in the cell cortex where they bundle and cross-link actin filaments in an anti-parallel fashion, and both localize to the cleavage furrow of dividing cells. We now report that the double deletion of ctxI and ctxII alters the actin cytoskeleton, with bundled actin filaments accumulating in the cell cortex, and substantially inhibits all the molecular responses to extracellular cAMP. Importantly, synthesis of cAR1 and ACA is not stimulated, ACA is not activated, and activation of RasC and RasG, phosphorylation of ERK2, activation of TORC2 and stimulation of actin polymerization are greatly reduced. As a consequence of the inhibition of cAMP signaling, both cell streaming and development of cells into mature fruiting bodies are completely blocked in ctxI/ctxII-null cells, although chemotaxis of individual cells is only slightly impaired. Expression of ACA-YFP in the ctxI/ctxII double null cells significantly rescues the wild type phenotype indicating that the primary chemotaxis defect in the ctxI/ctxII-null cells is ACA synthesis. These results emphasize the importance of a properly organized actin cytoskeleton for the cAMP signaling pathways and chemotaxis.

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Non-Gaussian motility in 2D and 3D.

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Trajectories of mesenchymal cells on substrates are typically assumed to be well described by persistent random walks, a mode of migration that would be well suited for efficient space exploration for food and associated with Gaussian distributions of displacements and flat distributions of angular turns. Here monitoring the migration normal and cancer mesenchymal cells, of different species, on 2D substrates and in 3D matrices, in the presence/absence of actomyosin inhibitors, or following shRNA-depletion of proteins regulating motility, over 2.5 temporal decades reveals that distributions of cell displacements do not follow Gaussian, power-law, or Levy-flights statistics. Rather they follow exponential distributions over >4 decades of occurrence. This is because these cells undergo “bursty” migration, with rare, large, and fast displacements whose occurrence is vastly underestimated by Gaussian fits, yet which greatly contribute to overall cell motility. A simple stochastic model of motility parameterizes these distributions and shows how motility parameters are exquisitely regulated by actomyosin contractility and the Arp2/3/N-WASp/Cdc42/Cortactin protein module.

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Cell Shape Dynamics: From Waves to Migration.

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The shape and motion of cells can yield significant insights into the internal operation of a cell. We present a simple, yet versatile framework that provides multiple metrics of cell shape and cell shape dynamics. Analysis of migrating Dictyostelium discoideum cells, a model system for the study of chemotaxis, shows that global and local metrics highlight distinct cellular processes. For example, a global measure of shape shows rhythmic oscillations suggestive of contractions, whereas a local measure of shape, the local curvature, shows wave-like dynamics indicative of actin polymerization waves. At the leading edge of adherent cells, curvature waves are associated with protrusive activity. Like regions of high curvature, protrusive activity travels along the boundary in a wave-like manner. The wave-like character of protrusions provides an alternative rationale for the zig-zagging of pseudopods, and explains how cells can move through viscous fluids and navigate complex three dimensional topography.

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The Role of Centrosomal Scaffolding Protein Cep192 in Migration.

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The centrosome has been studied for over a century yet has not been found to be exclusively essential for any one particular function, indicating it may play a role in several cellular processes. Indeed, the centrosome has already been shown to be functionally tied to bipolar spindle formation and cell-cycle signaling. Intriguingly, the centrosome is composed of over 500 temporally regulated proteins, all recruited to form an electron dense cloud known as the pericentriolar material (PCM). Our previous work has shown one component of the PCM, centrosomal protein of 192kDa (Cep192), to be a basal scaffolding component, functioning either with, or basal to, the hallmark PCM protein Pericentrin. Cep192 levels are up-regulated in

mitosis and knockdown disrupts maturation and functional spindle assembly by altering centrosomal protein localization and MT nucleation ability.
While Cep192 is most thoroughly studied in mitosis, it persists at the centrosome during interphase. The interphase centrosome provides an asymmetric radial array of MTs, believed to be important for cell polarization and migration. Consistent with this, we have found that U2OS cells depleted of Cep192 display a nearly 2-fold increase in migration rates, as assayed by a wound healing assay, and develop elongate protrusions, which in extreme cases resemble immature neuronal processes. We are currently testing the hypothesis that these phenotypes result because: 1) Cep192 normally ensures that the majority of microtubule nucleation occurs at the centrosome and thus its absence causes an substantial increase in non-centrosomal microtubules and 2) in a similar vein, the loss of Cep192 releases important regulators of microtubule dynamics, particularly Aurora A kinase, into the cytoplasm thereby altering microtubule (and actin dynamics at the cell periphery). Mislocalization of Aurora-A from the centrosome has been shown to increase migration rate in breast cancer cells.

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The Effect of a Peptide Mimetic of Heat Shock Protein β6 on Migration is Cell-Type Dependent.

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Previous research has shown that AZX100, a 24-amino acid phosphopeptide analogue of Heat Shock Protein β6 (HSPβ6 or HSP20) containing a protein transduction domain, reduces the amount of filamentous actin in myofibroblasts. In addition, AZX100-induced actin depolymerization has been shown to affect contractile gene expression in myofibroblasts and migration in smooth muscle cells. Both types of cells are integral to wound healing and scar resolution. Thus, investigating how modulation of filamentous actin can affect the migration of invasive fibroblasts as well as vascular cells may elucidate the role of AZX100 in dermal fibrosis.

The present study used two techniques to measure the effect of AZX100 on migration: an Electric Cell-substrate Impedance Sensing (ECIS) system and a Boyden chamber assay. In the ECIS system, cell migration is measured in real time via changes in resistance over electrodes in the bottom of the culture plate. Migration was analyzed in four different cell types: 3T3 fibroblasts, keloid fibroblasts (KF), smooth muscle cells (A7R5 line) and human umbilical vein endothelial cells (HUVEC). In fibroblasts, AZX100 treatment resulted in a dose-dependent decrease in migration compared to the TGFβ1 control. This effect was even greater in KFs, where AZX100 treatment reduced migration by 24%. In contrast, AZX100 treatment in HUVECs and A7R5s caused increased migration. In HUVECs, there was a 51% increase in migration at the highest dose of AZX100 compared to the VEGF control.

In conclusion, AZX100 has an unexpected differential effect in migration behavior of various cell types. The effect of AZX100 in migratory cells such as fibroblasts may be due to disassembly of actin filaments. However, in vascular cells, AZX100 restructuring of actin may trigger a cell response for migration by disrupting the adhesive state of the cell.

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Tissue geometry regulates collective cell motility.

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Collective cell migration plays an important role in embryonic development, wound repair and cancer invasion. Certain cell types have an intrinsic ability to organize themselves and move collectively when they are confined within monolayers. Here, we explored the role of tissue geometry on the collective motility of epithelial cells. In particular, we used microlithography and timelapse imaging to ask whether tissue geometry affects multicellular polarity and supracellular organization that are necessary for collective cell motion. We found that epithelial cells within monolayers tended to rotate as a group, and that increasing the size of the tissue increased the collectiveness of group rotation. The shape and boundary conditions of the tissue organized the motion of the cells by altering group rotation and coherence. The motility parameters of individual cells, including speed and persistence, were also affected by tissue geometry. Our results suggest that the overall architecture of the tissue in which cells reside instructs their movements with respect to each other within a collective. Accurate recapitulation of *in vivo* tissue structure will benefit future studies of processes which involve collective cell migration.

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Myosin II Isoform Switching Mediates Invasiveness following TGFβ-induced Epithelial-Mesenchymal Transition.

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An epithelial-mesenchymal transition (EMT) occurs when epithelial cells acquire the morphological and phenotypic properties of a mesenchymal cell, including increased migratory and invasive characteristics. TGFβ is a known inducer of EMT in many settings. EMT and EMT-like events are suggested to precede cancer cell metastasis. Despite functional significance of non-muscle myosin II in cell migration and invasion, its role in EMT or TGFβ signaling is unknown. The goal of these studies was to determine the roles and regulation of nonmuscle myosin II during TGFβ-induced EMT in mammary epithelial cells. Analysis of normal mammary gland expression revealed that myosin IIC is expressed in luminal cells while myosin IIB expression is up-regulated in myoepithelial cells that have more mesenchymal characteristics. Furthermore, TGFβ induction of EMT in non-transformed murine mammary gland (NMuMG) epithelial cells results in an isoform switch from myosin IIC to myosin IIB and increased phosphorylation of myosin heavy chain (MHC) IIA on target sites known to regulate filament dynamics (Ser1916, Ser1943). These expression and phosphorylation changes are downstream of heterogeneous nuclear ribonucleoprotein-E1 (E1), an effector of TGFβ signaling. E1 knockdown drives cells into a migratory, invasive mesenchymal state, and concomitantly upregulates MHC IIB expression and MHC IIA phosphorylation. Abrogation of myosin IIB expression in the E1 knockdown cells has no effect on 2D migration but significantly reduced transmigration and macrophage-stimulated collagen invasion. These studies indicate that

transition between myosin IIC/myosin IIB expression is a critical feature of EMT that contributes to increases in invasive behavior.

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Galectin 3 modulates the mast cells migration.

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Mast cells are imunorregulatory cells that participate in the defense of the organism and are known for exerting a fundamental role in asthma, allergy and inflammatory reactions, as well as expulsion of parasites. Galectins have a modulatory role in various cell types by regulating growth, adhesion, migration and cell proliferation. The aim of this study was to investigate the role of galectins 1 and 3 in mast cell function, using knockout mice for these galectins. The distribution of mast cells in the spleen, lungs and skin was characterized. The role of galectins in cell adhesion and migration was studied in vitro with bone marrow derived mast cells. The results showed that there were no differences in the number of mast cells in the spleen, skin and lungs between or between the knockout mice and the wild-type mice. Using Transwell assays, bone marrow derived mast cells from galectin-3 knockout mice migrate less in comparison with mast cells from galectin-1 knockout mice or wild-type mice, even in presence of the chemotactic factors IL-3 and SCF. On the other hand, no differences were observed in the adhesion of bone marrow derived mast cells from galectin-1 and 3 knockout mice as well as wild-type mice to components of the extracellular matrix such as fibronectin, laminin, collagen type I and IV. These results indicate that galectin-3 is important for the migration of mast cells, but the absence of galectin-3 does not alter the adhesion of these cells to extracellular matrix components.

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EB1-dependent microtubule +TIP complexes coordinate protrusion dynamics during 3D epithelial remodeling.

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Epithelial remodeling, in which apical-basal polarized cells switch to a migratory phenotype, plays essential roles in development and cancer progression. How microtubules are controlled or contribute to epithelial remodeling in a physiological three-dimensional (3D) environment is not understood. We analyzed microtubule function and dynamics using an epithelial cyst cell culture system in which polarized MDCK epithelial cells undergo a partial epithelial-to-mesenchymal transition (EMT) in response to hepatocyte growth factor (HGF), and cells extend, and migrate into the surrounding extracellular matrix (ECM). Using high-resolution confocal microscopy, we found that extensions at the basal surface of HGF-treated cysts were filled with dense microtubule bundles. Computational tracking of EB1-EGFP showed large numbers of microtubules growing persistently from the apical domain into these extensions, often deforming the extension tip, and an increase in microtubule growth rate in response to HGF before morphological changes were evident. Next, we tested the role of microtubule plus-end tracking protein (+TIP) complexes in HGF-induced migration in 3D by depleting cells of EB1, the central adaptor that mediates association of other +TIP proteins with growing microtubule plus-ends. In EB1-depleted cells, microtubules displayed rapid lateral and retrograde movements demonstrating that EB1 is required to anchor and stabilize microtubules in HGF-induced

extensions. EB1-depleted cysts formed shorter, more branched extensions further suggesting that EB1 is required for productive HGF-induced extension outgrowth. Analysis of cell-matrix interactions and F-actin dynamics revealed that control extensions progressively pulled on and deformed the ECM typically with one F-actin-rich protrusion near the tip. However, EB1-depleted cells produced multiple highly dynamic F-actin-rich protrusions that did not productively engage the matrix. This resulted in extensions that rapidly protruded, retracted, and changed direction. The inability to engage the matrix and stabilize a dominant protrusion was also associated with defects in cell-matrix adhesions. Although EB1-depleted extensions formed nascent adhesions, they never matured, were mislocalized, and were uncoordinated, in contrast to the highly coordinated adhesions of control cells. Together our findings indicate that EB1-dependent microtubule +TIP complexes are required to coordinate protrusion dynamics in migrating cells during 3D epithelial remodeling.

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Phosphoinositide Signaling Regulates the Exocyst Complex and Polarized Integrin Trafficking in Directionally Migrating Cells.

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Cell migration is essential for many biological processes. Cell migration hinges on the ability of cells to traffic signaling molecules and proteins toward the direction of migration, a process that requires the tight regulation of cytoskeletal and vesicle trafficking machineries. The trafficking of both newly synthesized and recycled integrin molecules to and from the leading edge plasma membrane is critical for directional cell migration. Here, we describe a novel role for the phosphatidylinositol-4,5-biphosphate (PIP2) synthesizing enzyme, PIPKI(i2, in modulating polarized integrin trafficking during cell migration. PIPKI(i2 knockdown impaired directional migration in multiple assays. Loss of PIPKI(i2 impaired Golgi and microtubule orientation towards the direction of cell migration and trafficking of (1 integrin to the leading edge.

PIP2 signaling specificity is defined by the interaction of PIP kinases with PIP2 effectors or compartments containing PIP2 effectors. We show that PIPKI(i2 interacts with the exocyst an evolutionarily conserved vesicle-trafficking protein complex required for polarized trafficking. In vitro binding study showed Sec6 and Exo70 as PIPKI(i2-interacting subunits of the exocyst complex. Upon initiation of directional migration, PIPKI(i2 associated with both the exocyst complex and ?1 integrin and this was dependent both on kinase activity of PIPKI(i2 and its ability to associate with talin.

The integrity of exocyst complex was pivotal for PIPKI(i2-regulated cell migration as knockdown of any exocyst complex components impaired cell migration including polarized trafficking of ?1 integrin to leading edge. Furthermore, the expression of Exo70 mutant defective in PIP2-binding abrogated the PIPKI(i2-regulated cell migration emphasizing the role of PIP2. PIPKI(i2 was specific isoforms to promote integrin-dependent cell migration as its expression specifically promoted the integration of exocyst complex into ?1 integrin-containing vesicle like intracellular compartment. We have discovered a fundamental role for PIP2 in polarized integrin trafficking required for directionally migrating cells.

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Myosin-II-mediated contractility coordinates cell movements during collective migration through cell-cell mechanotransduction.

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Collective migration of epithelial sheets is an important process in morphogenesis, tissue repair and tumor invasion. To achieve collective migration, individual cells must communicate with one

another and coordinate their migratory processes. The mechanisms underlying such coordination are not well understood. Using an automated, high-throughput time-lapse microscopy and cell tracking platform, we monitored the migration trajectories of ~0.5 million MCF10A cells undergoing collective migration in wound-healing assays. We used several parameters to assess coordination in cell movements, and found that migration persistence, as well as the correlation in migration velocities and polarization directions between individual cells, is dependent on myosin-II-mediated cell contractility. Decreasing cell contractility by decreasing substrate stiffness or by RNAi-mediated knockdown of myosin-IIA and myosin-IIB reduces coordination in cell motion during collective migration. Importantly, we discovered that contractility can affect coordinated motion by modulating mechanical forces communicated between cells through cadherin-mediated cell-cell adhesions. Using a novel traction force microscopy-based method to measure tension exerted on adherens junctions, we found that the forces transmitted across individual cell-cell junctions positively correlate with cellular myosin-II activity. Interestingly, a high level of actomyosin activity can also disrupt mechanical communication across multiple adjacent cells. These results reveal a complex relationship between actomyosin contractility, cell-cell mechanotransduction and coordination in cell movements during collective migration. Further characterization of this relationship will be an important step towards understanding collective migration, and may shed light on pathological developments such as cancer invasion, which is often associated with altered microenvironmental stiffness, cellular contractility and cell-cell adhesions.

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F-actin dynamics and actomyosin contraction during neutrophil motility in live zebrafish.

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Neutrophils are the most abundant white blood cell and are the first cells to respond to sites of tissue injury. The in vivo migration of neutrophils is highly dynamic and involves the generation of multiple leading edge pseudopodia. For directed motility, neutrophils maintain a dominant pseudopodium in the direction of movement while retracting other pseudopodia. Although uropod retraction is known to require myosin II contractility, the cytoskeletal mechanisms that govern pseudopodia selection at the front of the cell are not well understood. To investigate pseudopodia selection in vivo, we used time-lapse spinning-disk confocal microscopy of neutrophils with dual channel imaging of bioprobes specific for all (Lifeact-Ruby) and more stable (GFP-UtrCH) populations of F-actin. We show that dynamic F-actin is localized in the dominant pseudopodium while the more stable F-actin accumulates in the retracting pseudopodia and uropod during neutrophil migration. To determine the role of myosin II in pseudopodia selection, we performed ratiometric imaging of myosin regulatory light chain (MRLC) to a volume marker to allow visualization specifically of cytoskeleton-associated myosin II. This revealed localization of MRCL with the cytoskeleton in the uropod, as expected. However, MRLC also accumulated and localized to the leading edge of pseudopodia precisely at the time of retraction, and this accumulation increased as the retraction progressed. Taken together, we demonstrated that in an in vivo context, F-actin stabilization and myosin II accumulation occur in retracting pseudopodia, suggesting that actin stabilization and myosin II activity are regionally regulated during pseudopodia selection to guide directed neutrophil motility. Further studies will be needed to test if actomyosin contraction is required for biased pseudopodia retraction in directed motility during the immune response in vivo.

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Dynamic Cytoskeleton Organization Couple Cell Shape Variations with Migration Phenotypes in HL-60 Cells.

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The neutrophil-like HL60 cell is a common model system for the study of human neutrophil migration. A population of HL60 cells under constant conditions exhibits highly heterogeneous migration phenotypes, as characterized by their speed, shape, and frequency of polarity switching. These natural variations allow us to investigate the mechanism underlying shape determination and its correlation with migration phenotypes.

Well-differentiated HL-60 cells, stably expressing fluorescent protein-tagged actin or myosin, are migrating within uniform field of chemoattractant, and imaged with high spatiotemporal resolution. To mimic the neutrophil's native confined environment, the cells are plated in between the fibronectin-coated coverslip and an agarose pad. To identify important shape parameters, we apply principal component analysis on hundreds of cell contours. Interestingly, the first few principal modes are biologically meaningful. Variations in cell length, leading edge width, and left-right asymmetry can explain close to 70% of total shape variation. These three shape modes are also preserved in migrating human primary neutrophils. Cells with relatively wider leading edge migrate faster and switch polarity less frequently. Left-right asymmetry of cell shape correlates well with asymmetric distribution of myosin, as well as turns in cell trajectory, although interestingly asymmetric myosin accumulation lags behind cell turning.

Inhibition of actin polymerization by latrunculin reduces cell length and leading edge width, while inhibition of myosin by blebbistatin increases cell length and reduces leading edge width. This observation is consistent with our model using membrane tension as a limiting factor for extension of leading edge width and cell length.

Finally, we use the movement and position of cell nucleus relative to the leading edge and cell rear to probe for the cell's internal mechanical properties. Nocodazole treatment increases persistence of protrusion, and reduces the distance between leading edge and the nucleus, while taxol treatment decreases the persistence of protrusion. Variations in microtubule dynamics might affect the cell-nuclear coupling and contribute to variations in migration persistence.

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An in vivo tracking system to investigate the migration of bone marrow stem cells in an osteochondral defect model.

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An in vivo system was developed to monitor the migration of human bone marrow-derived MSCs (BMSCs) transplanted within the marrow cavity of athymic nude rats. The in vitro studies confirmed that PDGF (platelet-derived growth factor)-AA had the most potent chemotactic effect of the factors tested, and possessed the greatest number of receptors in BMSCs. In the in vivo study, the BMSCs were labeled with fluorescent nanoparticles and injected into the marrow cavity through an osteochondral defect created in the distal femur of rats. The defect was sealed with HCF (heparin-conjugated fibrin) or PDGF-AA-loaded HCF. In the HCF-only control group without PDGF-AA, the nanoparticle-labeled BMSCs were dispersed outside the marrow cavity within 3 days after the injection. In the PDGF-AA-loaded HCF group, the labeled cells moved time-dependently for 14 days toward the osteochondral defect. The in vivo tracking

patterns differed depending on the PDGF-AA concentration 21 days after injection: HCF-PDGF in low dose (8.5 ng/μl) was more effective than HCF-PDGF in high dose (17 ng/μl) in recruiting the BMSCs to the osteochondral defect. When the osteochondral defect was evaluated macroscopically and histologically, the defects treated with PDGF and TGF-β1-loaded HCF showed excellent cartilage repair compared with other groups. Further in vivo studies confirmed that this in vivo osteochondral BMSCs tracking system (IOBTS) worked for other chemo-attractants (CCL-2 and PDGF-BB). In conclusion, IOBTS can be a very useful tool for examining the effect of growth factors or chemokines on BMSC migration and cartilage repair.

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Manganese Superoxide Dismutase Inhibited Gastric Cancer Cellular Invasion.

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Reactive oxygen species (ROS) have been reported to play an important role for cancer cellular invasion. Recently, we have established a new cancerous cell-line RGK-1 from a rat gastric normal epithelial cell-line RGM-1. We also established a stable clone RGK-MnSOD, which overexpressed a manganese superoxide disumutase. In this study, we elucidated a role of ROS for cancer cellular invasion. [Methods] The ROS concentration and the cell membrane lipid peroxidation were determined using fluorescence probes. The kind of ROS was clarified with EPR. The invasion abilities were determined by a matrigel assay. [Results and Discussion] The ROS from cancer cell was O_2^-. Cancer cellular O_2^- concentration was significantly higher than in normal, and MnSOD significantly decreased the O_2^- concentration. The depth of cancer cellular invasion into matrigel was significantly longer than that of normal, and MnSOD also significantly inhibited the invasion. We concluded that cancer specific ROS derived from mitochondria involved the cell invasion ability because MnSOD is a mitochondrion-particular enzyme.

Dynamics of Focal Adhesions and Invadosomes

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A Molecular Model for How Increased pH Activates Focal Adhesion Kinase.

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Activity of focal adhesion kinase (FAK), a key regulator of focal adhesion turnover and cell migration, is dependent on autophosphorylation of Tyr397. The recently identified FAK crystal structure revealed that FAK activity is inhibited *in cis* by an interaction between its N-terminal FERM and C-terminal kinase domains. Release of this auto-inhibited conformation allows autophosphorylation of Tyr397 in a linker region between FERM and kinase domains, which docks Src kinases that phosphorylate Tyr576/577 in the kinase domain for full activation of FAK. However, how the initial step of releasing an auto-inhibited conformation is regulated remains unknown. We previously showed that increased intracellular pH from activation of the Na-H exchanger NHE1 is necessary for increased FAK-pY397, focal adhesion turnover, and directed cell migration. Our current data suggest that pH > 7.2 disrupts an auto-inhibited conformation of FAK to increase FAK-pY397. The FERM domain of FAK contains an unusually high number of seven conserved histidines compared with FERM domains in other proteins, and two histidines, His41 and His58 are at the interface of the FERM and kinase domains. We reasoned that pH-dependent charges on histidines in the FERM domain might regulate an electrostatic interaction

with the kinase domain. In support of this prediction, we used in vitro kinase assays with recombinant full-length FAK to show increased pY397 from pH 6.5 to 7.5. Increased pY397 at higher pH is dependent on the FERM domain because a truncated FAK lacking the FERM domain has constitutively high pY397 that is pH-independent. We also used separate FERM and kinase domain constructs to show binding *in trans* is markedly greater at pH 6.5 compared with pH 7.5, further suggesting that increased pH releases an auto-inhibited conformation. To test the role of His58 in pH-dependent pTyr397, we used a mutant full-length FAK-H58A to show constitutively high and pH-independent pY397 similar to a truncated FAK lacking the FERM domain. Our data suggest a molecular mechanism for pH-dependent activation of FAK that predicts increased pH > 7.2 and deprotonation of His58 releases an auto-inhibited charge interaction between FERM and kinase domains.

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A mechanochemistry model of focal adhesion dynamics in cell migration.

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Focal adhesions are essential to mediate cell extracellular matrix (ECM) adhesion and force transmission during cell motilities, which involve the crosstalk between physical signals such as contractile forces or membrane dynamics, and chemical signaling events such as focal adhesion kinase related regulation pathways. However, the underline mechanism of the biophysical regulations of force transmission among actin cytoskeleton, cell membrane, focal complex and ECM remains poorly understood. We constructed a mathematical model to understand the behavior of focal adhesion complex under different experimental conditions. By integrating the cell membrane dynamics, actin network fluid dynamics, and the mechanochemistry of focal complex, the model reveals itself the capability to capture the essential characteristics of focal adhesions in cell motility. In particular, the model explains the focal adhesion growth pattern at different ECM stiffness. The model thus provides a comprehensive vision of the focal adhesion dynamics.

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Matrix-properties dependent cell migration speed: characterization of the driving force of cell migration.

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It was shown that cell migration exhibits strong mechanosensitivity behaviors, e.g., the migration speed biphasically depends on the matrix rigidity. However, these behaviors have not been quantitatively understood. In this study, a mechano-chemical coupling model was developed for studying the cell migration behaviors by modeling the dynamics of focal adhesion and effect of cell shape on cell traction force distribution, in which not only the rigidity of matrix but also the concentration of ligands on matrix and the activity of myosin were considered. We showed that the cell migration behaviors depended on not only rigidity of matrix, but also the concentration of ligands in a biphasic manner. The underlying mechanism behind these biphasic behaviors is that these parameters can influence the stability of focal adhesions which are crucial for the creation of pulling force by stable focal adhesions at cell front and the cell detachment and retraction triggered by the destabilization of focal adhesion at cell rear. These results agree with the experimental observations. Furthermore, a motility factor was suggested for characterizing the driving force of cell migration which was mainly determined by the cell shape, matrix stiffness and concentration of ligands on matrix. This study provided a quantitative understanding of how cells control their migration behaviors.

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Direct observation of catch bonds in focal adhesions of living cells.

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Force spectroscopy measurements on isolated integrins revealed the counterintuitive effect that integrin binding with fibronectin strengthened with force. This so-called catch bond behavior has never been observed in living cells, however. The anchorage of cells to the extracellular matrix is established through a force-transmitting molecular chain of integrin-type adhesion receptors, proteins of the focal adhesion complex, and the cytoskeleton. The resulting adhesion strength is determined by a collective binding energy landscape which arises from the superposition of the energy landscapes of the individual molecular bonds along the force transmission chain. To measure this collective energy landscape, we used high-force magnetic tweezers to apply forces of up to 80 nN to 5 µm RGD coated beads bound to the cell. The forces were applied as a linear ramp (loading rates of 1...40 nN/s) or as staircase-like force steps. The average force at which the beads detached from the cell was recorded and was taken as a measure of bond stability. Bond stability increased with higher loading rates, as expected for thermally activated molecular bonds. Surprisingly, a staircase-like loading protocol further increased bond stability and bond lifetimes. Such behavior cannot be explained by active mechanotransduction processes or by thermally activated molecular bonds, but is consistent with the existence of force-strengthening catch-bonds. A possible molecular mechanism for catch-bond behavior are hidden binding domains in focal adhesion proteins that become available during force-induced unfolding.

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Lasp-2 Binds Vinculin and Paxillin in Focal Adhesions and Affects Cell Adhesion and Spreading.

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Focal adhesions are intricate protein complexes that facilitate cell attachment, migration and cellular communication. Lasp-2 (LIM-nebulette) is a recently identified member of the nebulin family of actin-binding proteins and appears to be an integral component of focal adhesions. The function of lasp-2 is currently viewed as a molecular scaffold capable of organizing and bundling actin filaments. To gain further insights into the functional role of lasp-2 at focal adhesions, we set out to identify additional binding partners of lasp-2 using yeast two-hybrid screens. Two new focal adhesion interacting partners, vinculin and paxillin, were identified. These novel interactions were confirmed using solid phase binding assays. Immunofluorescence microscopy demonstrated that Lasp-2 co-localizes with vinculin and paxillin in focal adhesions. Interestingly, overexpression of GFP-lasp-2 decreases cell adhesion and the number of vinculin-containing focal adhesions. Reduction of lasp-2 protein levels via siRNA knockdown reduces the ability for cells to spread. Taken together, these data suggest that lasp-2 has an important role in coordinating and regulating the composition and dynamics of focal adhesions.

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β-actin mRNA compartmentalization by ZBP1 controls focal adhesion stability and directed cell motility.

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Localization of β-actin mRNA is facilitated by the zipcode binding protein, ZBP1. Binding to the cognate zipcode within the 3'UTR of β-actin mRNA enables ZBP1 to asymmetrically distribute mRNA and control translation of its target, a process known to be necessary for directed cell motility. This study utilized a ZBP1 KO fibroblast cell line to compare β-actin mRNA dynamics to wild-type cells. TIRF microscopy and mRNA particle tracking in live cells enabled us to discover a specific compartment near focal adhesions where β-actin mRNA dwells for periods greater than one minute. In ZBP1 KO fibroblasts, the probability of tracking β-actin mRNA within the adhesion environment is significantly reduced. Consequently, adhesion lifetimes are reduced in ZBP1 KO cells. This supports the hypothesis that ZBP1 facilitates mRNA localization to strengthen focal adhesions and therefore direct cell migration. To test directly whether mRNA localization to adhesions can alter adhesion dynamics and cell motility we utilized a novel mRNA tethering technique. We tethered β-actin mRNA to focal adhesion complexes through MS2 stem loops in the 3'UTR of β-actin mRNA and the MS2 coat protein fused to vinculin. This produced a significant increase in adhesion lifetime and adhesion size in cells expressing MS2-β-actin mRNA. Additionally, cell velocity was significantly reduced in cells with tethered β-actin mRNA. These experimental results lead us to conclude that β-actin mRNA compartmentalization to adhesions, mediated by ZBP1, strengthen adhesions and therefore produce an asymmetric force distribution within the cell to control its directionality.

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β1- and β3-integrins move and function as distinct adhesion units inside cell adhesions.

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Focal adhesions (FAs) are adhesive structures linking the cell to the extracellular matrix (ECM) and constitute molecular platforms for biochemical and mechanical signals that control cell adhesion, migration, growth, differentiation and apoptosis. Integrin receptors are core components of FAs, where they trigger signaling and connect the ECM to the actin cytoskeleton (F-actin) via recruitment of intracellular scaffolds such as talin. Different classes of αβ-integrin heterodimers perform distinct functions and are simultaneously present in a FA. The static nanoscale organization of FAs has been recently described, with integrins being at the base of a vertical multilamelar protein organization ending with F-actin connection. However, the nanoscale dynamics of integrins within FAs is still unknown. Here we show, using super-resolution microscopy and single particle tracking, that integrins could move and function as nanoclusters within FAs. β3- and β1-integrins form distinct homotypic nanoclusters displaying specific dynamics within the same FA. β3-integrins undergo repeated activation cycles within a FA, seen as rapid switches between levels of free-diffusion, confinement, and immobilization

lasting less than a hundred seconds. Talin binding triggers immobilization more than interaction with the ECM, while both control immobilization duration, suggesting that talin and ECM cooperate to fully activate integrins inside FAs. Comparing β1- and β3-integrins, we showed an increased density of β3-integrin immobilization in FAs, supporting specific functions for each type of β-integrin in the same FA. Thus, this dynamic nanoscale partitioning of homotypic β-integrin nanoclusters within FAs could control local forces and signaling necessary for integrin-mediated cellular processes such as adhesion and migration.

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The Endosomal Signaling Adaptor APPL1 Impairs Cell Migration by Inhibiting the Turnover of Adhesions at the Leading Edge.

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Cell migration is a complex process that requires the coordination of signaling events that take place in distinct locations within the cell. Adaptor proteins are emerging as key modulators of spatially integrated processes because of their ability to localize to different subcellular compartments and bring together important signaling proteins at these sites. However, the role that adaptor proteins play in regulating cell migration is not well understood. Here, we show a novel function for the adaptor protein containing a pleckstrin homology (PH), phosphotyrosine binding (PTB), and leucine zipper motif denoted APPL1 in modulating cell migration. APPL1 impairs the turnover of adhesions at the leading edge of cells thereby inhibiting their migration. The ability of APPL1 to impair migration is dependent on its PTB domain, which interacts with the serine/threonine kinase Akt, suggesting the interaction of APPL1 with Akt is important for its effect on migration. Interestingly, APPL1 decreases the amount of active Akt in cells. Using a mutant-based approach, we further show that APPL1 modulates migration and adhesion dynamics via a mechanism that involves regulation of Akt function. An APPL1 mutant which is unable to localize to endosomal membranes no longer has an effect on migration or adhesion turnover, indicating that APPL1 endosomal localization is required for its ability to regulate these processes. Furthermore, APPL1 is found in vesicular structures containing the non-receptor tyrosine kinase Src. Src has been shown to regulate Akt function through the phosphorylation of two Akt tyrosine residues, and intriguingly, we have found that APPL1 reduces the tyrosine phosphorylation of Akt. Therefore, we propose a model in which APPL1 regulates adhesion dynamics and cell migration by altering Src-mediated tyrosine phosphorylation of Akt. Our results further underscore the importance of adaptor proteins in modulating the flow of information through signaling pathways by demonstrating a critical new function for APPL1 in regulating cell migration and adhesion turnover.

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TKS5 regulates invadopodium membrane anchoring in breast cancer cells.

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A growing body of research implicates invadopodia in cancer cell invasion and metastasis (Oser et al., 2009, J Cell Biochem.; Stylli et al., 2008, J Clin. Neurosci.). Invadopodia are protrusive structures of cancer cells, 0.5-1 micron in diameter and 1-10 microns long. The primary function of these structures is to degrade ECM and protrude into degraded spaces, which creates a passage (like a tunnel) through the extra-cellular space, which cells utilize to migrate from the site of primary tumor to enter the bloodstream and eventually metastasize at distant sites to make secondary tumors. To understand the signaling pathways during the assembly of invadopodia in breast cancer cells, we did live-cell time-lapse imaging to visualize the order of

arrival of the proteins of the invadopodium precursor, N-WASp, Cortactin and Tks5. Our data indicate that Cortactin and N-WASp arrive together to form the invadopodium precursor core, followed by Tks5 recruitment to the complex. We found that Tks5 is not required for invadopodium precursor formation but is needed for the stabilization and anchoring of invadopodium precursors. Tks5 has been shown to interact with 3' phosphoinositides (Abram et al., 2003, J Biol. Chem.), and recently PI(3,4,5)P3 was shown to localize at invadopodia (Yamaguchi et al., 2011, J. Cell Biol.). We show that PI(3,4)P2 localizes to invadopodial precursors and appears to be the main binding partner ofTks5 as a link between the precursor core and the cell membrane.

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A novel focal adhesion TRIM protein regulates focal adhesion disassembly.

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The tripartite motif (TRIM) family of proteins play important roles in diverse cellular functions such as cell differentiation, oncogenesis and innate immunity. Here we identify a TRIM protein as a novel component of focal adhesions. This TRIM protein is recruited to focal adhesions by an interaction between its coiled coil domain and the LD2 motif of paxillin. It is recruited to focal adhesions early in a myosin-II-independent manner. But unlike any other focal adhesion component, it remains stably bound, forming a long-lived focal adhesion scaffold. Cells lacking the TRIM protein are impaired in cell migration due to a defect in focal adhesion disassembly. We hypothesize that the TRIM protein senses cues delivered by microtubuli to initiate focal adhesion disassembly because microtubules correctly target focal adhesion, but fail to induce focal adhesion turnover. Given the importance of the TRIM protein in the dynamic turnover of focal adhesions, it is predicted to play a critical role in metastasis and oncogenesis.

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Fascin phosphorylation is necessary for efficient turnover of focal adhesions.

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Efficient cell migration depends on phospho-regulation of the actin bundling protein fascin. Cells expressing phospho-mimetic fascin mutant, S39E, had difficulties to polarize and protrude, while cells expressing non-phosphorylatable fascin mutant, S39A, had problems retracting the uropod (Hashimoto et al, 2007). Our hypothesis is that cycles of fascin phosphorylation/dephosphorylation are necessary for directional cell motility: non-phosphorylated fascin for filopodia formation and cell guidance and phosphorylated fascin for focal adhesions (FAs) turnover and cell body retraction. Using TIRF microscopy we found that GFP-tagged wt and S39A mutant fascin, but not S39E, were enriched in the FAs marked with Cherry-tagged vinculin, paxillin, α- actinin or zyxin. In fascin-depleted cells, FAs were thinner and longer with slower turnover rate. The FA turnover rate was rescued by expression of S39E mutant, but not with S39A, suggesting that fascin phosphorylation is required for rapid turnover of FAs. Using a FAs disassembly assay (Ezratty et al, 2005), we found that fascin phosphorylation is required for FAs disassembly after microtubule re-growth. Our current model is that fascin plays a role in FAs assembly by bundling actin filaments and its phosphorylation is required for efficient FAs disassembly.

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An integrin endocytic recycling pathway mediated by FAK and Src controls the polarized reassembly of focal adhesions after their disassembly.

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Cell migration is a multi-step process that involves focal adhesion (FA) turnover and recycling of integrins. FA disassembly is an endocytic process that involves focal adhesion kinase (FAK), dynamin and clathrin (Ezratty, E. et al., NCB, 2005; JCB, 2009; Chao, W. and Kunz, J., FEBS, 2009). Little is known about the recycling of integrin following FA disassembly and whether FA components might play a role in the trafficking of endocytosed integrin. We used microtubule regrowth to synchronously induce FA disassembly and integrin endocytosis to examine whether endocytic recycling is coupled to FA reassembly. Focal adhesion reassembly commenced 30 min after microtubule-induced FA disassembly and occurred preferentially at the leading edge of cells. Integrin and active FAK were detected in both Rab 5- and Rab11-positive endosomes and FA reassembly required both Rab 5 and Rab 11. Src kinase participates in FA reassembly following disassembly induced by microtubules (Yeo, M. et al., MBC, 2006), so we explored the possible link between FAK and Src during the disassembly/reassembly cycle. Notably, both Src inhibitor (PP2) and FAK inhibitor (PF228) reversely blocked FA reassembly (without affecting disassembly), but only PP2 blocked the recycling of integrins to the cell surface. Furthermore, FAK and Src colocalized at the Rab 11 endocytic recycling compartment in either PP2 or PF228 treated cells. FAK mutants FAK-Y397F (autophosphorylation/Src binding site mutant) and FAK-K454R (kinase dead) rescued FA disassembly but not reassembly in FAK-/- cells, further suggesting that FAK kinase activity is essential for FA reassembly but not for disassembly. Constitutively active (activation loop mutant K578E/K581E) but not wild type FAK rescued the reassembly defect in Src, Yes and Fyn null cells (SYF cells), showing that Src functioned in FA reassembly through FAK. Consistent with this interpretation Src was transiently associated with the newly formed FAs. These results provide the first evidence that FAK and Src kinase activities contribute to FA reassembly by controlling the recycling of integrins from a Rab 11 compartment. This recycling pathway may direct integrin recycling to the leading edge thus contributing to the polarized formation of FAs in migrating cells.

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Microenvironmental control of cell migration: Myosin IIA is required for efficient migration in fibrillar environments through control of cell adhesion dynamics.

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Recent evidence suggests that organization of the extracellular matrix (ECM) into aligned fibrils or fibril-like ECM topographies promotes rapid migration in fibroblasts. However, the mechanisms of cell migration altered by these changes in microenvironmental topography remain unknown. Here, using 1D fibrillar migration as a model system, we find that fibroblast leading edge dynamics are enhanced by 1D fibrillar micropatterns and demonstrate a dependence on the spatial positioning of cell adhesions. Although 1D, 2D, and 3D matrix adhesions have similar assembly kinetics, both 1D and 3D adhesions are stabilized for prolonged periods, while both paxillin and vinculin show slower turnover rates in 1D adhesions. Moreover, actin in 1D adhesions undergoes slower retrograde flow than the actin present in 2D lamellipodia, suggesting an increase in mechanical coupling between adhesions and protrusive machinery. Experimental reduction of contractility resulted in loss of 1D adhesion structure and

stability, with scattered small and unstable adhesions and an uncoupling of adhesion protein-integrin stability. Genetic ablation of myosin IIA or IIB isoforms revealed that myosin IIA is required for efficient migration in restricted environments as well as adhesion maturation, while myosin IIB helps to stabilize adhesions beneath the cell body. These data suggest that restricted cell environments such as 1D patterns require cellular contraction via myosin IIA to enhance adhesion stability and coupling to integrins behind the leading edge. This increase in mechanical coupling allows for greater leading edge protrusion and rapid cell migration.

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Adhesion and Protrusion Signatures Predict Cell-to-cell Variability in Response to EGF.

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Cell migration plays an essential role in many biological processes, such as cancer metastasis, wound healing and immune response. Migration occurs through autocatalytic cycles of protrusion, focal adhesion (FA) assembly, FA maturation, FA disassembly and retraction. Epidermal growth factor (EGF) has been shown to enhance the migration rate in many cell types; however it is not known how it regulates FA dynamics, spatial patterns of protrusion or the coupling between these processes. We use total internal reflection fluorescence (TIRF) microscopy and image analysis to quantify FA characteristics, FA dynamics and protrusion dynamics under different doses of EGF stimulation. In a metastatic rat adenocarcinoma cell line, we find that the average cell migration rate does not change dramatically with increasing EGF concentration. However, the distribution of cell migration rates becomes broader with increased EGF concentration. If the FA characteristics, FA dynamics and protrusion dynamics are grouped based on based on EGF concentration, there are few noticeable differences. However, when cells are grouped according to migration rate, dramatic differences in FA characteristics, FA dynamics and protrusion dynamics are observed. This indicates that EGF stimulates increased migration in a subpopulation of cells and this subpopulation of cells contains certain signatures in FA characteristics, FA dynamics and protrusion dynamics. First, fast moving cells form more FAs with intermediate size and speed. Second, fast moving cells form FAs with low intensity and short lifetime. Third, fast moving cells organize their protrusion into lateral waves, whereas slow moving cells generate very little spatial organization in their protrusion. Furthermore, EGF increases the fraction of fast moving cells with lateral waves. We propose that during cancer progression, EGF induces a subset of cells to enhance their migration rate and this subset of cells is characterized by these adhesion and protrusion signatures. These signatures will be informative in understanding how to control migration responses in fast moving subpopulations of cells.

Structure and Function of the Extracellular Matrix

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Growth of lung cancer cells in three-dimensional microenvironments reveals key features of tumor malignancy.

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Cultured human lung cancer cell lines have been used extensively to dissect signaling pathways underlying cancer malignancy, including proliferation and resistance to chemotherapeutic agents. However, the ability of malignant cells to grow and metastasize in vivo is dependent upon specific cell-cell and cell-extracellular matrix (ECM) interactions, many of which are absent

when cells are cultured on conventional tissue culture plastic. Previous studies have found that breast cancer cell lines show differential growth morphologies in three-dimensional (3D) gels of laminin-rich (lr) ECM, and that gene expression patterns associated with organized cell structure in 3D lrECM were associated with breast cancer patient prognosis. We show here that established lung cancer cell lines also can be classified by growth in lrECM into different morphological categories and that transcriptional alterations distinguishing growth on conventional tissue culture plastic from growth in 3D lrECM are reflective of tissue-specific differentiation. We further show that gene expression differences that distinguish lung cell lines that grow as smooth vs. branched structures in 3D lrECM can be used to stratify adenocarcinoma patients into prognostic groups with significantly different outcome, defining phenotypic response to 3D lrECM as a potential surrogate of lung cancer malignancy.

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Impact of tissue tension in pancreatic cancer progression.

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Pancreatic Ductal Adenocarcinomas (PDAC) present a strong desmoplastic reaction, characterized by significant changes in the tumor microenvironment and the extracellular matrix (ECM), which severely compromises their treatment and surgical resection. The Weaver lab and others have shown in other cancers, that tumor development is accompanied by increased stiffness of the ECM. Understanding the mechanisms by which ECM stiffness regulates tumor development may lead to novel targets for anti-cancer treatment as well as for the diagnosis and early detection of the disease. The objective of this work is to understand what factors might contribute to the stiffening of the ECM during the development of PDAC and whether there is a causal effect to this stiffening. We used the transgenic mice models of PDAC that incorporate the two most common mutations found in human PDAC: Kras activating mutation alone (KrasG12D, which develop precancerous lesions) or in combination with Tgfb-receptor 2 deletion (Tgfbr2+/-, which develop invasive PDAC), using pancreatic epithelial cells specific promoter driven Cre-recombinase mice (Ptf1a-Cre). Initial experiments focused on characterizing collagen status, mechanical properties and mechano-signaling at various stages of tumor progression. We also explored the role of stromal fibroblast in driving tissue tension and PDAC.

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How Deeply Cells Feel: Nuclear Phenotypes Defined by Matrix.

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Tissue cells constantly probe their surroundings. They lack eyes to see and ears to hear but sense their microenvironment by adhering and physically deforming, which allows cells to feel into the depths of a matrix. To address how deeply cells feel we cultured mesenchymal stem cells, as prototypical but particularly sensitive adhesive cells, on collagen-coated gels-made microfilms of controlled elasticity (E) and thickness (h). After just 36 hrs in culture, cell spread area was distinctively smaller on thick and soft compared to either thin or stiff films, correlating well with nuclei morphology. Transition from small-to-large spread area transition was obtained at <5 microns gel thickness, which defines a tactile length scale for mechanosensitivity. Matrix-dependent cytoskeletal organization exhibits thickness-dependent nematic and smectic-like assembly, and nuclear component Lamin-A (LMNA), which is widely implicated in cell differentiation, was also found to be different for thin films relative to thick gels of the same E. Transcriptional profiles of many nuclear genes, including LMNA and histones, also showed a

dependence on h. The changes suggest mechanical links to regulation of gene expression by matrix physics

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Effects of Substrate Rigidity on Fibronectin Matrix Assembly.

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Fibronectin (FN) is an abundant extracellular matrix protein that cells secrete, bind to and forcefully unfold to assemble into a fibrillar matrix. Increases in both FN production and tissue matrix stiffness have been associated with tumor malignancy and tissue fibrosis; however, little is known about the specific effects of rigidity on the process of cell-mediated FN matrix assembly. To address this question, polyacrylamide gels of varying stiffnesses were prepared to mimic the range of rigidities between softer normal tissues and stiffer diseased tissues, and gels were made cell-adhesive by crosslinking FN to the surfaces. NIH3T3 cells were grown on the gels, and FN matrix assembly was visualized by immunofluorescence or quantified by matrix insolubility in buffered deoxycholate (DOC) detergent at different time points. We found that FN matrix assembly is significantly reduced on softer substrates. To investigate the cause behind this rigidity-dependent decrease in FN matrix assembly, cells on soft substrates were treated with lysophosphatidic acid (LPA), a phospholipid known to increase cell contractility. Addition of LPA rescued FN assembly on soft substrates, indicating that FN matrix assembly can be promoted by increasing the contractile force a cell applies to the substrate. Since cells forcefully extend FN using integrin receptors, we stimulated integrin binding to FN with Mn^{2+} and found that FN matrix assembly was rescued on soft gels by this treatment. These results suggest that FN matrix assembly depends on the strength of integrin binding to FN, and binding may be weak on soft substrates. Integrin binding supports the extension of FN from a compact form to promote fibril formation, and integrin binding strength has been found to be affected by the conformation of FN. Binding of a conformation-dependent antibody against FN revealed that FN conformation varies with gel stiffness, and it appears to assume a more compact conformation on softer substrates. To test whether pre-extending substrate-bound FN on a soft gel affects matrix assembly, FN was partially unfolded by treatment with 1M guanidine hydrochloride. This treatment increased both FN matrix assembly and the number of matrix assembly sites formed by cells. Together, these findings show that cells assemble less FN matrix on softer substrates, and the difference between soft and stiff substrates is due to a deficiency in cells binding to and unfolding FN on softer substrates.

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Spatial organization of the extra-cellular matrix regulates cell-cell junction positioning.

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Organization of cells into epithelium depends on cell interaction with both the extracellular matrix (ECM) and adjacent cells. The role of cell-cell adhesion in the regulation of epithelial topology is well described. ECM is better known to promote cell migration and provide a structural scaffold for cell anchoring. We developed a minimal model system to investigate the contribution of ECM to the spatial organization of multicellular structures. Fibronectin micropatterns were used to constrain the location of cell-ECM adhesion and analyze its effect on intercellular junction positioning. We found that ECM geometry affects the degree of stability of intercellular junction positioning and the magnitude of intercellular forces. Intercellular junctions were permanently displaced and experienced large perpendicular tensional forces as long as they were positioned close to ECM. Remarkably, intercellular junctions remained stable solely in regions deprived of ECM where they were submitted to lower tensional forces. Finally,

cells seemed to adapt their position in order to minimize both intra and inter-cellular tension and thereby adopt stable configurations in which overall tension anisotropy was regulated by ECM geometry. These results uncover a new morphogenetic role for ECM in the mechanical regulation of cell positioning.

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Individual versus collective cell migration of corneal fibroblasts: regulation by matrix composition in 3D culture.

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Extracellular matrix (ECM) supplies both physical and chemical signals to cells and plays a critical role regulating cell migration during wound repair. To study how ECM composition regulates migration of corneal fibroblasts, we created a nested 3D matrix model. In this model, compressed cell-populated collagen buttons are embedded in cell-free collagen or fibrin matrices. Corneal fibroblasts cultured in PDGF migrated out of the button into the acellular outer matrix. Time-lapse DIC imaging and 3D confocal analysis showed that cells migrated into collagen more rapidly than they migrated into fibrin. Furthermore, fibroblasts migrated into and through collagen mostly as individual cells, whereas fibroblasts moved into fibrin as an interconnected meshwork. Confocal reflection microscopy showed that cells remodeled both collagen and fibrin matrices during migration. Addition of fibronectin to matrices increased the rate of cell migration into both collagen and fibrin matrices, but the differences in cell connectivity remained. Spreading and migration of fibroblasts cultured within and on top of un-nested collagen and fibrin matrices showed similar differences in cell connectivity. Cells migrated individually in collagen matrices, but gradually became interconnected and formed clusters in fibrin matrices. On fibrin matrices, cadherin was localized to cell-cell adhesions, and increased fibronectin was secreted as compared to cells on collagen matrices. Our novel findings show that matrix composition plays a critical role modulating the pattern and amount of cell migration in a 3D environment.

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Characterization of collagen gel pore sizes from confocal reflection images.

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The ability of tumor cells to invade connective tissue is crucial for the development of metastases. The mechanical tissue properties, in particular stiffness and pore size, crucially influence cell migration e.g. in collagen gel invasion assays and therefore need to be characterized. Here we present a simple and robust method to measure the pore size of collagen networks from confocal 3D image images. Images were recorded in reflection mode, which has the advantage that no fluorescent staining is necessary, but the disadvantage that collagen fibers that are oriented perpendicular to the imaging plane remain invisible. The resulting bias on the pore size can be corrected as follows. Images were binarized to define the solid and the liquid phase and to compute the distribution of distances between randomly chosen points of the liquid phase and their nearest solid obstacle. For collagen networks, this distribution follows a Rayleigh distribution from which the network line density, or average pore, size can be extracted. We show analytically that the fraction of invisible fibers corresponds to a shift of the distribution by a constant factor that only depends on the numerical aperture of the imaging objective and therefore can be easily corrected for. We then applied this method to a range of collagen gels with largely diverging pore sizes that were polymerized under different conditions including collagen concentration and temperature, with the aim to find an image binarization method that is robust against the influence of laser power, gain voltage, and fiber

density. We present an image normalization and thresholding (binarization) procedure that is robust against such variations of imaging parameters.

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Solid phase binding assays reveal cryptic molecular interactions between Fibronectin Type III modules.

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Fibronectin fibrillogenesis is a complex, multi-step process that involves the conversion of soluble, compact fibronectin into an insoluble fibrillar matrix. The formation, extension, and augmentation of fibronectin fibrils represent essential steps in the establishment of a mature matrix that is biochemically characterized by its insolubility in the detergent deoxycholate (DOC). These fibrils are formed by various intermolecular fibronectin-fibronectin interactions involving specific domains within fibronectin. Although several fibronectin self-binding sites have been identified, the domains responsible for matrix maturation are largely unknown. In an earlier study (*Mol. Biol. Cell.* 20:S, 454), proteomic analysis was used to identify FN types $III_{4\text{-}6}$ (Hep III) and $III_{12\text{-}14}$ (HepII) as domains potentially associated within small, DOC-insoluble proteolytic fibril fragments. Both domains contain putative FN self-binding sites. To further investigate interactions between $III_{4\text{-}6}$ and $III_{12\text{-}14}$, recombinant FN fragments containing each domain were used in solid phase binding assays. Under non-denaturing conditions, fragments displayed relatively weak binding, however under mildly denaturing conditions produced by addition of guanidine hydrochloride (GdnHCl), we noted a robust increase in binding affinity between $III_{4\text{-}6}$ and $III_{12\text{-}14}$. Peak absorption in the binding assay occurred between 1.5M and 2.0M GdnHCl. Binding was both concentration-dependent and saturable. The results from binding experiments taken together with the previously obtained proteomic data, suggest that these domains contain sites that are involved in fibronectin-fibronectin intermolecular interactions required for DOC-insolubility and matrix fibril stability.

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Fibronectin assembly and composite matrix remodeling within a micropatterened model of tissue morphogenesis.

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Encapsulations of cells in reconstituted collagen I matrices have served as in vitro models of tissue morphogenesis and cell migration for nearly 50 years. As cells remodel and compact the exogenous collagen matrix, they also assemble a dense network of cell derived fibronectin which has been shown to increase the efficiency of collagen compaction. Moreover, previous studies have shown that individual fibronectin modules within fibers can unfold in response to cell generated tension, revealing cryptic binding sites that may alter cell adhesion and fibronectin fibrillogenesis. Here, we extend previous 2D work using a FRET sensor of fibronectin conformation/tension by combining this assay with a micropatterned approach to culture large arrays of 3D microtissues with well defined mechanical gradients.

We find that as the microtissue remodels, the fibronectin matrix is increasingly unfolded. Surprisingly, the cell assembled fibronectin and the exogenous collagen matrix become spatially segregated rather than forming an intercalated network. Over the course of several days, the initial collagen matrix is compacted into a dense core around which the cells form a fibrous

shell. Moreover, across a single microtissue, we find gradients of F-actin, fibronectin and tenascin C that match the predicted gradients of mechanical stress.

Here, we expand upon recent techniques to measure fibronectin conformation and to culture arrays of micropatterened tissues in order to simultaneously image matrix reorganization, matrix conformation and overall tissue morphogenesis at the length scale of single cells.

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Study of the promoter of the cancer-associated extracellular matrix protein tenascin-W.

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The microenvironment hosting a tumor actively participates in the regulation of tumor cell proliferation, migration, and invasion. Among the extracellular matrix proteins enriched in the cancer stroma is tenascin-W, a member of tenascin family. Tenascin-W is expressed transiently in embryonic development and seems to have specific functions in osteogenesis. In the adult, Tenascin-W expression is strongly reduced, being restricted mainly to kidney and periosteum. However, in a large fraction of various types of human tumors tenascin-W is expressed, while in the corresponding normal tissue tenascin-W is not detectable. Immunohistochemistry of glioblastoma samples showed perivascular localization of tenascin-W. Moreover, we demonstrated that exogenous tenascin-W increases in vitro migration and sprouting of HUVEC, suggesting a possible role in tumor angiogenesis. More recently, the association of tenascin-W and tumor blood vessel has been observed in other types of tumor as well. Because of the distinct spatial and temporal changes in tenascin-W expression it is of interest to study its regulation.
The poster summarizes the recent study of the promoter region of human tenascin-W. Transfection of various deletion mutants of the human tenascin-W proximal promoter reporter constructs was performed in osteoblastoma and fibrosarcoma cell lines to identify regulatory elements. Deletion of a 12-bp sequence upstream of the published transcription start site led to a strong induction of the reporter activity.
DNA-pulldown experiments followed by mass spec analysis was performed to identify possible transcriptional repressors that binds to the 12-bp region. The effect of the downregulation by RNA interference of five putative repressors is currently under examination.

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Gene expression analysis of neutrophils cultured on collagen matrix lacking telopeptides.

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[Background] The neutrophils protect the host by constituting the first line of defense against bacterial and fungal invading. In chemoattractant responses, neutrophils migrate to an inflammatory site in the extracellular matrices packed with type I collagen fibril and others. In particular, neutrophils secrete widely various cytokines, chemokines, and other proteins to regulate immune system. It is worth to investigate whether the telopeptides in collagen fibrils regulate the mRNA expressions of neutrophils or not. In this study, we report that gene expression profiles of neutrophils cultured on actinidain-hydrolyzed collagen (AHCol) lacking of telopeptides by DNA microarray and qRT-PCR.
[Materials & Methods] Pepsin-hydrolyzed collagen (PHCol) and AHCol were prepared from chicken skin according to our procedures (1). Activated neutrophils were collected from a mouse abdominal cavity stimulated by casein solution, and subsequently were cultured on a plate pre-coated with the PHCol or the AHCol. After the incubation period of 1.5 h, the mRNAs

of neutrophils were extracted and analyzed by mouse gene expression microarrays (Agilent Technologies). To compare the activation levels of neutrophils, we quantified some specific mRNA expressions by real time RT-PCR with the specific primer sets.
[Results] The mRNA expression levels of chemokines of neutrophils cultured on the AHCol were drastically decreased than those on the PHCol. Similarly, important cytokines involved in inflammatory response were affected by contacting the AHCol matrix.
[Conclusion] We demonstrated that the scaffold by the AHCol matrix alters signals of immune network in neutrophils.

(1) Kunii, et al., J. Biol. Chem. 285, 2010

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The role of filamin in cytoskeletal dynamics of differentiated vascular smooth muscle cells.

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Filamin (FLN) is a large non-muscle actin crosslinking protein (MW 240-280 KD). It is known to crosslink actin filaments orthogonally and to stabilize 3D filament structure. Filamin is also known to act as a mechanotransducer, but its role in the vascular smooth muscle contractility is unclear. In this study we used cellular and molecular approaches to study the structure and function of the cortical cytoskeleton in differentiated vascular smooth muscle cells (dVSMC) in order to decipher the composition of filamin in this structure, identify its binding partners, and understand its possible involvement in regulating actin cytoskeleton connections to the adhesion plaques during the cells contractile response to the agonist phenylephrine.

We demonstrate that FLNa is highly expressed in the dVSMCs while FLNb expression is significantly lower. In addition by using deconvolution immunofluorescence experiments we have found that FLNa localizes in punctae consistent with focal adhesions and dense bodies. By quantitative differential centrifugation we have found that there is an agonist-induced redistribution of the FLNa from the cytosol fraction to the cytoskeleton fraction. Furthermore, by immunoprecipitation experiments, we have found that FLNa interacts with α, β, and γ- Actin as well as CaMKII in an agonist-dependent manner.

Our data suggest that FLNa may play a regulatory role in cytoskeletal dynamics during dVSMC contraction.

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A novel role for PI3K in mediating the reciprocal relationship between stem cell gene expression and remodeling of the stem cell microenvironment.

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Although there have been recent advances in the application of stem cell technologies, the cyclical relationships between changes in stem cell structure, cell function and the microenvironment remain largely undefined. As stem cells differentiate, cell structures adapt and cause changes to the microenvironment. Inversely, alterations to the stem cell microenvironment, or niche, induce a functional response leading to structural changes. Chemically altering functional protein pathways to activate cell structure changes and monitoring the resulting microenvironmental remodeling provides a method for analyzing cyclical relationship changes between stem cells and the stem cell niche. Phosphoinositide-3 Kinase (PI3K) transduces microenvironmental changes from ECM-binding, transmembrane

receptors through a variety of pathways, including the Rho-family of GTPases which regulate cytoskeletal organization. Inhibition of PI3K with the drug LY294-002 alters stem cell structure through remodeling of the actin cytoskeleton. Cytoskeletal remodeling was imaged using fluorescent confocal microscopy of human Mesenchymal Stem Cells (hMSC) seeded on collagen-I coated plates or encapsulated in 3D collagen-I hydrogels and treated with either LY294-002 or control media. Inhibiting PI3K causes a reciprocal change in the microenvironment due to the altered cellular structures and functions. Quantified changes in hydrogel compaction rates, along with elastic and viscous moduli collected using a rheometer capture altered physical and mechanical changes to the microenvironment of the 3D collagen-hMSC hydrogels. Microarray analysis of both the 2D and 3D treated and control samples revealed dimensionality-dependent changes in stem cell gene expression along five different mesenchymal lineages. Together, these results suggest that functional changes alone do not determine stem cell fate because the cyclical response from the surrounding microenvironment heavily impacts differentiation. Therefore, in addition to its well-known functions controlling the cell cycle and apoptosis, PI3K now plays a role in remodeling the ECM component of the stem cell niche.

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A role for estrogen receptor alpha (ERalpha) and estrogen receptor beta (ERbeta) in collagen biosynthesis in mouse skin.

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Objective: The goal of this study was to investigate the role of ERalpha and/or ERbeta on collagen biosynthesis in the skin using unique genetic models: knockout ERalpha (ERKO-alpha) and ERbeta (ERKO-beta) female mice.
Methods: We first investigated whether ERalpha and ERbetaƒninfluence collagen accumulation in the skin. The collagen content was assessed by the hydroxyproline assay .We also extracted the collagen by acetic acid method with addition of pepsin. Furthermore, we investigated the amounts of newly made soluble (non-crossed-linked) collagens that are not yet incorporated into the large fibrils using acetic acid extraction method. Using qRT-PCR method we measured mRNA levels of small leucine -rich proteoglycans (SLRP¡¦s) contributing to this process.
Results: The hydroxyproline content was two times higher in ERKO-alpha mice while in ERKO-beta mice was decreased (~30%) as compared to control animals. Furthermore, more collagen was extracted from the skin of ERKO-alpha mice (3.1 fold increase) as compared to controls. In contrast, the total collagen content decreased (~40%) in the skin of ERKO-beta mice. In ERKO-alpha mice, the soluble collagen was slightly elevated (1.6 fold increase) most likely reflecting increased synthesis of collagen. Alterations in collagen solubility have suggested changes in the expression level of SLRPs involved in fibrillogenesis. In ERKO-alpha mice, expression levels of Lumican and Decorin were significantly elevated (9.9 folds and 1.9 folds respectively). On the other hand, in ERKO-betaƒnmice, the expression levels of Lumican and Decorin was diminished (2.3 folds and 7.7 folds respectively).These data suggest that the ERbeta loss of function may lead to changes in collagen fibrils shape, diameter and assembly in these mice. Interestingly, we observed an increased mRNA and protein expression levels of MMP3, 8 and MT1, 2, 3-MMP in ERKO-beta mice suggesting elevated collagen degradation while MT2-MMP was significantly decreased in ERKO-alpha animals.
Conclusions: Our data show that the Estrogen Receptor pathway is involved in collagen biosynthesis. Furthermore, our results indicate that ERalpha plays an inhibitory role in collagen synthesis and fibrillogenesis, while data from ERKO-beta mice suggest that ERbeta may play an opposite role in these processes.

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Identification of biologically active site in the laminin gamma 1, 2, and 3 chain short arm regions.

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Laminins are major components of the basement membrane and have various biological activities such as promotion of cell adhesion, growth, migration, differentiation, and angiogenesis. Laminin is composed of three different subunits α chain, β chain, and γ chain. So far, five α (α1--α5), three β (β1--β3), and three γ (γ1-- γ3) chains were identified. At least sixteen laminin isoforms were reported with the combination of each subunit. These laminin isoforms expresses tissue- and/or developmental stage-specifically. Laminin γ chains have chain specific diverse biological functions. Each laminin γ chain is composed of N-terminus short arm region (EGF-like repeats and globular domains) and a coiled-coil domain, which forms triple-helical assembly. Here, we focused on the short arm region of the laminin γ1, 2, and 3 chains. We have constructed the synthetic peptide library derived from the laminin sequences, and identified cell adhesive peptides. We expanded the synthetic peptide library to add peptides covered short arm region of laminin γ chains sequences, and screened cell adhesive peptides in the library. We prepared synthetic peptide library including 256 peptides derived from three γ chains sequences. All peptides were synthesized by the 9-fluorenylmethoxycarbonyl strategy with C-terminal amide, and purified by reverse-phase HPLC. These peptides were generally 12 amino acids in length and overlapped with neighboring peptides by 4 amino acids. We screened cell attachment activity of the peptides by the peptide-coated plastic plate assay using human fibrosarcoma (HT-1080) cells. Four peptide derived γ1 chain sequence, C16 (KAFDITYVRLKF), C28 (TDIRVTLNRLNTF), C64 (SETTVKYIFRLHE), and C68 (TSIKIRGTYSER)[1], five peptide derived γ2 chain sequence, C2-25 (AKFLGNQQVSYG), C2-29 (VILEGAGLQIRAP), C2-31 (GITKTYTFRLNE) C2-35 (NLTALLIRATYG), and C2-37 (GYIDNVTLVSAR), and four peptides derived from γ3 sequence, C3-24 (APLNGGNVAFSTL), C3-28 (STDILISLDRLN), C3-65 (RVQLQFLLQETS), and C3-68 (AQRLLSNLTALSI), were identified as cell attachment peptides. The corresponding sites to these active peptides are important for the biological activities of the short arm regions of the laminin γ1, 2, and 3 chains.

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Corneal epithelial-like cells in total limbal stem cell deficiency in rabbit model.

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[Objective]

Corneal epithelial stem cells reside in the basal layer of limbus located at the transitional zone between cornea and conjunctiva. When limbal epithelial stem cells are destroyed, conjunctival tissue covers the surface of cornea, and this condition is known as limbal stem cell deficiency (LSCD). Previous study suggests that invaded abnormal conjunctival epithelial cells are transdifferentiated into corneal epithelial-like cells in rabbit total LSCD model. This study investigated the character of invaded conjunctival epithelial cells and basement membrane in rabbit total LSCD model.

[Methods]
Rabbit LSCD model was prepared by removing corneal and limbal epithelium surgically and chemically. The expressions of cytokeratin 4 (K4), K13, mucin 5 (Muc5), K12, and type IV collagen alpha-chains of the model were investigated for 6 and 12 months.
[Results and Discussions]
Neovascularization and cornea opacification were observed in the cornea surface of LSCD model. The integrity of LSCD model was characterized by observing the expression of conjunctival epithelial cell markers (K4 and K13), goblet cells marker (Muc5), and neovascularization in the corneal stroma. The central cornea of 6- and 12- months follow-up LSCD models were found to have corneal epithelial maker of K12 expressing cells, which were islanded without connecting to the limbal epithelial region.
Native conjunctival basement membrane consists of α1(IV)-α1(IV)-α2(IV) and α5(IV)-α5(IV)-α6(IV) collagens, and corneal basement membrane have α3(IV)-α4(IV)-α5(IV) and α5(IV)-α5(IV)-α6(IV) collagens. However, α2 (IV) collagen was found to be continuously expressed in the basement membrane from conjunctiva to central cornea, including the basement membrane of corneal epithelial-like cells of 6-month follow-up LSCD model. These results suggested that invaded conjunctival epithelial cells might be transdifferentiated into corneal epithelial-like cells. Whereas, in the 12-month follow-up LSCD model, α2 (IV) collagen expression under the corneal epithelial-like cells were weaker than that 6-month follow-up LSCD model. Interestingly, α5 (IV) collagen was strongly expressed under the corneal epithelial-like cells in 6-month follow-up LSCD, compared with conjunctival basement membrane. These observations suggested that basement membrane had a possible ability to regulate cell phenotype.
[Conclusion]
This study demonstrated that abnormal conjunctival cells on the corneal surface of LSCD model were transdefferentiated into corneal epithelial-like cells, and basement membrane had a possible ability to regulate cell phenotype. This study was beneficial to understand human LSCD, epithelial transdifferentiation, and stem cell maintenance.

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Orthogonal and reversible control of physical and optical properties of polyethylene glycol (PEG) hydrogel matrix.

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Recent studies suggest that dynamic sensing and regulation of the stiffness and viscosity of the extracellular matrix is required for stem cell differentiation and tumor metastasis. An understanding of how changes in physical properties of the micro-environment regulate cell function and behavior requires new experimental approaches that allow for dynamic, high spatial and temporal resolution of physico-chemical properties of the cell matrix. In this study we describe new probes that provide for high fidelity, reversible optical control of the physical and optical properties of soft gel matrices and other biomaterials. Thiol-functinalized 4-arm polyethylene glycol (PEG) monomers are crosslinked using a thiol-reactive homo-bifunctional optical switch, spironaphthoxazine (NISO), to form transparent gels of defined stiffness. The populations of the colorless spiro (SP) and the intensely blue colored merocyanine (MC) states of NISO within the gel are controlled via reversible and efficient optical or opto-thermal driven isomerisation reactions. The thermodynamically-stable SP state of the gel is rapidly turned the MC-state on exposure to 365 nm or 405 nm light. The MC to SP transition is quickly realized by exposing the same MC state to visible light or via a slower thermally-driven isomerisation. NISO-PEG gels are shown to undergo many cycles of high-fidelity, opto-thermal switching between the SP and MC states with little to no evidence of fatigue. Incorporation of collagen or fibronectin onto the surface of, or throughout, the gel supports the growth and microscope imaging of cells while having no impact on the optical switching properties. These new gels

allow for high-resolution and multiscale analyses of the relationship between changes in physical properties of the matrix and cell mechanics and behavior.

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Asymmetric Expression/Distribution of Connexins is Essential for Contractile Function in the Mammary Gland.

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Intercellular communication is essential for glandular functions and homeostasis. Gap junctions couple cells homotypically and heterotypically and coordinate reciprocal responses between the different cell types. Connexins (Cxs) are the main mammalian gap junction proteins, and their distribution in the heterotypic gap junctions is not always symmetrical; in the mammary gland, Cx26, Cx30 and Cx32 are expressed in the luminal epithelial cells and Cx43 in myoepithelial cells. Expression of all four Cxs peaks during late pregnancy until late lactation suggesting essential roles for these proteins in functional differentiation of the gland. When Cx26 was expressed driven by the keratin 5 promoter in a transgenic mouse model designed to study Cx26 over-expression in skin, K5-Cx26 dams had an unexpected mammary phenotype whereby transgenic dams were unable to feed their pups to weaning age, leading to litter starvation and demise in early to mid-lactation. We thus asked why ectopic expression of the luminal Cx26 to mammary myoepithelial cells impairs mammary function, and by what mechanism. The mammary glands of K5-Cx26 female mice develop normally but pups are unable to receive milk. This is despite the normal levels of beta casein and whey acidic protein present in the mammary glands of transgenic mice, and suggesting a defect in delivery rather than milk production. Primary mammary organoids, isolated from wild-type FVB females, contracted in culture upon treatment with oxytocin; however, primary mammary organoids from the transgenic mice failed to respond to oxytocin. Interestingly, we found that ectopic expression of Cx26 to myoepithelial cells alters the expression of endogenous Cx43 and inhibits gap junction-mediated dye coupling in myoepithelial cells expressing high levels of Cx26. The mammary contractile defect is thus due to Cx26 acting as a dominant negative to Cx43 function and impairing gap junctional coupling, since inhibition of gap junctional communication and knock-down of Cx43 in wild-type organoids similarly impairs contraction in response to oxytocin in culture.

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The Cytoplasmic Tail of Connexin32 and the Assembly of Gap Junctions.

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The spatio-temporal events and cellular factors required for the de novo formation of a nascent gap junction plaque, its growth, and disassembly are poorly understood. The assembly of gap junctions is a multi-step process, and is initiated when six connexin(Cx)s oligomerize to form connexons, which dock with connexons on contiguous cells to form intercellular channels. A gap junction plaque is formed when several intercellular channels cluster. We previously showed that the carboxy tail (CT) of Cx32, a Cx highly expressed in well-differentiated and polarized cells, may be required to initiate the formation of a gap junction plaque and/or its subsequent

growth. To explore the role of Cx32-CT further, a CT-truncated Cx32 (Cx32Δ220) as well as full length Cx32 (Cx32-FL) were retrovirally introduced in three separate cell-culture model systems. As assessed by quantitative immunocytochemical analyses, our results showed that Cx32Δ220 showed 2-3 fold decrease in gap junction size compared to Cx32-FL. Cell surface biotinylation showed that Cx32Δ220 trafficked normally to the cell surface, however, it was inefficiently assembled into gap junctions as assessed by detergent-solubility assay. Immunocytochemical analysis showed that Cx32Δ220 did not colocalize with markers of the secretory and endocytic pathway. Moreover, expression of Cx32-FL in cells stably expressing Cx32Δ220 increased the size of gap junctions, implying the direct involvement of Cx32-CT in this process. To define which domain of Cx32-CT determined the size of gap junctions, a series of deletion mutants, with progressive truncation of 20 amino acids, was retrovirally expressed in human prostate cancer cell line, LNCaP, in pancreatic cancer cell line, BxPC3, and in RL-CL9 cells. These experiments showed that the domain that determined the size of gap junctions lied between amino-acids 230-250 of Cx32-CT. A protein motif search identified two PKA phosphorylation motifs: RKGS and RLS, with serine residues at positions 233 and 240 that might be phosphorylated. Protein kinase A mediated phosphorylation has previously been shown to increase clustering of cell-cell channels. Our findings thus imply that the PKA phosphorylation motifs in Cx32-CT may regulate gap junction size and growth.

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The C-Terminus is essential for functional expression and internalization of rCx46.

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Recent results have shown that a total ablation of the C-terminus of rat lens connexin46 altered formation of functional rCx46 hemichannels. We combined molecular biological, cell imaging and dye transfer techniques to characterize the structures of the C-terminus that are involved in expression and internalization of rCx46. Six mutants rCx4630.3, rCx4630.9, rCx4632.6, rCx4633.5, rCx4635.3, and rCx4636.4 were generated by truncation of the C-terminus at different positions. All mutants were labeled with EGFP and expressed in HeLa cells to analyze the expression and internalization behavior using a confocal laser scanning microscope. The results showed that the rCx46 variants can be classified into three groups, according to the increasing length of the C-terminus. The variants of the first group (rCx4628.2, rCx4630.3, and rCx4630.9) did not form functional gap junction channels, they were found neither in large vesicles nor in cellular compartments. rCx4632.6 and rCx4633.5 form the second group. They formed functional gap junction channels which were clustered in large gap junction plaques. But they were not found in large vesicles and their compartmentalization seemed altered. The last group is composed of rCx4635.3 and longer rCx46 variants. They formed large gap junction plaques and functional gap junction channels as well. They were found in compartments and could be detected in large vesicles. Moreover HeLa cells expressing rCx4635.3 showed a 5.78 times higher rate of vesicle budding from the gap junction plaques in comparison to the 29 amino acid residues shorter variant rCx4632.6. Additionally, in cells pairs expressing rCx4632.6, only 2.91 annular junctions per cell pair were detected while cell pairs expressing rCx4635.3 showed an average of 6.70 annular junctions per cell pair. The results suggest that the first 81 amino acid residues of the C-terminus are necessary for a correct compartmentalization and large vesicle formation of rCx46. Within these 83 residues, the first 51 residues of the C-terminus are enough for formation of functional gap junction channels. However rCx4632.6 showed a reduced vesicle budding and formation of annular junctions compared to rCx4635.3 , we propose that the first 81 amino acid residues in the C-terminus are essential for an annular junction dependent removal of rCx46 gap junction channels.

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Ultrastructural and Live-Cell Analysis of Dynamin's Role in Gap Junction Plaque Endoexocytosis.

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Gap junction-mediated cell-cell communication modulates cell function in most tissues of the body. Such communication is regulated, in part, by the rate of gap junction plaque assembly and removal from the cell surface. While the assembly process has been extensively studied, much less is known about gap junction plaque disassembly. It has been established however that gap junction plaques can be removed from the cell surface by an internalization process (a unique combination of endocytosis and exocytosis, termed “endoexocytosis”) which results in the formation of a cytoplasmic annular gap junction in one of two contacting cells. Both clathrin and dynamin have been demonstrated to play a role in this endoexocytotic process. To increase our knowledge of the details and molecular machinery involved in gap junction plaque endoexocytosis, immunocytochemical, time lapse, and quantum dot transmission electron microscopic (TEM) techniques were used to image SW-13 adrenal cells, which spontaneously internalize connexin 43 (Cx43) gap junction plaques. Ultrastructural evaluation of cell populations in which dynamin was inhibited by treatment with a dynamin GTPase inhibitor (dynasore), or with siRNA knockdown techniques, revealed gap junction plaques with large invaginations. While invaginated gap junction plaque membrane was seen in the diluent-treated control populations, this was rare compared to the number and the size of invaginations observed in dynamin-inhibited cells. Most of the invaginations were not bud-like but instead formed U-shaped structures which had a wide base. These TEM observations would be consistent with failure of the gap junction plaque to form a bud with a constricted neck, in the dynamin inhibited populations. Time lapse imaging however was used to monitor and to confirm that gap junction “buds” formed in the dynamin inhibited populations. These buds failed to be scissored from the plasma membrane. Over time the buds converted to the U-shaped invaginations similar to the structures seen with TEM. The U-shaped areas remained in that configuration for several hours although the morphology of the invagination was altered. While annular gap junctions were observed in both control and dynamin-inhibited cell populations, they were more frequent in control populations. Here we describe, in detail, for the first time the ultrastructural changes during endoexocytosis and demonstrate the changes that occur when dynamin is inhibited. We suggest gap junction buds can form in dynamin inhibited populations but that dynamin plays a pivotal role in scissoring of these buds from the plasma membrane and thus the formation of the annular gap junction vesicle. Information on the molecular machinery needed for gap junction plaque removal from the cell membrane is thought to be critical to an understanding of gap junction processing and cell communication. NSF Grant # MCB- 1023144

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Endocytosis of Connexin43 and the Dynamics of Gap Junction Assembly.

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Connexin(Cx)s, the constituent proteins of gap junctions, are expressed both in a tissue-specific manner as well as redundantly. Redundant and tissue-specific expression implies that distinct mechanisms must exist to regulate the assembly of each Cx into gap junctions. The importance of understanding this is underscored by the fact that the trafficking and assembly of Cxs into gap junctions are defective in different cancers. To explore how the assembly of Cxs is regulated upon arrival at the cell surface, we used two pancreatic cancer cell lines, BxPC3, in which Cx43 is inefficiently assembled and remains intracellular while Cx26 is efficiently

assembled into gap junctions, and Capan-1, in which both Cxs are inefficiently assembled. Cell surface biotinylation showed that Cx43 trafficked normally to the cell surface and was endocytosed by the clathrin-mediated pathway in a Rab5-dependent manner before its assembly into gap junctions. Yeast two hybrid analysis showed that Cx43 interacted with the μ2 subunit of the AP-2 complex via a consensus sorting signal, YKLV, residing in its cytoplasmic tail between amino acid residues 286-289. Retrovirus mediated transduction of mutant Cx43Y286A/V289D, but not of wild type Cx43, induced gap junction assembly in BxPC3 and Capan-1 cells. Phosphorylation of serines in the vicinity of YKLV has been shown to modulate the interaction between the cargo and the AP-2 complex. As assessed by immunocytochemical analysis, we found that transient transfection of mutants Cx43S279A and Cx43S282A, but not of Cx43S279D and Cx43S282D, also induced gap junction assembly in both cell lines. Transient transfection of mutants Cx43Y286/V289D, Cx43S279A and Cx43S282A also prevented endocytosis of Cx43 in single cells. Finally, retrovirally-introduced Cx32 was assembled efficiently into gap junctions in BxPC3 cells. Our findings document that the assembly of Cxs into gap junctions upon arrival at the cell surface is subject to regulatory mechanisms at the site of cell-cell contact that are likely to be Cx specific.

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Acetylation Modulates Connexin32 Ubiquitination and Turnover.

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Gap junctions (GJ) are dynamic hexameric channels comprised of connexins that allow neighboring cells to communicate through intercellular passage of small molecules such as ions and metabolites. It has long been known that connexins are relatively short half-lived ($t_{1/2}$ =1.5-6h), but the pathways that regulate connexin protein level in order to maintain normal GJ communication under a variety of physiological conditions remain unknown. Using Connexin32 (Cx32) as a model, we are investigating the crosstalk between connexin acetylation and ubiquitination in the regulation of connexin protein level. We have determined that acetylated Cx32 is found both at the cell surface and in the cytoplasm, suggesting that the modification is present for most of the life cycle of Cx32 protein. Our earlier work indicated that pharmacological inhibition of HDACs resulted in increased Cx32 protein level that is independent of transcriptional alterations; further investigation revealed that HDAC6 inhibition in particular plays a role in regulating Cx32 protein level. Pharmacological inhibition of HDAC6 activity results in increased Cx32 at the cell surface, suggesting that deacetylation of Cx32 may be important for Cx32 endocytosis or that Cx32 acetylation promotes trafficking of Cx32 to the cell surface. Cx32 acetylation is increased and ubiquitination is decreased when HDAC6 expression is reduced, suggesting that deacetylation of Cx32 enhances ubiquitination. Analysis of K→R mutants, which eliminate acetylation sites but are unlikely to alter protein structure, has allowed us to identify several acetylated lysines in cytoplasmic domains of Cx32. In K→R mutants, ubiquitination was significantly reduced compared to WT, whereas in K→Q mutants in which Q mimics constitutive acetylation, ubiquitination levels resembled that of WT Cx32. This suggests that acetylation helps recruit ubiquitination machinery to Cx32. Based upon these results, we hypothesize that HDAC6 binding to acetylated Cx32 concurrently recruits ubiquitination machinery that ubiquitinates Cx32 at residues other than the acetylated lysines and augments Cx32 degradation. Current experiments are exploring the possibility of HDAC6-mediated regulation of GJ based cell-cell communication.

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Internalized Gap Junctions are Degraded by Autophagy.

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Direct intercellular communication mediated by gap junction (GJ) channels is a hallmark of normal cell and tissue physiology. In addition, GJs significantly contribute to physical cell-to-cell adhesion. Clearly, these cellular functions require precise modulation. Typically, GJs represent arrays of hundreds to thousands of densely packed channels, each one assembled from two half-channels (connexons), that dock head-on in the extracellular space to form the channel arrays (termed GJ plaques) that link neighboring cells together. Interestingly, docked GJ channels cannot be separated into connexons under physiological conditions, posing potential challenges to GJ channel function and renewal, and to physical cell-cell separation. We described previously that cells can efficiently internalize entire GJs in a clathrin-mediated endocytic process (Piehl et al., 2007, Mol. Biol. Cell, 18:337-347; Gumpert et al., 2008, FEBS Lett., 582:2887-2892), thus enabling these critical cellular functions. GJ internalization generates unique cytoplasmic double-membrane vesicles, described and termed earlier annular GJs (AGJs) or connexosomes. Here, using expression of fluorescent-tagged Cx43 in HeLa cells, ultrastructural analyses, confocal colocalization microscopy, pharmacological and molecular biological RNA-interference approaches depleting cells of key-autophagic proteins, we provide compelling evidence that GJs, following internalization, are degraded by autophagy. The ubiquitin-binding protein p62/SQSTM1 (sequestosome 1) was identified in targeting internalized GJ vesicles to autophagic degradation. While previous studies identified proteasomal and endo-/lysosomal pathways in GJ degradation, our study provides novel molecular and mechanistic insights into an alternative GJ degradation pathway. Its recent link to health and disease lends additional importance to this GJ degradation mechanism and to autophagy in general.

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Molecular Mechanisms of Gap Junction Internalization: Involvement of the C-Terminal Domain of Cx43.

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Gap Junctions (GJs) electrically and chemically couple neighboring cells by forming clusters of hydrophilic membrane channels. Connexins (Cxs) are the trans-membrane proteins that form these channels. Complete double-membrane spanning GJ channels are formed when two hexameric hemi-channels (termed connexons) dock in the extracellular space. In addition, GJs significantly contribute to physical cell-to-cell adhesion. Interestingly, docked GJ channels cannot be separated into hemi-channels under physiological conditions, and we have shown that GJs are internalized as a whole, in a clathrin-mediated endocytic process (Piehl et al., 2007; Gumpert et al., 2008). It is known that Cx43 is differentially phosphorylated during its life cycle. For example, mitogen (e.g. EGF) treatment of mouse embryonic stem cells (mESCs) results in increased phosphorylation of Cx43 and a reduction in GJ intercellular communication, suggesting that phosphorylation flags/activates Cx43 for internalization. Clathrin normally does not bind directly to its cargo. Instead, it requires adaptor proteins to link the cargo to clathrin molecules. We found several potential binding sites for (I) the classical clathrin-adaptor protein complex AP-2 (binding motif YXXF), and (II) the alternative clathrin-adaptor Dab2 (binding motif NPXY) in the cytoplasmic regulatory C-terminal domain of Cx43, both shown to be involved in GJ internalization. To characterize the Cx43 internalization signal/s, we mutated the potential binding sites of both adaptor proteins by site-specific mutagenesis. We have generated 9 different Cx43 mutants including 4 tyrosine point mutations (Y to H), 3 small (3-7 amino acids)

and 2 large (36 and 128 amino acid) deletion mutants. Live cell imaging of HeLa cells expressing the mutants exhibit very different cell behavior. Half-live studies revealed that Cx43-delL254-290-GFP and Cx43-delL254-CT-GFP have a significantly longer half-live than full length. Immuno-coprecipitation experiments show that Cx43-delL254-290-GFP and Cx43-delL254-CT-GFP have lost the ability to interact with AP-2, Dab2, clathrin, and dynamin-2. These, and additional analyses elucidate how, mechanistically, clathrin can internalize such large plasma membrane structures, and whether phosphorylation is the signal that triggers GJ internalization.

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Regulation of photoreceptor gap junction coupling by dopamine and adenosine.

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Gap junctions in retinal photoreceptors serve to suppress voltage noise and to facilitate entry of rod signals into the cone pathway when rod synapses approach saturation. These synapses are highly plastic and are regulated by light and circadian rhythms. The current study investigates the molecular mechanisms that regulate photoreceptor coupling. Recent studies have revealed an important role of Cx36 phosphorylation at two regulatory sites in regulating cell-cell coupling. Dopamine, as a light signal, has been suggested to cause uncoupling between photoreceptors via its action on D4 receptors, which inhibit adenylyl cyclase and PKA activity. We hypothesized that adenosine, with its extracellular level peaking in the darkness and at night, may serve as a dark signal and co-regulate photoreceptor coupling.

Adult C57BL/6 mice, as well as A1-/- and A2a-/- mice, were maintained under a 12/12h L/D cycle. Some animals were dark-adapted for 6h starting at ZT2h; Corresponding light-adaptation experiments were conducted at the same time. Eyecups were incubated for 30min in oxygenated Ames' medium alone or supplemented with the following drugs: dopamine D4 receptor agonist PD 168077 or antagonist L-745870, adenosine A2a receptor agonist CGS21680 or antagonist 8-(3-Chlorostyryl)caffeine, A1 receptor agonist 2'-MeCCPA or antagonist DPCPX, PKA activator Sp-8-cpt-cAMPS or inhibitor Rp-8-cpt-cAMPS. Cx36 phosphorylation level was evaluated by immunohistochemistry using antibodies for phospho-Cx36 and pan-Cx36. Dopamine and adenosine receptors were localized in retina by in situ hybridization.

Dark-adaptation, in the daytime and at night, increased Cx36 phosphorylation in photoreceptor gap junctions, while light-adaptation decreased phosphorylation. This predicts low photoreceptor coupling in light and enhanced coupling upon dark adaptation, as is observed with other species. Inhibiting D4 receptor activity elevated Cx36 phosphorylation in the light-adapted retina, and this increase was blocked by a PKA antagonist, suggesting that dopamine suppresses coupling by downregulating PKA activity. Activating D4 in dark-adapted retina diminished Cx36 phosphorylation, which was restored by a PKA agonist, in agreement with the previous conclusion. A broad-specificity adenosine receptor agonist CGS21680 increased Cx36 phosphorylation in light-adapted retina. This increase, as well as the increase in Cx36 phosphorylation caused by dark adaptation, were prevented in A1-/- mice. In contrast, in A2a-/- mice, Cx36 phosphorylation was abnormally high in light-adapted retina, but CGS21680 application further enhanced phosphorylation. In situ hybridization experiments showed that both A1 and A2a mRNA were present in inner retinal neurons, but little if any signal was present in photoreceptors. We conclude that adenosine receptors play a role in regulating photoreceptor coupling, but that their actions are at least partially indirect, resulting from regulation of dopamine secretion in the inner retina.

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Increase of Gap Junction Coupling in Aortic Vascular Smooth Muscle and Endothelial Cells by Dipyridamole.

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Dipyridamole is used in the prevention of primary and secondary stroke as well as for transient ischemic attack. We applied the scrape loading/dye transfer technique on the bovine aortic endothelial cell line GM-7373 and the rat aortic smooth muscle cell line A-10 to analyze whether dipyridamole affects the gap junction coupling of vascular cells. For cells cultivated under control conditions, a Lucifer Yellow diffusion distance of 93 pixels and 81 pixels was found for GM-7373 endothelial cells and A-10 smooth muscle cells, respectively. Presence of 1, 5, 10, 25, 50, 75 and 100 µM dipyridamole for 24 h increased the relative diffusion distance up to 127%, 130%, 137%, 153%, 160%, 173% and 146% in endothelial cells and up to 105%, 117%, 131%, 139%, 161%, 168% and 181% in smooth muscle cells. A significant dipyridamole induced increase of gap junction coupling could be observed after an incubation time of at least 3 h in both cell lines. Within an incubation time of 24 h, the effect of dipyridamole on gap junction coupling increased in both cell types. In order to determine the intracellular mediators of the dipyridamole related effect on gap junction coupling, protein kinase A (PKA) inhibitors like Rp-cAMPS or adenylyl cyclase and PKA activators like forskolin and 8-Br-cAMP were used. We found that presence of 200 µM Rp-cAMPS reduced the dipyridamole evoked increase of the dye diffusion distance. In contrast, activation of adenylyl cyclase or PKA with 100 µM forskolin and 1 mM 8-Br-cAMP, respectively amplified the dye diffusion distance similar to dipyridamole. Accordingly, we propose that dipyridamole increases gap junction coupling by activating a cAMP/PKA dependent mechanism. Whether PKA activates the transcription of connexins, the transport along the ER-Golgi network or modifies the opening state of gap junction channels is under investigation. But preliminary results indicate an effect of dipyridamole on expression of connexin 43 (Cx43) in smooth muscle cells.

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Multiple binding domains within the ZO-1 scaffolding protein are required for proper junction assembly and function.

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ZO-1 and ZO-2 are cytosolic, multi-domain scaffolding proteins necessary for tight junction (TJ) assembly. They also bind directly to F-actin and numerous cytoskeletal proteins, but the functional relevance of these domains and interactions is poorly understood. In this study we demonstrate that the depletion of both ZO-1 and ZO-2 in Madin-Darby Canine Kidney (MDCK) cells with shRNA constructs leads to three distinct phenotypes: mis-localization of TJ proteins, an increase in paracellular permeability and a dramatic expansion of the contractile array of actomyosin filaments within the Zonula Adherens (ZA). This latter observation suggests the novel hypothesis that assembly and contractility of the ZA is negatively regulated by tight junction ZO proteins. To better understand the synergistic relationship between the tight junction and zonula adherens, we have expressed ZO-1 rescue transgenes in the dKD cells to identify the protein-binding domains that are required for normal TJ and/or ZA assembly. We find that expression of the N-terminal half of ZO-1 protein is sufficient to rescue both TJ and ZA phenotypes observed in the ZO dKD MDCK cells, indicating that direct binding of F-actin to ZO-1 is not required for either TJ or ZA cytoskeletal organization. The PDZ1, PDZ2, SH3 and U6

domains are all required for normal TJ assembly, whereas only the PDZ1 and SH3 domains are necessary for proper ZA assembly. Interestingly, we found that the PDZ3 and GUK domains were not required for the assembly of either junction complex. Based on our results, we conclude that the assembly of a fully functional TJ is dependent on the coordinated interaction of multiple proteins with the N-terminus of ZO-1, and that a smaller subset of these domains also regulates the assembly of the ZA cytoskeleton.

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Postranslational modifications regulate the subcellular localization of the tight junction protein ZO-2.

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ZO-2 protein has a dual localization: the tight junction and the nucleus. Here we asked if in confluent cultures ZO-2 travels directly to the plasma membrane and if the putative nuclear localization signals (NLS) present in the sequence of the protein are functional. Employing permeable peptides, one that functions as nPKCε activator (ψεRACK) and other as inhibitor (εV1-2), we show that in sparse cultures the nuclei are respectively devoid or full of ZO-2, confirming the previous observation that nuclear exportation is regulated by nPKCε phosphorylation of nuclear export signal 1. In contrast, treatment of confluent MDCK monolayers with the nPKCå inhibitor does not trigger the accumulation of ZO-2 at the nucleus, suggesting that in confluent cultures the protein is not travelling to the nucleus before reaching the plasma membrane. To test the functionality of the bipartite (bp) NLSs of ZO-2 we microinjected the cytoplasm of sparse cultures with peptides homologous to NLSs chemically coupled to the reporter protein ovalbumin. We observe that while bpNLS1 accumulates ovalbumin at the nucleus, bpNLS2 does not. In silico analysis reveals that S257, S259 and S261 present within bpNLS2 are putative PKC phosphorylation sites, whereas S257 is also a O-GlcNAc target. We demonstrate that nuclear ZO-2 and the amino segment of the protein that contains the NLSs are O-GlcNAc, and show that inhibition of O-GlcNAc glycosidase with PUGNAc diminishes the cellular amount of ZO-2 through a process sensitive to proteosome inhibition. These results suggest that the function of bpNLS2 might be regulated by phosphorylation or O-GlcNAc and that the latter postranslational modification promotes the degradation of the protein at the proteosome.

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Tubulobulbar Complexes in Primary Cultures of Rat Sertoli Cells Contain Intercellular Junction Molecules.

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During spermatogenesis, the regulated turnover of intercellular junctions is crucial for the translocation of spermatocytes from basal to adluminal compartments of the seminiferous epithelium. Tubulobulbar complexes (TBCs) are unique subcellular structures that develop in association with basal junction complexes between Sertoli cells and have been proposed to facilitate junction turnover. We hypothesize that TBCs internalize components of the basal junction complexes, including tight, gap and adhesion junction elements. To test this hypothesis, we have established a primary Sertoli cell culture system in which TBCs or TBC-like structures form in the vicinity of junction complexes between Sertoli cells. Using this culture system, "rod-like" structures that are immunopositive for the intercellular junction elements Claudin-11, Connexin-43 and Nectin-2 occur that extend into Sertoli cells from the cell periphery. These

structures resemble TBCs that form in the intact seminiferous epithelium. These structures also co-localize with F-actin, which is known to associate with the proximal tubular part of TBCs and can be used as a marker for TBCs. Significantly, similar structures in cultured Sertoli cells that express GFP-tagged Claudin-11 and Connexin-43 extend into neighboring non-transfected Sertoli cells. Our results are consistent both with the conclusion that junction elements from neighboring Sertoli cells are found together within TBCs, and with the hypothesis that tubulobulbar complexes may play a role in the turnover of intercellular junctions. Supported by an NSERC Discovery Grant to AWV.

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DUSP23 controls the maturation state of cell-cell adhesions by regulating the phosphorylation-dependent dissociation of α-catenin and β-catenin.

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Cadherin-mediated cell-cell adhesion is dynamically regulated by interactions between cadherin-associated proteins β-catenin and α-catenin. These interactions couple E-cadherin to the actomyosin tension-sensing machinery, permitting strengthening and maturation of cell-cell junctions in response to force. A previous screen for genes that affect collective migration in MCF10A cells revealed that knockdown of the 17 kDa dual-specific phosphatase, DUSP23, results in loss of cell-cell adhesion as the cells migrate to fill a scratch wound. In follow-up studies to determine the interaction networks of proteins that regulate cell-cell adhesion using IP/mass spectrometry, we determined that DUSP23 interacts with the cadherin/catenin complex. Furthermore, knockdown of DUSP23 enhances phosphorylation of β-catenin at Tyr 142, and decreases the interaction between α-catenin and β-catenin. Consistent with our observation, biochemical and structural studies from other groups have previously demonstrated that phosphorylation of β-catenin at Tyr 142 disrupts the interaction between α-catenin and β-catenin. Analysis of cell-cell junctions in MCF10A cells depleted of DUSP23 compared to control cells reveals that a significantly higher percentage of DUSP23-depleted cells fail to mature their cell-cell adhesions into linear junctions and instead they remain as immature, zipper-like junctions. Thus, we have revealed a role for the smallest known phosphatase, DUSP23, in regulating the maturation of cell-cell adhesions through modulating the interaction between α-catenin and β-catenin. Studies are ongoing to determine how DUSP23 targets specifically to the adhesion complex, and also to investigate whether the weakening of cell-cell adhesion through loss of DUSP23-dependent regulation of the interaction between α-catenin and β-catenin contributes to invasive activity in 3D culture models and in vivo. Furthermore, we plan to use additional mass spectrometric studies to determine whether other proteins in the DUSP23 interaction network, including EGFR, are DUSP23 substrates.

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Role of the cytoskeletal protein alphaT-catenin in the heart.

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Members of the cadherin family of cell adhesion molecules are located in the adherens junction. They interact homophilically to mediate strong cell-cell adhesion and play a key role in the maintenance of tissue structure. AlphaT-catenin is a newly identified cadherin-associated protein that mediates linkage of the cadherin–catenin complex to the actin cytoskeleton. In the

mammalian heart, mixed-type junctional structures (*areae compositae)* are present at the intercalated discs (ICD). We observed alphaT-catenin localized in this novel type of junction, where it associates with endogenous plakophilin-2. To investigate the function of alphaT-catenin in the heart, a murine loss-of-function model was generated.
AlphaT-catenin-null mice are viable but exhibit progressive cardiomyopathy. It has been reported that suppression of PKP-2 activity in cultured cardiomyocytes leads to reduction in Cx43, which affects gap junctional communication. We observed reduced expression of PKP-2 in the ICD of alphaT-catenin-null myocardium. Furthermore, Cx43 was reduced at the ICD, and its co-localization with N-cadherin was also reduced. This indicates that alphaT-catenin might act synergistically with desmosomal PKP-2 to stabilize gap junctions at the *area composita*. This gap junction remodeling was associated with an increase in the incidence of ventricular arrhythmias in alphaT-catenin knockout mice subjected to acute ischemia.
The human alphaT-catenin gene, *CTNNA3*, has been mapped to chromosome 10q21, a region linked to autosomal-dominant dilated cardiomyopathy (DCM). Though genetic screening has not detected an association between *CTNNA3* mutations and DCM to date, *CTNNA3* is considered a candidate cause of this disease.

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Rac1 Promotes Formation of VE-cadherin Adhesion via IQGAP1-Dependent Mechanism.

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The endothelium lining the blood vessel wall forms a semi-permeable barrier that separates plasma proteins and cells from underlying tissue. Dysfunction of endothelial barrier leads to permeability increase accompanied by protein-rich edema as well as infiltration of blood cells into the interstitial space. The monomeric RhoA-GTPases Rac1, Cdc42, and RhoA play a critical role in regulating endothelial barrier function. Stable Vascular Endothelium (VE)-cadherin homophilic interaction, the main adhesive complex of adherens junction (AJ), causes an increase in the activity of Rac1 and Cdc42, whereas activation of RhoA leads to destabilization of AJs. The purpose of this study was to determine the causal relationship between Rac1 activity at AJs and stability of VE-cadherin mediated adhesion. We utilized a photo-activatable Rac1 probe (mCherry-PA-Rac1), consisting of a constitutively active Rac1 (V12) mutant fused to a photo-activatable LOV (Light Oxygen Voltage) domain of phototropin1 from *Avena Sativa*, as a tool to activate Rac1 locally. We demonstrated that photo-activation of PA-Rac1, but not a light insensitive probe bearing a mutation in the LOV domain (PA-Rac1-C450A), induced VE-cadherin clustering with a rate constant of 0.258 min^{-1} in human dermal microvascular endothelial cells. Interestingly, photo-activation of PA-Rac1 also resulted in junctional accumulation of IQGAP1, a scaffold protein that sequesters GTP-Rac1 and prevents GTP hydrolysis, suggesting that these two events might occur in concert. Furthermore, down regulation of IQGAP1 using the siRNA technique completely inhibited clustering of VE-cadherin upon PA-Rac1 photo-activation although IQGAP1-depleted cells displayed an overall increase in Rac1 activity as demonstrated by Rac1 activation assay. These findings suggest that clustering of VE-cadherin at AJs requires spatial activity of Rac1 and that Rac1 regulates the integrity of VE-cadherin adhesion in an IQGAP1-dependent manner.

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Effects of Fluid Shear on Myocytes Expressing ARVC-Causing Mutant Plakoglobin.

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Introduction: Arrhythmogenic right ventricular cardiomyopathy (ARVC) is typically an autosomal dominant disease characterized by a particularly high incidence of lethal ventricular arrhythmias. Several molecular mechanisms have been proposed for the progressive degeneration of myocardium associated with ARVC. For example, nuclear translocation of plakoglobin (JUP), a structural homologue of ß-catenin, may suppress the canonical Wnt / TCF-Lef pathway, resulting in cell apoptosis. In contrast, the effects of mechanical shear, a force likely experienced by collagen-coupled myocardial sheets in vivo, remains largely unexplored.
Objective: To investigate the effects of steady and oscillatory fluid shear on neonatal rat ventricular myocytes that express ARVC-causing mutant JUP.
Methods: Neonatal rat ventricular myocytes were isolated following established protocol, and cultured on collagen-coated glass slides. Adenoviral infection was used to generate ARVC-expressing cell models, with either wild-type (WT) or mutant plakoglobin (JUP). Cells were sheared under steady and oscillatory flow in a parallel-plate shear chamber at 0.06 Pascal (Pa), and immunostained for JUP or N-cadherin (N-Cad) using established protocol. Samples were imaged on an Olympus IX-81 confocal microscope, and analyzed using quantitative confocal microscopy. Immunoblotting was performed on cardiac myocyte lysates separated by PAGE.
Results & Conclusions: Cardiac cells were responsive to fluid shear stress. Control (no infection) and wild-type (WT) infected cardiac myocytes exhibited junctional reinforcement (i.e. – an increase in the expression level of proteins at cell-cell junctions) of both JUP and PC in response to both steady and oscillatory fluid shear, in addition to ERK 1/2 upregulation. These results are consistent with those previously established using mechanical stretch and demonstrate that fluid shear is a potent mechanostimuli that cardiac cells respond to. We next used mutant JUP to assess whether induction of ARVC-mutant JUP affects mechanoresponse of these cells to fluid shear. In contrast to WT, cells expressing ARVC-causing mutant JUP showed a diminished or totally inhibited ability for junctional remodeling. However, ERK1/2 activation remained unaffected, suggesting that some mechanically-sensitive pathways are not dependent on JUP. Unable to reinforce cell-cell junctions, we hypothesize that myocytes expressing mutant plakoglobin may be more susceptible to alterations in cell-cell interactions when exposed to shear forces, but that not all mechanosensitive pathways are affected in ARVC.

Glycobiology

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Micromembrane interactions of Galectin-3.

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The β-galactoside-binding lectin galectin-3 is involved in a variety of cellular processes including tumor development, immune-regulation or targeted protein sorting to the apical membrane of polarized epithelial cells. Here, we focused on the membrane interaction of galectin-3 and first employed giant plasma membrane vesicles (GPMVs) from Madine Darby canine kidney (MDCK) cells and large unilamellar vesicles (LUVs). Recombinant galectin-3 binds to GPMVs

and accumulates in liquid disordered domains in a cholesterol-dependent manner. The lectin also interacts with LUVs containing lactosylceramide and cholesterol, which suggests that even in the absence of any glycoprotein galectin-3 binds to lipid domains. In vivo, total internal reflection fluorescence microscopy (TIRFM) depicts galectin-3 binding with caveolin- or flotillin-enriched domains at the apical plasma membrane. This transient interaction with liquid ordered membrane domains is pH-dependent and involves sugar binding, which indicates that the binding of galectin-3 to membrane domains is modulated in living cells. Further studies on galectin-3-glycoprotein binding or oligomerization into cross-linking complexes with multivalent carbohydrates revealed that the environmental pH especially influences lectin-oligomerization with a maximum at pH 6.5. To conclude, interaction of galectin-3 with membrane domains of artificially generated vesicles or with the apical plasma membrane depends on membrane composition and pH.

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Polymorphic Salivary PNA-Binding Proteins.

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Polymorphisms were characterized in salivary glycoproteins binding to the galactose-binding lectin peanut agglutinin. Little knowledge exists on buccal cell glycoproteins and both buccal cell and salivary fluid glycoproteins may be studied as disease biomarkers. PNA reactivity of buccal proteins, cells and salivary fluid was examined by SDS gel electrophoresis and lectin blotting, microtiter plate binding assay and fluorescence microscopy of buccal cells.

In 53 native salivary fluid samples, 84.9% contained an approximately 150,000 Dalton PNA-reactive protein and 15.1% did not. This 150,000 Dalton protein had slightly different electrophoretic mobilities in different samples, being close to 150,000 Daltons in some, greater than 150,000 Daltons or less than 150,000 Daltons in others and sometimes consisted of two closely spaced bands. This protein had different staining intensities in different saliva samples.

Before neuraminidase treatment, only 3 out of 8 salivary fluid samples tested had 150,000 Dalton PNA-binding proteins. After neuraminidase treatment, 7 out of the same 8 samples had PNA-binding proteins and staining of previously reactive samples was increased. No shift in electrophoretic mobility was observed for the 150,000 Dalton protein in reducing conditions versus non-reducing conditions. Microtiter plate assays showed that PNA bound to both buccal cells and salivary fluid. Fluorescence microscopy confirmed that all buccal cells tested bound PNA to their surfaces. It was concluded that buccal cells and salivary fluid contain 150,000 Daltons glycoproteins that vary in amounts of terminal galactose and molecular mass among individuals. These proteins may be further examined for use as biomarkers.

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Metabolic changes in sialic acid synthesis pathway in DMRV/hIBM model mice with long-term sialic acid treatment.

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Distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy (DMRV/hIBM) is an autosomal recessive disorder characterized by muscle atrophy, weakness that initially involves the distal muscles, and presence of accumulated proteins and rimmed vacuoles in myofibers. DMRV/hIBM is secondary to mutations in the *GN*E gene, which encodes an essential enzyme in sialic acid biosynthesis. We recently showed that muscle atrophy and weakness were completely prevented in the DMRV/hIBM mouse after treatment with sialic acid (NeuAc and sialyllactose) and its precursor (ManNAc). In a recent report of neonatal suckling rats, the endogeneous sialic acid synthesis in colon is either inactivated or activated depending on milk

sialic acid level. This finding implies that long-term treatment of DMRV/hIBM patients with sialic acid may affect sialic acid biosynthetic pathway.

In this study, we first analysed the expression of genes encoding the enzymes and transporter in sialic acid biosynthesis pathway in various organs of non-treated wild mouse. The genes involved in synthesis are highly expressed in liver, while those involved in degradation are high in kidney, suggesting that liver is an anabolic organ and kidney is a catabolic organ for sialic acid synthesis. We then examined the expression of genes in skeletal muscle, liver and kidney of DMRV/hIBM mouse after long-term treatment with NeuAc and sialyllactose or ManNAc for more than 300 days and found that the genes for catabolic enzymes were further up-regulated in kidney, while the genes for anabolic enzymes were down-regulated in skeletal muscle. These results suggest that the long-term sialic acid administration may variably influence the effect of treatment depending on the type of compounds administered. We will also discuss the effects of inhibiting catabolic enzymes on sialic acid synthesis in DMRV/hIBM cells.

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Peracetylated N-acetylmannosamine, a synthetic sugar molecule, unravels important biomarkers in a mouse model of sialic acid deficient myopathy.

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Distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy (DMRV/hIBM), characterized by progressive muscle atrophy, weakness and degeneration, is due to mutations in GNE, a gene encoding a bifunctional enzyme critical in sialic acid biosynthesis. In the DMRV/hIBM mouse model, which exhibited hyposialylation in various tissue in addition to muscle atrophy, weakness, and degeneration, we recently have demonstrated that the myopathic phenotype was prevented by oral administration of N-acetylneuraminic acid, N-acetylmannosamine and sialyllactose, underscoring the crucial role of hyposialylation in disease pathomechanism. The choice for the preferred molecule, however, was limited probably by the complex pharmacokinetics of sialic acids, and the lack of biomarkers which could clearly show dose-response. To address these issues, we screened several synthetic sugar compounds that could increase sialylation more remarkably and allow demonstration of measurable effects in the DMRV/hIBM mice. In this study, we found that tetra-O-acetylated ManNAc (Ac4ManNAc) increased cell sialylation most efficiently, and in vivo evaluation in DMRV/hIBM mice revealed more dramatic, measurable effect and improvement in muscle phenotype, enabling us to establish analysis of protein biomarkers that can be used for assessing response to treatment. Our results provide a proof of concept in sialic acid-related molecular therapy with synthetic monosaccharides.

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The bile acid Deoxycholic acid impairs N-linked glycosylation and fucosylation processes in oesophageal epithelial cells.

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It is generally accepted that oesophageal adenocarcinoma arises from a Barrett's metaplastic lesion. Deoxycholic acid (DCA) is a bile acid present in gastric refluxate that alters many cell signalling pathways implicated in oesophageal cancer progression. Altered glycoprotein expression has been demonstrated in tissue from patients with Barrett's oesophagus and oesophageal cancer but the mechanisms regarding such changes are unknown. We had

previously demonstrated DCA-induction of Golgi fragmentation in the HCT116 colonic cell line, therefore we hypothesised DCA could induce Golgi fragmentation in oesophageal cells and impair protein processing, glycosylation and secretion.
We have demonstrated that DCA disrupts Golgi structure and affects protein secretion and glycosylation processes in cell lines derived from normal squamous epithelium (HET1-A) and Barrett's metaplastic epithelium (QH or CP-A). Protein secretion was monitored using a Gaussia Luciferase contruct. Cell surface expression of glycans were identified using carbohydrate-specific probes (WGA, ConA, PNA and UEA) that monitored N-glycosylation, O-glycosylation and core fucosylation in resting and DCA-treated cells. DCA caused a reduction in cell surface expression of (GlcNAc)n,Galß4GlcNAcß6Gal (WGA), GlcNAcßMana6(GlcNAcß2Mana3) Manß4GlcNAc (ConA) and Fuca2Galß4GLcNAcß6r (UEA-1) and altered subcellular localisation of (GlcNAc)n,Galß4GlcNAcß6Gal and GlcNAcßMana6(GlcNAcß2Mana3)Manß4GlcNAc in both cell lines. Furthermore, DCA reduced the expression and cellular localisation of E-cadherin in a manner analogous to treatment of cells with the N-glycan biosynthesis inhibitor tunicamycin. This is the first study to identify an altered Golgi structure and glycomic profile in response to DCA in oesophageal epithelial cells, a process which could potentially contribute to metaplasia, dysplasia and cancer of the oesophagus.

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Cell surface Calreticulin is a possible ligand for rArtinM on the mast cell surface.

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Mast cells are essential cells in IgE-associated immune responses. FcεRI crosslinking induces mast cell degranulation and release of mediators. We have previously shown that the lectin ArtinM induces mast cell activation but the mechanisms involved in this activity remain unknown. The aim of this work is to identify the ArtinM ligands present on the mast cell surface and to evaluate the role of these ligands in mast cell activation. For these studies, the rat mast cell line RBL-2H3 and recombinant rArtinM (rArtinM, a monomer) were used. The ability of rArtinM to induce mast cell degranulation was assessed by measuring β-hexosaminidase activity liberation. It was observed that in the presence of IgE, rArtinM was able to induce mast cell degranulation. To identify possible cell surface rArtinM ligands binding assays were performed using plasma membrane enriched cell lysate. This enriched lysate was incubated with biotinylated rArtinM and its ligands were precipitated using agarose-streptavidin. SDS-PAGE analysis revealed a few protein bands, which were extracted from the gel and digested by trypsin. The peptide sequences from these proteins were determined by MS/MS. Sequencing analysis revealed one specific rArtinM-associated protein, cell surface calreticulin (csCRT). It has been shown that csCRT is an important innate immune receptor that serves as a signal transduction receptor for mannose binding lectins. The interaction between csCRT and rArtinM was confirmed by Western Blot and the localization of csCRT was confirmed by immunofluorescence assays. These results demonstrate the presence of csCRT on the mast cell surface and also suggest that rArtinM binds specifically to csCRT. Such an interaction between ArtinM and csCRT may be important in mast cell immune responses and could explain the effects of ArtinM on mast cells.

Vesicle Docking and Fusion

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Rab6, Rab8, and MICAL3 cooperate in controlling docking and fusion of exocytotic carriers.

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Constitutive exocytosis delivers newly synthesized proteins and lipids from the Golgi apparatus to the cell surface. This process is mediated by vesicles, which bud off from the Golgi apparatus, move along cytoskeletal filaments and fuse with the plasma membrane. We use live cell imaging of individual vesicles with a high spatiotemporal resolution in combination with fluorescence recovery after photobleaching (FRAP) and photoactivation approaches to investigate the involvement of Rab GTPases and their effectors in fusion of secretory vesicles with the plasma membrane. We find that transport and fusion of secretory carriers is controlled by two Rab GTPases, Rab6 and Rab8. Rab6 is a conserved small GTPase that localizes to the Golgi apparatus and cytoplasmic vesicles and controls transport and fusion of secretory carriers. Rab8 is another Rab implicated in trafficking from the trans-Golgi to the plasma membrane. Both Rabs stably associate with exocytotic vesicles but their functions are different: Rab6 regulates vesicle transport and Rab8 recruitment, while, in turn, Rab8 is required for vesicle docking and fusion. We show that Rab8A stably associates with exocytotic vesicles in a Rab6-dependent manner. Rab8A function is not needed for budding or motility of exocytotic carriers but is required for their docking and fusion. These processes also depend on the Rab6-interacting cortical factor ELKS, suggesting that Rab8A and ELKS act in the same pathway. Rab8A and ELKS can be linked by the Rab8 effector, MICAL3, a member of the MICAL family of flavoprotein monooxygenases. Expression of a MICAL3 mutant with an inactive monooxygenase domain resulted in a strong accumulation of secretory vesicles that were docked at the cell cortex but failed to fuse with the plasma membrane, an effect that correlated with the strongly reduced mobility of MICAL3. FRAP-based analysis of protein turnover suggested that the redox activity of MICAL3 is needed to regulate its own turnover and the concomitant remodelling of vesicle-docking protein complexes in which it is engaged. Taken together, our study illustrates the cooperation of two Rab proteins in constitutive exocytosis and implicates a redox enzyme in this process.

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Mechanisms Controlling the Motility and Fusion of Rab6-positive Exocytotic Carriers.

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Constitutive exocytosis is an essential cellular process responsible for transport of newly synthesized proteins and other cellular components to the plasma membrane. It is mediated by Golgi-derived vesicles, which move along cytoskeletal filaments and fuse with the plasma membrane. We have previously shown that the small GTPase Rab6 marks the carriers of constitutive secretion, regulating their transport and fusion with the plasma membrane. The

Kinesin-1 Kif5B was shown to stimulate processive transport of Rab6-positive vesicles. Now we show that the kinesin-3 family members Kif1B and Kif1C also play a role in this process, and that in the absence of these kinesins, cytoplasmic dynein assumes a more prominent role, driving faster movements.
Vesicular carriers utilize a complex network of factors, which promote their specific interactions and fusion with the target membranes. Among these factors, a family of proteins called soluble N-ethylmaleimide-sensitive factor (NSF) adaptor proteins receptors (SNAREs) are considered essential. There are three groups of SNAREs: VAMPs, SNAP-25 homologues, and syntaxins; each of the groups contains several mammalian homologues involved in fusion steps between different compartments. Previous studies in our laboratory have shown that the coiled coil protein ELKS/Rab6IP2, which resides in cortical patches localized at the leading edges of migrating cells, promotes docking and fusion of Rab6 vesicles. Interestingly, in the absence of Rab6, the fusion of secretion carriers is accelerated but is much less selective with respect to cell location. This suggests that ELKS/Rab6IP2 and Rab6 cooperate in some specific way with the protein fusion machinery and are likely to interact, directly or indirectly, with SNARE proteins. We are now testing the involvement of specific SNAREs in the fusion of Rab6-positive vesicles, and we have found that specific members of the VAMP (v-SNARE), and SNAP (t-SNARE) families are necessary for fusion of exocytotic carriers.

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Munc18b is Essential for Regulated Mucin Secretion in Lung Epithelium.

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A tight homeostasis of mucus secretion in respiratory airways is fundamental to ensure the protection against inhaled pathogens and particulates and at the same time avoid airway diseases due to hypersecretion.
The molecular mechanism of regulated mucin secretion in lung epithelium is not yet completely understood. In eukaryotic cells Sec/Munc18 proteins are essential for regulated exocytosis; we thus utilized a genetic strategy to investigate in lung epithelium the role of Munc18b, the only isoform expressed at the apical membrane of airway mucous cells. We first generated constitutive and conditional mouse lines null for Munc18b (loss-of-function, LOF) and transgenic mice overexpressing a gain-of-function (GOF) Munc18b; we then measured airway epithelial mucin content by histochemical (Periodic Acid Fluorescent Schiff staining - PAFS) and biochemical (vacuum blot) techniques. We tested spontaneous mucin accumulation (basal secretory defect) in the naïve mice or ATP-induced degranulation (stimulated secretory defect) in mice sensitized and aerosol-challenged with ovalbumin to induce strong mucin production (metaplasia).
Since homozygous Munc18b null mice die at E9.5, we first studied heterozygous mice, detecting a 50% reduction in stimulated mucin secretion, but no mucin accumulation in naïve mice. In order to establish the role of Munc18b in basal secretion, we crossed the conditional allele to CCSP-Cre to delete Munc18b in airway mucous cells. Conditional Munc18b null mice displayed spontaneous mucin accumulation suggesting that Munc18b is essential for basal secretion. This conclusion is supported by the ability of GOF mice to fast resolve metaplasia by basal secretion. Together, these data indicate that Munc18b is a key regulator of mucin secretion in lung epithelium.

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The Exocyst Subunit Sec6 and the SM Protein Sec1 Act in Concert to Modulate Exocytosis in Yeast.

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Trafficking of protein and lipid cargo through the secretory pathway in eukaryotic cells is mediated by membrane-bound vesicles. Secretory vesicle targeting and fusion require the cooperation of multiple protein families. These molecules mediate the recognition step, ensure the fidelity of the process and the subsequent cellular activities, such as proper cell organization, cellular growth and secretion. Membrane fusion is carried out by SNARE complexes, which are formed by SNARE proteins localized on the vesicle and the target membrane. SNARE complex assembly is monitored and regulated by several factors, including the multisubunit tethering complexes and the Sec1/Munc18 family (SM) proteins. Regulation of the yeast secretory pathway relies on critical interactions between the SM protein Sec1, the exocyst complex and SNARE proteins. Our studies show that the exocyst is directly involved in regulating SNARE complexes and membrane fusion through interactions between the Sec6 subunit and the plasma membrane SNARE protein, Sec9. Moreover, we reveal another facet of Sec6 function—it directly binds Sec1, a SNARE regulator of the SM family. The Sec6-Sec1 binding is exclusive of Sec6-Sec9, but compatible with Sec6-exocyst assembly. In contrast, the Sec6-exocyst interaction is incompatible with Sec6-Sec9. Therefore, upon vesicle arrival, Sec6 is proposed to release Sec9 in favor of Sec6-exocyst assembly and to simultaneously recruit Sec1 to sites of secretion for coordinated SNARE complex formation and membrane fusion. Cooperation between the tethering complexes and the SM protein have been shown to be important for proofreading SNARE complexes and/or for SNARE complex formation, suggesting that Sec1-Sec6-exocyst may add further regulation and specificity to the fusion process.

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Mechanisms of collective force production by SNAREpin clusters on the pathway to fusion.

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SNARE proteins are thought to constitute the core of the intracellular fusion machinery which additionally may involve several accessory proteins (e.g. Sec/Munc proteins, synaptotagmin). Evidence suggests that target- and vesicle-SNAREs engage one another to assemble single SNARE complexes through a zipping mechanism which pulls the anchoring membranes together, while clusters of multiple SNARE complexes are thought to be required to drive fusion [Montecucco et al, Trends in Biochem. Sci., 2005]. In order to isolate SNARE mechanisms in controlled experiments many studies have employed the vesicle-supported bilayer (SBL) assay [Fix et al, Proc. Nat. Acad. Sci., 2004]. Here we present a combination of modeling of basic SNARE mechanisms with image analysis which allows us to quantitatively relate experiment to the model. The model determines the collective force exerted by a cluster of SNARE complexes based on the coarse-grained SNARE structure and the complexation energy previously measured. We propose a cluster drives fusion if it contains enough SNAREs to produce sufficient force above a threshold value, estimated from the threshold forces that drove fusion in protein-free, soft-supported SBL-SBL systems [Wong et al, Biophys. J., 1999]. Hence we determined the number of SNARE complexes required for fusion and find it depends strongly on the vesicle size. The model predicts the SNARE complex requirement for fusion of a 50-nm

vesicle is consistent with the reported requirement of 5-11 complexes [Karatekin et al, Proc. Nat. Acad. Sci., 2010]. We extended the model of the experiments to calculate the opened and closed times of a flickering fusion pore based on the measured lipid flux from the vesicle into the SBL. To test the model, quantitative image analysis of the experiments was required. We developed an image analysis routine which accounts for TIRF effects, polarization, and fluorophore quenching and bleaching which allowed us to precisely measure (i) vesicle size, (ii) fusion times and (iii) lipid flux. We thus measured the dependence of both the fusion times and the subsequent lipid flux on vesicle size and the presence of cholesterol. The model is fit to the lipid flux data to infer that fusion pores in cholesterol-free systems flicker over lipid mixing times of ~10-100 ms, but spend more time closed than open. With cholesterol, lipid mixing was somewhat slower, suggesting that cholesterol favors closed fusion pores.

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Loss-of-function mutations in SNAP29 result in defects in endocytic recycling and cell motility.

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Synaptosomal-associated protein SNAP29 is the most ubiquitous target SNARE among the SNAP protein family. Membrane anchored SNAREs assemble into SNARE complexes that bring membranes together to promote fusion. Loss of functional SNAP29 results in the human CEDNIK syndrome (Cerebral Dysgenesis, Neuropathy, Ichthyosis and Keratoderma). Thus far, we identified two mutations among CEDNIK patients: 1. A 1 bp deletion (c. 222delG of the cDNA), found in consanguineous Arab Muslim families from northern Israel, resulting in a premature termination, 75 amino acids downstream the initiator ATG; 2. A 1 bp insertion (c.486insA) found in two sibs from consanguineous parents originating from the Kashmiri region of Pakistan, resulting in a premature termination 5 amino acids downstream the mutation and a truncated protein of approximately 19 kDa.
In SNAP29 deficient cells recycling of transferrin and β1-integrin is impaired. Consequently, impaired β1-integrin recycling affects cell motility as reflected by changes in cell spreading and wound healing. No major changes are detected in exocytosis of VSVG protein from the Golgi apparatus, although the Golgi system acquires a dispersed morphology in SNAP29 deficient cells. Our results emphasize the importance of SNAP29 mediated membrane fusion in endocytic recycling and cell motility.

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A Novel Role of Syntaxin 3 as a Transcription Regulator.

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SNARE proteins mediate membrane fusion events in virtually all cellular membrane trafficking pathways. We have discovered an unexpected, novel function of the SNARE protein syntaxin 3. Syntaxin 3 normally has a C-terminal trans-membrane anchor and is involved in trafficking to the apical plasma membrane domain of polarized epithelial cells. We found that syntaxin 3 undergoes cleavage at an extremely conserved glutamine residue, which removes its trans-membrane domain resulting in a soluble fragment. Furthermore, a novel splice-isoform of syntaxin 3 lacks the trans-membrane anchor, and is expressed in human kidneys. Both, the cleavage fragment and the soluble isoform bind to the nuclear import factor RanBP5, target to the nucleus and co-activate several transcription factors including ETV4. ETV4 is required for

branching morphogenesis in kidney development, and associated with carcinogenesis and tumor metastasis. We found that kidneys from autosomal dominant polycystic kidney disease (ADPKD) patients express a small syntaxin 3 fragment – consistent with soluble syntaxin 3. We hypothesize that cleavage and transcriptional regulation in the nucleus is a novel function and common feature of syntaxin members of SNARE proteins. This may be a novel signaling mechanism that transduces information from cytoplasmic membrane trafficking events to the nucleus to affect changes in gene expression, which introduces a new paradigm of SNARE function.

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Impaired Transcytosis in Ca^{2+}-depleted Cells Correlates with Decreased Apical Labeling of MAL2 and Syntaxins 2 and 3.

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Over 15 years ago, transcytosis in MDCK cells was reported to be Ca^{2+}-dependent: thapsigargin enhanced transcytosis while BAPTA impaired it. Furthermore, calmodulin (CaM) antagonists were found to impair the basolateral endosome exit of transcytosing cargo. To date, the Ca^{2+}-dependence of basolateral to apical transcytosis has not been examined in polarized hepatocytes, cells that rely heavily on the transcytotic pathway for the delivery of newly-synthesized apical proteins. We determined that the transcytosis of three classes of newly synthesized apical proteins (GPI-anchored, single spanning and polymeric IgA receptor) was impaired by Ca^{2+} chelation with BAPTA in polarized WIF-B cells. As in MDCK cells, treatment with W7 (CaM antagonist) led to the accumulation of the transcytosing cargo in basolateral endosomes. Because MAL2 regulates transcytosis from the basolateral endosome to the sub-apical compartment, we examined its distributions in Ca^{2+}-depleted cells. After treatment with BAPTA for 1 h, nearly all MAL2 apical labeling was lost. The extent of MAL2 loss from the apical surface markedly coincided with impaired transcytosis. To determine whether diminished apical labeling was specific to MAL2 or was reflecting a general disruption of apical protein retention mechanisms or the disruption of tight junctions, we examined the distributions of a number of apical proteins, basolateral residents and tight junction-associated proteins in BAPTA-treated cells. There were no changes in the distributions of the tight junction markers or basolateral proteins indicating that membrane polarity was maintained in the BAPTA-treated cells. Although most apical proteins examined maintained their steady state apical distributions in Ca^{2+}-depleted cells, the apical labeling of syntaxins 2 and 3 was lost. Because protein levels did not change in BAPTA-treated cells, the decreased MAL2 and synataxins 2 and 3 labeling was not due increased protein degradation, but rather redistribution. Treatment with W7 and KN93 (a CaM kinase II inhibitor) also led to decreased apical labeling of MAL2 and the syntaxins and impaired transcytosis. Together these results suggest that CaM kinase II activation is required for the MAL2-mediated fusion of apically-targeted vesicles with syntaxins 2 and 3. We are actively testing this hypothesis.

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Otoferlin is a calcium sensor that directly regulates SNARE-mediated membrane fusion.

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Otoferlin is a large multi–C2 domain protein proposed to act as a calcium sensor that regulates synaptic vesicle exocytosis in cochlear hair cells. Although mutations in otoferlin have been associated with deafness, its contribution to neurotransmitter release is unresolved. Using recombinant proteins, we demonstrate that the C2 domains of otoferlin sense calcium, and in the presence of membranes, the apparent binding affinities increase by up to sevenfold. Using a

reconstituted membrane fusion assay, we found that five of the six C2 domains of otoferlin stimulate membrane fusion in a calcium-dependent manner. We also demonstrate that a calcium binding–deficient form of the C2C domain is incapable of stimulating membrane fusion, further underscoring the importance of calcium for the protein's function. These results demonstrate for the first time that otoferlin is a calcium sensor that can directly regulate SNARE membrane fusion reactions.

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Quantitative analysis of syntaxin clusters during insulin granule docking.

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For insulin to be secreted from pancreatic beta-cells, secretory granules must translocate to and then dock at the plasma membrane. Secretagogues can then trigger exocytosis of these granules, resulting in release of insulin. Only a limited number of granules can dock at any time, suggesting the existence of specialized docking sites where the exocytosis machinery is assembled. This is supported by the finding that SNARE proteins cluster in raft-like nanodomains near docked granules. We have used TIRF imaging and quantitative image analysis to study the association of GFP-labeled syntaxin-1 and L-type Ca^{2+}-channels (CaV1.3) with granules in live Ins1-cells. Both proteins formed small clusters (<100 nm) in the plasma membrane that associated with a subset of the docked granules (~50%). Clusters were stationary over several minutes, but exhibited striking intensity variations with a period of a few seconds. Single-molecule imaging showed that individual molecules of both proteins moved rapidly in the plasma membrane and were occasionally captured beneath a granule, for a short time (<1s). Granules that were associated with a syntaxin cluster were less likely to spontaneously undock, but more likely to undergo exocytosis during stimulation. Syntaxin was recruited to the granule site during docking, and lost during undocking and exocytosis. In summary, granule-associated nanodomains correlate with granule docking and exocytosis. This remarkably dynamic organization is established during or just after granule docking, which may suggest that granules induce the formation of their own docking site. Association of exocytosis-related proteins with granules occurs on a timescale consistent with rapid cellular signaling, and may be important for the short-term regulation of insulin secretion.

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Identifying New Sites of Insulin Action in GLUT4 Vesicle Exocytosis.

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Insulin increases glucose uptake in muscle and adipose tissues, which is accomplished through the mobilization of intracellular GLUT4 storage vesicles (GSVs) to the cell surface. Although insulin has been shown to promote GLUT4 translocation, precisely where insulin acts to control trafficking of GSVs is debated. In this study, by using ultrasensitive total internal reflection fluorescence microscopy (TIRFM) imaging, we quantified the size, dynamics, frequency and spatial localization of single vesicle exocytosis in 3T3-L1 adipocytes. We validated and used VAMP2-pHluorin as a new probe to visualize GSV and studied the spatial distribution of GSV fusion relative to endocytic machinery (clathrin and dynamin). We showed that in unstimulated

TUG shRNA cells, the exocytic rate is similar to that in insulin-stimulated control cells; insulin triggered a transient, two-fold burst of exocytosis in TUG shRNA cells, which was rescued by overexpression of shRNA-resistant TUG. Surprisingly, insulin promoted fusion pore expansion and increased full vesicle fusion, which were blocked by acute perturbation of phospholipase D, reflecting both properties intrinsic to the mobilized vesicles and a novel regulatory site at the fusion pore. Prolonged stimulation caused cargo to switch from 60 nm GSVs to larger exocytic vesicles (~150 nm) characteristic of endosomes, and increased accumulation of GLUT4 and VAMP2 with clathrin patches on the plasma membrane. Dual-color imaging showed that majority of GSVs (~95%) are fuse outside of clathrin and dynamin structures, suggesting a rate limiting endocytic step of regulation by insulin. In summary, our results support a dual brake-accelerator model of GSV translocation, whereby insulin promotes exocytic flux primarily by releasing an intracellular brake (TUG), but also by accelerating plasma membrane fusion and switching vesicle traffic between two distinct circuits. Our data unite the static retention and dynamic equilibrium models, and reveal the fusion pore as a novel site of insulin action, which may be perturbed in type 2 diabetes.

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The Potential Roles for PLD-derived Phosphatidic Acid in the Late Stages of Exocytosis.

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Understanding molecular mechanisms underlying multiple steps of regulated exocytosis has been a major focus for decades. Although essential roles of phospholipases have been excluded from the fusion step per se [1], their upstream and/or modulatory effects have received far less attention. In this regard, phospholipase D (PLD) and its product phosphatidic acid (PA) are of particular interest as the latter may define binding sites for critical proteins. Here, integrating acute treatments [1-6] and selective pharmacological manipulations of sea urchin eggs[7] with quantitative functional/molecular assays [3-7], we have addressed the potential roles for PLD-derived PA in the late stages of exocytosis, including docking and Ca2+triggered fusion. Our data indicate a selective inhibitory effect on the fusion rate when isolated cortical vesicles (CV) are treated with structurally diverse PLD inhibitors, but a consistent inhibitory effect on the extent, Ca2+sensitivity and rate of fusion when these inhibitors are used to treat intact eggs. Docking assays link this inhibition to an alteration in inter-membrane attachment, indicating that a PLD activity may promote CV docking to the plasma membrane (PM). To determine the localization of PLD activity, we labelled sheets of PM with associated docked CV - cell surface complexes (CSC) - and isolated CV with a fluorescent PLD substrate, NBD-PC. High performance thin layer chromatography identified NBD-PA only in the CSC, indicating a PLD activity localized to the inner monolayer of the PM, and presumably closely associated with CV docking sites. This was also consistent with the highest PLD activity being detected on CSC, relative to the intact egg, cytosol or CV membrane when using an alternate assay. Overall, this work with PLD inhibitors indicates upstream roles for PA in docking and/or late priming steps rather than directly in fusion and shed light on the localization of PLD activity with CV docking sites. Alterations in local PA composition may thus modulate fusion, in particular kinetics, through late effects on docking.

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Expression of Clusterin decreases with skin dryness and is stimulated by phospholipids.

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Clusterin is a 80 kDa glycoprotein widely distributed in mammalian tissues and biological fluids and is implicated in a variety of physiological and pathological processes. It acts as a molecular chaperone to stabilize proteins in a folding competent state and has a role in scavenging extracellular misfolded proteins produced following stress-induced injury. Clusterin gene expression is elevated at sites of tissue remodelling, such as differentiation and apoptosis and has also a role in the clearance of cellular debris and phagocytosis promotion.
Skin dryness is the result of multiparametric disorder implicating the cutaneous barrier, the water transport of and lipidic organization.

Objective

The purpose of this study was, first to demonstrate the effect of cellular debris on gene expression implicated in epidermis differentiation, hydration and inflammation, then characterize the clusterin expression in dry skin versus normal skin and finally modulate this clusterin expression using vesicles containing phosphatidylserin.

Methods

Normal human Keratinocytes (NHK) in culture, were incubated in the presence cellular debris or with vesicular structures (micelles) composed of a mixture of phosphatidyl choline and phosphatidylserine 70% and 30% respectively in weight, during various times. Clusterin expression was quantified by RT-QPCR and western Blot. Gene expression is studied by RT-QPCR.
Tissue clusterin expression is studied on normal skin biopsies issues of patients with clinical normal skin or dry skin. Punch biopsies were performed for further immunohistochemistry analysis. The clusterin immunostaining was performed on cryo-sections with a polyclonal rabbit anti-clusterin antibody, and alexafluor 546 Goat anti-rabbit secondary antibody. The labelled digital pictures obtained by confocal microscopy were quantified by image analysis.

Results

The results of this study highlight a significant effect of the cellular debris on the expression of five genes: a significant decrease of CD44 (hyaluronic acid receptor), filaggrin and aquaporine 9 and a increase in IL1alpha and MMP9 genes. Moreover, immunohistological studies showed that clusterin, which is expressed in the basal layer of the epidermis, is statistically decreased in the dry skin in comparison with the normal skins (- 75.9%).
Cell culture study demonstrated that vesicular structures, are able to stimulate the expression of clusterin at mRNA level (2 times increase at 24h) and protein level (1.8 times increase at 72h).

Conclusion

These results demonstrate that cellular debris are able to modulate genes which are involve in skin differentiation, water homeostasis and inflammation. Moreover, Clusterin expression is significally decreases with dryness and a phospholipids mixture could be used to stimulate its expression to counteract deleterious effects of skin dryness on clusterin and of cellular debris.

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Cholesterol and low temperature enhance fusion of vesicles to a planar membrane.

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Lipid composition plays an important role in fusion of vesicles to membranes, an essential process for exocytosis. Lipid head group, tail structure, and sterol content all impact the complex phase behavior of membranes. To determine the effect of lipids on fusion rate, we utilized the nystatin/ergosterol fusion assay and stimulated fusion with an osmotic gradient. Using PE/PC planar membranes with increasing concentrations of cholesterol, and keeping vesicle lipids constant (PE/PC/PS/ERG), we observed significantly higher fusion rates. We also observed that lipid phase affects fusion rates by forming DPPC membranes on 5ul glass capillary pipets. Significantly different fusion rates were observed at temperatures above and below the DPPC transition temperature (T_t) of 41°C. Decreasing the temperature below T_t increased the fusion rate. These data are consistent with the hypothesis that vesicle fusion with a membrane is suppressed in a liquid-disordered lipid phase (and/or enhanced by the ripple phase), and suggest cells may have adjusted membrane lipids to reduce membrane fusion.

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Role for the acto-myosin complex in regulated exocytosis revealed by intravital microscopy.

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The regulation and the dynamics of membrane trafficking events have been studied primarily in in vitro models that often do not fully reflect the functional complexity found in a living multicellular organism. We have used intravital microscopy in the salivary glands of live rodents to investigate regulated exocytosis, a fundamental process in all the secretory organs. Specifically, we focused on the dynamics and the machinery regulating the fate of large secretory granules (SCGs) after their fusion with the apical plasma membrane (APM). To this aim, we employed a combination of transgenic mice and gene transfection into the SGs of live rats, which enabled us to visualize the SCGs, their limiting membranes, their content and the actin cytoskeleton. We found that after fusion with the plasma membrane, SCGs gradually collapse releasing their content into the lumen of the acinar canaliculi. Furthermrore, we found that after fusion with the APM, F-actin and two of the isoforms of nonmuscle myosin II (IIa and IIb) are recruited onto the SCGs and form a contractile scaffold that is necessary to complete the exocytic process. Our results provide novel information on the machinery controlling regulated secretion and show that intravital microscopy provides unique opportunities to address fundamental questions in cell biology under physiological conditions.

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A1 adenosine receptor-mediated modulation of membrane trafficking requires activation of ADAM17 and the subsequent transactivation of EGFR.

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The umbrella cells form the outermost layer of the uroepithelium that lines the renal pelvis, ureters and the urinary bladder. We previously showed that apical addition of adenosine induced apical exocytosis; however, the signaling pathway is not known. Here, we report that specific activation of apically localized A1 adenosine receptors (A1ar) using the agonist CCPA stimulated exocytosis at the apical surface of umbrella cells. The activation of A1ar caused

phosphorylation of epidermal growth factor receptor (EGFR) at tyrosine residue 1173 (Y-1173) and inhibiting EGFR with the small molecule inhibitor AG1478 blocked the exocytosis. The transactivation pathway required HB-EGF as CRM197 an HB-EGF specific inhibitor blocked A1ar-induced exocytosis. A1ar-mediated EGFR- transactivation was also blocked by inhibitors of Gi-proteins, PLC, PKC, MAPK and protein synthesis. Furthermore, by immunolabeling, we show that a disintegrin and a metalloproteinase-17 (ADAM17/ TACE) is richly distributed at the apical domain of umbrella cells and broad-spectrum metalloproteinase inhibitor GM6001 and ADAM17 selective inhibitor Tapi-2 blocked A1AR-mediated vesicular exocytosis. We propose a model whereby activation of Gi-protein coupled A1ar triggers PLC-mediated generation of IP3, which increases intracellular calcium and leads to PKC-mediated activation of ADAM17. Activated ADAM17 in-turn cleaves HB-EGF and triggers EGFR activation leading to MAPK-mediated protein synthesis and exocytosis.

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Kv2.1 cell surface clusters are insertion and retrieval platforms for ion channel trafficking at the plasma membrane.

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The Kv2.1 delayed rectifier K+ channel regulates electrical activity in nerve and muscle and has been postulated to play a non-conducting role in SNARE-mediated protein fusion in neuroendocrine cells. Kv2.1 is unusual among voltage-gated K+ channels in that it localizes to micron-sized clusters on the cell surface of neurons, atrial myocytes and transfected HEK cells. Within these clusters Kv2.1 is non-conducting. Here we examined the hypothesis that these surface structures are specialized platforms involved in the trafficking of membrane proteins to and from the cell surface. TIRF-based studies indicated that GFP-Kv2.1 containing vesicles directly tether to and deliver cargo in a discrete fashion to the Kv2.1 surface clusters in both transfected HEK and cultured hippocampal neurons. Qdot-based single molecule experiments indicated that the delivery and surface retrieval of Kv2.1 occurs at the perimeter of the surface clusters. Overall, 85±8.4% of newly synthesized channels in HEK cells and 84.9±10.4% in hippocampal neurons were inserted within 0.5 microns of the cluster perimeter even though the Kv2.1 clusters represent only 21.4 ±3.8% of the basal cell surface. When 132 continuously recycling Kv2.1 channels in HEK cells were examined, 96.2% were also inserted at the cluster perimeter. Unlike Kv2.1, the Kv1.4 K+ channel has a homogeneous cell surface expression in transfected cells. Demonstrating that the Kv2.1 clusters represent cell surface platforms for more than just Kv2.1 insertion and retrieval, the non-clustering Kv1.4 K+ channel also inserted into the HEK cell plasma membrane at the Kv2.1 cluster perimeter. Kv1.4 endocytosis also occurred at this region. Together, these results indicate that a non-conducting function of Kv2.1 is to form specialized cell surface microdomains which are involved in membrane protein trafficking. This study is the first to identify stable cell surface sites for membrane protein delivery and retrieval in mammalian cells.

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Near-membrane dynamics of TRPM8 channels and the effect of agonist stimulation.

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Modulation of TRP channel distribution and availability by vesicular translocation has been reported as a regulatory mechanism for TRP channel function. Plasma membrane (PM) vesicle insertion regulates the number of functional thereby dictating total ionic flux and the cellular

consequences of TRP channel function. These consequences will depend on both, the amplitude of current and its ionic composition. Na+ current drives membrane depolarization and may be a trigger for opening other voltage-dependent ion channels, whereas Ca2+ influx drives fundamental cellular signaling cascades that control secretion, protein translocation, and vesicular fusion. Therefore, a tight control of TRP channel trafficking and delivery to various membrane compartments is thus a potentially critical step in regulating the flow of sensory information. Here we show whether agonist treatments affect the membrane dynamics of cold receptors (TRPM8 ion channels). TRPM8-GFP fusion channels were transfected on F11 cells. Through-the-objective TIRF microscopy was used to perform single particle tracking and to record changes in mobility. As the tracking and subsequent analysis of the MSD was slow and tedious, we build our own automatized software for image analysis. We present here a detailed diffusion analysis based on single particle measurements. Our data clearly show that TRPM8 agonists stimulate an increase in receptor density at the PM, and the effect on single vesicle dynamics. These changes are calcium-dependent. Importantly, by the use of the selective blocker BCTC we show that this process is dependent on TRPM8 channel activity. We picture that stimulation of vesicle exocytosis by TRPM8-mediated Ca2+ flux may increase cell-surface expression in a positive feedback loop. Such amplification power may represent a central theme in their function.
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The fifth Adaptin and the evolution of the late secretory compartments.

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Adaptor protein (AP) complexes sort cargo into vesicles for transport from one membrane compartment of the cell to another. Four distinct AP complexes have been identified, which are present in most eukaryotes.

We report the existence of a fifth AP complex, AP-5. Tagged AP-5 localises to a late endosomal compartment in HeLa cells. AP-5 does not associate with clathrin and is insensitive to brefeldin A. Knocking down AP-5 subunits interferes with the trafficking of the cation-independent mannose 6-phosphate receptor and causes the cell to form swollen endosomal structures with emanating tubules. AP-5 subunits can be found in all five eukaryotic supergroups, but they have been co-ordinately lost in many organisms. Concatenated phylogenetic analysis provides robust resolution, for the first time, into the evolutionary order of emergence of the adaptor subunit families, showing AP-3 as the basal complex, followed by AP-5, AP-4, and AP-1 and AP-2.

This provides not only a newly reported, ancient, cellular complex in eukaryotic cells, but insight into the earliest stages of the late secretory system.

ER and Golgi Transport I

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In vivo crystallization of human IgG in the endoplasmic reticulum of engineered CHO cells.

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Protein synthesis and secretion are essential to cellular life. Although secretory activities may vary in different cell types, what determines the maximum secretory capacity is inherently difficult to study. Increasing protein synthesis until reaching the limit of secretory capacity is one strategy to address this key issue. Under highly optimized growth conditions, recombinant CHO cells engineered to produce a model human IgG clone started housing rod-shaped crystals in the endoplasmic reticulum (ER) lumen. The intra-ER crystal growth was accompanied by cell enlargement and multi-nucleation and continued until crystals outgrew cell size to breach membrane integrity. The intra-ER crystals were composed of correctly folded, endoglycosidase H-sensitive IgG. Crystallizing propensity was due to the intrinsic physicochemical properties of the model IgG, and the crystallization was reproduced in vitro by exposing a high concentration of IgG to a near neutral pH. The striking cellular phenotype implicated the efficiency of IgG protein synthesis and oxidative folding exceeded the capacity of ER export machinery. As a result, export-ready IgG accumulated progressively in the ER lumen until a threshold concentration was reached to nucleate crystals. Using an in vivo system that reports accumulation of correctly folded IgG, we showed that the ER-to-Golgi transport steps became rate-limiting in cells with high secretory activity.

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Sec16B is involved in the ER export of the peroxisomal membrane proteins Pex3 and Pex16 in mammalian cells.

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Recent work has suggested that some peroxisomal membrane proteins (PMPs), including the PMP import receptors Pex3 and Pex16, are, at least partly, transported from the endoplasmic reticulum (ER) to peroxisomes. The molecular machinery involved in the transport from the ER remains to be identified.

Sec16 is a key molecule in the formation of COPII vesicles, which mediate protein transport from the ER to the Golgi apparatus. Mammals have two Sec16 isoforms: Sec16A, which is a longer primary ortholog of yeast Sec16, and Sec16B, which is a shorter distant ortholog. Previous studies have shown that Sec16B, as well as Sec16A, defines ER exit sites, where COPII vesicles are formed in mammalian cells, and is involved in the transport from the ER to the Golgi apparatus. In this study, we showed that Sec16B plays an important role in the transport of Pex3 and Pex16 from the ER to peroxisomes in mammalian cells.

When overexpressed in HeLa cells, Sec16B was targeted to the entire ER, whereas Sec16A was mostly cytosolic. Overexpression of Sec16B caused Pex3 and Pex16 to be redistributed from peroxisomes to Sec16B-positive ER membranes, concomitantly with a reduction in the number of peroxisomes. Knockdown of Sec16B but not Sec16A by RNA interference inhibited the transport of Pex16 from the ER to peroxisomes, suppressed expression of Pex3, and

caused elongation of peroxisomes. These phenotypes were significantly reversed by the expression of RNAi-resistant Sec16B. Deletion of the C-terminal 348 amino acids of Sec16B abrogated the ability to rescue the Sec16B depletion phenotype, suggesting the importance of the C-terminal region in peroxisome biogenesis. These results suggest that Sec16B is involved in peroxisome biogenesis dependent on the PMP transport pathway from the ER.

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The delayed ER export of HLA-G is due to its lack of ER export signal, but not to its potential ER retention signal.

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The cell surface expression of HLA-G, the non-classical major histocompatibility complex class I (MHC-I) molecule specifically expressed in placenta, was reported to be regulated by its dilysine motif present in the cytosolic domain. In contrast to the previous study, we find that the dilysine of HLA-G is not a functional ER retrieval motif as a HLA-G mutant, whose dilysine motif was replaced with alanine residues, did not accelerate its intracellular trafficking. Instead, another HLA-G mutant (HLA-G/A-tail), whose cytosolic tail was replaced with that of HLA-A2.1 with a functional ER export signal, traffics significantly faster than wild-type HLA-G. Interestingly, addition of an ER retention motif at the C-terminal end of HLA-G/A-tail leads to complete ER retention. Finally, addition of 6 amino acids, which includes a functional ER retention motif (KKXX) at the C-terminus, to HLA-G (RAASSD) fully retains HLA-G in the ER. These results are consistent with a previous model whereby a functional dilysine ER retention motif needs to be placed at least 8 amino acids apart from the membrane for stable COPI binding. Therefore, our current study also raises a question on the model where the ER retention motif of HLA-G functions in a peptide-quality-dependent manner.

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The Dendritic Endoplasmic Reticulum and Conventional Kinesin Define a Non Canonical Trafficking Modality for GABAB Receptors.

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The availability of neurotransmitter receptors is regulated by coordinated mechanisms that deliver newly synthesized receptors to the plasma membrane and remove them for storage, recycling or degradation. In neurons, a complex spatial distribution of secretory organelles defines local, non-canonical protein trafficking pathways that comprise the dendritic endoplasmic reticulum (ER) and satellite Golgi outposts. Metabotropic $GABA_B$Rs regulate the efficacy of synaptic transmission throughout the brain, but the mechanisms controlling their biosynthetic route and plasma membrane delivery remain unclear. In this study we examined the trafficking modality of $GABA_B$Rs in dendrites and evaluated the roles of the ER and kinesin-I in this process. The localization, colocalization and mobility of $GABA_B$Rs were evaluated using fixed and live-cell imaging in primary cultures of rat hippocampal neurons under control conditions or after blockade of intracellular trafficking. *De novo* receptor insertion was determined using α-bungarotoxin labeling and single molecule tracking was performed using photoactivated localization microscopy. We show that $GABA_B$Rs insert throughout the somatodenritic plasma membrane and traffic through satellite Golgi outposts. $GABA_B$R1, the

subunit that contains an ER retention motif, colocalizes preferentially with the ER in dendrites and is mobile within this compartment. The transport of dendritic $GABA_BR1$ has a modal velocity of 200 nm/sec and depends on kinesin-I and microtubule acetylation. Taken together, these results indicate that $GABA_BRs$ utilize a non-canonical secretory route in dendrites and uncover a novel role for kinesin-I in the transport of cargo along the dendritic ER. (Supported by Fondecyt 1100137 and ICM P-09-015F. JIV supported by CONICYT).

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Live-cell imaging of organelle trafficking and before, during and after mitosis.

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In mammalian cells the process of mitosis requires substantial trafficking of organelles both before mitotic onset and following separation of daughter cells. Complex organelle morphologies and interactions need to be disassembled then reassembled. Furthermore many organelles show cell-cycle dependent-activity. Intracellular organelles were labeled with targeted fluorescent proteins. Aspects of mitosis were reported by targeted fluorescent proteins to cytoskeletal elements and cell-cycle progression was imaged using FUCCI fluorescent protein biosensor. Cells expressing a fluorescent protein targeted to a particular organelle were combined with either a cytoskeletal targeted fluorescent protein or one of the FUCCI constructs (to delineate G1/S or S/G2/M). Cells were imaged for 24 hours to correlate organelle dynamics with mitotic dynamics, daughter cell separation or cell-cycle progression. Using this approach we show the disassembly of the Golgi apparatus during mitosis and the timing of reformation in G1. Furthermore we show the segregation of endosomal and mitochondrial structures during mitosis and trafficking to reestablish organelle morphology in G1.

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Mechanisms of Proglucagon Sorting to the Regulated Secretory Pathway.

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Introduction: Prohormones are synthesized in the endoplasmic reticulum, transported through the Golgi and sorted to secretory granules (SGs), where they are processed to mature peptide hormones and stored until a stimulus triggers their release, a process known as regulated secretion. There are several mechanisms by which prohormones are sorted to SGs. One prohormone sorting hypothesis is “sorting-by-entry” whereby prohormones are sorted to SGs via a sorting signal, such as an amphipathic α-helix or di-basic amino acids. Proglucagon is a prohormone that is processed in a tissue-dependent manner, to yield glucagon in pancreatic α-cells, and glucagon-like peptide-1 (GLP-1) and GLP-2 in intestinal L cells and brainstem. We are examining the molecular mechanisms underlying the sorting of proglucagon to SGs. We hypothesize that proglucagon is sorted to SGs via sorting signals comprising either the α-helix structure in glucagon, GLP-1, or GLP-2, or the di-basic amino acid sequence in glucagon.

Results: In order to identify sorting signals within the sequence of glucagon, we have generated fusion constructs, composed of a reporter molecule, Fc, and either wild-type (WT) or mutant glucagon. Glucagon mutations were designed to disrupt either the α-helix (L14P/L26P; LP-Fc) or di-basic amino acids (R17R18Q; RQ-Fc). Stable transfectants using the neuroendocrine cell line, PC12, were generated and verified by Western blot analyses. To measure the extent of regulated secretion, Fc release in response to 55 mM K+ was determined by immunoprecipatation/Western blot. For subcellular localization in SGs, we conducted

fluorescence immunocytochemistry for Fc and an SG marker, chromogranin A. The LP-Fc mutant showed no significant difference in K+-stimulated secretion compared to basal (n=3, t-test), while the RQ-Fc mutant showed a 3-fold increase in K+-stimulated secretion (n=3, $p<0.05$, t-test). Fluorescence immunocytochemistry showed that both WT-Fc glucagon and RQ-Fc mutant significantly co-localized with chromogranin A, while the LP-Fc was localized exclusively to the Golgi. These results indicate that both the WT-Fc glucagon and RQ-Fc mutant are sorted to the regulated secretory pathway while the sorting of the LP-Fc mutant is disrupted.

Conclusion: WT glucagon contains sufficient information for sorting proglucagon to SGs. Specifically, the α-helix within glucagon is a sorting signal while the di-basic amino acid sequence may not be as important for regulated secretion. Further experiments will test the α-helices within GLP-1 and GLP-2 as putative proglucagon sorting signals.

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PLD2 is important for glycoconjugate trafficking in mast cells.

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PLD has been shown to play an important role in vesicle trafficking along the secretory pathway. PLD2, a PLD isoform, was investigated in order to assess its involvement in transport of glycoproteins through the secretory pathway. The mast cell line RBL-2H3 and RBL-2H3 cells that overexpress catalytically active (PLD2CA) and inactive (PLD2CI) forms of PLD2 were used in the study. The glycosylation pathway was investigated using N-acetylmanosamine-azide (ManNAz), which is a metabolic precursor for sialic acid, which was detected with Alexa Fluor 488-alkyne. The cells were pulse labeled with ManNAz for 1h and chased for up to 3 days and the labeling intensity at the plasma membrane was examined. In RBL-2H3 cells the plasma membrane was labeled with ManNAz after 12h of chase, and the labeling remained unchanged until 36h. After 36h of chase, the ManNAz labeling decreased. In CA cells the modified sugar labeled the cell membrane at 12h and the labeling intensity remained the same until 36h of chase. After 36h the modified sugar decreased its labeling intensity in the cell membrane. In CI cells, ManNAz intensity in the cell membrane was higher than the other cell lines after 12h of chase. After this time the labeling intensity decreased, but still had more labeling in the cell membrane than the other cell lines. The same pulse-chase experiment was repeated, but the cells were permeabilized in order to visualize the localization of the modified sugar inside the cells. In RBL-2H3 cells the ManNAz was present in the juxtanuclear region, most probably in the Golgi complex, at 12h. This labeling persisted until 36h and then decreased. In the CA cells the modified sugar was present juxtanuclearly, after the first 12h of chase. After 24h, the intensity of the juxtanuclear labeling decreased, but persisted until 48h. In the CI cells, after the first 12h of chase there was labeling in the juxtanuclear region which remained with the same intensity until 36h of chase. With 60h of chase the intensity of the labeled ManNAz at the cell membrane was the highest when compared to the other cell lines. These observations indicate that in CI cells there is a delay in the synthesis and/or trafficking of the glycosylated proteins when compared to the wild type RBL-2H3 and CA cells. Thus, PLD2 appears to play an important role in glycosylation in RBL-2H3 mast cells. These results provide insights for future studies to elucidate the molecular mechanisms underlying intra-Golgi transport.

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ALPS motifs and alpha-synuclein bind selectively to different types of vesicles in vitro and in cells.

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Membrane curvature sensors have different structures and chemistries. ALPS motifs bind specifically to highly curved membranes due to the fact that they are unbalanced lipid-binding amphipathic helices, having a well-developed hydrophobic face but few charged residues on their polar face. Numerous ALPS motifs are found in proteins that function in the early secretory pathway. A second type of amphipathic helical curvature sensor is exemplified by alpha-synuclein, a causative agent of Parkinson's disease that is localized to synaptic vesicles. alpha-synuclein is also an unbalanced amphipathic helix, but opposite to ALPS motifs, has a well developed polar face and a poor hydrophobic face. Hence both ALPS motifs and alpha-synuclein have a "weakness" that makes positive membrane curvature mandatory for their binding. However, these curvature sensors have opposite chemistries, and direct in vitro comparisons of ALPS motifs and alpha-synuclein demonstrate that they have different lipid requirements for binding, preferring lipid compositions complementary to each one's distinct chemical properties. When expressed in yeast cells, these two curvature sensors were targeted to different classes of vesicles, those of the early secretory pathway for ALPS motifs and to negatively charged endocytic/post-Golgi vesicles in the case of alpha-synuclein. The lipid composition of the vesicles that alpha-synuclein and ALPS motifs associate with in cells correlate well with their in vitro lipid binding properties. These findings show that complementary curvature sensing mechanisms operate in the two major lipid environments of the cell, and that curvature sensors can discriminate between vesicles of different lipid composition.

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A Yeast Genomic Screen to Identify Negative Regulators of Kir2.1 Plasma Membrane Residence.

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The inward rectifying potassium channel, Kir2.1, functions at the plasma membrane of muscle and neuronal tissues to maintain membrane potential. Gain or loss of function mutations in Kir2.1 cause diseases such as Anderson-Tawil syndrome and short QT syndrome, respectively. Like other complex ion channels, a significant percentage of Kir2.1 is also targeted for ER associated degradation (ERAD). However, the spectrum of cellular determinants that assemble, deliver, and maintain channel plasma membrane residence remain largely unknown.

As in human cells, the yeast, Saccharomyces cerevisiae, must maintain intracellular potassium levels. Yeast express two potassium transporters (Trk1 and Trk2), but in the absence of Trk1 and Trk2 the cells can utilize an exogenously expressed potassium channel to grow on low potassium media.

To identify factors necessary for Kir2.1 plasma membrane residence, we engineered a trk1Î" trk2Î" yeast strain in which Kir2.1 restores growth on low potassium. Thus, yeast growth on low potassium media provides a direct readout for Kir2.1 plasma membrane residence. As a proof of principle that factors could be identified that affect Kir2.1 trafficking, the reporter strain was

crossed to five strains deleted for genes hypothesized to be important for Kir2.1 plasma membrane residence. Yeast deleted for DOA10 grew better and yeast deleted for EMP24 grew less well on low potassium media, suggesting a screen for effectors of Kir2.1 trafficking could be identified. The trk1Î" trk2Î" strain expressing Kir2.1 was then crossed to the yeast deletion collection and the resulting strains were screened on low potassium. Initial results from the screen uncovered nearly all members of the Endosomal Sorting Complex Required for Transport (ESCRT) and the entire retromer complex, as well as members of the transport protein particle (TRAPP) and Conserved Oligomeric Golgi (COG) complexes. Surprisingly, ERAD-associated factors were not identified. Together, these data suggest that Kir2.1 function at the plasma membrane is controlled mainly by later steps in the secretory pathway and not by ERAD.

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Profragment Chain Topology Controls Early Folding Events of a Nascent Membrane-bound Hydrolase.

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Many eucaryotic proteins have a complex three-dimensional structure with more than one structural domain and each domain plays a particular role within the intramolecular organization of the polypeptide. The transport-competent precursor of human intestinal lactase phlorizin hydrolase, pro-LPH, encompasses four homologous domains, which presumably have evolved from two subsequent duplications of one ancestral gene. Pro-LPH comprises two intramolecular chaperones, one in its profragment - LPHα - containing domains I and II and one within domain III of the active brush border LPHβ region. The profragment is proteolytically removed in two steps within the Golgi apparatus and by luminal pancreatic trypsin. The role of each profragment domain for the attainment of LPHα chaperone function and the interrelationship between both intramolecular chaperones in the nascent pro-LPH polypeptide has not been elucidated so far. Therefore, we analyzed the early events of its biosynthesis by directed restructuring of the domain composition. Expression in COS-1 cells revealed that removal of domain II results in a diminished phlorizin hydrolase and absent lactase activity, lack of dimerization in the ER, but normal transport kinetics from the ER to the Golgi apparatus. By contrast, deletion of domain I generates a malfolded protein that is blocked in the ER. Moreover, expression of domains I, II, and the profragment alone results in ER-resident proteins. However, the protein containing domains I, II, and III is transport-competent and efficiently secreted. The data presented here propose a model of the early folding events of nascent pro-LPH in which the profragment topological organization precedes the hierarchical intramolecular organization of the whole protein. To our knowledge, this is the first cell biological and biochemical analysis of the early organizational events of a nascent membrane-anchored multi-domain polypeptide.

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SEC23B is required for development of the murine pancreas, salivary glands and gastric glands.

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In eukaryotic cells, COPII (coat protein complex II) captures cargo into vesicles and mediates vesicle budding from the endoplasmic reticulum (ER). COPII contains 5 core components: SAR1, SEC23, SEC24, SEC13 and SEC31. Mammalian cells express multiple paralogs of COPII proteins, including two SEC23 paralogs, SEC23A and SEC23B. SEC23 is a GTPase-

activating protein (GAP) that activates the SAR1 GTPase and also plays a role in cargo recognition. Recently two human genetic disorders have been associated with defects in SEC23 genes. Cranio-lenticulo-sutural dysplasia (CLSD) is characterized by craniofacial and skeletal malformation due to homozygosity for one of two missense mutations in SEC23A. Mutations in SEC23B cause congenital dyserythropoietic anemia type II (CDAII), defined by moderate anemia, multinucleated erythroblasts, ineffective erythropoiesis and aberrant glycosylation of specific red blood cell membrane proteins. How SEC23B deficiency leads to this selective red blood cell defect in humans remains unclear. Homozygous SEC23B null mutations have never been identified in CDAII patients, suggesting that total loss of SEC23B may be lethal. The objective of this study is to characterize mice completely deficient for SEC23B. Sec23b heterozygous null mice were generated from a gene-trap embryonic stem cell clone. Homozogous Sec23b null pups die shortly after birth, with degeneration of the pancreas, salivary glands and gastric glands. The typical grape-like clusters of acinar cells of the WT pancreas are lost in the Sec23b deficient pancreas, with no discernible islet structure. Zymogen granules are nearly absent in Sec23b deficient pancreatic acini and greatly reduced in salivary glands. The ER is severely distended due to the accumulation of proteins in the lumen. The pro-apoptotic branch of the unfolded protein response pathway is selectively activated in SEC23B deficient pancreas, gastric glands and salivary glands, and these tissues also exhibit positive staining for caspase 3 and TUNEL, suggesting a central role for apoptosis in the degeneration of these tissues in Sec23b deficient embryos. The affected tissues all express high levels of SEC23B, suggesting that professional secretory cells in the mouse may be particularly dependent on SEC23B function. These results suggest distinct and non-overlapping functions for mammalian SEC23A and SEC23B.

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Biological role and significance of the PAR1 signal peptide.

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The G protein-coupled protease-activated receptor 1 (PAR1) is activated by cleavage of its N terminus by thrombin which in turn uncovers a tethered ligand transactivating the receptor. Thrombin cleaves between amino acid residues Arg41 and Ser42 and releases an N-terminal peptide, namely parstatin, which was recently shown to have a regulatory function in angiogenesis and in myocardial ischemia and reperfusion injury. However, the actual length of parstatin was unclear since the receptor also possesses a putative signal peptide (residues 1-21) in its amino terminus according to prediction programs. If functional, this signal peptide should be removed from the receptor following insertion into the membrane of the endoplasmic reticulum and parstatin released from plasma membrane PAR1 would be shorter. Here, we studied the cleavage of the signal peptide of PAR1 and show that it is indeed removed from the N tail resolving the question of parstatin length. Moreover, we show that deletion of the sequence encoding the signal peptide leads surprisingly to a strong decrease in the amount PAR1 mRNA. Transcription itself was not affected and the sequence thus seems to play a role in stabilization of the mRNA secondary structure, a function which was never described before for a G protein-coupled receptor.

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Novel Regulation of the Golgi Complex and Endosomes by the Cytoplasmic Phospholipase A2 Enzyme PAFAH Ib.

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Previous studies have shown that membrane tubule-mediated export from the Golgi complex and endosomal compartments requires cytoplasmic phospholipase A_2 (PLA_2) activity. Here we report that the cytoplasmic PLA_2 enzyme complex Platelet Activating Factor Acetylhydrolase (PAFAH) Ib regulates the trafficking and organization of the mammalian Golgi complex and endosomes by mediating the formation of membrane tubules. PAFAH Ib is comprised of two PLA_2 subunits, α1 and α2, and a dimer of the dynein regulator LIS1. Both α1 and α2 partially localize on Golgi and endosome membranes. Using a variety of in vivo and in vitro approaches, we conclude that the formation of PAFAH Ib-dependent membrane tubules is important for the biogenesis and maintenance of an intact Golgi ribbon, anterograde trafficking through the Golgi stack, and export of itinerant receptors from various endosomal compartments. Although α1 and α2 can form functional homo- and heterodimers, we found that the α1 and α2 catalytic subunits do not have identical functions. Knockdown of the subunits individually had different effects on Golgi structure and function. Finally, we found that α1 and α2 expression levels affect Golgi and endosome organization through interactions with LIS1. Our results demonstrate a cooperative interplay between the PLA_2 activity of α1 and α2 with LIS1 to facilitate the functional organization of the Golgi complex and endosomes, thereby suggesting a model that links continual phospholipid remodeling and membrane tubule formation to dynein-dependent transport. Work supported by NIH grant DK51596 to WJB.

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Analysis of the retrotranslocated pool of the ERAD substrate CD3delta.

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The quality control mechanism encompassing protein retrotranslocation from the ER and disposal of the faulty polypeptides in the cytosol is known as ER-associated degradation (ERAD). Inefficient degradation of the dislocated proteins by the proteasome has been linked to several disorders, like Alzheimer's disease and the prion diseases. Here, we present data on the retrotranslocated pool of a well-established ERAD substrate, the T cell receptor subunit CD3delta. By applying mono- and dual-labeling approaches, i.e. CD3delta tagged to either one or two fluorescent proteins, the retrotranslocation of CD3delta and the dynamics of its cytosolic pool could be examined by live cell imaging techniques and fluorescence correlation spectroscopy (FCS). Upon dislocation of the type 1 membrane protein, the cytosolic location and aggregation status of CD3delta were investigated. By using reversible proteasome inhibitors the final degradation of cytosolic CD3delta by the recovering proteasome system could be monitored and quantified. The results presented here demonstrate the power of fluorescent protein-based strategies as tools to examine protein turnover pathways.

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Rabankyrin-5 regulates retromer function through an interaction with EHD1.

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Rabankyrin-5 (Rank-5) has been implicated as an effector of the small GTPase Rab5 and plays an important role in macropinocytosis. We have identified Rank-5 as an interaction partner for the recycling regulatory protein EHD1. We have shown this interaction by GST-pulldown, yeast two-hybrid, isothermal calorimetry, and co-immunoprecipitation and found that the binding occurs between the EH-domain of EHD1 and the NPFED motif of Rank-5. Similar to EHD1 and EHD3, Rank-5 co-localizes and interacts with components of the retromer complex and depletion of Rank-5 causes mislocalization of the retromer and affects biosynthetic transport. The effect of Rank-5 on the retromer depends upon the interaction between Rank-5 and EHD1, as rescue experiments with Rank-5 containing mutations in its NPF motif fail to mitigate retromer mislocalization. Finally, we show that depletion of either Rank-5 or EHD1 impairs secretion of VSV-G. Overall, our data indentify a new role of Rank-5 in interacting with EHD1 to regulate retromer function.

Endocytic Trafficking

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RNAi-based Functional Profiling Identifies Novel Disease Related Proteins with a Role in Low-density Lipoprotein (LDL) Uptake and Cholesterol Trafficking.

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Elevated serum lipid levels are a major risk factor for atherosclerosis and coronary heart disease. To date, over ten proteins which function in cholesterol uptake or trafficking are known to cause lipid disorders such as hypercholesterolemia. Recently, over 120 loci associated for lipid traits and cardiovascular disease were identified in genome-wide association studies (GWAS). The majority of these loci are novel. Variants regulating expression or function of genes encoded in these loci are suspected of being causative in perturbing blood lipid levels in humans. However, most of these genes are uncharacterized or have no known functional role in cellular cholesterol regulation or trafficking. Identifying and characterizing causative genes promises to shed light both on the cell biology of cholesterol trafficking as well as on the pathogenesis of cardiovascular disease.
We have established a high-content screening platform with two functional assays for measuring low-density lipoprotein (LDL) uptake and cellular free cholesterol in tissue culture cells. The combination of automated fluorescent microscopy and image analysis allowed us to obtain multiparametric profiles of siRNA treated cells. Cellular free cholesterol or LDL uptake was affected with at least 2 siRNAs for 40 of the 109 genes encoded in associated loci. Analysis of LDL receptor (LDLR) expression upon siRNA knockdown indicates that most of our candidates exert their effect via pathways independent of LDLR expression level regulation. For 25 of the 40 candidates, our results represent the first demonstration of a functional role in cholesterol metabolism. To further characterize the novel candidates, synthetic genetic interactions were assessed by treating cells with multiple siRNAs.

Our data now allows us to generate hypotheses about how these genes may affect lipid regulatory mechanisms in cells.

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Rapid Assembly and Internalization of Caveolae Promotes Plasma Membrane Repair in Injured Cells and Muscle Fibers.

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Plasma membrane repair is essential for cellular survival after wounding. Muscle tissue is particularly susceptible to injury, and defects in maintaining sarcolemmal integrity lead to severe forms of muscular dystrophy. Mutations in caveolin-3 cause a range of muscle diseases including cardiomyopathy, and muscle fibers from some forms of muscular dystrophy show an increased number of caveolae. The mechanism underlying these observations is still obscure. In this study we show that myotubes share with other cell types a Ca2+ dependent plasma membrane repair mechanism that involves exocytosis of lysosomes, secretion of acid sphingomyelinase, and rapid endocytosis. A few minutes after wounding, injury-induced endosomes appear as large vesicles with properties of early endosomes, and then mature into late endosomes/lysosomes. However, the nature of the vesicles formed immediately after plasma membrane disruption was unknown. By examining fibroblasts and myotubes shortly after wounding, we observed massive de novo formation and internalization of caveolae. This process is required for plasma membrane resealing, and is also triggered by exposure to purified sphingomyelinase, in the absence of wounding. Thus, the accumulation of caveolae observed in muscle fibers from muscular dystrophy patients is likely to reflect an ongoing process of sarcolemmal injury and repair. These findings identify sphingomyelinase-induced caveolae as dynamic endocytic structures with a fundamental role in the restoration of plasma membrane integrity.

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Knockdown of all Rab5 isoforms in the liver leads to loss of endosomes and a mis-organization of hepatocellular polarity.

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The liver is a multi-functional organ participating in an immense number of physiological processes, e.g. regeneration, toxicology, bile formation, and lipid and glucose metabolism. All those functions are mainly carried out by hepatocytes, which exhibit a very complex and peculiar polarity with several apical and basolateral sides per cell. Specific trafficking routes and retention mechanisms are necessary to ensure proper function of these distinct membrane domains. The endosomal system plays a key role in maintaining this functional asymmetry or polarity by the formation of spatially distinct intracellular, endocytic networks, which secure the correct sorting and spatial temporal regulation of different transporters and signaling receptors in the liver. We set out at a systems level to dissect the interaction between endocytosis, metabolism and signaling in the liver. Here, we used the state-of-the-art technology of RNA interference in vivo in the liver of adult mice to specifically downregulate all three Rab5 isoforms. By knocking down Rab5, the central component of the endosomal system, we wanted to investigate the effect of alterations in the endocytic network on cellular signaling, hepatocellular polarity, and whole body metabolism. We find that knockdown of Rab5 in vivo leads to a loss of endosomes and to a mis-organization of hepatocellular polarity resulting in a cholestatic phenotype in these mice.

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Connections between endocytic membrane trafficking, GPCR signaling and cytokinesis revealed by the small molecule XZ1.

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Endocytosis and cytokinesis are distinct cellular processes that share certain sub-reactions, but this connection is poorly understood. Endocytic membrane trafficking is essential for cytokinesis and several proteins have been implicated in both processes, including the ESCRT complex, which was discovered in the context of endosomal sorting and is required for abscission, the final step in cytokinesis. We report here new insights into connections between endocytosis and cytokinesis revealed by the small molecule XZ1 and we show that these connections are mediated by G-protein coupled receptor (GPCR) signaling. XZ-1 inhibits cytokinesis, causes tubulation of endocytic compartments and aggregates the ESCRTIII complex. RNAi depletion of GPCR signaling components or the ESCRTIII adaptor protein Vsp4 inhibit XZ1's effects on both cytokinesis and endocytosis, as does inhibition of the vacuolar ATPase, suggesting a common interaction during endocytic sorting. XZ-1 thus reveals, and will help to further elucidate, a complex interaction between cell surface receptors and cytokinesis, mediated by endocytosis.

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Endocytic Recycling of the Transferrin Receptor is Regulated by c-Abl Kinase.

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Clathrin-mediated endocytosis (CME) of transferrin (Tf) and its cognate receptor (TfR) is a central pathway supporting the cellular internalization of trophic iron. It has generally been assumed that this uptake is a constitutive process that is independent of kinase regulation. Recently we tested if pharmacological inhibition of different non-receptor tyrosine kinases might attenuate TfR endocytosis and found that Tf ligand activates the Src-mediated phosphorylation of a dynamin-cortactin complex to facilitate the internalization of the ligand-receptor. In addition to this finding, a significant endocytic block was observed by treating cells with “Gleevec”, a well-characterized inhibitor of the c-Abl family of tyrosine kinases. Therefore, the **Goal of this study** was to define the role of this kinase family in TfR internalization and traffic. To confirm these initial observations, we subsequently utilized cultured cells transfected to express either WT or kinase dead (K290R and Y412F) c-Abl or c-Abl-/-, Arg-/- cells isolated from KO mice models. Importantly, ligand and TfR internalization were reduced significantly (60-70%) in all of these cell models while re-expression of WT c-Abl restored normal uptake levels in c-Abl-/-, Arg-/- cells. Surprisingly, this attenuated Tf/TfR endocytosis was due largely to a substantial drop (~80%) in the cellular and surface receptor levels, suggesting that the recycling of internalized receptor is compromised. Indeed, IF staining of cells in which c-Abl was inhibited revealed a remarkable colocalization of internalized TfR with the lysosome indicating a mis-sorting step leading to receptor degradation. In support of this finding, TfR levels were increased substantially by treating the kinase inhibited cells with the lysosomal protease inhibitor Leupeptin. These findings are the first to implicate a tyrosine kinase (c-Abl) that insures the normal recycling of the TfR away from the lysosome and back to the cell surface.

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Cellular Trafficking of Helical Rosette Nanotubes in Dendritic Cells.

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Biologically inspired, self-assembling helical rosette nanotubes (HRNs), composed of cytosine and guanine, have multiple desired therapeutic applications including drug delivery and vascular stents. HRN interactions with dendritic cells (DCs), sentinels of the immune system thus potentially the first cell type to encounter HRN upon exposure, are key in determining safety and application viability. Splenic classical and plasmacytoid DCs were isolated from male C57/BL6 mice using magnetic cell sorting. HRN functionalized with the RGD (arginine, glycine, aspartic acid) peptide and coupled with the fluorophore FITC in a ratio of 1:10μM of RGDSK/FITC HRN were delivered to the isolated DCs for 15 minutes at a further 1:10μM dilution in media. Confocal microscopy was used to determine which endocytic pathway (receptor-mediated, clathrin or caveolin-dependent) was utilized and whether the process was energy-dependent. 3-D imaging to characterize HRN intracellular localization and live imaging to view the uptake process in real-time also were completed. HRNs were able to utilize multiple uptake pathways to take residence within clathrin and caveolin-coated vesicles, early endosomes, and lysosomes. In addition, the RGD peptide on the HRN was recognized by αVβ3, an integrin expressed in various cell types including some DC subsets and tumor cells, demonstrating receptor-mediated endocytosis. HRNs co-localized with the major histocompatibility complexes (MHC) I and II indicating possible direct interaction leading to antigen presentation. DC maturation occurred upon HRN exposure as illustrated by the formation of dendrites and presence of maturation markers (such as CD40 and CD83). Cell viability was confirmed through a commercially available kit and visually through microscopy and DAPI staining. These data demonstrate that HRN engage integrin αVβ3 to enter DCs through multiple, presumably redundant, pathways, interact with antigen-presenting molecules and induce maturation of DCs.

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Protein Kinase C Delta Regulates Endosomal Trafficking and Volume Recovery in Response to Osmotic Swelling.

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The metabolic activities of the liver generate intracellular osmotic loads that evoke rounds of swelling and volume recovery with corresponding membrane expansion and compensatory retrieval. How the responsible trafficking events are coordinated is uncertain. We sought to determine the cytoskeletal/membrane regulators in a model of hypotonic challenge in HTC hepatoma cells. In live imaging studies, labeled membrane, internalized after swelling, localized to actin-coated endosomes that were also positive for the trafficking regulator Arf6. In addition, pharmacological inhibition of an Arf6 effector, phospholipase D (PLD), attenuated volume recovery. Because the protein kinase C isoform PKCδ can regulate PLD as part of a complex with Arf6 and PLD, we investigated its actions during osmotic stress. On cell swelling, PKCδ transiently increased in membrane fractions, and rottlerin, a PKCδ inhibitor, blocked volume recovery. Swelling also evoked transient association of GFP-PKCδ with endosomes positive for the membrane marker RFP-farnesyl, or with endosomes positive for RFP-actin. Such endosomes could undergo actin-associated morphologic change after PKCδ association. Since PKCδ can regulate actin polymerization on endosomal membranes through an association with cortactin, we tested PKCδ immunoprecipitates and found cortactin present. PKCδ can be activated directly by Src, which we have shown to be a central cell volume regulator. We have also shown that Src activates the phospholipase C isoform PLCγ, which can activate PKCδ

through lipid hydrolysis. We therefore tested whether Src regulates volume-sensitive membrane trafficking by direct interaction with PKCδ. In immunoprecipitates, PKCδ did not associate with Src but did with focal adhesion kinase (FAK). In addition, immunofluorescence studies showed colocalization of GFP-PKCδ in endosomes with the tyrosine phosphorylated forms of FAK and PLCγ. In live imaging studies, GFP-PKCδ transiently colocalized with an RFP fusion that included the pleckstrin-binding domain of PLCγ. This colocalization was attenuated by the Src inhibitor PP2. By contrast, the PKCδ inhibitor rottlerin did not affect endosomal targeting of PLCγ but suppressed endosomal association of PKCδ and endosomal motility. These findings suggest that Src contributes to hepatocyte volume regulation in part via PLCγ-mediated activation of PKCδ and by regulated motility of actin-coated endosomes. Since PKCδ can modulate actin polymerization through interactions with FAK and with cortactin, we speculate that such interactions coordinate actin and membrane dynamics required for endosomal trafficking during osmotic stress.

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Src-Mediated Phosphorylation of CIN85 Regulates Trafficking of the EGF Receptor at the Level of Early Endosomes.

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Ubiquitination of the epidermal growth factor receptor (EGFR) by cbl and its cognate adaptor CIN85 (cbl interacting protein of 85 kDa) is known to play an essential role in directing this receptor to the lysosome for degradation. Currently, it is unclear where in the endocytic pathway this ubiquitin modification occurs or how it is regulated in response to ligand. In this study we show that EGFR activation leads to a pronounced Src-mediated tyrosine phosphorylation of CIN85. This CIN85 modification is essential for EGFR ubiquitination as impairment of CIN85 phosphorylation by the expression of a phospho-defective mutant (CIN85-4F) inhibits its ability to interact with-cbl and causes a significant reduction in receptor ubiquitination. Importantly, phospho-CIN85 interacts with the Rab5-positive endosome where it mediates the sequestration of the ubiquitinated receptor into intra-luminal vesicles (ILVs) for subsequent degradation. Expression of the phospho-defective CIN85-4F mutant interferes with this process leading to retention of the EGFR in the early endosome and a substantial delay in EGFR degradation. These findings provide novel insights into how CIN85 regulates EGFR traffic at the early endosome in conjunction with the Ub-ligase cbl and how this complex might affect cellular growth. This study was supported by DK44650 and CA104125 to MAM.

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A Novel Mechanism Regulates EGF Receptor Trafficking.

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The ubiquitin ligase c-Cbl controls epidermal growth factor receptor (EGF-R) signaling by enhancing receptor ubiquitination, downregulation, and lysosomal degradation. Our laboratory previously reported that Cbl does this by controlling EGFR's passage through an intracellular trafficking checkpoint at the limiting membrane of early endosomes. Here, we report further studies of the molecular mechanism by which Cbl controls EGFR fate at endosome limiting membranes. We tested three hypotheses: 1) that ubiquitin in complex with EGFR is sufficient to move receptors from the limiting membranes of endosomes to their luminal vesicles, in the absence of Cbl's ubiquitin ligase activity; 2) that forced retention of ligand-activated EGFR at the limiting membrane of early endosomes prevents their degradation, regardless of whether the receptors are associated with ubiquitin; and 3) that the evolutionarily non-conserved Cbl

sequences C-terminal to amino acid 436 have no impact on EGFR fate at the endosomal trafficking checkpoint. Our studies employed HEK 293 and COS-7 cell systems, EGFR and Cbl expression constructs, ubiquitin fusion proteins, and GFP-tagged wild type and mutant Cbl proteins in assays of receptor downregulation, recycling, marker colocalization, trafficking, and degradation. Based on our data, we reject all three hypotheses. 1) Ubiquitin in complex with EGFR is not sufficient to move receptors from the limiting membranes of endosomes onto their luminal vesicles. This finding contradicts the outcome that was predicted on the basis of yeast trafficking investigations. 2) Forced retention of ligand-activated EGFR at the limiting membrane of early endosomes fails to prevent its degradation, in the absence of additional inhibitory signals. This is consistent with a report that EGFR delivery to the MVB lumen is not essential for receptor degradation. 3) Non-conserved residues in the Cbl C-terminus block EGFR degradation at the endosomal trafficking checkpoint, unless their activity is countered by a conserved functional domain within Cbl 1-436. Through our studies, we have identified a new regulatory mechanism controlling EGFR trafficking and fate.

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The Saccharomyces cerevisiae Na+ ATPase exporter ENa1 is a yeast epsin-specific endocytic cargo.

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The Epsins are an essential family of endocytic adaptors conserved across evolution. Their endocytic functions have been proposed to be redundant with the ones from other adaptor proteins such as the Ap180s (Maldonado-Baez, Dores). Therefore, to date no physiologically relevant cargo has been identified to depend exclusively on the yeast epsins for internalization. In contrast, mammalian epsin has been reported to be important for the endocytosis of the epithelial sodium channel (ENaC) and epidermal growth factor receptor (EGFR). Although there are no obvious EGFR-homologs in yeast, several ion transporters have been identified, including sodium pumps. Here we report that in budding yeast, internalization of the Na+ ATPase exporter ENa1 (Exitus Natru 1) depends specifically on epsins. Specifically, we determined that while in wild type cells a major fraction of ENa1 is localized in internal structures, in cells lacking both yeast epsins, the transporter predominantly accumulated at the plasma membrane. In contrast, in cells lacking both Ap180s, localization of ENa1 was indistinguishable from wild type. Importantly, the ENa1 localization defect observed in epsin knock-outs could be rescued by re-introduction and expression of either of the two yeast epsin paralogs, Ent1 or Ent2. Further, we established that the C-terminal epsin endocytic determinants are necessary for ENa1 internalization. We anticipate that this finding will provide the basis for more detailed studies of the role of the epsin protein family in endocytosis.

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Common Membrane Trafficking Defects of Disease Associated Dynamin 2 Mutations.

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Dynamin (Dyn) is a multidomain and multifunctional GTPase best known for its essential role in clathrin-mediated endocytosis (CME). Dyn2 mutations have been linked to two human diseases, Centronuclear Myopathy (CNM) and Charcot-Marie-Tooth (CMT) disease. Paradoxically, although Dyn2 is ubiquitously expressed and essential for embryonic development, the disease-associated Dyn2 mutants are autosomal dominant, but result in slowly progressing and tissue-specific diseases. Thus, although the cellular defects that cause disease remain unclear, they are expected to be mild. To gain new insight into potential pathogenic mechanisms we utilized mouse Dyn2 conditional knock-out cells combined with retroviral-mediated reconstitution to

mimic both heterozygous and homozygous states and characterized cellular phenotypes using quantitative assays for several membrane trafficking events. Surprisingly, none of the four mutants studied exhibited a defect in CME, but all were impaired in their ability to support p75/neurotrophin receptor export from the Golgi, the raft-dependent endocytosis of cholera toxin, and clathrin-independent endocytosis of EGFR. While it will be important to study these mutants in disease-relevant muscle and neuronal cells, given the importance of neurotrophic factors and lipid rafts in muscle physiology, we speculate that these common cellular defects might contribute to the tissue-specific diseases caused by a ubiquitously expressed protein.

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Late stages of antibody transcytosis in the mammalian neonatal small intestine.

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Humoral immunity in neonatal mammals is conferred from mother to young by transport of maternal antibodies in milk across the intestinal epithelium to the bloodstream. This transcytosis is directed by the neonatal Fc receptor (FcRn) in specific cells of the proximal small intestine (duodenum and jejunum). In earlier work (He et al (2008)) we defined structural features of early events in the FcRn transcytotic pathway using electron tomography to follow enhanced, nanogold-labeled Fc in the jejunum of neonatal rats. In conjunction with immuno-EM, this approach allowed us to resolve and identify an intricate series of interconnecting vesicles and tubules through which the Fc-FcRn complex travels from the apical (luminal) to the basolateral (bloodstream) side of the epithelium. We are now using these methods and large-area electron tomography to characterize later stages of FcRn transport and observe where and how Fc exits the basolateral epithelium in route to the bloodstream. Neonatal rats were fed nanogold-tagged Fc. Following short (~10-15min) chase times, samples of jejunum were excised and rapidly cryo-immobilized by high-pressure freezing. Nanogold was enhanced at low temperature during subsequent freeze-substitution (He et al (2007)). Tomography of the embedded tissue detected enhanced Fc-gold in membranous compartments distal from the apical surface. These include multivesicular bodies and tubulovesicular elements analogous to those reported in the earlier study. Gold was detected in lateral intercellular spaces (LIS), but pools of gold were often concentrated in dilated regions of the LIS, typically at the junction of three epithelial cells. As the basolateral LIS is open to the highly vascular lamina propria, these dilated regions may contain Fc exit domains and the pooling effect may be due to eddys in the bulk flow of liquid through the channel.

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The Insulin Receptor Translocates to the Nucleus to Regulate Cell Proliferation and Liver Regeneration.

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Insulin regulates such varied processes in the liver as glucose metabolism and cell proliferation. Insulin's biological actions are mediated by the Insulin Receptor (IR), a Receptor Tyrosine Kinase. The IR translocates to the hepatocyte nucleus upon stimulation with insulin to initiate inositol 1,4,5-trisphosphate (InsP3)-mediated calcium (Ca2+) signals. The aims of the present

study were to determine the mechanism by which the receptor reaches the nucleus and to understand the physiological role of these InsP3-dependent nuclear Ca2+ signals. Western Blot of non-nuclear and nuclear fractions and confocal immunofluorescence were used to detect the translocation of the receptor to the nucleus of SkHep-1 cells. Cell surface biotinylation and streptavidin pull-down were performed to determine whether the receptors that reach the nucleus originated at the plasma membrane. siRNA knockdown studies were performed to establish the endocytic mechanism of translocation. Nuclear and cytosolic Ca2+ signals were monitored by time-lapse confocal microscopy under control and nuclear InsP3 buffering conditions to assess the importance of nuclear InsP3 in insulin-induced Ca2+ signals. BrdU and partial hepatectomy studies were carried out in rats under control and nuclear InsP3 buffering conditions to analyze the importance of nuclear InsP3 in insulin-induced cell proliferation and liver regeneration. Biotinylated IR was found in the nuclear fractions after insulin stimulation, supporting the conclusion that the receptor translocates from the plasma membrane to the nucleus of SkHep-1 cells. Clathrin heavy chain and caveolin-1 siRNA knockdown each resulted in a decrease in nuclear IR after insulin stimulation, and simultaneous knockdown of both had an additive effect, suggesting that either of these endocytic pathways can participate in translocation of the receptor to the nucleus. Nuclear InsP3 buffering lead to the inhibition of insulin-induced Ca2+ signals throughout the cell. Furthermore, nuclear InsP3 buffering resulted in a decrease in BrdU uptake as well as in liver weight after partial hepatectomy, providing evidence that insulin-induced Ca2+signals depend on nuclear InsP3 and that the Ca2+ signals generated in the nucleus regulate insulin-induced cell proliferation and liver regeneration. Together, these observations demonstrate that insulin induces a subpopulation of IR to move from the plasma membrane to the nucleus in order to mediate insulin’s effects on liver growth and regeneration, and that InsP3 and Ca2+ signaling pathways within the nucleus are responsible for these particular actions of insulin.

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Cross-talk between Clathrin-dependent and Clathrin-independent Endocytic Entry and Trafficking Pathways.

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Endocytosis can be divided into two types based on the involvement or not of the clathrin coat. Clathrin-dependent endocytosis (CDE) is reliant on clathrin, adaptor proteins such as AP2 and dynamin whereas clathrin-independent endocytosis (CIE) occurs independently of clathrin, AP2 and dynamin. Much is known about CDE, the molecular machinery and the cargo carried, but increased attention is focusing on CIE, identifying the cargo carried and their intracellular itinerary. We have been studying CIE in human cells and have identified an endosomal sorting system that takes place in HeLa cells that sorts CIE cargo after endocytosis. CIE cargo proteins such as the major histocompatibility complex Class I protein (MHCI) are sorted into endosomes associated with EEA1 and containing transferrin receptor and then are trafficked to late endosomes for degradation or recycled back to the PM via tubular carriers. In contrast, other CIE cargo proteins, such as CD44, CD98 and CD147, avoid trafficking to EEA1-associated endosomes and move directly into recycling tubules. There is some evidence from work in *C. elegans* that these parallel endosomal membrane systems (loaded by CDE and CIE) might communicate and coordinate to maintain plasma membrane homeostasis. Therefore, we set out to examine the effects of blocking CDE on CIE and the subsequent trafficking of CIE cargo proteins. We blocked CDE by expression of the dominant negative form of the adaptor protein AP180 or by si-RNA-mediated depletion of clathrin heavy chain or the mu subunit of AP2. Any of these treatments blocked CDE as assessed by transferrin internalization without affecting internalization of CIE cargo proteins. However, subsequent trafficking of CIE cargo was affected

in cells where CDE was impaired. Most notably the recycling tubules were absent and CD44, CD98, and CD147 now trafficked to EEA1-associated endosomes along with MHCI. They also exhibited increased trafficking to LAMP1-positive compartments and reduced surface expression in cells where CDE was blocked. Similar results of altered trafficking of CIE cargo were observed in the lung epithelial BEAS2B cells. We did not observe any direct colocalization of clathrin or AP2 with CIE endosomal system including the tubules. These observations imply that input from CDE membrane systems affects sorting and itinerary of CIE cargo proteins, supporting the notion of a complex coordination of endosomal trafficking pathways.

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PKC-Driven Feedback Control of Rapid mu-Opioid Receptor Recycling.

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Post-endocytic sorting and recycling of G-protein coupled signaling receptors (GPCRs) are critical the re-sensitization of cells to signals. However, little is known about the mechanisms and signals that regulate GPCR recycling.

We have monitored the regulation of individual receptor recycling events mediating the surface insertion of the mu opioid receptor (MOR), a recycling receptor critical for the action of opioid neuropeptides and abused drugs, using Total Internal Reflection Fluorescence (TIRF) Microscopy and multi-color live cell confocal imaging. We determined that Phospholipase C signaling through Protein Kinase C (PKC) regulates the sorting and recycling of MOR. This effect was specific, as PKC signaling did not alter the recycling of other signaling receptors. Further, we have identified the receptor itself as a target for this regulation. Together, our results implicate a PKC-driven positive feedback loop in controlling opioid recycling, and suggest that biochemically diverse mechanisms regulate the post-endocytic sorting of GPCRs in the endosome.

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NEEP21 and EEA1 early endosome regulators work together to ensure proper trafficking of L1/NgCAM and L1-mediated axon outgrowth.

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Neuronal endosomal system is essential for membrane receptor trafficking to and within dendrites and axons, by which it participates in various neuronal functions, such as synaptic plasticity and axon outgrowth. The neuronal endosomal system differs from the canonical endosomal system in other cell types due to unique neuronal function and morphology. Neuronal specializations of the endosomal system include adaptations of cannonical endosomal regulators to neuronal-specific functions as well as introduction of neuronal-specific endosomal regulators. EEA1 (early endosome antigen 1) is an example of a cannonical endosomal regulator, localized to cytoplasmic side of early endosomes, important for homotypic early endosomal fusion in non-neuronal cells. In neurons, EEA1 is localized to the somatodendritic domain where it colocalizes with endocytosed AMPA receptors and endocytosed L1/NgCAM adhesion molecules. NEEP21 (neuron-enriched endosomal protein of 21 kD), an example of a transmembrane neuronal-specific early endosomal regulator localized to Golgi and endosomal compartments in somatodendritic domain, is important for trafficking of L1/NgCAM, AMPAR, and APP. What is the relationship between canonical EEA1-positive endosomes and neuronal-specific NEEP21-positive endosomes, and how they work together to traffic cargo, is not known. To address those questions we carefuly analyzed the colocalization and dynamics of EEA1- and NEEP21-positive compartments as well as dissected the role of those two regulators in trafficking of L1/NgCAM axonal adhesion molecule. We chose L1/NgCAM for our study based

on our previous observations that L1/NgCAM endocytosed in somatodendritic domain, on its way to the axon, colocalizes with both EEA1 and NEEP21 and that NEEP21 function is essential for proper trafficking of L1/NgCAM to the axon. Since L1/NgCAM is known to promote axon outgrowth, a crucial process during development of the nervous system, we used here axon outgrowth as our readout for the role of EEA1 and NEEP21 in L1/NgCAM trafficking. We showed that EEA1 and NEEP21 are localized to two, largerly distinct, populations of early endosomal compartments. EEA1 and NEEP21 labeled compartments have different dynamics, with EEA1-positive endosomes being mostly stationary and NEEP21-positive compartments consisting of both stationary and motile populations. However, we also observed a pool of endosomes containing both EEA1 and NEEP as well as pool of compartments where EEA1 and NEEP21 were positioned adjacent to each other. Live imaging further demonstrated interactions between EEA1-positive and NEEP-positive compartments such as budding/fusion (in case of NEEP21) and recruitment/dissociation (in case of EEA1). Our experiments also show that endocytosed L1/NgCAM colocalizes with both EEA1 and NEEP21 stationary compartments and that both EEA1- and NEEP21-positive endosomes are involved in trafficking of L1/NgCAM from somatodendritic to axonal domain. Expression of dominant negative EEA1 construct, which is known to interfere with EEA1-based homotypic fusion, led to accumulation of L1/NgCAM in somatodendritic endosomes and its misslocalization to somatodendritic membrane. Finally, both, interference with EEA1 and downregulation of NEEP21 led to inhibition of L1-mediated neurite outgrowth, which further demonstrated how the proper trafficking of L1/NgCAM through EEA1- positive and NEEP21-positive somatodendritic endosomal compartments is important for axon outgrowth. In conclusion, these findings point to the existence of neuronal adaptations of early endosomal compartments and the importance of somatodendritic endosomal trafficking of L1/NgCAM in L1-mediated axon outgrowth.

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Effect of modulating PIP2 on cargo endocytosis and clathrin adaptor recruitment.

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Localized synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2) at clathrin coated pits is crucial for the recruitment of adaptors and other components of the internalization machinery, as well as in regulating actin dynamics during endocytosis. Plasma membrane PIP_2 in nonpolarized cells is generated by 5' phosphorylation of phosphatidylinositol 4-phosphate mediated by any of three phosphatidylinositol 5-kinase type I (PIP5KI) isoforms (α, β, or γ). We found that PIP5KIβ over-expression increased apical endocytosis of the polymeric immunoglobulin receptor (pIgR), but had no effect on basolateral endocytosis. In contrast, overexpression of PIP5KIα and γ had no effect on endocytosis of pIgR from either cell surface domain. Overexpression of each isoform significantly increased PIP_2 levels in non-polarized cells, however only PIP5KIβ significantly increased PIP_2 levels in polarized cells. Increasing PIP_2 by overexpression of PIP5KIβ also decreased current of the apical sodium epithelial channel (ENaC) in polarized cortical collecting duct cells, consistent with an increase in channel endocytosis. In contract, PIP5KIβ increased surface expression of another channel, ROMK (Weixel et al., 2007, JBC, 282, 36534–36542). We hypothesized that differential recruitment of ENaC and ROMK clathrin adaptors to the apical surface in response to elevated PIP_2 could account for this difference in channel response. To test this we performed liposome sedimentation assays with purified recombinant PIP_2 binding domains of epsin and ARH, the ENTH and PTB domains, respectively. Preliminary data indicate that the PTB domain bound to PIP_2 containing liposomes with higher affinity than the ENTH domain, suggesting that increases in PIP_2 may selectively enhance ENTH domain recruitment to membranes.

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Rab7 mutants involved in Charcot-Marie-Tooth disease exhibit delayed growth factor receptor transport and altered endosomal signaling.

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Rab7 belongs to the family of small GTPases and is a master regulator of early to late endocytic membrane transport. Four missense mutations in the late endosomal Rab7 GTPase i.e.L129F, K157N, N161T and V162M cause the autosomal dominant peripheral neuropathy Charcot-Marie- Tooth type 2B (CMT2B) disease. As yet, the pathological mechanisms connecting mutant Rab7 protein expression to altered neuronal function are undefined. Here, we analyze the effects of Rab7 CMT2B mutants on epidermal growth factor (EGF) dependent intracellular signaling and trafficking. HeLa cells stably expressing Rab7 CMT2B mutants stimulated with Alexa 555 labeled EGF exhibited delayed association with LAMP1-positive late endosomes and lysosomes and slowed EGF receptor degradation following EGF stimulation. Expression of all Rab7 CMT2B mutants enhanced and prolonged EGFR activation as well as enhancing Erk1/2 activation. Rab7 CMT2B mutant proteins colocalized with the late endosomal/lysosomal marker LAMP1 and exhibited a tight, perinuclear clustering of the endosomes. Interestingly, the nuclear activation of Elk-1, a downstream target of Erk1/2 was decreased by the disease mutants similar to what is reported for cells overexpressing the constitutively active Rab7Q67L mutant. Our results suggest that in the disease state, Rab7 CMT2B mutants impair growth factor receptor trafficking and in turn down-regulate nuclear signaling. In addition, intracellular positioning of signaling endosomes plays a key role in determining the altered endosomal signaling. In conclusion, the data demonstrate a mechanistic link between impaired trafficking by Rab7 CMT2B mutants and altered Erk1/2 signaling from perinuclear, clustered signaling endosomes as a causal effect of disease pathogenesis.

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The Exocyst Component Exo84 and EHD3 Cooperate in Endosome Recycling.

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The exocyst is an octameric protein complex mediating the tethering of secretory vesicles to the plasma membrane before fusion. We report that the exocyst subunit, Exo84, interacts with the dynamin-like EHD proteins both in vitro and in cells. Exo84 contains a specific motif with amino acids NPFEDE that is responsible for binding to a hydrophobic pocket within the EH domain of EHD3. In addition, Exo84 and EHD3 co-localize on Rab8-containing endosome membrane tubules, and EHD3 is needed for the recruitment of Exo84 to these tubular structures. Exo84 is known to be a direct downstream effector of the GTPase RalA. Our data show that when RalA is pre-bound to Exo84, Exo84 can no longer bind to EHD3 indicating that RalA and EHD3 compete for Exo84 binding. The interactions and activities of RalA, Exo84, and EHD3 are important for endosome localization and trafficking. We propose a sequence of activation and recruitment of Exo84 to the highly-curved tubular domains of endosomes where fission of transport carriers is coordinated with the targeting and tethering functions of Exo84.

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A Model System to Study Turnover of Plasma Membrane Proteins.

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The turnover of plasma membrane (PM) proteins is an essential and regulated function of cells. Cytoplasmic sorting signals, such as ubiquitination, facilitate the down-regulation of cell surface receptors resulting in their delivery to late endosomal compartments for degradation. Typically, measuring turnover of cell surface proteins entails non-specific labeling of the entire cell surface with biotin, followed by immunoprecipitation and detection with protein-specific antibodies. Here, we take advantage of the SNAP-tag technology that allows labeling of specific fusion proteins on the cell surface. The SNAP-tag is a variant of a 20 kDa DNA repair protein (O6-alkylguanine-DNA alkyltransferase), which can be covalently labeled with fluorescently tagged benzylguanine derivatives (BG). The interleukin 2 receptor alpha subunit/CD25 (also known as Tac) is a type I membrane protein that has been used to identify cytoplasmic sorting sequences in a variety of membrane trafficking studies. When expressed in cells CD25 enters by clathrin-independent endocytosis (CIE), but uses clathrin-dependent endocytosis (CDE) when the C-terminus is modified with dileucine/isoleucine residues. Chimeras of the SNAP-tag with CD25 were expressed in HeLa cells, and an initial PM pool of BG-labeled SNAP-CD25 was followed by immunofluorescence microscopy (with BG-Alexa Fluor 488), and by gel electrophoresis (with BG-IR800) to monitor its degradation in pulse-chase studies. By immunofluorescence microscopy SNAP-CD25 entered cells by CIE, whereas SNAP-CD25-LI entered cells by CDE. SNAP-CD25 exhibited a half-life of 5 h, whereas SNAP-CD25-LI had a half-life of 2.5 h, both of which were extended in the presence of lysosomal inhibitors. Turnover of PM proteins is regulated in part by ubiquitination, typically on cytoplasmic lysine residues. CD25 contains one lysine residue in its cytoplasmic tail, which we mutated to arginine (K to R). SNAP-CD25R entered cells through CIE but its half-life was greater than 8 h. Interestingly, the turnover of SNAP-CD25-GPI, a chimera anchored to the membrane through a GPI anchor and thus lacking a cytoplasmic domain, was also greater than 8 h. These results support a role for ubiquitination in the turnover of CD25. We have extended these assays to examine the turnover of a number of proteins that enter cells by CIE and found that coexpression of known interacting partners of selected cell surface proteins can greatly influence their rates of degradation.

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Shank2 traffics with albumin-laden endosomes in human podocytes.

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Podocytes play a key role in forming the glomerular filtration barrier and restricting the unimpeded flux of serum proteins into the nephron. Podocyte clearance of albumin from the glomerular basement membrane is also thought to play an essential role in maintaining the glomerular filtration characteristics but the capacity and mechanisms for albumin clearance by podocytes is undetermined. The present study shows that over 30 min, cultured human podocytes take up significant amounts of albumin in a temperature sensitive manner. Confocal microscopy shows that FITC-albumin co-localize with megalin and the neonatal Fc receptor (FcRn), two receptors implicated in albumin uptake and translocation in renal proximal tubules cells. FITC-albumin also co-localizes with markers for early endosomes (EEA1) and lysosomes (LAMP1). Shank2, a PDZ protein implicated in coordinating epithelial cell endocytosis also co-localizes with FITC-albumin containing endosomes. Image analysis at different focal planes from the basal membrane to the top of the cell after 0, 30, 60 and 120 min following a 10 min FITC-albumin pulse revealed distinct spatiotemporal patterns of Shank2-associated FITC-albumin endosomes with FcRn. These observations indicate that podocytes have a definitive

pathway for albumin endocytosis and that Shank2 is positioned to direct the trafficking of albumin-laden endosomes.

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Trafficking of GluA2 flip receptors and stargazin upon application of glutamate.

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AMPA receptors are post-synaptic, ionotropic glutamate receptors that play a key role in fast excitatory neurotransmission. Individual receptors consist of 4 subunits (GluA1-4) that are one of two splice isoforms: flip or flop. The presence and composition of AMPA receptors at synapses is dynamic, and contributes to synaptic plasticity, long-term potentiation and long-term depression. Altering the normal synaptic composition of AMPA receptors can lead to pathological conditions. For example, decreasing the GluA2 component of AMPA receptors may lead to increased calcium flow into cells, excitotoxicity and subsequent cell death. Abnormal targeting of AMPARs to the cell membrane impacts neuronal signaling and can be caused by defects in transmembrane AMPA receptor regulatory proteins (TARPs) such as stargazin, that normally target the receptors to the membrane. Exposure of AMPA expressing cells to glutamate or other agonists also affects the distribution of AMPARs and provides a way to study receptor and TARP trafficking in response to external stimuli. Previous biochemical analyses suggested that exposure to agonist differentially affects the distribution of AMPA receptors and stargazin, and that glutamate receptors and stargazin separate upon application of agonist (Tomita et al., 2004). To investigate the trafficking of AMPARs and stargazin more closely, we used confocal microscopy of HEK cells expressing yellow fluorescent protein-tagged GluA2 flip wild-type receptors (GluA2i-wt-yfp) and cyan fluorescent protein-tagged stargazin (stg-cfp) and observed the changes in protein distribution following a brief exposure to glutamate mimicking synaptic events. In HEK cells transiently expressing stg-cfp and GluA2i-yfp, stargazin was primarily associated with the membrane, whereas the glutamate receptor was found both cytoplasmically and associated with the membrane. In approximately 50% of the cells examined, the pattern of fluorescence for both proteins remained stable following application of glutamate, resembling the fluorescence pattern of control cells over time. In the remaining cells, the patterns of stg-cfp and GluA2i-wt-yfp changed following exposure to glutamate; membrane-associated GluA2 and stargazin expression was minimal at 10 seconds and 1 minute. GluA2-yfp at the membrane peaked at 5-7 minutes and stargazin-cfp peaked 2-6 minutes following exposure to glutamate. Within the intracellular compartment, the fluorescence intensities of the proteins changed in the presence of glutamate, presumably reflecting the movement of proteins into and out of intracellular compartments. These results suggest dynamic regulation of AMPARs and stargazin trafficking in HEK cells and studies are underway to confirm these findings in neurons.

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GIV Spatially Coordinates Gαs and Gαi Signaling to Downregulate EGFR.

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The heterotrimeric G-proteins Gαs and Gαi regulate the endocytic trafficking of activated EGFR and thereby control the strength, compartmentalization, and duration of signaling. Gαi3 forms a complex with activated EGFR at the PM via GIV, a GEF that activates Gαi3 and regulates receptor dynamics at the PM and endosomes. Gαs also binds GIV, facilitates EGFR degradation, and localizes to endosomes. Here we investigated whether Gαi3 and Gαs differentially regulate EGFR dynamics at the PM and endosomes via interaction with GIV. Depletion of Gαs delays endosome maturation with prolonged retention of EGFR at EEA1 early

endosomes, enhanced membrane association of EEA1, and delayed EGFR degradation. The prolonged stay of EGFR in endosomes results in increased endosome-based signaling including EGFR autophosphorylation, recruitment of SH2-adaptors, and phosphorylation of downstream kinases. The effects of Gαs depletion are reversed by an inactive but not an active Gαs mutant and Gαs-GDP directly binds GIV which localizes to endosomes. Depletion of GIV (like depletion of Gαs) increases the membrane association of EEA1 reflecting a predominant role for the interaction between GDP-bound Gαs and GIV in enhancement of trafficking of EGFR through EEA1 early endosomes. In keeping with previous findings, depleting Gαi3 increases early EGFR signaling at the PM and does not affect signaling from endosomes or the membrane association of EEA1, reflecting Gαi3's predominant role in early EGFR dynamics at the PM. These data support a model in which GIV regulates early, PM-based EGFR dynamics via interaction with Gαi3 and later, endosome-based dynamics via interaction with Gαs.

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Cortical actin modulates trafficking of the KV2.1 channel to the cell surface.

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The voltage gated potassium channel Kv2.1 forms stable 0.5 – 0.15 μm sized clusters on the cell surface of mammalian cells with a density of 15 – 70 molecules/μm^2. Here we are studying the role of the actin cytoskeleton in cluster maintenance and trafficking. Quantum dot (QD) labeled channels allow us to track individual channels with nanometer accuracy. By simultaneously tagging the channel with GFP describes the characteristics of the channels as an ensemble. We have observed both endocytic and exocytic events with fluorescently labeled Kv2.1 channels. We are able to quantify these events by localizing surface channels manually and automatically using a multi-target tracing algorithm. We see that 96% of the insertion sites of recycled QD labeled channels are localized to the edge of Kv2.1 clusters (n = 127). The few channels that do not arrive directly to a cluster diffuse into a cluster where they become trapped within 10 seconds. Actin depolymerizing agents such as swinholide A and cytochalasin D are seen able to have a direct effect on channel localization and targeting. After the application of 5 μM cytochalasin D channels are no longer targeted to clusters (n = 18). Using 80 μM swinholide A, intact clusters dissociate and therefore newly inserted channels arrive outside of clusters. We further investigate channel trafficking by inhibiting clathrin-dependent endocytic events with 80 μM dynasore. We observe that the rate of recycling events is dramatically reduced with the application of dynasore. Analysis of channel lifetime, i.e. the amount of time a channel spends on the cell membrane shows that recycled channels spend significantly less time on the cell surface than newly synthesized channels, both with and without actin depolymerizers and endocytosis inhibitors. After dynasore is applied the time a channel spends on the membrane is increased by 60% (n = 60) showing that trafficking is mediated by clathrin dependent pathways. In cells with no actin or endocytic inhibitors we see the lifetime of a recycled channel is reduced by 27% as compared to newly synthesized channels. Recycled channels in cells treated with cytochalasin D and swinholide A have lifetimes reduced by 28% and 46%, respectively. These results reveal interesting details about Kv2.1 channel trafficking and targeting as well as the maintenance of clusters.

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Both class 1A PI3K Subunits, p85 and p110, Regulate Rab-mediated PDGFR Signaling and Trafficking.

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Activated receptor tyrosine kinases recruit many signaling proteins to activate downstream cell proliferation and survival pathways, including phosphatidylinositol 3-kinase (PI3K) consisting of a p85 regulatory protein and a p110 catalytic protein. We have previously shown the p85α protein has GTPase activating protein (GAP) activity towards Rab5 and Rab4, small GTPases that regulate vesicle trafficking events for activated receptors. Expression of a GAP-defective mutant, p85R274A, caused sustained levels of activated platelet-derived growth factor receptors (PDGFRs), enhanced downstream signaling, and resulted in cellular transformation, through alterations in receptor trafficking. Our objective was to determine if p110 also contributed GAP activity towards Rab5, with p85 providing a catalytic arginine residue (R274) and p110β providing switch stabilization functions specific to the GTP-bound state. To accomplish this goal we set out to compare the results from cells expressing individual p85 defects (lacking GAP activity, R274A, generated previously; or lacking p110-binding ability through deletion of residues 478-513, Δ110) with results from cells expressing a double mutant missing both functions. The enhanced and sustained receptor activation (pTyr) and PDGFR levels observed for late times after PDGF stimulation (60 and 120 min) was the most pronounced in cells expressing the p85Δ110+R274A mutation. In contrast, the sustained MAPK activation (pMAPK) noted in p85R274A-expressing cells, was not observed in p85Δ110+R274A expressing cells, and p85Δ110 expression caused a more transient pMAPK activation profile. We propose that p110β may regulate PDGFR trafficking by providing Rab5-GTP switch stabilization that complements the catalytic arginine residue (R274) within p85, and that p85α and p110β work together as a Rab5 GAP. Supported by the CCS (grant #019040).

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Identification of a point mutation in Rab11-FIP1A as an inhibitor of plasma membrane recycling.

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Protein trafficking and transport are vital aspects of cellular function as cells interact with their environment by internalization and release of cell surface proteins. Aberrant trafficking contributes to diseases including diabetes insipidus, cystic fibrosis, and Alzheimer's disease. Even though different cargoes, such as channels and receptors, are unique to different cell types, plasma membrane recycling is highly conserved among mammals and regulated by a common set of trafficking proteins including Rab small GTPases and their effectors. Rab11a is involved in recycling and interacts with the Rab11-Family-Interacting-Proteins (FIPs). To study how Rab11-FIP1A interacts with other proteins we identified Threonine 197 as a putative 14-3-3 binding site. Mutating Thr197 to an Alanine caused a dramatic dominant negative phenotype in HeLa cells. The normally perinuclear distribution of GFP-FIP1A was condensed into a membranous cisternum with almost no GFP-FIP1A remaining outside of this site. Also, this condensed GFP-FIP1A (T197A) altered the distribution of proteins in the recycling pathway including endogenous Rab4, Rab5, Rab11a, FIP1C, FIP5, and EEA1. This dominant negative mutation also affects transferrin trafficking in HeLas. While transferrin was taken up and recycled out of cells within 1 hour in non-transfected cells, in GFP-FIP1A transfected cells,

transferrin remained in a perinuclear complex with GFP-FIP1A (T197A). In order to understand this mutation and what role it may have on the structure of the protein, we performed Circular Dichroism (CD) Spectroscopy on recombinant purified FIP1A and FIP1A (T197A). Purified FIP1A (T197A) showed a remarkable change in the structure of FIP1A. The T197A mutation shows a more pronounced alpha helical structure than the wildtype protein, which appears to be comprised of a much more random coil structure. The Rab11-FIP proteins in general have flexible or disordered structures. The results here suggest that the T197A mutant may confer a more ordered confirmation that may reflect an "activated" conformation that is dominant negative for trafficking.

Lipids and Membrane Microdomains

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Investigating the organisation of lipid rafts in mammalian cells.

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The lipid raft hypothesis "proposes that the lipid bilayer is not a structurally passive solvent, but that the preferential association between sphingolipids, sterols, and specific proteins bestows cell membranes with lateral segregation potential"1. The physical existence of lipid rafts was demonstrated some time ago2 and there is extensive evidence that lipid rafts are small, and that some types might be highly dynamic (reviewed in1,3,4), with rafts providing physical platforms for a diverse range of fundamental and essential cellular processes. Here, we present evidence that the protein tetherin (aka CD317, HM1.24 antigen, BST-2) is an organiser of lipid rafts. Tetherin possesses a short amino-terminal cytosolic domain that interacts indirectly with the actin cytoskeleton via the proteins RICH2, EBP50 and ezrin5, a conventional non-raft localised transmembrane domain and raft localised carboxyl-terminal glycophosphatidylinositol (GPI) anchor6; the two membrane anchors linked by an extracellular region containing a coiled-coil domain7. Tetherin thus provides a mechanical link between lipid rafts and the actin cytoskeleton5. Fluorescence recovery after photobleaching (FRAP) assays have demonstrated that the lateral diffusion mobility of a GPI anchored reporter protein (YFP-GPI) is increased in cells in which tetherin expression has been knocked down. Consistent with this, data obtained using the fluorescent membrane probe Laurdan have shown that a reduction in membrane order is observed in cells in which tetherin expression has been knocked down. These findings suggest that tetherin limits the lateral diffusional mobility of a raft-localised reporter and that tetherin is playing a role in the organisation of more liquid-ordered membrane domains. The results of single particle tracking studies (presented here) designed to monitor the movement of individual reporter proteins within the plane of the lipid bilayer in control cells and in cells in which the expression of tetherin has been knocked down using siRNA are consistent with tetherin playing a role in organising membrane microdomains.

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Tetherin is an organiser of lipid rafts.

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Tetherin (Bst2, CD317) has been implicated in preventing the spread of HIV1 virions from infected cells by tethering them to the cell surface. The cytosolic domain of tetherin interacts indirectly with the actin cytoskeleton, and its knockdown in polarised epithelial cells leads to a loss of the sub-apical actin network. Whilst the N-terminus of tetherin interacts with the actin cytoskeleton, the glycophosphatidylinositol (GPI)-anchored C-terminus is localised to lipid rafts; this unusual topology of tetherin enables it to link the actin cytoskeleton to lipid rafts. Moreover, the extracellular region of tetherin forms a parallel coiled-coil dimer, which is likely to limit the free diffusion of proteins into and out of lipid rafts, thereby organising lipid rafts. We therefore set out to examine whether tetherin is involved in lipid raft organisation, and here we present evidence that tetherin is indeed involved in the organisation of lipid rafts. Tetherin has previously been shown to activate the NF-κB pathway and, in line with this, increased expression of tetherin upregulates the activity of an NF-κB activated luciferase reporter, consistent with increased expression of tetherin stabilising lipid raft domains and the signalling complexes associated with them. In addition, using a GPI-YFP reporter construct (a lipid raft marker) in concert with fluorescence recovery after photobleaching (FRAP) assays, the lateral diffusion mobility of GPI-YFP was shown to be increased in cells in which tetherin expression had been knocked down – a result consistent with tetherin playing a role in lipid raft organisation. Further, we have employed the membrane dye laurdan in combination with confocal microscopy to demonstrate that a loss of tetherin expression leads to a reduction in membrane order. Together, the results from FRAP, laurdan and NF-κB activation experiments are consistent with tetherin being a regulator of lipid raft organisation.

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Clustering and Internalization of Toxic Amylin Oligomers in Pancreatic Cells Requires Plasma Membrane Cholesterol.

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Self-assembly of the human pancreatic hormone amylin into toxic oligomers and aggregates is linked to the dysfunction of islet beta-cells and pathogenesis of type-2 Diabetes Mellitus (TTDM). Recent evidence suggests that cholesterol, an essential component of eukaryotic cells membranes, controls amylin oligomerization and aggregation on model membranes. However, the question as to whether cholesterol may also influence amylin interactions with the native

pancreatic membranes and cells remains open. In view of this, the current study was designed to investigate amylin binding and internalization pathways in pancreatic rat insulinoma (RIN-m5F) and human islet cells with depleted, normal and enriched plasma membrane (PM) cholesterol levels, and explored the extent to which PM cholesterol and lipid rafts contribute to amylin uptake and toxicity in these cells. Modulatory effects of cholesterol depleting agents, lovostatin (Lov) and methyl-beta cyclodextrin (BCD), or water soluble cholesterol on PM and cellular cholesterol levels were quantified by the Filipin and Amplex Red fluorometric assays, respectively. The extent of amylin toxicity in controls and treatments were quantified and analyzed using 3-(4, 5-Di methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction, Lactate Dehydrogenase (LDH) release and Caspase-3/Annexin V apoptotic assays. Immunofluroscence confocal microscopy and western blot analysis were used to study the internalization and accumulation of amylin monomers and oligomers in cells. Depletion of PM cholesterol by lipid raft disrupting agents, BCD and Lov inhibited the internalization of amylin oligomers in both cell types, which in turn enhanced extracellular oligomer accumulation and potentiated amylin toxicity. Confocal microscopy confirmed binding and accumulation of amylin oligomers and to lesser extent amylin monomers in the lipid rafts of cholesterol-enriched but not cholesterol-depleted cells. Biochemical studies demonstrated the accumulation of toxic amylin oligomers in the medium following depletion of PM cholesterol. Replenishment of PM cholesterol from intracellular cholesterol stores, or by addition of water soluble cholesterol restored amylin oligomer clustering at the PM and internalization, which consequently diminished the cell surface coverage and toxicity of amylin oligomers. In contrast to oligomers, amylin monomers followed clathrin-dependent endocytosis, which is not sensitive to cholesterol depletion. Our studies identify lipid raft-mediated and cholesterol-dependent mechanism for selective uptake and clearance of amylin oligomers, impairment of which greatly potentiates amylin toxicity in pancreatic cells.

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The first 5 seconds in the life of an endocytic clathrin coated pit.

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Formation of endocytic clathrin coated pits and vesicles requires the rapid and highly orchestrated generation of a scaffold made of a very large number of interacting proteins whose assembly results in entrapment of cargoes and membrane deformation. Key for this process is the organized recruitment of clathrin triskelions and AP-2 adaptors. In order to understand the molecular events occurring at the early stages of pit formation we developed a method applicable to living cells and based on total internal reflection fluorescence microscopy that allows visualization and tracking with single-molecule sensitivity and 100 millisecond temporal resolution of clathrin and AP-2 as they are recruited during the first few seconds in the life of a coated pit. By combining this imaging method with newly developed analytical tools to cells expressing fluorescently tagged clathrin and AP-2, we established that coated pits start by the simultaneous recruitment of one clathrin triskelion and two AP-2s. This first recruitment step typically lasts ~2 s, followed by a second step of similar characteristics. We also found that there is no need to pre-cluster AP-2s at the site of coated pit formation and that the presence of the phosphoinositide PIP2 is essential for pit initiation.

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LC3II content is increased in livers and lipid droplets (LDs) of ethanol-fed rats.

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Autophagy is a major intracellular catabolic pathway that is critical to cellular quality control and is altered in a variety of pathological states. We are examining the trafficking of lipid droplets (LDs) in liver cells during the development of alcohol-induced fatty liver. Our previous work has demonstrated that ethanol administration slows the rate of protein trafficking in liver cells. Because autophagy involves the movement and fusion of lipid droplets with lysosomes and autophagosomes we examined whether chronic ethanol consumption alters lipophagy (i.e. the autophagic sequestration of lipid droplets). **Methods:** Male, Wistar rats were pair-fed a liquid diet containing either ethanol as 36% of calories or isocaloric carbohydrate for 4-6 wk. Rat liver homogenates were fractionated into post-nuclear supernatants (PNS), mitochondria/lysosomes, cytosols, and microsomes, while LDs were separately isolated from the remaining liver. All fractions were subjected to SDS-PAGE and immunoblotting and probed for the lipidated form of the autophagy marker, Atg 8, the microtubule associated protein 1 light chain 3, known as LC3II. **Results:** In ethanol-fed animals, steatosis was clearly evident, as total liver triglyceride was increased 2-3 fold over pair-fed controls. Chronic ethanol feeding significantly increased LC3II content in liver PNS and microsomal fractions by 30% ($p<0.05$) over those of controls; no change was evident in other subcellular fractions. Additionally, LC3II content associated with LDs from ethanol-fed animals was enhanced 5-fold or more over that of controls. Our findings suggest that chronic ethanol feeding to rats activates autophagosome formation in liver ER membranes, as indicated by a rise in LC3II in total liver and microsome fractions. We propose that the large increase in LD-associated LC3II reflects an attempt by the autophagic machinery to sequester lipid droplets for eventual lipolysis.

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Lipid droplets are polarized in fission yeast during G2 phase.

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Lipid droplets (LDs) are now being appreciated as active organelles involved in a variety of cellular processes and not just inert storage depots for neutral lipids. There is evidence that LD dynamics are linked to cell cycle progression in both budding and fission yeast. In fission yeast, S. pombe, the number of lipid droplets follows an oscillatory cycle where the number increases during G2 phase and is roughly halved when the septum forms after M phase. We observe that LDs are strongly polarized to the cell tips in a temperature sensitive mutant (cdc25), whose cell cycle is arrested in late G2 phase at 36 °C well before formation of the septum. Here, we investigate the mechanisms for this distribution. Since LDs associate with microtubules in fly embryos, we tested if these mechanisms were related by disrupting the microtubules with nocodazole. LDs were still polarized in cdc25 after addition of nocodazole. However, the addition of latrunculin A to cdc25 cells caused the LDs to be dispersed throughout the cytoplasm. We conclude that LDs are being actively polarized via the actin cytoskeleton. Future experiments will determine whether this is a myosin-based mechanism, and if so which genes are regulating LD-myosin binding.

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Acyl-CoA synthetase 3 is dynamically localized to lipid droplets/ER and functions in fatty acid uptake.

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Cytosolic lipid droplets are storage organelles for neutral lipids derived from endogenous metabolism. Acyl-CoA synthetase family proteins are essential enzymes in this biosynthetic pathway, contributing activated fatty acids.

Immunofluorescence microscopy showed that ACSL3 is localized to both the endoplasmic reticulum and lipid droplets, with the distribution dependent on the cell type and the supply of fatty acids. ACSL3 was effectively translocated from the ER to nascent lipid droplets as observed by live microscopy. Analysis of the N-terminal hydrophobic domain indicates that it forms an amphipathic helix restricted to the cytosolic leaflet of the ER membrane. N-terminal deletion mutants reduced the efficiency of targeting to lipid droplets. Cellular fatty acid uptake was increased by overexpression and reduced by RNA interference of ACSL3.

In conclusion, the structural organization of ACSL3 allows the fast and efficient movement from the ER to emerging lipid droplets. ACSL3 not only esterifies fatty acids with CoA but is also involved in the cellular uptake of fatty acids, presumably indirectly by metabolic trapping. The unique localization of the acyl-CoA synthetase ACSL3 on lipid droplets suggests a function in the local synthesis of lipids.

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Testing the effect of drugs on arrays of NPC1 mutations.

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95% of Niemann–Pick disease type C (NPC) disease is caused by mutations in a transmembrane protein, NPC1, that block efflux of cholesterol from late endosomes and lysosomes. This efflux mechanism keeps the cholesterol content in organelles and organelle membranes low in normal cells. Previous studies have found that histone deacetylase inhibitors (such as LBH589) are able to correct the NPC1 phenotype in human fibroblast cells with an NPC1 I1061T mutation. *NPC1*^{I1061T} is the most common mutation observed in NPC1 patients. However, there are more than 100 different clinical mutations in NPC1 patients. The effects of drugs on NPC1 mutations other than NPC1 I1061T are still unclear. In order to examine the effectiveness of histone deacetylase inhibitor treatment on hundreds of different NPC1 mutations simultaneously, a 384-well plate screen has been designed. The NPC1-null human osteosarcoma cell line (U2OS_shNPC1) is used in the screen. U2OS_shNPC1 cells are transfected with mutated NPC1 and GFP coexpression vector (pMIEG3-NPC1) by reverse transfection, which has been optimized in respect to concentration of DNA and transfection reactant. The mutated NPC1 protein is expressed in GFP positive cells. Using this system, we can test the effects of drug treatments on many different NPC1 mutations. The first application will be to determine which NPC1 mutations can be corrected by histone deacetylase inhibitors. This should help in evaluating whether or not histone deacetylase inhibitor treatment is suitable for patients carrying these mutations.

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A novel, apical ceramide-enriched compartment (ACEC) regulates ciliogenesis through a complex with ceramide-associated atypical PKC.

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The primary cilium is an important sensory organelle, the regulation of which is not fully understood. Using an antibody against the sphingolipid ceramide, we found that in polarized Madin-Darby Canine Kidney (MDCK) cells, an apical ceramide-enriched compartment (ACEC) is located at the base of primary cilia. This novel compartment immunostained for the centrosome marker γ-tubulin, the Rho type GTPase cell division cycle 42 (Cdc42), and atypical protein kinase Cζ/λ (aPKC), a kinase activated by ceramide and associated with a polarity protein complex consisting of partitioning defective Par6 and Cdc42. Inhibition of ceramide biosynthesis with Fumonisin B1 prevented co-distribution of aPKC and Cdc42 in the ACEC and severely impaired ciliogenesis. Cilium formation and co-distribution of aPKC and Cdc42 were restored by incubation with N-acetyl or N-palmitoyl sphingosine (C2 or C16 ceramide), or the ceramide analog N-oleoyl serinol (S18). Using a novel isolation method termed "Lipid-vesicle mediated magnetic activated cell sorting" (LIMACS) we isolated ceramide vesicles from ciliated MDCK cells. Mass spectrometric analysis (sphingolipidomics) showed that these vesicles were highly enriched with ceramide, suggesting that the ACEC is functionally involved in ciliogenesis. We also found that ceramide vesicles were enriched with aPKC, consistent with the observation that aPKC co-distributed with the ACEC at the base of the cilium. Expression of a phosphorylation-incompetent but ceramide-binding mutant of aPKC prevented ciliogenesis, which suggested that binding of aPKC to ceramide at the ACEC is critical for cilium formation or elongation. We will now further determine how the ACEC is formed and how the ceramide-aPKC interaction regulates ciliogenesis. This work is supported by NIH and NSF.

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The lipid flippase TAT-5 regulates the budding of extracellular vesicles in *C. elegans* embryos.

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During cytokinesis and morphogenesis, embryos undergo dramatic changes in cell shape. While the role of the cytoskeleton in regulating these processes is well known, we understand less about the role of the lipid bilayer in modulating cell shape. For example, the asymmetric partitioning of phosphatidylserine (PS) and phosphatidylethanolamine (PE) to one leaflet of the bilayer can affect membrane curvature and influence dynamic membrane events such as cytokinesis. We identified TAT-5, a P4 ATPase predicted to flip phospholipids to the cytoplasmic leaflet of the bilayer, in an RNAi screen for essential regulators of cell contact-induced polarity in *C. elegans*. Loss of TAT-5 also resulted in defects in plasma membrane dynamics during cytokinesis and morphogenesis.
GFP-tagged TAT-5 localized to the plasma membrane and TAT-5 prevented the externalization of PE, but not PS, on the surface of cells. Since TAT-5 acts at the cell surface, we used electron tomography to examine the three-dimensional structure of the plasma membrane at high resolution. Strikingly, loss of TAT-5 caused the large-scale shedding of budding vesicles from the plasma membrane, which disrupted the structure of cell contacts and likely explains the defects in morphogenesis that we observed in *tat-5* embryos. The robust production of extracellular vesicles in *tat-5* embryos depended on the function of RAB-11, the recycling endosome-associated GTPase, as well as ESCRT complex proteins, which normally function in the formation of multivesicular bodies. Homologs of these proteins regulate viral budding,

suggesting mechanistic similarities between these topologically similar membrane budding events. Our findings define the essential role of a P4 ATPase in the regulation of PE asymmetry and extracellular vesicle budding. Our results also suggest that PE externalization could influence dynamic remodeling of the plasma membrane. No proteins were previously known to regulate extracellular vesicle budding, and our findings may provide novel insights into diseases influenced by extracellular vesicles, including viral spread, blood clotting disorders, and tumor metastasis.

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Monitoring of Radioprotective Effects of Amifostine on Rat Liver Microsomal Membrane and Brain Tissue Lipids by FTIR Spectroscopy and Microspectroscopy.

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Amifostine is the only approved radioprotective agent by the Food and Drug Administration for reducing the damaging effects of radiation on healthy tissues. The purpose of this study was to investigate the protective effect of amifostine against the deleterious effects of ionizing radiation on rat liver microsomal membranes and white matter (WM) and grey matter (GM) regions of brain tissues at molecular level. Sprague-Dawley rats, which were administered amifostine or not, were whole-body irradiated with a single dose of 800 cGy, decapitated after 24 h and liver microsomal membranes and brain tissues of these rats were analyzed using FTIR spectroscopy and FTIR microspectroscopy. The results of the liver microsomal membrane study [1] revealed that radiation caused a significant decrease in the lipid to protein ratio and the degradation of lipids into smaller fragments that contain less CH2 and more carbonyl esters, olefinic=CH and CH3 groups, which could be interpreted as a result of lipid peroxidation. In addition, radiation altered the secondary structure of proteins by inducing a decrease in the â-sheet structures and an increase in the turns and random coil structures. Moreover, a dramatic increase in lipid order and a significant decrease in the membrane dynamics were observed in the irradiated group. The administration of amifostine before ionizing radiation inhibited all the radiation induced compositional, structural and functional damages in the liver microsomal membranes [1]. Brain FTIR imaging results showed that the total lipid content and CH2 groups of lipids decreased significantly and the carbonyl esters, olefinic=CH, and CH3 groups of lipids increased significantly in the WM and GM after exposure to ionizing radiation, which were interpreted as the byproducts of lipid peroxidation. These changes were more prominent in the WM of the brain. The administration of amifostine before ionizing radiation caused significant protective effect against the radiation induced lipid peroxidation in the WM and GM of the brain. In conclusion, this study indicated that amifostine administration to the rats prior to whole body irradiation protects liver microsomal membrane and brain tissue against the radiation-induced damages. In addition, these results suggest that FTIR spectroscopy together with microspectroscopy provides a novel approach to monitor ionizing radiation induced-damage on biological systems. Supported by TUBITAK (SBAG-2939) and by the METU (BAP-2006-07-02-0001).

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Investigation of the Cell Membrane Architecture by Single-Molecule Tracking of Peptidic Toxins.

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The cellular membrane is a vital part of the cell, which plays a crucial role in many cellular processes, such as, signaling and trafficking, and pathologies. This study aims at investigating the membrane architecture by probing the motion of two membrane receptors that are exploited by bacterial toxins.

To investigate the membrane, lanthanide doped nanoparticles ($Y_{0.6}Eu_{0.4}VO_4$) are coupled to two different peptidic pore-forming toxins, the α-toxin of *C. septicum* and the ε-toxin of *C. perfingens* [1]. Single molecule tracking (SMT) of receptor bound labeled toxins in the apical membrane of MDCK cells in a wide-field microscope reveals the receptor motion with sub-diffraction resolution of down to 10 nm. The α and ε-toxin receptors both undergo confined diffusion with similar diffusion coefficients of 0.16 ± 0.14 $\mu m^2/s$ in stable domains of 0.5 μm^2 [2].

To analyze the receptor trajectories, we introduced a novel approach based on an inference method [3]. Our only assumption is that the receptor moves according to the Langevin equation of motion. This method exploits the information of the ensemble of the trajectory and the quality of the extracted values is verified through simulations. Both receptors are confined in a spring-like potential with a spring constant of 0.45 pN/µm.

Tracking after cholesterol depletion by cholesterol oxidase and cytoskeleton depolymerization by Latrunculin B, shows that confinement of single receptors is cholesterol dependent and actin depolymerization does not influence the confinement. We propose that the receptors are confined in lipid rafts and that hydrophobic coupling between the receptor and the surrounding lipids or proteins are sufficient to lead to the observed confinement potentials.

Using the nanoparticle labels as a hydrodynamic force amplifier in a liquid flow, tests the response of the receptor to an external force. SMT shows mainly elastic displacements of the receptor over distances up to 10 times the confining domain diameter. Once the flow stops, the receptors return to their initial position indicating attachment to the cytoskeleton.

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Characterization of the ezrin/periaxin/desmoyokin/spectrin/ankyrin-B-based macromolecular complexes in lens fiber cells and their potential role in plasma membrane organization and stability.

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Purpose: To identify and characterize the molecular mechanisms regulating fiber cell plasma membrane organization and stability in normal and cataract lenses.

Methods: Lens fiber cell cytoskeletal-protein enriched fractions, immunoprecipitations assays, mass spectrometric–based protein identification and immunofluorescence-based co-localization analyses were performed to isolate and characterize the mouse lens fiber cell membrane-associated macromolecule protein complexes.
Results: Lens fibers of post-natal and adult mice revealed a maturation dependent redistribution of periaxin, a myelin sheath stabilizing protein, clustering discretely at the tricellular junctions in mature fiber cells. Periaxin and desmoyokin, the PDZ-domain proteins were found to co-exist in a macromolecular complex with proteins involved in membrane subdomain organization including NrCAM, ankyrin B, β-spectrin, ezrin, filensin and phakanin. Moreover, lens fiber cells were found to express and distribute contactin-1, Caspr2, βIV-spectrin, dystrophin-glycoprotein complex proteins and ion-channel proteins involved in membrane organization and cytoskeletal scaffolding in neuronal cells.
Conclusions: Taken together, these observations provide some key pieces of evidence in support of a critical scaffolding role for the ezrin/periaxin/desmoyokin/ankyrin-b/spectrin-based macromolecular complexes in regulating membrane cytoarchitecture, tri-cellular junctional stability and membrane subdomain organization in lens fibers. Additionally, these observations paved the way for the intriguing and previously unsuspected possibility of conserved parallel molecular mechanisms regulating membrane stability and organization in lens fibers and myelinating neurons.

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Membrane potential-gated re-organization of plasma membrane.

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The existence of highly ordered, sphingolipid-enriched gel microdomains in the plasma membrane of baker's yeast S. cerevisiae was recently proposed [1]. Using membrane order/fluidity reporter dyes trans-Parinaric Acid (tPA) and 1,6-Diphenyl-1,3,5-Hexatriene (DPH), we studied the stability of these membrane microdomains in response to plasma membrane depolarization. A clear effect on fluorescence lifetime of tPA following various depolarization treatments of living BY4742 S. cerevisiae was detected. DPH fluorescence anisotropy, in addition, reported an increase of the global membrane fluidity in the treated cells – a clear indication of homogenization of the dye microenvironment in the depolarized membrane. We conclude that the non-zero transmembrane potential across the membrane is a prerequisite for the existence of gel sphingolipid microdomains in the yeast plasma membrane. This conclusion complements our earlier observation of membrane depolarization-gated decomposition of ergosterol-rich MCC (Membrane Compartment of Can1) microdomains [2] and accents the primary role of membrane potential in plasma membrane microdomain organization.

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Caveolin Targeting to Endo-lysosomal Membranes is Induced by Serum Starvation and Perturbations of Lysosomal pH and Cholesterol Content.

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Caveolin-1 is an integral membrane protein of plasma membrane caveolae, which represent one of the clathrin independent endocytic pathways. Here we report that caveolin-1 collects at the cytosolic surface of lysosomal membranes when cells are serum starved. We show this is due to an elevation of the intra-lysosomal pH and that ionophores and proton pump inhibitors that dissipate the lysosomal pH gradient also trapped caveolin-1 on LE/lysosomes. Accumulation is both saturable and reversible. At least a portion of the caveolin-1 goes to the plasma membrane upon reversal. Several studies have suggested that caveolin-1 is involved in cholesterol transport or homeostasis within the cell. Strikingly, we find that caveolin-1 also accumulates on LE/lysosomal membranes when cholesterol homeostasis is perturbed either by low concentrations of cyclodextrin or when the export of lysosomal cholesterol is blocked using progesterone or U18666A. Under these conditions, live cell imaging shows cavicles actively docking with lysosomes, suggesting these structures may be involved in delivering caveolin-1. A recent paper suggested that caveolin translocates to lysosomes primarily to be degraded. While this is undoubtedly true when tagged caveolins are transiently over expressed, or when PTRF/cavin1 is knocked down and caveolin enters the cell via the bulk endocytic pathway, this is not the case here. Endogenous caveolin is degraded very slowly (t1/2 ~ 23 hours) and is not normally seen on LE/lysosomes in our cells except after these treatments. In addition we show that neither serum starvation nor U18666A change the turnover rate of the endogenous protein or caveolin-1-GFP that is stably expressed in cells confirming that the accumulation on late endosomes/lysosomes is not a consequence of blocked degradation. Cavicles normally shuttle between membranes without a loss of domain identity and when they accumulate on LE/lysosomes after progesterone or U18666A treatment they appear to retain their identity as punctate structures. In contrast, when lysosomal pH is high the caveolin that is associated with LE/lysosomes is evenly distributed over the membrane surface suggesting the structure of the cavicle/caveolae is significantly different. In addition, when the association of caveolin-1 with LE/lysosomes is reversed after serum starvation caveolin-1 leaves by a process that appears not to involve cavicles and is not inhibited by genistein whereas this inhibitor blocks trafficking to the LE/lysosome. We conclude that caveolin-1 normally traffics to and from the cytoplasmic surface of lysosomes during intracellular cholesterol trafficking. A recent paper questioned the existence of the caveosome and suggested that they were seeing caveolin on LE/lysosomes. In stable cell lines we still find structures that are not labeled with either dextran or lysotracker and behave as described previously. These are independent of the LE/lysosomal structures that were observed in treated cells and suggest that the death of the caveosome may be greatly exaggerated.

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Cholera toxin B-subunit assembles slowly diffusing domains and induces tubulation of the plasma membrane of intact cells.

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Shiga toxin and cholera toxin have the intrinsic capacity to crosslink glycolipids via their multivalent membrane binding B-subunits. Recent models suggest toxin binding induces the formation of membrane domains that drive toxin uptake into host cells by a novel toxin-dependent endocytic pathway. How the structure and dynamics of the toxin-dependent domains

that trigger this pathway are regulated is still poorly understood, especially in intact cells. To address this question, we investigated the properties of toxin/glycolipid complexes formed by cholera toxin B-subunit (CTxB) binding to its glycolipid receptor GM1. As predicted by current models, CTxB accumulated in tubular plasma membrane invaginations in cells under conditions where tubule scission is inhibited by actin disruption or ATP depletion. However, these treatments led to the formation of toxin-independent invaginations and protrusions as well, indicating that multiple mechanisms of membrane deformation are initiated under these conditions. Next, we characterized the dynamics of the CTxB/GM1 complex within the plasma membrane prior to its internalization using confocal FRAP. Compared to the dynamics of a representative GPI-anchored protein, transmembrane protein, and lipid probe, the diffusional mobility of CTxB was highly constrained at the cell surface. We show that actin and ATP-dependent processes, but not caveolae act as major regulators of motion of the CTxB/GM1 complex within the plane of the membrane. These findings identify CTxB-induced domains as slowly diffusing structures, which are strongly confined by the actin cytoskeleton. We speculate that coupling of the CTxB/GM1 complex to actin may regulate its uptake into cells by a toxin-dependent mechanism.

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Control of membrane traffic and synaptic growth signaling by the neuronal F-BAR/SH3 domain protein Nervous Wreck.

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Internalized receptors exhibit subcellular location-dependent signaling properties as they are trafficked through endosomal compartments. Therefore, modulating the machinery that controls receptor traffic between compartments represents a novel mechanism to tune signal output. Nervous Wreck (NWK) encodes an F-BAR/SH3 protein that regulates the traffic and signaling output of synaptic growth receptors at the *Drosophila* neuromuscular junction through its interactions with the membrane, actin nucleation machinery, and other endocytic proteins including dynamin and Dap160/Intersectin. Recently, we reported that Nwk promotes synaptic membrane traffic through a physical interaction with Sorting Nexin 16 (Snx16), a conserved early endosomal membrane-binding protein. Snx16 promotes synaptic growth signaling by the Wingless and BMP pathways, and our live imaging of *Drosophila* larvae reveals that Snx16-positive early endosomes interact transiently with Nwk-containing recycling endosomes in nerve terminals, leading to signal attenuation (Rodal et al., 2011; J. Cell Biol.). Understanding these cellular dynamics will require a deeper analysis of the membrane deforming activities of Nwk and Snx16. Here, we have further addressed the mechanisms involved, and found that the N-terminal F-BAR domain of Nwk has a novel membrane deforming activity that distinguishes it from two other presynaptically expressed F-BAR proteins in *Drosophila*, dCip4 and Syndapin. We have identified unique structural features of the Nwk F-BAR domain, which may confer its special membrane bending properties. Further, we found that the activities of the Nwk F-BAR domain are regulated by intramolecular interactions and by phosphorylation of the F-BAR domain, suggesting that multiple regulatory inputs into Nwk could serve as a knob or switch for modulating intracellular membrane traffic at synapses.

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'Lipid Raft' Association Determines the Recycling Kinetics but not the Late Endosomal Trafficking of Endocytosed GPI-Anchored Proteins in Mammalian Fibroblasts.

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We have examined the post-endocytic trafficking of phosphatidylethanolamine-polyethylene (PE-PEG-) -anchored protein conjugates, incorporated into CHO and BHK fibroblasts, to test previous proposals that ordered lipid microdomains play important roles in glycosylphosphatidylinositol-anchored (GPI-) protein sorting within endosomal compartments. In CHO cells internalized PE-PEG-anchored proteins, like GPI-proteins and the transmembrane transferrin receptor, are efficiently returned to the cell surface via recycling endosomes. However, PE-PEG-protein conjugates with 'raft-phobic' short-chain or unsaturated lipid anchors recycle rapidly to the plasma membrane at the fast rate observed for the transferrin receptor, while analogous conjugates with 'raft-philic' long-chain saturated lipid anchors recycle at the much slower rate observed for the GPI-anchored folate receptor. Our findings strongly support previous suggestions that the slow kinetics of surface recycling of endocytosed GPI-proteins rests on their potential to associate with ordered lipid microdomains ('rafts').

In BHK cells GPI-proteins initially present at the cell surface gradually accumulate over long time courses (several hours) in late endosomes/lysosomes, trafficking to which has also been proposed to depend on the 'raft' association of GPI-proteins. We show that after internalization, the GPI-anchored folate receptor is largely recycled to the cell surface but that a smaller but significant fraction is instead trafficked to late endosomes/lysosomes; this 'bifurcated' sorting explains the slow net rate at which GPI-proteins from the cell surface accumulate in late endosomes/lysosomes. PE-PEG-protein conjugates with either long-chain saturated or unsaturated lipid anchors both traffic very similarly to GPI-proteins in these cells, suggesting that 'raft' association does not play a significant role in GPI-protein trafficking to late endosomes/lysosomes in these cells. (Supported by the Canadian Institutes for Health Research).

The Nuclear Envelope and Nuclear Pore Complexes

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Membrane proteins of the endoplasmic reticulum are required for nuclear pore complex and spindle pole body assembly and function.

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As a critical step in gene expression, mRNA must travel from the nucleus to the cytosol for translation. Movement across the nuclear envelope (NE) requires transport through nuclear pore complexes (NPCs), large macromolecular protein complexes that span NE pores. Indeed, all cellular functions are in part regulated by transport of factors through NPCs. While molecules smaller than ~30kDa diffuse freely through NPCs, large proteins and RNA move through the NPC in a directed manner by active transport. During a cell's lifetime, new NPCs are generated in response to the need for survival and function. Without proper NPC formation, neither RNA nor proteins can be correctly transported through the NE. For a new NPC to form in an intact double membrane NE, the fusion of the inner and outer membrane leaflets must occur. Membrane proteins are predicted to mediate this fusion step. The resulting curved pore membrane is thought to be stabilized by structural NPC components which also provide a

scaffold for additional functional components to assemble. While a small number of membrane proteins have been identified as stable components of the NPC, the mechanism by which these proteins function has yet to be elucidated. Previously, we found that Rtn1 and Yop1, membrane bending proteins of the endoplasmic reticulum (ER), play a role in NPC assembly. S. cerevisiae cells lacking Rtn1 and Yop1 have NPC defects including a clustering to one region of the NE. As the spindle pole body (SPB) is also NE embedded in *S. cerevisiae*, we examined *rtn1Δyop1Δ* cells for altered SPB structure and function. Electron microscope images of *rtn1Δyop1Δ* cells revealed abnormalities in SPB structure. Furthermore, mitotic spindles appeared on average shorter and misoriented in dividing cells. These observations potentially reflect the known dual requirement of the essential membrane protein Ndc1 in both NPC and SPB assembly. Recently, others suggested that NPC and SPB biogenesis could act antagonistically through the recruitment and sequestering of common components such as Ndc1. Using *rtn1Δ yop1Δ* cells, we examined this relationship between SPBs and NPCs. Increased expression of Ndc1 rescued defects in both NPCs and SPBs of *rtn1Δ yop1Δ* cells. However, increased expression of NPC specific interaction partners of Ndc1only rescued defects in NPCs while worsening defects in SPB function. We are currently investigating the roles by which these different ER membrane proteins influence NPC stability and potentially initiate NE fusion for pore formation.

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The SUMO proteases SENP1 and SENP2 bind to Nup153 and control a cycle of SUMO modification of this nucleoporin.

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The nuclear rim houses a variety of proteins with diverse regulatory functions, ranging from nucleocytoplasmic trafficking, transcription regulation to coordination of cell cycle progression. Numerous enzymes of the mammalian SUMO modification pathway, including two members of the SUMO protease family, SENP2 and SENP1, localize to the nuclear periphery. The SUMO proteases play roles both in processing SUMO during the biogenesis of this peptide moiety and also in reversing SUMO modification on specific targets to control the activities conferred by this post-translational modification. Although interaction with the C-terminal domain of the nucleoporin Nup153 is thought to contribute to SENP2 localization to the nuclear pore complex, little is known about the binding partners of SENP1 at the nuclear periphery. We have found that Nup153 binds to both SENP1 and SENP2 and does so through a dual platform, involving both the unique N-terminal domain of Nup153 and a specific region within the C-terminal FG-rich region. We have further found that Nup153 is a substrate for sumoylation, with this modification kept in check by these two SUMO proteases. Specifically, either RNAi depletion of SENP1/SENP2 or expression of dominantly interfering mutants of these proteins results in increased sumoylation of endogenous Nup153. SENP1 and SENP2 are themselves subjected to dynamic SUMO modification regulated by their own catalytic activity. Interestingly, whereas sumoylation of Nup153 promotes its interaction with SENP1/2, sumoylation of the protease appears to prevent this interaction. These studies reveal a dynamic mechanism that likely contributes to concentrating SENP1 and SENP2 in proximity to the nuclear pore complex. In the case of SENP2, its association with the pore has been previously shown to restrict its enzymatic activity, regulating its ability to target nucleoplasmic SUMO substrates. We are working to further understand the functional implications of the partnerships between Nup153 and SENP1 and SENP2.

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Distribution and Quantification of the Nucleolar Channel System of Human Endometrium.

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The Nucleolar Channel System (NCS) is a membranous organelle unique to the nuclei of human endometrial epithelial cells. NCSs are limited to days 19-24 of an idealized 28-day menstrual cycle, which overlaps with the implantation window. NCS presence is robust and independent of fertility status. Overall, NCSs are specific to healthy human endometrium of the secretory phase; they are absent from proliferative endometrium, from other hormonally sensitive human tissue such as breast tissue, from endometrial carcinoma specimens, or from baboon endometrium. Having recently identified molecular markers for these enigmatic structures revealed a unique composition of only a subset of nuclear envelope antigens, e.g. only some nuclear pore complex and inner membrane proteins. To these we now add the nuclear transport factors Ran, karyopherin α, and karyopherin β1, and, as a first component of NCS cores, γ-tubulin despite the absence of α- and β-tubulin. Whether these recent additions are important for NCS formation and/or their function is under investigation.

A major hurdle in NCS study has been their detection and quantification. The former we have overcome using indirect immunofluorescence against specific nuclear pore complex proteins enriched in NCSs. Quantification of NCSs has been hampered by their small, 1µ-diameter and peak appearance in only about 45% of endometrial epithelial cell nuclei. Thus, grading of uterine specimens for NCS presence is done semi-quantitatively as normal versus low or absent. In this manner, we show that NCSs are distributed uniformly throughout the upper endometrial cavity in the 9 NCS-positive specimens out of 42 uteri analyzed. Specifically, the fundus, left and right cornua, and anterior and posterior body exhibited between 89% and 100% of normal NCS presence. In contrast, the lower uterine segment contained significantly fewer NCSs compared to the other five regions (56% vs. 93%, $P<0.01$). This uniform distribution is consistent with a preferred implantation rate reported for the upper part of the uterus. To study NCS distribution at the level of endometrial biopsies, consecutive paraffin sections were stained for NCSs and for histologic assessment; each containing between 20 and 60 glandular cross-sections. Surprisingly, the number of NCSs varied randomly from gland to gland without a trace of a gradient or pattern. Our findings demonstrate a large local variability of NCS numbers despite their even distribution across the upper endometrial cavity. Although some glands were devoid of NCSs, neighboring glands contained NCSs paving the way for automated NCS counting algorithms on 5 to 10 glandular cross-sections. Presently, we are devising an NCS counting tool that determines the number of NCSs per epithelial cell nuclei by analyzing deconvolved Z-series of glands. This will create the first quantitative and unbiased understanding of NCSs within human endometrium.

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Oxidative Stress Signaling to the SUMOylation Machinery in Hutchinson-Gilford Progeria syndrome.

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We recently reported that expression of the Progerin form of Lamin A in Hutchinson-Gilford Progeria Syndrome (HGPS) reduces nuclear levels of the SUMO E2, Ubc9, as well as nuclear levels of SUMO2/3-modified proteins (Kelley et al. 2011). Given the important role of SUMOylation in nuclear function, we have studied how Progerin inhibits Ubc9 import by analyzing two, inter-related mechanisms: oxidative stress signaling and disruption of the Ran GTPase gradient. Fibroblasts from HGPS patients have elevated levels of reactive oxygen

species (ROS) as detected with dichlorofluorescein (DCF). Induction of oxidative stress by H_2O_2 causes a rapid and reversible redistribution of Ubc9 to the cytoplasm. We therefore tested whether Progerin affects Ubc9 distribution via oxidative stress signaling using Chinese Hamster Ovary cells pre-adapted to oxidative stress (CHO^r). Progerin expression as well as H_2O_2 treatment in the parental CHO line caused Ubc9 to redistribute to the cytoplasm, while the pre-adapted CHO^r line was resistant to the effects of both. Oxidative stress is known to inhibit Ubc9 activity via oxidation of a catalytic Cys93 and subsequent formation of a thioester with the E2 Uba2. Ubc9 with a Cys93Ser substitution is localized to the nucleus and undergoes redistribution to the cytoplasm in response to Progerin and H_2O_2 addition, suggesting that catalytic function is not required for Ubc9 import and cytoplasmic localization is not caused by oxidation-induced heterodimerization with Uba2. In heterokaryon experiments, Ubc9 from Progerin-expressing "donor" cells was competent for nuclear import into nuclei of "acceptor" cells, consistent with the interpretation that it does not undergo assembly into a cytoplasmic E1-E2 complex that precludes nuclear import. Ubc9 localization defects in HGPS could reflect a response to disruption of the Ran GTPase gradient, since Ubc9 import is Ran-dependent and the Ran gradient is disrupted in HGPS. We tested this hypothesis by siRNA depletion of the Ran import factor NTF2, which resulted in a significant reduction in the nucleocytoplasmic Ran gradient and concomitant inhibition of Ubc9 import. Finally, oxidative stress is sufficient to disrupt the Ran gradient. Our observations suggest the model that Progerin effects on nuclear SUMOylation in HGPS are mediated by oxidative stress signaling (ROS) to the Ran GTPase system and inhibition of Ubc9 import. According to our model, drugs that restore the redox state of the cell are predicted to reverse Progerin effects on the Ran System and SUMOylation.

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Membrane Attachment of Lamin A Reduces Histone H3 Tail Phosphorylation in Progeria.

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Fibroblasts from patients with Hutchinson-Gilford Progeria Syndrome (HGPS) have a disruption in the nucleocytoplasmic gradient of the Ran GTPase (Kelley et al., 2011). Ran gradient disruption, which can be recapitulated in various cell types by expressing the Progerin form of lamin A, is strictly dependent on Progerin attachment to the inner nuclear membrane, since farnesyl transferase inhibition or mutation of the CAAX motif prevent the effect. The Ran gradient defect in Progerin-expressing nuclei persists in heterokaryon fusions with normal cells, suggesting the molecular defect resides within the nucleus and is not transduced through the cytoplasm, and is relatively stable. A strong candidate for transducing the effect of Progerin on Ran is the nucleotide exchange factor RCC1, a nuclear protein that is required to establish the Ran gradient in both interphase and mitotic cells. FRAP analysis of GFP-RCC1 revealed reduced mobility in response to Progerin expression, suggesting the RCC1 chromatin-binding cycle (which is linked to nucleotide exchange) is altered by Progerin. RCC1 interactions with chromatin can be modulated by posttranslational modifications of histones. Since Progerin expression is known to reduce the levels of epigenetic marks on the N-terminal tail of histone H3, we examined whether Progerin expression affects the level of H3 Ser10 phosphorylation in interphase cells. H3Ser10 phosphorylation was dramatically reduced by Progerin transfection in both HeLa and Cos cells. H3Ser10 phosphorylation was reduced in fibroblasts from 3/3 HGPS patients examined ($p\text{-value}<10^{-4}$), while normal levels of the epigenetic mark were observed in fibroblasts from an unaffected parent of a patient. H3Ser10 phosphorylation was also reduced by inhibiting lamin A cleavage with lopinavir, and Progerin that contains a Cys-Ser substitution in the CAAX motif failed to reduce H3Ser10 phosphorylation. Thus, the Progerin-dependent reduction in H3Ser10 phosphorylation is mediated by constitutive membrane attachment of lamin A. Nuclear levels of RSK2, one of the kinases that deposits the phospho-Ser10 mark in interphase, were reduced only slightly by Progerin. Disruption of the Ran gradient by siRNA

depletion of its import factor NTF2 did not reduce H3 Ser10 phosphorylation, suggesting the effect of Progerin on this chromatin mark is not caused simply by defective import of RSK2 or another H3 Ser10 kinase. Our data on H3 Ser10 together with work from other groups on H3 Lys9 and H3 Lys27 trimethylation shows that lamins attached to the inner nuclear membrane can regulate the epigenetic state of chromatin throughout the nucleus.

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Trafficking and retention of the *Caenorhabditis elegans* SUN protein UNC-84.

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Nuclear envelope SUN/KASH bridges are conserved across eukaryotes and function to maintain nuclear envelope integrity, transmit forces generated in the cytoplasm to the nuclear lamina, and control the stiffness of the global cytoskeleton. Central to all of these functions is the localization and retention of SUN proteins at the inner nuclear membrane. However, the molecular mechanisms used to actively traffic integral membrane proteins from the peripheral ER to the inner nuclear membrane, and retain them there are mostly unknown. In our model of nuclear migration, the *Caenorhabditis elegans* SUN protein, UNC-84, localizes to the inner nuclear membrane and is retained, prior to recruiting the KASH protein, UNC-83, to the outer nuclear membrane.
To better understand how functional SUN-KASH bridges are assembled, we have identified three types of targeting signals (cNLS, SUN-NELS, and INM-SM) in the cytoplasmic/nucleoplasmic domain of UNC-84 that facilitate its localization and function at the inner nuclear membrane. To distinguish whether these signals function in ER to nuclear envelope targeting and/or retention at the inner nuclear membrane, we are examining the kinetics of ER to NE trafficking of both wild-type and mutant versions of UNC-84 using photoactivation, and examining their mobility at the nuclear envelope in HeLa cells. To identify proteins that interact with the cytoplasmic/nucleoplasmic domain of UNC-84, a yeast two-hybrid screen was performed using UNC-84 1-510 and UNC-84 1-385 as baits. The UNC-84 candidate interacting proteins identified have been implicated in ER to Golgi transport, nuclear architecture, nuclear signaling, and pro-nuclear migration. Elucidating these mechanisms in *C. elegans* and mammalian cell culture will provide novel insight into how nuclear membrane biogenesis relates to normal development and the progression of human disease.

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Functional Analysis of the Lumenal Domain of the SUN Protein UNC-84.

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Proper positioning of the nucleus is required for development, cell polarization, fertilization, cell motility, and cell division. In most eukaryotes, a nuclear envelope bridge consisting of an inner nuclear membrane SUN protein and an outer nuclear membrane KASH protein form the LINC complex (for linker of the nucleoskeleton to the cytoskeleton) to link the cytoskeleton to the nuclear lamina. Very little is known about how KASH and SUN protein interact in the perinuclear space or how SUN proteins span the perinuclear space to connect the inner and outer nuclear membranes. We hypothesize that during nuclear migration, the forces generated by microtubule motors are transduced through KASH-SUN bridges across the nuclear envelope to the nuclear lamina. We take advantage of a unique model system that allows quantitative observation of nuclear migration events, hypodermal nuclear migration in *C. elegans*. In this system, the KASH protein UNC-83 and the SUN protein UNC-84 bridge the nuclear envelope and recruit microtubule motors to the surface of the nucleus. Here we report the results of mutational

analysis of the large lumenal domain and SUN domain of UNC-84. We find that the majority of the linker domain between the *trans*-membrane domain and SUN domain is not required to facilitate nuclear migration. However the minimal domain required for function contains a predicted coiled-coil, suggesting that in agreement with the recently solved structure of mammalian Sun2, UNC-84 may function as an oligomer. Based on homology modeling of UNC-84 with the structure of the mammalian Sun2 in complex with the nesprin-2 KASH domain, we have undertaken a structure-function study of the SUN domain of UNC-84. We have mutated several conserved residues in the SUN domain of UNC-84 predicted to mediate interaction with KASH domains. We are currently testing the ability of these mutant SUN proteins to be properly targeted to the nuclear envelope and function during nuclear migration. Understanding how nuclear positioning is accomplished will lead to greater insight into organelle positioning in general, as well as potential therapeutic targets for human diseases.

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Modeling the interplay between nuclear pore complex assembly and the cell cycle.

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Nuclear pore complexes (NPCs) perforate the nuclear envelope (NE) of all eukaryotic cells and control traffic between the cytoplasm and nucleus. Pore assembly is spatially restricted to the NE membrane and temporally restricted to the end of mitosis and to interphase. However, it remains unclear how pore assembly is tuned to the cell cycle to achieve a reproducible density of NPCs. Recent work identifies the core nucleoporin Nup96 as a possible bidirectional regulator of cell cycle progression and nuclear pore complex assembly (1). Nup96 is degraded by the anaphase-promoting complex (APC) during mitosis. Since the APC is activated by cyclin-dependent kinase (Cdk) at the G2/M transition, Nup96 levels are linked to Cdk activity. Conversely, Nup96 inhibits the nuclear export of cyclin D and Cdk6 mRNAs. To understand how Nup96 might influence the oscillations of the cell cycle, we modeled the interactions of Nup96, APC, and activated Cdk using a system of three ordinary differential equations, based on a recent minimal model of the cell cycle (2). We find that a negative feedback loop consisting of APC inhibiting Nup96 and Nup96 inhibiting active Cdk slows the period of the cell cycle. Our model recapitulates the experimentally observed cyclical fluctuations in Nup96 levels and Cdk activity. Nup96 levels plummet during mitosis, but rise again through G1 phase after the APC is inactivated. Activated Cdk accumulates while Nup96 is low but begins to plateau as Nup96 levels rise, with the consequence that higher Nup96 levels slow the cell cycle. Above a certain threshold level of Nup96, the oscillations of Cdk activity halt. This outcome predicts that elevated Nup96 may promote exit from the cell cycle. We next incorporated NPC assembly kinetics into the model, using two additional ordinary differential equations to account for total NPCs and NPCs containing Nup96. Interestingly, we find that the model only recapitulates experimental results showing that Nup96 levels tune the cell cycle if Nup96 can incorporate into all NPCs: those assembled postmitotically and those assembled in interphase. Finally, our model predicts that when the cell cycle is longer, a greater number of NPCs will assemble on the nuclear surface. The predictions of this model have interesting implications for our understanding of Nup96 incorporation into NPCs, regulation of NPC density, and regulation of the cell cycle.

1. Chakraborty et al Dev Cell 2008
2. Ferrell et al Cell 2011

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Nesprin-1-dependent recruitment of centrosome proteins to the nuclear surface is essential for nuclear alignment in myotubes.

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In skeletal muscle cells undergoing differentiation, centrosome proteins relocalize from the pericentriolar material to the outer surface of the nucleus. Using cultures of differentiating mouse C2C12 myoblasts, we are addressing the functional significance of this relocalization: During muscle formation, myoblasts fuse into syncytial myotubes. The multiple nuclei in myotubes are positioned evenly along the fibre, with the exception of clusters of nuclei at the neuromuscular junction. Nuclear positioning is believed to be important for correct muscle function and requires the nuclear envelope protein nesprin-1, since defective nuclear positioning has been found in mice lacking functional nesprin-1, mimicking aspects of Emery-Dreifuss muscular dystrophy. Using RNA silencing of differentiating C2C12 cells, we show that nuclear positioning requires the centrosome protein PCM-1, which is recruited early in differentiation to the nuclear envelope in a nesprin-1-dependent manner. PCM-1 is in turn necessary for the recruitment of components of the dynein/dynactin and kinesin motor complexes, thus suggesting that microtubule motors, bound to the nuclear envelope, transport nuclei along microtubules to position them in the myotube.

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Brambleberry, a novel nuclear envelope associated protein, regulates nuclear membrane fusion during cleavage stage development.

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Compared to later stages, early blastomeres employ modified cell division mechanics presumably to accommodate the nature of the large cells present following zygote formation. During the cleavage stage in zebrafish, karyokinesis involves a step not found at later stages in development whereby individual chromatin masses surrounded by nuclear envelope, called karyomeres, fuse to form a single mononucleus. Using a genetic approach in zebrafish, we identified *brambleberry* (*bmb*), a maternal-effect mutant that disrupts karyomere fusion resulting in the formation of multiple micronuclei. Positional cloning reveals that *bmb* encodes a conserved novel protein with homology to Kar5p, a protein required for pronuclear fusion in yeast. Similar to yeast, Bmb contains a predicted coiled-coiled domain and two C-terminal transmembrane domains. However, unlike yeast, Bmb contains a highly conserved 69-residue domain near the N-terminus that has not been previously described in any other protein, but is present in many potential Bmb homologues in both invertebrates and vertebrates. To gain further insight into how Bmb functions, we generated Bmb polyclonal antibodies. Immunofluorescence experiments reveal dynamic Bmb localization throughout the cell cycle during the cleavage stage. At late prophase, Bmb protein becomes concentrated adjacent to the condensing chromatin. Subsequently, during metaphase Bmb foci are more evenly distributed in the vicinity of the mitotic spindle and ER. Putative Bmb-associated vesicles then assemble on the separating chromosomes during anaphase. As karyomeres form, Bmb protein is found at the nuclear envelope with prominent puncta evident near karyomere-karyomere interfaces corresponding to pre-, post- and active fusion sites. Our studies identify the first factor acting in karyomere fusion and suggest that specialized proteins and mechanisms are necessary for proper nuclear division in large dividing blastomeres. Moreover, since the nuclear envelope is composed of two distinct membranes, the discovery of Bmb has significant implications for the

study of membrane fusion, as most of our molecular understanding in metazoans is limited to fusion of single membrane structures.

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Embryonic nucleus formation requires chromatin-induced microtubule disassembly.

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Chromatin-dependent signals control a variety of vital processes in mitosis. Here we report a novel chromosome-binding protein, Vespera, from *Xenopus* egg extracts, which is exclusively expressed in the early frog embryo and is the homolog of mammalian pluripotency genes *Dppa2* and *Dppa4*. Vespera is SUMOylated upon binding to chromatin by the nuclear matrix-associated E3 ligase PIASy, and this modification is essential for Vespera's activity. Specifically, SUMOylated Vespera destabilizes microtubules in the vicinity of chromosomes, and overexpression of Vespera abolishes metaphase spindle assembly. When Vespera-depleted metaphase extracts are released into interphase to initiate nucleus formation, persistent microtubules are observed, leading to defective nucleus assembly, pinched, lobed nuclear morphology and severely retarded nuclear expansion. Removing persistent microtubules using the drug nocodazole rescues nuclear morphology, and moreover induction of ectopic microtubules using the drug taxol causes the same nucleus assembly defects in control extracts, while it causes no additional defect in Vespera-depleted extracts. Together, these activities of Vespera functionally oppose those of the kinase Aurora B, which instead stabilizes microtubules and inhibits nucleus assembly. Indeed, co-depletion of Aurora B rescues the nucleus assembly defects of Vespera depletion. Similarly, spindle assembly is normally abolished in extracts depleted of Aurora B, but is partially restored by co-depletion of Vespera. Hence, chromatin-induced activation of the antagonistic activities of Vespera and Aurora B finely balances the regulation of microtubule dynamics at mitotic exit, ensuring the critical integrity of nuclear division in the embryo.

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Do Nuclear Proteins Reorganize During Mitosis to Form an Elastic, Gel-like Spindle Matrix?

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The idea of a spindle matrix has long been proposed in order to account for poorly understood features of mitosis. However, its molecular nature and structural composition have remained elusive. In *Drosophila* we have recently identified four nuclear proteins, Skeletor, Chromator, Megator, and EAST that interact with each other and that redistribute during prophase to form a fusiform molecular complex that persists in the absence of polymerized tubulin. Dual-imaging studies of Chromator-GFP and α-tubulin-mCherry suggests that whereas Chromator reorganizes away from the condensing chromosomes at prophase and fills the entire nuclear space, it does not attain an obvious spindle-like morphology until the microtubules begin invading the nuclear space. This suggests that the spindle matrix may exist as a malleable gel-like structure that reorganizes in response to the incoming microtubules, thereby giving rise to a spindle-like appearance. Furthermore, if microtubules are depolymerized with colchicine just prior to metaphase, we find that the spindle matrix decompresses and coalesces around the chromosomes suggesting that microtubules acts as "struts" stretching the spindle matrix toward the poles with the matrix conferring an elastic inward force. Measurements of fluorescence

intensities across the width of an individual spindle structure immunostained with both anti-α-tubulin and antibodies to the spindle matrix protein Skeletor revealed that the peaks of tubulin labeling are notably distinct from the peaks of Skeletor labeling consistent with what might be observed for a gel-like matrix that embeds the invading microtubule spindle structure. Interestingly, a C-terminal deletion construct of the spindle matrix protein Megator diffuses away from the spindle region during mitosis, indicating that Megator requires its coiled-coil N-terminal domain for spindle matrix localization. In addition, this finding suggests that specific interactions between spindle matrix molecules are necessary for them to form a complex confined to the spindle region. Supported by NSF grant MCB0817107.

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The structure and dynamics of nuclear pore proteins in living cells.

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Transport across the nuclear membrane occurs through the nuclear pore complex (NPC), which is a large multiprotein complex with a molecular mass greater than 50MDa. The structure of the NPC has been characterized by electron microscopy but the orientation of individual proteins within the complex is unknown. The transport through the NPC is both selective and rapid. This transport involves the phenylalanine glycine repeat nucleoporins (FG nups). However the mechanism by which the FG-nups selectively transport cargo remains unclear. We have developed a theoretical framework which exploits the symmetry of the NPC and its organization in the nuclear envelope in order to resolve the order and disorder of individual protein domains within the complex. Specific domains of individual nucleoporins (nups) were tagged with GFP and examined using fluorescence polarization microscopy to determine their orientation and dynamics. We characterized the domain organization of the FG nups, which are required for transport of cargo through the NPC. This approach revealed both rigid and flexible domains: the tips of the FG domains are relatively disordered compared to the more ordered NPC-anchored domains. We also determined the orientation of several structural nucleoporins with in NPC. This technique allows the collection of structural information in vivo with the ability to probe the organization of protein domains within the NPC. This has particular relevance for the FG domain nups, which are implicated in the mechanism of cargo transport.

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Sm protein down-regulation leads to defects in nuclear pore complex disassembly and distribution in C. elegans embryos.

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Nuclear pore complexes (NPCs) are large macromolecular structures embedded in the nuclear envelope (NE), where they facilitate exchange of molecules between the cytoplasm and the nucleoplasm. In most cell types, NPCs are evenly distributed around the NE. However, the mechanisms dictating NPC distribution are largely unknown. Here, we used the model organism C. elegans to identify genes that affect NPC distribution during early embryonic divisions. We found that down-regulation of the Sm proteins, which are core components of the spliceosome, but not down-regulation of other splicing factors, led to clustering of NPCs. Down-regulation of Sm proteins also led to incomplete disassembly of NPCs during mitosis, but had no effect on

lamina disassembly, suggesting that the defect in NPC disassembly was not due to a general defect in nuclear envelope breakdown. We further found that these mitotic NPC remnants persisted on an ER membrane that juxtaposes the mitotic spindle. At the end of mitosis, the remnant NPCs moved toward the chromatin and the reforming NE, where they ultimately became NPC clusters. Our results suggest a novel, splicing-independent, role for Sm proteins in NPC distribution and disassembly, and point to a possible link between NPC disassembly in mitosis and NPC distribution in the subsequent interphase.

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The CTD Phosphatase SCPL-2 regulates the lipid phosphatase Lipin at the nuclear envelope to control nuclear envelope dynamics.

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In budding yeast, the phosphatidic acid phosphatase Pah1, which converts phosphatidic acid to diacylglycerol, and its activating protein phosphatase Nem1 (a member of the small CTD phosphatase family) are required to maintain a spherical nuclear shape. Here, we examine the roles of the metazoan homologs of these enzymes in controlling nuclear structure, using the early *C. elegans* embryo as a model. Prior work in this system showed that partial inhibition of the phosphatidic acid phosphatase LPIN-1^{Pah1} affects nuclear envelope disassembly during mitotic entry (Golden et al. 2009; Gorjánácz and Mattaj 2009). We find that penetrant inhibition of LPIN-1^{Pah1} results in profound defects in endoplasmic reticulum (ER) structure, embryo production, and germline development. Consistent with this, LPIN-1^{Pah1}localizes throughout the ER, including the ER-contiguous nuclear envelope. By contrast, the LPIN-1^{Pah1} activating protein phosphatase SCPL-2^{Nem1} specifically localizes to the nuclear envelope, suggesting that it controls LPIN-1^{Pah1} activity at this subcellular location. Consistent with this, inhibition of SCPL-2^{Nem1} by either RNAi or mutation results in a specific defect in nuclear envelope disassembly resembling that in weak LPIN-1^{Pah1} inhibitions. Quantitative imaging of SCPL-2^{Nem1}-inhibited embryos revealed slowed kinetics of lamin removal and formation of abnormally shaped nuclei after chromosome segregation. However, SCPL-2^{Nem1} inhibition did not significantly affect dispersal of inner nuclear membrane proteins into the ER during envelope disassembly. Prior work in *C. elegans* embryos implicated the transmembrane nucleoporin NPP-12^{gp210} in inner nuclear membrane protein dispersal and nuclear envelope disassembly (Audhya et al. 2007; Galy et al. 2008). Double inhibitions of SCPL-2^{Nem1} and NPP-12^{gp210} showed a strongly additive effect on nuclear envelope disassembly, with lamina and envelope components persisting between the separating chromosomes and blocking cytokinesis. Based on these results, we propose that nuclear envelope-localized SCPL-2^{Nem1} locally controls the lipid phosphatase LPIN-1^{Pah1} and that mitotic entry-coupled regulation of this pathway works in parallel to NPP-12^{gp210}-mediated inner nuclear membrane protein dispersal to ensure timely nuclear envelope breakdown.

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Association of persistent DNA breaks with the LINC complex and cytoplasmic microtubules contributes to repair outcome.

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Proteins embedded in the inner and outer nuclear membrane form a complex (the LINC complex) that physically couples the nuclear interior to the cytoplasm. We have shown previously that the nuclear aspect of the LINC complex can associate with chromatin in fission

yeast, mechanically coupling it to the cytoplasmic cytoskeleton. We now show that two types of microtubule-associated LINC complexes exist in *S. pombe*: a centrosomal complex composed of the SUN protein Sad1 and the KASH protein Kms2, and a non-centrosomal complex composed of Sad1 and the KASH protein Kms1. Here, we draw on recent studies in *S. cerevisiae* demonstrating that SUN-domain proteins associate with persistent double-stranded DNA breaks (DSBs) to investigate a potential role for the cytoplasmic cytoskeleton in DNA repair. We show that DNA damage induces a proliferation of non-centrosomal LINC complexes that colocalize with repair foci. Thus, upon LINC complex association, persistent DSBs are subject to both microtubule-dependent mobility and microtubule-generated forces. In the absence of Kms1, the non-centrosomal KASH protein necessary for cytoplasmic microtubule association, DSB repair by homologous recombination becomes less efficient. Further, our genetic analysis shows synthetic enhancing interactions between Kms1 and mediators of homologous recombination as well as synthetic suppressive interactions with other repair pathways. Together, our data support a model in which association of DSBs with the LINC complex regulates the repair process through changes in chromatin mobility and/or associated forces.

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The C terminal domain of Nup93 is essential for assembly of the structural backbone of the nuclear pore complexes.

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Nuclear pore complexes (NPCs) are large macromolecular assemblies that act as gatekeepers for nucleocytoplasmic exchange. They also restrict access to the nucleus by forming a permeability barrier. NPCs are composed of approximately 30 proteins called as nucleoporins (nups). These can be categorized into ones forming the structural skeleton of the pore and others forming the central channel thus imparting functional properties to the pore. During open mitosis in metazoans the nuclear envelope and the NPC breaks down and at the end of mitosis this process is reversed wherein the nucleoporins assemble in an ordered and regulated fashion to form the intact NPC.

We are interested in functionally characterizing the role of one of the structural complexes, the Nup93 complex in NPC assembly. This complex in vertebrates is composed of nucleoporins Nup93, Nup205, Nup188, Nup53 and Nup155. It is known that Nup93 forms two distinct complexes: Nup93-188 and Nup93-205. When depleted individually these complexes are not essential for NPC assembly.

The objective of this study was to know what happens when we deplete both Nup93 containing complexes together, which we did by immunodepleting all three proteins from *Xenopus laevis* egg extracts using antibody against Nup93. This leads to a block in the NE and NPC assembly. This phenotype observed could either be a direct consequence of absence of Nup93 or resulting from co depletion of Nup188 and Nup205. To distinguish these two scenarios we did add back experiments with recombinant Nup93 and concluded that rescue in the block is due to Nup93 alone and not due to the two sub complexes implying that Nup188 and Nup205 together dispensable for NPC assembly.

To know which region of Nup93 contributes to the NPC assembly and function we added back different fragments of Nup93. Surprisingly, the C terminus of Nup93 alone could rescue the phenotype. (Closed nuclear envelope and formation of structural backbone of NPC)

As its known that p62 complex lining the central channel imparts the functional properties to the pore and it interacts with the N terminus of Nup93, we checked this by nuclear import and size exclusion assays. Nuclei where only the C terminus of Nup93 was present were not competent for import and lost their permeability barrier. In contrast nuclei where nup93 full length was added back were transport competent.

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Phosphorylation of FG nucleoporins by ERK p38 MAP kinases is involved in the regulation of nucleocytoplasmic transport.

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In eukaryotic cells, the nuclear pore complex (NPC) is a highly selective, bidirectional transporter for a wide range of material between the nucleus and cytoplasm. It is composed of ~30 different nucleoporins (Nups), and approximately one-third of these Nups are classified as FG Nups due to their content of phenylalanine-glycine repeats, which provide binding sites for nuclear transport receptors (NTRs) during the NPC passage.

Recently, our multi-step phosphoproteomic approach combining IMAC (immobilized metal affinity chromatography) and 2D-DIGE (two-dimensional difference gel electrophoresis) revealed a number of new ERK MAP kinase targets including Nup50. ERK phosphorylation of the FG repeat region of Nup50 reduced its affinity for importin-β family proteins and regulated the permeability properties of the NPC.

Here we show that both ERK and p38 MAP kinases efficiently phosphorylate various FG and non-FG Nups *in vitro*. Phosphate-affinity SDS-PAGE using the Phos-tag ligand followed by Western blotting revealed that these Nups are stoichiometrically phosphorylated by ERK and p38 *in vivo*. Then effects of phosphorylation of different FG repeat regions on interaction with importin-β were analyzed qualitatively and quantitatively. GST pull-down assays showed that interactions of FG Nups with various importin-β family proteins but not with other Nups are sensitive to phosphorylation. Furthermore, MAP kinase activation inhibited nucleocytoplasmic localization of fluorescent reporters in cells. Signal transduction-mediated phosphorylation and regulation of multiple FG Nups may have a broad impact on cellular physiology.

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Overlapping functions of MAN1 and emerin in Xenopus laevis.

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Members of the family of LEM-domain containing proteins are involved in gene regulation by associations with chromatin, the nuclear lamina and proteins of several signal pathways. The most prominent and eponymous members of this protein family are LAP2, emerin and MAN1, which are integral membrane proteins of the inner nuclear membrane and share the so called LEM-domain.
In human, emerin is encoded by the EMD gene which has been identified as the gene mutated in patients suffering from Emery-Dreifuss muscular dystrophy (EDMD). This disease is characterized by contractions of main tendons, muscle wasting and cardiac arrhythmias. The human protein MAN1 is encoded by the LEMD3 gene. Mutations in this gene cause sclerodermatous bone dysplasias, characterized by increased bone density and skin

aberrations. The physiological role of MAN1 is still unclear but there is evidence that MAN1 has an important role in transforming growth factor-beta signalling.
Apart from their specific roles, it is increasingly supposed that LEM-domain proteins share partially overlapping functions. Here we analyzed the possibility of overlapping functions of MAN1 and emerin during the embryogenesis of Xenopus laevis which is a well established model organism.
Because no commercial antibodies are available for Xenopus specific MAN1 (XMAN1), we first generated specific antibodies against XMAN1. As expected after immunostaining, MAN1 was localized to the inner nuclear membrane of somatic and germline Xenopus nuclei. As shown by immunoblot analysis, XMAN1 is detectable in oocytes and all developmental stages during early embryogenesis of X. laevis. Interestingly, prior studies illustrated that Xemerin is absent in germline and is first detectable at stage 41 of Xenopus development (Gareiss et al., 2005).
Whole-mount immunolocalization experiments showed that at stage 41 onwards both emerin and MAN1 are predominantly colocalized in the somites and the neural tube of embryos. Furthermore it was shown by co-immunoprecipitation studies that Xemerin is able to interact with XMAN1 in vitro (Gareiss et al., 2005). Following it is tempting to speculate that XMAN1 and Xemerin share partially overlapping functions during embryogenesis.
In order to analyze overlapping functions of both proteins during the early development of X. laevis, we performed Knock-down studies by microinjection of Oligo-Morpholinos into a blastomere of stage-2-embryos. We therefore registered a delay in embryonic development and abnormal formations in the caudal limb. However co-injections of truncated isoforms of Xemerin revealed a normal embryogenesis. Our results thus show that emerin and MAN1 have overlapping functions during embryogenesis. This enables an insight into the crosstalk of nuclear envelope proteins.

Gene Structure and Transcription

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The action of the sulfur metabolic transcription factors is context-dependent in budding yeast.

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The sulfur metabolic genes are responsible for the conversion of extracellular sulfur-containing molecules into usable sulfur for the cell. The expression of these genes is coordinated by four DNA-binding cofactors (Cbf1p, Met28p, Met31p, Met32p), and a strong transcriptional activator, Met4p, which lacks a DNA-binding domain. Recently, we characterized a gene induction system, which, upon the addition of estradiol to the culture, results in near-immediate transcription of a single gene of interest with minimal background expression changes. We've constructed strains in which we can induce expression of a single sulfur metabolic transcription factor in both prototroph and methionine-auxotroph backgrounds with this system. Direct targets of these transcription factors are determined from microarray expression profiles as those genes whose expression changes immediately following transcription factor induction. We demonstrate that this rapid induction approach can be used as a discovery tool for establishing a causative regulatory relationship between a factor and its targets. By performing experiments in chemostats, we are able to 1) choose the identity of a single limiting nutrient (i.e., phosphate or methionine) and 2) maintain the culture at a constant growth rate. With this approach we establish that the identity and strength of target regulation can depend on the choice of nutrient limitation. By identifying changes in expression, we are able to improve upon the static

regulatory information provided by other methods and directly probe the existence, strength, and direction of regulation between the sulfur metabolic transcription factors and their targets.

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Visualization of Promoter-Enhancer Interactions in silent and active loci of a Drosophila Hox gene.

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In eukaryotes, gene transcription is controlled by regulatory regions (enhancers), which are often located at significant distances from the promoters they regulate. Multiple lines of evidence support the idea that enhancers interact with promoters, while the intervening DNA loops out, however the exact mechanism and the frequency of this interaction are not fully understood. *Drosophila* Hox gene *Ultrabithorax* (*Ubx*) is regulated by multiple enhancers. Two of these enhancers, *bx* and *abx*, are located 34 kb and 51 kb downstream of the transcription start site, respectively. Using small (less than 4.5 kb) DNA probes and 3-D multiplex in situ hybridization (M-FISH), we detect the interactions between the *bx* and *abx* enhancers and *Ubx* promoter in *Drosophila* embryos, while simultaneously detecting *Ubx* transcription from the locus. In the region where *Ubx* is actively transcribed, *Ubx* locus appears relaxed, consistently with the “beads on string”, i.e. nucleosome-only, level of chromatin compaction, while in the regions where *Ubx* is not transcribed, the chromatin appears significantly more condensed. Looping of the *Ubx* locus that is consistent with enhancer-promoter interactions is detected at a high frequency (60-70% of the loci) in the actively transcribing *Ubx* alleles.

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Intragenic Transcription and the Regulation of Protein Function.

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The function of a given gene can be regulated at transcriptional, translational and post-translational levels. Intragenic transcription occurs when initiation of transcription starts from within the coding region but the significance and regulation of intragenic transcription is not well understood. Using budding yeast as a model organism, we found smaller protein isoforms of the conserved spindle midzone protein Ase1 when cells are treated with Hydroxyurea (HU), a drug that inhibits DNA replication and arrests cells in S-phase. We demonstrated that the protein isoforms are not the result of cleavage but of intragenic transcription using Northern Blotting and 5’ Rapid Amplification of cDNA Ends (5’ RACE). After HU treatment, the full length *ASE1* mRNA disappears and a shorter mRNA transcript is present. The result from 5’RACE indicates that the intragenic transcription initiates 756-766 nucleotides downstream of the first start codon. Mutation of the two start codons downstream of the intragenic initiation site abolish generation of the shorter Ase1 isoforms. Interestingly, intragenic transcription of *ASE1* depends on the S-phase checkpoint indicating that it is a regulated process.
Ase1 promotes spindle elongation during anaphase by bundling and stabilizing antiparallel microtubules (MTs) at the spindle midzone. Ase1 proteins can form a homodimer and the domain responsible for dimerization is located within the N-terminal region, while the MT binding domain is at the C-terminal region. The shorter Ase1 protein isoforms resulting from intragenic transcription lack the dimerization domain but contain the intact MT-binding domain. Therefore, we speculated that the shorter Ase1 isoforms play a dominant negative role by competing with the full length Ase1 for MT binding, thereby preventing premature spindle elongation when DNA synthesis is blocked. Using fluorescence microscopy we have shown that cells exhibit slower growth as well as a spindle elongation defect after overexpression of the short isoform of Ase1.

Our results support the hypothesis that intragenic transcription of *ASE1* is induced upon HU treatment in an S-phase checkpoint dependent manner in order to counteract full length Ase1 function. We propose that intragenic transcription may be a new and general way to down-regulate the function of a group of genes in response to growth stress. We have performed a microarray to identify other genes subjected to this regulation and analysis of candidate genes is currently in progress.

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Transcriptional regulation of Kindlin-2.

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Introduction: Kindlin family consists of three integrin interacting proteins namely Kindlin-1, Kindlin-2 and Kindlin-3. Kindlin-2 is the most conserved protein in Kindlin family. In contrast to its two tissue specific paralogs, Kindlin-2 is expressed ubiquitously in different parts of the body. Kindlin-2 gene was primarily identified as Mitogen inducible gene (MIG-2) because of its early induction after serum treatment. Kindlin-2 is involved in a number of important processes i.e. cell to cell contact, cell migration, cell cycle regulation, cancer progression and cancer metastasis. **Objective:** Much of the work has been done to explore the functional aspects of Kindlins, however their transcriptional regulation have not been investigated yet. In this study we are trying to elucidate the mechanism of serum based induction of Kindlin-2 and its physiological as well as pathological implications. **Methods:** The main methods we use are the one used commonly in cell signaling and gene regulation based research projects i.e. routine cell culturing, transfection and drug treatments, Luciferase assay, realtime PCR, western blot, flow cytometry and microscopy etc. **Results:** We identified that IGF1 plays an important role in Kindlin-2 transcriptional regulation. Knocking down of IGF1R showed that IGFI induces Kindlin-2 expression through IGF1 receptor. In addition, the preliminary data suggests that IGF1 based Kindlin-2 expression is controlled by P13 kinase pathway. The Luciferase assay for Kindlin-2 promoter in normal as well as cancer cells shows that transcription factor SP1 is responsible for the basal expression of Kindlin-2. However, it seems that Kindlin-2 expression through SP1 dependent core promoter activation is not involved in the serum or IGF1 based Kindlin-2 induction. In addition, we see a peculiar expression pattern of Kindlin-2 gene in prostate cancer (PC3 cells) cells where serum starvation does not seem to affect Kindlin-2 expression, suggesting other factors controlling Kindlin-2 expression in certain types of cancer. **Conclusion:** IGF1 plays important role in regulation of Kindlin-2 expression through PI3-Kinase pathway. The regulation of Kindlin-2 expression through serum as well as IGF1 does not seem to be controlled at transcriptional level. **Future planning:** Currently we are looking into post transcriptional and post translational modifications which may account for serum or growth factor based induction of Kindlin-2. We hope that elucidation of the mechanism of Kindlin-2 regulation at transcriptional, post-transcriptional and post-translational level will open new avenues in delineating the physiological roles of Kindlin-2 i.e. cell migration, cell adhesion, cell cycle, cancer progression and cancer metastasis.

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Conservation and diversification of cis-regulatory mechanisms of the pax2/5/8 paralog group in chordates.

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During the chordate evolution, the genome duplications produced a number of paralogous genes. While the evolution of their coding sequences has been extensively studied, it remains unclear how the evolution of their cis-regulatory elements occurred and how such cis-regulatory evolution contributed to the development of gene regulatory networks in modern vertebrates. To address these questions, we are studying regulatory mechanisms of vertebrate pax2, pax5 and pax8 genes. These genes have evolved from a single ancestral gene of early cephalochordates, and exhibit overlapping, yet distinct, expression in the developing neural and nephric tissues, including the ear, brain and pronephros.

We compared human, chicken and Xenopus genomic sequences of pax2/5/8 loci, and identified more than 80 conserved non-coding elements (CNEs). We then subjected them to transgenic Xenopusassay, and detected enhancer activities in 16 CNEs of pax2, 3 CNEs of pax5, and 1 CNE of pax8. These enhancer activities are similar and pleiotropic in the neural and nephric tissues, in spite of the divergent expression of endogenous pax2/5/8 genes, suggesting the conservation of upstream genetic pathways for activation of the paralog members. We also found that one of the pax2 enhancers increases its activity in the pronephros in response to both the down-regulation of pax2 and hypersalinity of the culture medium. We are currently investigating whether other enhancers of pax2/5/8 genes possess similar functions to protect embryos from genetic and/or environmental disturbance.

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Structural and functional characterization of DAWDLE in *Arabidopsis thaliana*.

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DAWDLE (DDL) is one of the eighteen genes that encode a protein with a Fork-Head Associated (FHA) domain in *Arabidopsis thaliana*. FHA is a phospho-threonine binding domain found in plant and animal proteins that function in DNA repair and cell cycle regulation. DDL contains an arginine-rich N terminal domain that has sequence similarity with proteins involved in RNA processing. DDL is localized to the nucleus and regulates the level of several small RNAs including microRNAs. Smad Nuclear Interacting Protein 1 (SNIP1), the DDL ortholog in humans and known to regulate RNA stability, is also essential for the production of microRNAs in humans. Our aim is to understand the function of DDL in RNA metabolism in *Arabidopsis*. Two *ddl* T-DNA insertion alleles in the WS-2 ecotype exhibit a strong phenotype that includes pleiotropic developmental defects such as short root and hypocotyl, reduced fertility, and distorted organs. This developmentally delayed phenotype is the result of a reduction of accumulation of small RNAs. In contrast a *ddl* T-DNA insertion allele in the Columbia (Col) ecotype exhibits a weak *ddl* phenotype. The difference of *ddl* phenotype between the two ecotypes is due to a single gene. This natural variation between Col and WS-2 has been mapped to an interval of 0.48 centimorgan on the 5th chromosome. To study the structure-function of DDL, twelve point mutations spanning *DDL* were isolated by Targeting Induced Local Lesions in Genomes (TILLING) analysis and the severity of each point mutation allele is being compared to T-DNA alleles. Traits for comparison include hypocotyl and root development, fertility and microRNA accumulation. To identify DDL interactors, we carried out a suppressor screen and identified a strong, and two weak suppressors of *ddl* that are currently being

mapped. In conclusion, DDL protein being conserved in both animals and plants is expected to play a crucial role in RNA metabolism including that of small RNAs.

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Isolation and Purification of HAP1 Promoter, and Transformation into GFP Producing Plasmid.

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Huntington's disease (HD) is an autosomal dominant neurological disorder, leading to progressive degeneration of the nervous system. Usually becoming apparent in mid-life and progressing for 15-20 years, with increased motor and cognitive impairment. Huntington's disease is a type I polyglutamine(CAG) expansion disease. The disease causes severe changes in the striatum Huntington's disease is associated with a mutant huntingtin gene, responsible for producing the huntingtin protein, since HD is a dominant allele disorder it exhibits mendelian inheritance . The focus of the research conducted in the lab was on the HAP1 promoter gene. HAP1 was one of the first proteins identified to interact with mutant huntingtin protein (htt).

HAP1, or htt-associated protein-1 is ubiquitous in the brain and colocalizes with htt in the cytoplasm of neurons. HAP1 and htt are believed to be associated with intracellular trafficking and endocytosis due to their involvement with other trafficking proteins such as dynactin p150 and kinesin light chain. HAP1 is primarily found to be involved with axonal trafficking and endocytosis possibly playing the role as an adaptor protein between cargos and intracellular transporters. It has been found that in some mutations of the disease mutant huntingtin protein binds more tightly to HAP1 and can cause variation in the onset of the disease .

The promoter region governs where initial transcription will begin for a gene. Through the use of PCR, restriction digests, ligation and transformation of bacteria we can learn much more about the promoter of the HAP1 gene. To find the promoter region of the HAP1 gene, we isolate bands of DNA from an agarose gel using electrophoresis. The isolated DNA is then placed into constructs, transformed into bacteria and transfected into cells for viewing fluorescence.

The focus of research for this gene is whether removing its promoter region will still allow this gene to transcribe the protein. To test this idea we will use several different sizes of Rat DNA that contains the HAP-1 gene and insert them into our plasmid to identify whether or not the GFP(Green Fluorescent Protein)gene will be expressed.

Following the transformation, DNA was extracted and a restriction digest performed. By comparing these results to the initial plasmid, we are in the process of determining which region of the HAP1 gene was successfully transformed into bacterial cells. We will then determine which of the constructs retained functional protein production from the HAP1 gene.

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Extended Mutation Spectrum of Usher syndrome in Finland.

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The distribution of the clinical Usher syndrome (USH) types in Finland is 40% USH3, 34% USH1, and 12 % USH2. All USH3 patients carry the founder mutation in clarin 1 (CLRN1) making the molecular diagnostics of USH3 patients simple. However, recently we reported three novel myosin VIIA (MYO7A) mutations in two unrelated USH1 patients. This study was done to further investigate the USH mutation spectrum in Finnish patients. The Asper Ophthalmics USH mutation chip was used to search for known mutations, and to evaluate the chip in molecular diagnostics of Finnish patients. We analyzed samples of nine unrelated USH patients/families without known mutations and two USH3 families with atypically severe phenotype. The chip revealed a heterozygous usherin (USH2A) mutation, p.N346H, in one patient. Further studies done by sequencing of MYO7A and/or USH2A in three index patients revealed two novel heterozygous mutations, p.R873W in MYO7A and c.14343+2T>C in USH2A. We did not identify definite pathogenic second mutations in the patients, but identified several probably non-pathogenic variations that may modify the disease phenotype. For example, we studied a MYO7A splice site change characterized as a polymorphism that, however, leads to altered splicing patterns in the studied region. In four cases, we could not exclude the possibility of digenisms when variations/mutations occurred in two proteins involved in the USH protein interactome. We identified two families segregating genomic variations in both MYO7A and USH2A, and two families with variations in CLRN1 and USH2A. We conclude that compared with the USH3 founder mutation identified in all Finnish USH3 patients, there is considerable genetic heterogeneity of USH1 and USH2 in Finland, making molecular diagnostics and genetic counseling of patients and families challenging.

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RTR functions in transcriptional silencing during the division phase of male gametophyte development of *Marsilea vestita.*

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The male gametophyte of the water fern *Marsilea vestita* develops from a single cell contained in a desiccated microspore. Development is initiated when the dry spore is placed in water. The single cell in the spore, a meiotic product, then undergoes 9 rapid rounds of mitotic divisions to produce 7 sterile cells and 32 spermatids in less than 5 hours. During the next 5 hours, the spermatids undergo maturation, which culminates in the formation of a complex cytoskeleton and ciliary apparatus. The entire process reaches completion in 11 hours and depends almost entirely on stored transcript and proteins; alpha-amanitin treated spores develop without any anomalies while cycloheximide treated spores fail to progress through the first division cycle. This rapid process depends on precise temporal regulation of translation for the formation of specific proteins required during different phases of development, and on the degradation of proteins that are no longer needed for gamete development. In this study, we describe a protein

we have named RTR, which functions as an essential transcriptional regulator for the temporal and spatial control of transcript abundance during rapid development.

We isolated a transcript for a protein containing a RRM-1 domain (MvU620). Knockdowns of MvU620 by RNAi, resulted in multiple division defects. Cytokinesis was anomalous as the division planes were misplaced, of incorrect shape, incomplete, or absent. Cells with multiple nuclei suggest nuclear division often continued. Despite division defects, the development of spermatogenous cells progressed as cells became rounded, small starch-bearing plastids appeared and gamete nuclei started to elongate. qPCR analyses of RNA isolated early in the division phase showed that in the knockdowns, transcripts of alpha tubulin, G3PDH and a putative nuclear movement protein were up-regulated. Staining with a dichromatic dye, GRSafe, showed a dramatic increase in the abundance of single stranded RNA in the knockdowns, but when spores were treated with the broad-spectrum transcriptional inhibitor Actinomycin D (ActD), or with ActD and dsRNA made from MvU620, the staining patterns were identical to those in untreated cells. These data suggest that MvU620 is involved in transcriptional inhibition during the division phase of spermiogenesis in the male gametophyte, and that in the absence of transcriptional inhibition, early development in the gametophyte is anomalous. We renamed the MvU620 RRM-1 domain containing Transcriptional Regulator RTR. This research was supported by NSF grant 0842525 to S.M.W.

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Microarray Analysis to Probe Gene Expression in [PSI+], a Prion-Containing Yeast Cell.

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Prions are infectious, self-propagating, aggregated particles devoid of nucleic acid which are composed entirely of abnormally-folded proteins. They are examples of epigenetic protein inheritance. Mammalian prions cause fatal neurodegenerative diseases like Creutzfeldt–Jakob disease (humans) and bovine spongiform encephalopathy (cattle).
Yeast prions model some aspects of mammalian prions and allow for extensive studies of basic mechanisms involving prion-cell interactions. We are using the [PSI+] yeast prion which is a epigenetic modifier of translation termination fidelity resulting in nonsense suppression. [PSI+] is a self-perpetuating amyloid-forming conformation of the eRF3 protein and is coded for by the Sup35 gene
The overall goal is to determine whether the presence of a prion protein in a cell affects the mRNA types and levels present in the cell. Transcriptional (mRNA) profiles were obtained using microarrays. Previous work had not shown large (2x) changes in mRNA when the cells were grown in rich YPD media.
Studies by others have shown that the presence of the [PSI+] prion modulates colony morphology and survivability in a variety of (stressful) growth conditions. Our studies were done in minimal medium containing ornithine, a poor nitrogen source, in an attempt to induce stress in the cells. Microarrays done comparing the transcriptional profile of [PSI+] growing in YPD versus ornithine minimal media showed many transcriptional changes many of which were 2x or greater. When the transcriptional profiles of [PSI+] growing in ornithine minimal medium was compared with that of [psi-], several significant two-fold mRNA changes were observed. These results suggest that the effects of a prion protein on a cell's transcriptional profile may be manifested more in stressful medium conditions. The observed mRNA transcriptional differences may help answer what effect a yeast prion protein has on a cell.

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Differential role of transcription factor Nkx2-5 in activation of the ANF gene in developing vs. failing heart.

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Background: Atrial natriuretic factor (ANF) is abundantly expressed in atrial cardiomyocytes throughout ontogeny and in ventricular cardiomyocytes in the developing heart. However, during cardiac failure and hypertrophy, ANF expression can reappear in adult ventricular cardiomyocytes (“re-activation of a fetal cardiac gene program”). Transcription factor Nkx2-5 is one of the major transactivators of the ANF gene during development; but whether Nkx2-5 regulates ANF during cardiac hypertrophy and failure, as well as the identification of its responsive elements in vivo, remain to be elucidated.

Methods and Results: Neonatal cardiomyocytes either expressing or lacking Nkx2-5 isolated from Nkx2-5flox/flox or Nkx2-5flox/flox/Cre mice after maternal injection of tamoxifen were utilized for chromatin immunoprecipitation (ChIP) assay. We identified Nkx2-5 binding to three 5’ regulatory elements (-34, -31 and -21 kb) and the proximal ANF promoter. Chromosome conformation capture (3C) analysis revealed close proximity between the distal elements and the promoter region. A 5.8-kb fragment consisting of these elements transactivated a reporter gene in vivo recapitulating endogenous ANF expression in the inner trabecular layer, which was markedly reduced in Nkx2-5-ablated mice. However, expression of a reporter gene was increased and expanded toward the outer compact-layer in the absence of transcription repressor Hey2 similar to endogenous ANF expression. Functional Nkx2-5 and Hey2 binding sites separated by 59 bp were identified in the -34 kb element in neonatal cardiomyocytes. In adult hearts, this fragment did not respond to pressure-overload, and ANF was induced in the absence of Nkx2-5.

Conclusion: We established a strategy to identify physiologically significant regulatory elements in the ANF gene by ChIP assays with anti-Nkx2-5 antibody. Nkx2-5 likely mediates formation of a chromatin hub in the ANF genomic locus in the developing heart, but this active chromatin hub is not sufficient for the reactivation of ANF expression in the pressure-overloaded heart or during heart failure.

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Molecular Mechanisms for the Synchronized Transcription of the Three Complement C1q Subunit Genes in Dendritic Cells and Macrophages.

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Hereditary homozygous C1q deficiency is rare but it almost certainly causes systemic lupus erythematosus (SLE). On the other hand, C1q levels can decline in SLE patients without apparent C1q gene defects and the versatility in C1q production is a likely cause. As an 18-subunit protein, C1q is assembled in 1:1:1 ratio from three different subunits. The three human C1q genes are closely bundled on chromosome 1 (C1qA-C1qC-C1qB) and their basal and IFNγ-stimulated expression, largely restricted to macrophages and dendritic cells (DCs), is apparently synchronized. We cloned the three gene promoters and observed that, while the C1qB promoter exhibited basal and IFNγ-stimulated activities consistent with the endogenous

C1qB gene, the activities of the cloned C1qA and C1qC promoters were suppressed by IFNγ. To certain extents, these were corrected when the C1qB promoter was cloned at the 3Œ end across the luciferase reporter gene. A 53-bp element is essential to the activities of the C1qB promoter and the transcription factors PU.1 and IRF8 bound to this region. By chromatin immunoprecipitation, the C1qB promoter was co-precipitated with PU.1 and IRF8. shRNA knockdown of PU.1 and IRF8 diminished C1qB promoter response to IFNγ. STAT1 instead regulated C1qB promoter through IRF8 induction. Collectively, our results reveal a novel transcriptional mechanism by which the expression of the three C1q genes is synchronized.

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Clock Gene Expression in Human Keratinocytes.

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Objectives: Living organisms have developed complex timekeeping systems organized into a circadian clock network allowing their physiology to adapt to the daily environmental changes dictated by the solar cycle. Circadian clocks are cell autonomous systems based on clock proteins interacting within negative feedback loops. They have been described in neuronal and non-neuronal cells and are supposed to ensure adequate daily timing of cellular functions. Several skin functions display daily rhythms: e.g. cell renewal has a maximum at night, but little is known about their control. Here, we investigated the expression of the clock genes *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *RevErb-alpha*, *Ror-alpha* and *Ror-beta* in human keratinocytes.
Methods: Primary cultures were established from keratinocytes derived from an abdominal skin biopsy of a healthy 36 year-old donor. Expression profiles of the core clock gene transcripts were assessed over 52 h by qPCR. Data for each gene were fitted to a cosinor-derived sinewave function. All sinewave functions were simultaneously fitted by a non linear least squares regression algorithm which imposed a common endogenous period.
Results: All clock genes (except *Ror-beta*) were expressed in the tested keratinocytes and the expression patterns of their transcripts showed robust circadian rhythmicity (except *Clock*). A common period of 23.06 ± 0.22 h was calculated for the rhythmically expressed genes. Also, as reported for most molecular clockworks, *Bmal1* transcripts oscillated in antiphase with respect to *Per* transcripts. The phases of *Cry* transcripts were opposed approximately 12 h with respect to *Bmal1* while the phase of *Cry1* was 8.5 h phase advanced and the phases of *RevErb-alpha* and *Ror-alpha* were respectively 8 h- and 11 h-delayed.
Conclusion: Our results indicate the presence of an autonomous clock in the keratinocytes. Roles of this clock in the physiology of keratinocytes and skin, as well as the mechanisms by which it is synchronized with environemental changes remain to be determined.

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Clock Gene Expression in Human Melanocytes.

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Objectives: Day-time dependent variations were reported in skin functions. The complex cellular organization of skin and the rhythmic processes that take place in this tissue suggest the potential presence of several molecular clocks in the epidermal layer, localized at the

interface between external and internal environments of the body.We investigated the expression of the clock genes *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, Cry2, *RevErb-alpha* and *Ror-beta* in human epidermal melanocytes.
Methods: Primary cultures were established from melanocytes isolated from an abdominal skin biopsy of a healthy 36 year-old donor. Expression profiles of the clock gene transcripts were assessed over 52 h by qPCR. Data for each gene were fitted to a cosinor-derived sinewave function. All sinewave functions were simultaneously fitted by a non linear least squares regression algorithm which imposed a common endogenous period.
Results: Our results show that all clock genes are expressed in the human melanocytes. The expression patterns of *Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2* and *RevErb-alpha* transcripts showed robust circadian rhythmicity with a common period of 25.22 ± 0.48 h. Phase analysis by adjusting the period to 24 circadian hours showed a phase advance of approximately 7-8 h for *Per1*, *Per2* and *Cry2* and 3 h for *Cry1* as compared to *Bmal1* and a phase delay of approximately 8 h for *RevErb-alpha*.
Conclusion: Our findings indicate the presence of a molecular clock in the human melanocytes that might act locally and possibly interact with other epidermal and/or dermal skin clocks. The role of this circadian clock in the epidermal physiology remains to be determined

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Clock Gene Expression in Human Fibroblasts.

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Objectives: Skin performs functions which are critical for the preservation of body homeostasis, in coordination with the environmental changes. Some of these functions undergo daily variations and it is believed that they are controlled by the circadian clocks which are based on negative feedback loop interactions between specific clock proteins. Recent in vitro studies showed circadian expression of a few clock genes in human dermal fibroblasts. We investigated the expression of the all known clock genes *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *RevErb-alpha* and *Ror-beta* in human dermal fibroblasts.
Methods: Primary cultures were established from fibroblasts isolated from an abdominal skin biopsy of a healthy 36 year-old donor. Expression profiles of the clock gene transcripts were assessed over 52 h by qPCR. Data for each gene were fitted to a cosinor-derived sinewave function. All sinewave functions were simultaneously fitted by a non linear least squares regression algorithm which imposed a common endogenous period.
Results: Our results show that all canonical clock genes are expressed in the human fibroblasts. The expression patterns of *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2* transcripts showed robust circadian rhythmicity with a common period of 22.24 ± 0.39 h. Also, phase analysis showed 11 h delay of *Per1* transcripts as compared to *Bmal1*, while *Per2*, *Per3* and *Cry2* transcripts showed approximately 10 h phase advance and *Cry1* 6.8 h phase advance.
Conclusion: We confirm the presence of a molecular clock in the human fibroblasts, similar to human melanocytes and keratinocytes, and show which are the clock genes involved in time tracking in human fibroblasts. The role of this circadian clock in the dermal (skin) physiology remains to be determined.

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The Wnt5a alternative promoters A and B are differentially regulated by TNFalpha.

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WNT5A is an extracellular glycoprotein that activates both canonical and noncanonical Wnt signaling pathways important in development, differentiation, and tissue homeostasis. Wnt5a expression is often misregulated in cancer. The Wnt5A gene of both human and mouse generates multiple transcripts derived, in part, from alternative promoters. In this study, we analyzed the regulation of two of the Wnt5a alternative promoters we term A and B. 2178 bp and 1981 bp of sequences upstream from the human promoters A and B, respectively, were separately cloned in to a luciferase reporter vector and stably transfected in to NIH3T3 cells, allowing for individual analysis of the promoters. The p2178 promoter A and p1981 promoter B cell lines were treated with 5 ng/mL of TNFalpha for 6 and 12 hrs and luciferase activity determined. Results show that at 6 hr there was no significant increase in promoter A activity, whereas a significant increase of approximately 1.46-fold in promoter B activity was measured. At 12 hrs post TNFalpha treatment, promoter A activity increased 1.2-fold and promoter B activity 2-fold. To determine the involvement of the transcription factor NFkappaB and various effector kinases in the response of the promoters to TNFalpha, NIH3T3 promoter A and B reporter cells stimulated for 6 and 12 hrs with TNFalpha were treated with a NFkappaB inhibitor (JSH-23, 30 μM), ERK inhibitor (U0126, 10 μM), p38 inhibitor (SB203850, 10 μM), or JNK inhibitor (SP600125, 20 μM). At 6 hours for promoter B, the NFkappaB and ERK inhibitors caused a reduction in TNFalpha stimulated activity. At 12 hours for both promoters A and B, only the ERK and p38 inhibitors significantly reduced activity relative to the TNFalpha treated control cells. We designed Taqman primer/probe pairs for selective qRT-PCR analysis of promoter A and B transcripts derived from the endogenous Wnt5a gene. An increase in both promoter A and B relative transcripts levels was detected at 6 hr post TNFalpha treatment. The NFkappaB, ERK, and p38 inhibitors reduced the promoter B transcript levels in the TNFalpha treated cells but promoter A transcript levels were unaffected. Together these data indicate that promoter A and B are differentially affected by TNFalpha. The effect on promoter B involves NFkappaB, ERK, and p38, whereas promoter A is less affected by TNFalpha and the response involves ERK and p38. These results suggest a model for how the alternative promoters could provide a more complex pattern of regulation for the Wnt5a gene.

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Structure and function analysis of TEF and NFκB interaction in regulation of HIF-1α.

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Background: The transcriptional enhancer factor (TEF) family is responsible for the regulation of many genes expressed in cardiac, smooth muscle, and endothelial cells. Our lab previously demonstrated that Related Transcription Enhancer Factor-1 (RTEF-1) can increase angiogenesis via transcriptional regulation of HIF-1a and VEGF in endothelial cells. However, whether RTEF-1 utilizes a cofactor to aid in transcriptional regulation is still unclear. Methods and Results: TEF-1 (TEAD1) or RTEF-1 increased HIF-1a promoter activity by 1.88 ±0.12 and 3.73 ±0.23 fold, respectively, demonstrated by luciferase assay. Introduction of NFκB subunit p50 does not affect TEF-induced HIF-1a promoter activity, however, the introduction of subunit p65 or both p50 and p65 together significantly blocked the activity. Additionally, the physical interaction between TEF-1/RTEF-1 and NFκB were confirmed by co-immunoprecipitation with both p50 and p65-specific antibody. Crystal structure of human TEF-1 and RTEF-1 revealed a

conserved surface interaction with the helix domain of p65 but no interaction with p50 by Rosettadock software, in which the specific NFκB binding domain on TEF sequences were also determined. Moreover, GST pull-down assays indicated that the specific interaction area of NFκB was homologous between TEF-1 and RTEF-1. Conclusion: Both RTEF-1 and NFκB transcriptionally regulate the HIF-1 gene and NFκB may be a possible cofactor for RTEF-1. The structure of the NFκB-binding domain was found in human TEF sequences, and the competitive and negative regulation of the TEF family and NFκB requires HIF-1a transcriptional regulation. This finding may have therapeutic potential for hypoxia-/ischemia disorders.

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Interaction of human Sin3B with Transcription Factor KLF11.

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Sin3 is a global corepressor protein essential for a diverse array of cellular functions. It has established roles in transcription, cell cycle regulation, embryonic development and differentiation. Sin3 is known to regulate transcription of important genes. Well conserved from yeast to human, Sin3 exists as two isoforms in humans namely, hSin3A and hSin3B. The modular nature of Sin3 characterized by the presence of four Paired Amphipathic Helices or PAH domains allow it to serve as a scaffolding protein for the assembly of chromatin remodeling proteins and other proteins. Sin3 proteins do not have a DNA binding domain and access the promoter regions of various genes by binding to suitable transcription factors. While a lot of work has been done to understand protein-protein interactions involving Sin3A in the cell, human Sin3B has not been as well characterized. Our study focused on investigating interacting proteins for hSin3B. KLF11 (Kruppel like factor 11) is one such protein that was already known to interact with Sin3A. Using co-immunoprecipitation experiments in HEK293 cell line, we have found that it also associates with hSin3B. In addition, our yeast two hybrid experiments have shown that the N-terminal region of hSin3B from amino acids 1 to 397, harbouring the three PAH domains, interacts very strongly with KLF11. We also studied the contribution to the interaction by the individual PAH domains by yeast two hybrid experiments and found that it is the first PAH domain of hSin3B (PAH1) that largely contributes to the interaction. In comparison, the hSin3B C-terminal region (from amino acids 448 to 1177) had weak interaction with KLF11. In addition, we found that not only is hSin3B recruited onto the KLF11 binding site on the promoter of Manganese Superoxide Dismutase 2 (MnSOD2), a well-established target gene of KLF11, it also modulated the mRNA expression level of MnSOD2 in mammalian cells. Thus, interaction of the corepressor hSin3B with KLF11 transcription factor is likely to have important implications in control of cell growth.

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The Role of Human Spt6 in Transcription Regulation by RNA Polymerase II.

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Spt6 is a conserved histone chaperone that functions in control of transcription elongation and mRNA metabolism. Spt6 has been previously shown to have interactions with RNA polymerase II, Ref1/Aly, Iws1, and subunits of the exosome. These interactions of Spt6 suggest that all the functions for this protein have not yet been determined. Most of the current characterization of Spt6 has been done in yeast and Drosophila. The purpose of our study is to characterize the role of human Spt6 (huSpt6. We have made a specific antibody for huSpt6 that we will use to perform chromatin immunoprecipitation (ChIP) analysis to map huSpt6 across human genes. To further elucidate the function and importance of huSpt6, we have developed a panel of inducible

Spt6 shRNA lentiviruses. We will use these shRNA to analyze how huSpt6 knockdown affects RNA polymerase II distribution on human genes. This study will provide further insight into the function of Spt6 in relation to regulation of transcription initiation, elongation, and termination.

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Identification of AtWRKY46, a novel microgravity-specific transcription factor, by in silico and gene expression analysis.

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Agilent 43 K *Arabidopsis thaliana* DNA chips were used to detect genes specifically expressed under simulated microgravity stress (SMS). Ten SMS-inducible genes were selected from the DNA chip data and these 10 genes were found to be abundantly expressed in both 6-day-old seedlings and 3-week-old plants. Nine out of the 10 SMS-inducible genes were also expressed in response to the three abiotic stresses of drought, touch, and wounding in 3-week-old *Arabidopsis* plants; WRKY46, however, was elevated only in response to SMS. Six other WRKY genes did not respond to SMS. To clarify the characteristics of the genes expressed at high levels in response to SMS, 20 *cis*-elements in the promoters of the 40 selected genes including the 10 SMS-inducible genes, the 6 WRKY genes, and abiotic stress-inducible genes were analyzed and their spatial positions on each promoter determined. Four *cis*-elements (MYB1AT or TATABOX5, GT1CONSENSUS, TATABOX5, and POLASIG1) showed a unique spatial arrangement in most SMS-inducible genes including WRKY46. In addition, another sequence of six *cis*-elements (SORLIP2AT, ELRECOREPCRP1, two GT1CONSENSUSs, ANAERO1CONSENSUS, and IBOX) were identified only in WRKY46 and CML38, which are both SMS-responsive genes. Taken together, our data indicate that WRKY46 is a novel microgravity-specific transcription factor and the unique spatial arrangement of *cis*-elements in the WRKY46 promoter appears to be important for its response to SMS.

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Insights into Nuclear Reprogramming via Heterokaryon RNA Sequencing.

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Induced pluripotent stem cells (iPS) can be produced from virtually any somatic cell by the overexpression of a few transcription factors, a process termed “nuclear reprogramming”. At present, the molecular mechanisms underlying reprogramming are not well understood, in large part due to an inability to analyze early stages of reprogramming at the molecular level in iPS.
We hypothesized that there are as yet unidentified molecular regulators critical to the early onset of reprogramming. To identify key regulators of reprogramming we developed a synchronous, high efficiency, rapid reprogramming approach consisting of heterokaryons (interspecies multinucleate fused cells) in which activation of human pluripotency genes occurs rapidly (24hrs) and efficiently (70% of single heterokaryons), enabling early mechanistic studies.
We conducted a transcriptome-wide investigation of heterokaryon reprogramming using high throughput RNA sequencing. Heterokaryons were isolated over a three day time-course and subject to RNA sequencing to characterize the early gene expression dynamics of the mouse and human transcriptome during the early stages of nuclear reprogramming. Our results show significant changes in transcriptional output of both the mouse and human nucleus, including the induction of key human pluripotency genes and chromatin remodelers. Our results also validate using heterokaryon RNA sequencing as a discovery tool via the identification of

previously published molecules known to have a role in reprogramming towards pluripotency. In conclusion, the speed and efficiency of reprogramming in the heterokaryon system provides a means to identify critical transcription factors and epigenetic regulators via RNAseq, providing temporal information and transcriptional insights into mechanisms of reprogramming and cell fate.

Cytokinesis I

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Orc6 interacts directly with the Chromosomal Passenger Proteins.

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The origin of replication complex (ORC) is best known for its essential role in replication where it binds directly to DNA to initiate its replication at specific start sites. The ORC is comprised of six subunits, Orcs1-6, which are essential, conserved and constitutively expressed. While in yeast the ORC remains bound to DNA throughout the cell cycle, it is beginning to emerge that the individual ORC subunits have additional roles outwith S-phase in higher eukaryotes. Of particular note, in Drosophila and vertebrate cells Orc6 associates only transiently with the core ORC proteins, and is found on the centromeres in mitotic cells, and in the midbody during cell cleavage. Work from the Stillman lab has shown that siRNA mediated depletion of Orc6 leads to defects in chromosome movements and cytokinesis, a phenotype similar to that exhibited when activity of the mitotic chromosomal passenger complex (CPC) is compromised. In this project we ask whether Orc6 contributes to mitotic events by interacting with the CPC proteins, aurora-B, borealin, INCENP and survivin. We report that Orc6 can bind directly to all CPC proteins in vitro, map their interactions and begin to investigate how Orc6 contributes to late mitotic events in cultured human cells.

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Identification and characterization of novel proteins involved in cytokinesis in C. elegans embryos.

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Cytokinesis is a highly regulated, robust process that involves a large number of proteins. Genome-wide screening in *C. elegans* has probably enabled the identification of the majority of proteins that are essential for cytokinesis. However, due to the redundant mechanisms for cleavage furrow establishment, we postulate that some proteins involved in cytokinesis remain to be identified and studied. To identify some of these proteins, we used an existing allele, *nop-1(it142),* that is defective in pseudocleavage (nop-1: no pseudocleavage), to generate a sensitized background for genome-wide RNAi screening. By high-throughput RNAi screening, we identified thirty candidate genes which, upon depletion, showed embryonic lethality in *nop-1* but not in wild type worms. Seven of these candidate genes, show obvious cytokinesis defects by a secondary screen using time lapse DIC microscopy. These defects include shallow furrow ingression and regression, symmetric division, polar body extrusion, and mixed phenotypes. Here, we report that one of the candidate proteins that shows a synthetic cytokinetic phenotype with NOP-1 regulates microtubules during cytokinesis. Embryos lacking this gene product have several phenotypes that are consistent with depletion of microtubule regulators including a pronuclei rotation defect, chromosome congression and segregation defects, and loss of central spindle integrity. In addition, the extent of pseudocleavage ingression is reduced when this gene

product is depleted. Furthermore, depletion of this gene product results in smaller centrosomes, loss of centrosome integrity, and a slower rate of microtubule nucleation. Thus, we provisionally conclude that this gene product may contribute to the formation and maintenance of the microtubule organizing center (MTOC) during cytokinesis. We conclude that either this gene product independently controls cortical contractility and MTOC organization or even subtle changes in organization of microtubules can affect cortical contractility during pseudocleavage and cytokinesis.

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Structural Transitions in the Centralspindlin Complex Required for Microtubule Bundling During Cytokinesis.

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At anaphase onset, a set of overlapping, antiparallel, non-kinetochore microtubules become bundled to give rise to the central spindle. This structure is essential for the completion of cytokinesis and can dictate the position of the division plane. Central spindle assembly is controlled by the concerted actions of microtubule associated proteins and kinesin family motor proteins, most notably centralspindlin. The centralspindlin heterotetramer is comprised of two molecules each of a kinesin-6 family motor protein, ZEN-4, and a Rho GTPase activating protein CYK-4. Through its targeted localization to a narrow region of antiparallel microtubule overlap immediately following chromosome segregation, centralspindlin initiates central spindle assembly. Although it is known that microtubule bundling by centralspindlin requires the presence of both ZEN-4 and CYK-4, and their ability to interact, the mechanism of action of CYK-4 in microtubule bundling is not clear. Intriguingly, CYK-4 binds ZEN-4 in the linker region between the motor domain and the coiled coil, a key region for motility of other kinesins. This raises the possibility that CYK-4 binding allosterically modifies the structural and/or biochemical properties of ZEN-4. Consistent with this model, CYK-4 binding decreases the rate of motility of ZEN-4 in microtubule gliding assays. To directly address whether CYK-4 binding induces conformational changes in ZEN-4, we used EPR spectroscopy. ZEN-4 molecules comprised of the minimal CYK-4 binding region and the coiled-coil were spin-labeled at specific positions and the distances between sites determined in the presence and absence of CYK-4. We found that CYK-4 binding induces a global structural rearrangement in ZEN-4 that results in a dramatic stabilization of the relative positions of the two linker regions. Additionally, our structural studies indicate that the linker region of ZEN-4 is likely to be folded in both the bound and unbound states. We are currently investigating whether CYK-4 binding alters the positions of the motor domains of ZEN-4 in the presence and absence of microtubules, which could greatly impact the mechanism of motility of ZEN-4 in vivo. Combining these data with other structural considerations, we have developed a working model for the architecture of the centralspindlin complex and are examining the functional implications of these structural changes in ZEN-4 at the single-molecule level.

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Localization of cytokinesis factors to the future cell division site by microtubule-dependent transport.

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The mechanism by which spindle microtubules (MTs) determine the site of cell division in animal cells is still highly controversial. Putative cytokinetic “signals” have been proposed to be positioned by spindle MTs at equatorial cortical regions to increase contractility, and/or at polar

regions to decrease contractility. How factors are localized to the equatorial regions of the cortex is not understood. Here, we test cytokinesis models using computational modeling. We present a simple lattice-based model in which signal-kinesin complexes move by transient plus-end directed movements on MTs interspersed with occasions of uniform diffusion in the cytoplasm. In simulations, we find that these complexes distribute themselves initially at the spindle midzone and then move on astral MTs to accumulate with time at the equatorial cortex, much like central spindilin and chromosome passenger complexes. Simulations accurately predict cleavage patterns of cells with different geometries and MT arrangements and elucidate several experimental observations that have defied easy explanation by previous models. The organization of MTs, rather than differences in MT density, may be a critical determinant of cleavage furrow placement. Thus, our modeling suggests a simple mechanism for how spindle MTs localize signals that stimulate contractility to the future equatorial division site.

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Fission yeast UCS protein Rng3p stabilizes the activity of myosin-II motors during contractile ring assembly.

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UCS (Unc-45-/Cro1-/She4-related) domain proteins constitute a family of myosin regulators conserved from yeast to humans. Yeast UCS proteins are made up of the conserved central and UCS (myosin-binding) domains. Unlike fungal UCS proteins, higher eukaryotes have adapted to associate with Hsp90 through the inclusion of an N-terminal TPR domain. In muscle, UCS proteins have been shown to operate as co-chaperones that function with Hsp90 during the *de novo* folding of myosin-II motors. The fission yeast UCS protein (Rng3p) localizes to actomyosin contractile rings and is essential for cytokinesis and cell growth. Through careful analysis of actomyosin ring dynamics we show that the primary role of Rng3p lies in promoting the activity of the myosin-II (Myo2p) motor during actomyosin ring assembly. Isolation of Myo2p in the absence of Rng3p showed that the UCS protein is not required for its actin binding, but is essential for the motility and actin-activated ATPase activity of Myo2p. Similarly, a mutant form of Myo2p previously shown to be hypersensitive to loss of Rng3p function (Myo2-E1p) binds actin filaments effectively yet lacks motility and ATPase activity. Compared with wild-type Myo2p motors, mutant Myo2-E1p motors were more sensitive to limited proteolysis with trypsin, indicating defects in structural conformation. Rng3p has been proposed to function in the *de novo* folding of Myo2p and all other fission yeast myosin heavy chains. In contrast, our *in vivo* and *in vitro* analysis of fission yeast myosin-I (Myo1p) and myosin-V (Myo52p) indicates Rng3p is not required for the function of these unconventional myosins. Our work suggests Rng3p specifically activates Myo2p motors during contractile ring assembly. We propose a second role for UCS proteins in establishing and promoting myosin motor activity at appropriate actomyosin structures. This role involves maintenance of conformational stability, but is independent of a general role in myosin motor folding involving the TPR domain and Hsp90.

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A transglutaminase-like protein participates in cytokinesis and cell morphogenesis in fission yeast.

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The transglutaminase family, with notable members such as factor XIII, represents a conserved class of enzymes responsible for covalent cross-linking between amines (i.e. lysine side chain) and glutamine residues. Here, we report the identification of a transglutaminase-like protein (Cyk3p) that functions in morphogenesis in fission yeast. The phenotype of a cyk3 knockout

strain indicated a primary role for Cyk3p in cytokinesis. Correspondingly, Cyk3p localizes both to the actomyosin contractile ring and the division septum, promoting ring constriction and subsequent cell separation following ring disassembly. In addition, Cyk3p also appears to play a role in cell shape and integrity, reflected by its localization to polarized growth sites and the stationary phase cell cortex. Furthermore, over-expression of Cyk3p results in a loss of cell polarity and obvious defects in cell shape. Intriguingly, while Cyk3p possesses a transglutaminase domain, it lacks the active site. However, mutagenesis at conserved sites within the transglutaminase catalytic core indicated that this enzymatically inactive domain was essential for Cyk3p function. Collectively, our results and the conservation of Cyk3p across fungi points to a role in cell wall remodeling. In a broader sense, our work illustrates the physiological importance of inactive members of the transglutaminase family which are found throughout eukaryotes. We predict that the proposed evolution of animal transglutaminase cross-linking activity from ancestral bacterial thiol proteases was accompanied by the emergence of a subclass of transglutaminases whose cellular function does not depend on enzymatic activity.

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Cortical Ect2 is recruited by RhoA to potentiate its activity for successful cytokinesis.

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Cytokinesis occurs by the ingression of an actin-myosin ring to divide a cell into two daughter cells. This process requires RhoA activation by the GEF Ect2, which forms a phospho-dependent complex with MgcRacGAP/Cyk-4. This interaction recruits Ect2 to the central spindle, and potentiates its activation, possibly by altering its conformation to relieve autoinhibition of its GEF domain. Since the central spindle forms in anaphase, Ect2's recruitment to the central spindle coordinates RhoA activation with chromosome segregation. However, active RhoA forms a tight zone of localization around the cortex, and it is not clear how this zone is established when the bulk of Ect2 is on the central spindle. Ect2's GEF domain is comprised of conserved DH and PH regions. Although the DH region exchanges nucleotide on RhoA, the PH region has no known function. We found that a subset of human Ect2 is enriched at the equatorial cortex during furrow ingression. Its PH region mediates its cortical localization and is required for RhoA activation. Furthermore, Ect2's cortical recruitment is RhoA-dependent, suggesting a feed-forward pathway promotes Ect2 activation during cytokinesis. We propose that full Ect2 activation requires several steps. First, Ect2 binds to Cyk-4, which alters its conformation to make its GEF domain more accessible to interaction with other proteins and/or lipids, including its substrate, RhoA. Second, active RhoA recruits Ect2 to the cortex where it becomes fully active to generate more active RhoA. At the cortex, Ect2 also interacts with the contractile ring protein anillin, an effector for RhoA and a crosslinker for actin and myosin. In anillin-depleted cells there are fewer microtubules at the equatorial cortex and in Drosophila, an anillin-RacGAP50C/Cyk-4 complex was hypothesized to stabilize central spindle-cortical interactions to maintain the division plane. Drosophila anillin does not interact with the Ect2 homologue, Pebble, while human anillin does not interact with Cyk-4. We hypothesize that the human anillin-Ect2 interaction is functionally analogous to the Drosophila anillin-Cyk-4 interaction.

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p27Kip1 Regulates Cytokinesis via the Control of Citron-Kinase Activation.

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The cell cycle inhibitor p27Kip1 (p27) also has cyclin-CDK independent functions. For instance, p27 binds to and prevents the activation of the GTPase RhoA, thereby regulating cell migration and actin cytoskeleton dynamics. Genetic evidence for these functions was provided by p27CK- knockin mice in which p27 cannot bind or inhibit cyclin-CDK complexes. The p27CK- mutation dominantly causes hyperplastic lesions and tumors in multiple organs compared to wild-type and p27-/- mice, revealing an oncogenic role for p27.

We identified a phenotype of multinucleation and polyploidy in p27CK- mice not present in p27-/- animals, suggesting an important role for p27 in G2/M that is independent of cyclin-CDK regulation. Further analysis revealed that p27CK- caused a cytokinesis and abscission defect. We identified the Rho effector Citron-Kinase as a p27-interacting protein in a mass spectrometry screen. The interaction of p27 and Citron-Kinase was confirmed in vitro and in vivo and both proteins colocalized at the contractile ring and midbody during telophase and cytokinesis. Moreover, overexpression of the minimal p27-binding domain of Citron-Kinase was sufficient to rescue the phenotype caused by p27CK-. Finally, we find that by binding to Citron-Kinase, p27 prevented the interaction of Citron-Kinase with its activator RhoA. Altogether, we propose a novel role for p27 during cytokinesis via the regulation of Citron-Kinase activity.

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Citron kinase is an abscission regulator that controls the localization of anillin and of active RhoA at the midbody.

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Citron kinase (CIT-K) is a conserved protein required for cytokinesis. It has been previously proposed that this protein may function in cleavage furrow ingression downstream of active RhoA. However, it is still not well understood by which mechanisms this protein deploys its function.

In contrast with previous hypothesis, we report here that CIT-K is not required for cleavage furrow ingression, but for abscission. HeLa cells depleted of CIT-K show midbody instability and abscission failure. On the contrary, the CIT-K overexpression leads to midbody stabilization and abscission delay. Studies in CIT-K knockout mice indicate that, in vivo, the protein is required for cytokinesis only in specific cell types, such as the cerebellar granule precursor cells (GPCs). Importantly, we observed the same abscission failure phenotype in primary cultures of GPCs from CIT-K knockout mice.

We show that these defects can be explained mechanistically by the functional interaction of CIT-K with two key players of cytokinesis: RhoA and Anillin.

CIT-K forms a physical complex with Anillin and is required for the localization of this protein at the midbody. Quite surprisingly, we also found that, in late cytokinesis, CIT-K functions upstream of RhoA: it positively regulates RhoA activation state and retains active RhoA at the midbody; in contrast, late CIT-K localization is independent of active RhoA. Finally, RhoA inactivation rescues the abscission delay produced by CIT-K overexpression.

In conclusion, we propose that CIT-K is a crucial abscission regulator that promotes midbody stability through Anillin and active RhoA.

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Mitochondria in Cytokinesis.

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Cytokinesis is the final challenge faced by the dividing cell. In animal cells, cytokinesis begins in anaphase shortly after chromosome separation with the appearance of a cleavage furrow at the equator of the cell. Progressive advancement of the furrow causes the cell equator to become increasingly constricted and eventually leads to division. Mitochondria are organelles that function in energy production and calcium homeostasis. Whether mitochondria function at the cleavage furrow to provide local ATP supply and/or calcium signalling and thereby drive cytokinesis is completely unknown. In order to address this question, we performed live imaging of mammalian cells to determine the localization of mitochondria during cytokinesis. We observe that mitochondria localize to the cleavage furrow after anaphase onset and remain enriched at the cell equator until abscission. Using pharmacological inhibitors of cytoskeleton dynamics, we show that the equatorial localization of mitochondria is dependent on microtubules but not on actin. We also demonstrate that mitochondrial recruitment to the cell equator requires RhoA activity. This project will establish novel roles for mitochondria in cytokinesis and will provide critical insights into the molecular basis of cytokinesis. Such work is of dual importance for cell division research and for the development of improved cancer therapies.

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Role of Survivin in cytokinesis revealed by a separation-of-function allele.

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The Chromosomal Passenger Complex (CPC), containing Aurora B kinase, INCENP, Survivin, and Borealin, regulates chromosome condensation and interaction between kinetochores and microtubules at metaphase, then relocalizes to midzone microtubules at anaphase and regulates central spindle organization and cytokinesis However, the precise role played by the CPC in anaphase have been obscured by its prior functions in metaphase. We have identified a missense allele of *Drosophila* Survivin that allows CPC localization and function during metaphase, but not cytokinesis. Analysis of mutant cells showed that Survivin is essential to target the CPC and the MKLP1 ortholog Pavarotti to the central spindle and equatorial cell cortex during anaphase in both larval neuroblasts and spermatocytes. Survivin also enabled localization of Polo kinase and Rho at the equatorial cortex in spermatocytes, critical for contractile ring assembly. In neuroblasts, in contrast, Survivin function was not required for localization of Rho, Polo, or Myosin II to a broad equatorial cortical band, but was required for Myosin II to transition to a compact, fully constricted ring. Analysis of this “separation-of-function” allele demonstrates the direct role of Survivin and the Chromosomal Passenger Complex in cytokinesis and highlights striking differences in regulation of cytokinesis in different cell systems.

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Anillin acts as a bifunctional linker coordinating midbody ring biogenesis and stable anchoring of the plasma membrane during cytokinesis.

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During cytokinesis, an actomyosin contractile ring drives furrow ingression before transforming itself into a stable midbody ring. How this transformation occurs and how plasma membrane anchoring is maintained throughout cytokinesis is unknown. We have tested the potential contributions from the multidomain scaffolding protein, Anillin.
Coupling timelapse fluorescence microscopy with RNAi-mediated depletions in *Drosophila* S2 cells, we show that Anillin is required for complete closure of the contractile ring and the formation of the midbody ring. Cells depleted of Anillin failed cytokinesis in two distinct ways: some were characterized by oscillating cleavage furrows that failed to stabilize and regressed, as previously described in *Drosophila* (Hickson and O'Farrell, 2008 JCB 180:285-94) and human cells (Straight et al., 2005, MBoC, 16:193-201; Zhao and Fang, 2005, JBC, 280:33516-24; Piekny and Glotzer, 2008, Curr Biol 18:30-6); but the majority formed semi-stable furrows that ingressed almost to completion but still failed to form a midbody ring. Expression of Anillin truncations revealed that Anillin N- and C-termini are independently targeted to the equatorial cortex and perform distinct functions. Anillin N-termini (lacking their C-terminal Anillin homology and PH domains) connected with the actomyosin contractile ring and supported formation of stable midbody ring-like structures, but could not maintain anchoring of the plasma membrane. Conversely, Anillin C-termini (comprising the Anillin homology and PH domains) failed to connect with ring structures but recruited the septin, Peanut, to form ectopic membrane-anchored structures at the equatorial cortex. Anillin and septins were dispensable for membrane anchoring at the contractile ring stage, but essential at the midbody ring stage.
These data demonstrate that Anillin coordinates the transition from contractile ring to midbody ring, and that it does so by controlling and linking two distinct cortical cytoskeletal elements and by promoting stable anchoring of the plasma membrane.

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Anillin mediates feedback between the cortex and microtubules to define the division plane.

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During cytokinesis, communication between the mitotic spindle and the cortex ensures that the division plane bisects segregated chromosomes. Previous studies showed that changing the position of the mitotic spindle causes the formation of a new division plane, even after the onset of furrowing. However, the molecular mechanism(s) that mediates spindle-cortical communication is not fully understood, as there are cues associated with both the astral and central spindle microtubules. Furthermore, it is not clear if there is feedback from the cortex to the mitotic spindle to regulate microtubule stability. Anillin is a scaffold protein that helps maintain the division plane by crosslinking several components of the cell, including the actin-myosin cytoskeleton, septins and RhoA. Recent studies using *C. elegans* embryos showed that anillin also interacts with astral microtubules, which removes it from the cortex to form cortical domains. Furthermore, anillin depletion in combination with central spindle disruption blocks furrowing, even though depletion of each alone does not block furrowing. These results imply that anillin is a key communicator between the mitotic spindle and cortex. We found that although human anillin is highly cortical during cytokinesis, it also localizes to microtubules, and this localization is enhanced after perturbations that disrupt RhoA activity or stabilize

microtubules. Depletion of anillin in combination with central spindle disruption blocks furrowing in human cells. These results suggest that human anillin may function similar to *C. elegans* anillin to model cortical domains via microtubule interactions. However, anillin may also regulate feedback from the cortex to the mitotic spindle. In anillin-depleted cells, there is an overall decrease in microtubule staining in comparison to control cells, which is not phenocopied by depletion of the Rho GEF Ect2. This result suggests that anillin may influence microtubule stability independent of active RhoA. Furthermore, anillin depletion blocks polarization of monopolar cells (where cortical feedback may be more pronounced vs. bipolar cells), and central spindle proteins fail to localize. This data suggests that anillin acts as a communicator between the cortex and mitotic spindle to influence microtubule stability, a key step in promoting symmetry break.

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Regulation of syncytium formation and cell ploidy by a short anillin isoform.

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Cytokinesis is the last step of cell division, in which the two daughter cells physically separate. During certain developmental stages such as germline development, complete partitioning of the cytoplasm and plasma membrane do not occur. Instead, cells undergo incomplete divisions and form a syncytium, wherein multiple nuclei share a common cytoplasm. The molecular basis of syncytium formation is not well known. The *C. elegans* adult germline is a syncytium whose cortex is highly enriched in components of the actomyosin cytoskeleton. Among these is ANI-2, one of the three *C. elegans* homologs of anillin, a conserved multidomain scaffolding protein that plays a fundamental role in cytokinesis. ANI-2 is required in the germline for proper oocyte individualization. Its activity in the embryo is negatively regulated by the polarity kinase PAR-4/LKB1. We hypothesize that ANI-2 promotes germline syncytium formation in *C. elegans* by inhibiting cytokinesis and that PAR-4 functions upstream of ANI-2 in this process. To test this idea, we first analyzed the structure of the oogenic gonad in homozygous *ani-2* mutants and observed germ cell multinucleation, defects in oocyte differentiation and an increase of germ cell apoptosis. Analysis of heterozygous *ani-2* mutants, which have half the normal levels of ANI-2 protein, revealed that the openings between germ cells and the syncytium are significantly larger and that oocytes are individualized precociously. We found that in wild-type gonads, PAR-4 localizes to the nuclei of germ cells progressing through meiotic prophase, but, interestingly to the cortex of mitotic germ cells and of cellularized oocytes. This cortical localization correlates with stages of oogenesis during which cortical dynamics occur and suggests a causative role. Loss of PAR-4 function by mutation reduces the size of syncytial openings and reverts the precocious oocyte individualization defect observed in *ani-2* heterozygous mutants. Together, our results suggest that ANI-2 and PAR-4 have opposite contributions to germline syncytium formation in *C. elegans*.

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Truncation proteins allow dissection of the roles of Anillin in C. elegans zygote cytokinesis.

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Anillin is a conserved multi-domain protein that enriches in the contractile ring during cytokinesis and is implicated in crosslinking multiple regulatory and structural elements including the septins, non-muscle myosin II and actin filaments. When Anillin is depleted by RNA-mediated

interference from the C. elegans early embryo, the contractile ring displays several abnormalities, including abnormal circumferential symmetry. Our in silico model of cytokinesis proposes that feedback among cytoskeletal filament alignment, contractility and membrane curvature drive non-concentric (asymmetric) furrow ingression, thus promoting energy efficiency. The model predicts that Anillin contributes to furrow asymmetry by bundling actin, aligning actin with other cytoskeletal elements, or by coupling the cytoskeleton to the plasma membrane (directly or indirectly). To test what domains of Anillin are responsible for its contributions to cytokinesis, we generated C. elegans strains stably expressing GFP-tagged Anillin truncations. Because these transgenes are not expressed at levels equal to endogenous Anillin, nor do their expression levels respond consistently to depletion of endogenous Anillin, it was necessary to first generate a standard curve of a quantitative phenotype as a function of Anillin protein level. The severity and frequency of the abnormal furrow symmetry phenotype scaled linearly with protein levels. Using our Anillin level standard curve, we found that the predicted myosin-binding domain is not required for furrow asymmetry. In contrast, the C-terminal Anillin and Pleckstrin homology (AH and PH) domains, predicted to associate with Rho, a RhoGAP, the septins and lipids, are required for asymmetry. Truncations encoding the actin bundling domain (ABD) alone or a nearly full-length protein lacking half the ABD are expressed at levels too low to compare with the performance of endogenous Anillin. However, the latter truncation, and one lacking the AH and PH domains, perturb furrow asymmetry even in the presence of endogenous Anillin. The common feature of these constructs is that they are predicted to uncouple actin from the membrane. Together, these results support the idea that by bundling actin and linking the actomyosin cytoskeleton to the membrane, Anillin helps ensure proper furrow geometry and therefore energy efficiency.

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Mechanistic model for ESCRT mediated cytokinetic abscission.

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The ESCRT machinery have recently been shown to be directly involved in the late events of cytokinetic abscission leading to final separation between two daughter cells. Specifically, components of the ESCRT-III complex has been localized to both the midbody dark zone, found at the center of the intercellular bridge connecting the daughter cells, and the narrow constriction site, where abscission occurs. Moreover, the arrival of the ESCRT-III complex to the abscission site correlated temporally with the abscission event itself. This spatio-temporal characterization of the ESCRT machinery in cytokinesis provides for the first time a platform to understand the mechanistic basis of ESCRT mediated cytokinesis. In this work, we integrated biophysical modeling with experimental data to generate a detailed mechanism of action for ESCRTs in cytokinetic abscission. Based on our computational and experimental data we propose that polymerization of the ESCRT-III helix initiate at the edge of the midbody dark zone. VPS4 mediated breakage of the growing ESCRT-III helix enables formation of a separate helix whose diameter relaxes to the ESCRT-III spontaneous diameter of 50 nm. The latter drives constriction of the intercellular bridge membrane that is attached to the surface of the separate ESCRT-III helix, via electrostatic interactions, and in parallel the movement of the separate ESCRT-III helix away from the midbody dark zone. Interestingly, our computational analysis shows that there is an equilibrium distance from the midbody dark zone that is energetically favorable for constriction of the ESCRT-III separate helix. Our experimental measurements of that distance fits very well with the computed distance therefore supporting and validating our model. These finding further suggest that the conserved distance between the midbody dark zone and the abscission site, observed in cytokinesis, is directly determined by the ESCRT-III

complex. This work provides the first mechanistic model for cytokinetic abscission as well as for any specific ESCRT-III mediated process. Its significance to other ESCRT-III mediated events is yet to be discovered.

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Temporal regulation of cytokinetic abscission by mechanical clues.

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The very last step of cytokinesis in animal cells consists in the cleavage of an intercellular bridge containing stable microtubule bundles connecting the two daughter cells. Recently, several studies have described the microtubules organization and the implication of ESCRT components in membrane fission, but neither the triggering signal of the abscission nor the function of the bridge are known yet.
We have been asking whether the abscission process is regulated in time by extrinsic factors of the cell micro-environment. We analyzed the time from anaphase to abscission (abscission timing) in cells plated on different adhesive substrates (glass, fibronectin-coated glass or micro-patterned surfaces). We observed that abscission timing was shorter when cells were more confined. When cells were not confined, we found that motility of the daughter cells inversely correlated with abscission timing: the faster cells migrated, the longer abscission was delayed. Therefore, we hypothesized that tension in the intercellular bridge negatively regulated abscission. To directly demonstrate the inhibitory role of tension on abscission, we performed laser ablation experiments. Stereotyped abscission occurs by severing of microtubules on one side of the central piece (midbody), followed by severing on the other side. We cut microtubules on one side with the laser and found that this induced severing on the other side with the same average time delay observed between severing of both sides in control abscission. Importantly, this induced severing was inhibited by Spastin depletion. Experiments aimed at studying dynamic localization of ESCRT components in the process are underway. Finally we will present data showing that daughter cells have a correlated migration path as long as the intercellular bridge is present, suggesting an interesting function for the bridge in allowing relative positioning of daughter cells following division.
In conclusion, we propose that forces exerted on each other by daughter cells inhibit abscission probably by affecting ESCRT and Spastin localization and/or activity. This would allow daughter cells to remain connected until they reach independent mechanical equilibrium and, in some particular situation, this would allow them to move in a coordinated way until they reach their final position.

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The novel MIT-domain-containing protein MITD1 interacts with ESCRT-III proteins and functions in cytokinesis.

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Endosomal sorting complex required for transport (ESCRT) proteins are required for terminal membrane scission events in several important biological processes, including endosomal intraluminal vesicle formation, viral budding and cytokinesis. Unlike ESCRT-I and –II that are stable heteropolymeric complexes, ESCRT-III proteins assemble into large complexes at membrane target sites. Midbody recruitment of the ESCRT-III proteins are regulated by interactions with a series of microtubule interacting and transport (MIT)-domain-containing proteins including VPS4, spastin and AMSH. Even though many MIT-domain-containing proteins have been identified, it is still not fully understood whether they also function in the ESCRT-III recruitment to the midbody. Here we describe the ESCRT-III interactions of MIT-

domain containing protein 1 (MITD1), a 28-kDa protein that contains an N-terminal MIT domain. MITD1 localizes to the peripheral midbody during cytokinesis, and the MIT domain is critical for its midbody localization. Yeast-two hybrid analyses and co-immunoprecipitation assays demonstrated that MITD1 can self-assemble through its C-terminus, and gel-exclusion chromatography and chemical cross-linking studies showed that MITD1 exists as an oligomer in vivo. Interactions of MITD1 with all the ESCRT-III proteins were examined by yeast-two hybrid assay. Among them, only CHMP1B, CHMP2A and IST1 show strong interactions. Interestingly, sequence analysis revealed that the MIT domain of MITD1 is very similar to that of VPS4 proteins. The VPS4 residues identified by structure studies as required for interaction with ESCRT-III proteins are highly conserved in MITD1. Mutations in these residues of MITD1 inhibit both the interactions with ESCRT-III subunits and its localization to the midbody. We also investigated a role of MITD1 in cytokinesis in HeLa cells using siRNA transfection. We found that depletion of MITD1 increases the number of multinucleated cells, reflecting impaired cytokinesis. The recruitment of CHMP1B and IST1 to the midbody is also inhibited by MITD1 depletion. Taken together, our findings will provide a better understanding of the functional interactions of MIT-domain-containing proteins and ESCRT complexes as well as new insights into their roles in cell division.

Mitosis and Meiosis I

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Purification of CPC from Mitotic Chromosomes.

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Mitosis is regulated at both global levels and local level. While global regulation drives mitotic progression, the localized regulation fine tunes mitosis by turning on and off various activities at specific times and locations. The chromosome passenger complex (CPC) is the best characterized mitotic regulator that operates at the local level. The localization of the CPC is dynamic throughout mitosis, which corresponds to its various mitotic activities. During premetaphase and metaphase the CPC localizes at inner centromere where it corrects misattachment of microtubule and kinetochore and generates spindle checkpoint signals. Little is known about how and where the CPC is targeted to the inner centromere. To address this question, we have established 3 stable lines expressing LAP-Aurora-B, LAP-Borealin and LAP-Survivin respectively. Using immunofluorescence microscopy, we confirmed these LAP-tagged proteins localize properly in cells. The expression level of each tagged protein is about 0.5-1 fold of each endogenous protein. Using these lines we have successfully purified CPC from mitotic chromosomes digested with MNase. By MudPit analysis, we identified 122 proteins as CPC associated proteins at the inner centromere. Among these 122 proteins, only ten have known mitotic functions. Surprisingly, nine of the 122 proteins are involved in DNA repair and sixteen are implicated in apoptosis. The biochemical and biological significance of these interactions are under investigation.

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WNK1 is required for mitosis and abscission.

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WNK (with no lysine (K)) protein kinases are found in all sequenced multicellular and many unicellular organisms. WNKs influence ion balance. Two WNK family members are associated with a single gene form of hypertension. RNA interference screens have implicated WNKs in survival and growth, and WNK1 is essential for viability of mice. We found that in resting cells the majority of WNK1 is localized on cytoplasmic puncta. During cell division, WNK1 localizes to mitotic spindles. Therefore, we analyzed mitotic phenotypes in WNK1 knockdown cells. A large percentage of WNK1 knockdown cells fail to complete cell division, displaying defects in mitotic spindles and also in abscission and cell survival. One of the best characterized WNK1 targets is the protein kinase OSR1 (oxidative stress responsive 1). OSR1 regulates ion cotransporters, is activated in response to osmotic stress by WNK family members, and is largely associated with WNK1. In resting cells, the majority of OSR1, like WNK1, is on cytoplasmic puncta. OSR1 is also in nuclei. In contrast to WNK1, however, OSR1 does not concentrate around spindles during mitosis and does not show a WNK1-like localization pattern in mitotic cells. Knockdown of OSR1 has only a modest effect on cell survival and does not lead to spindle defects. As we are seeking the potential mechanism by which WNK1 may regulate spindle formation and abscission, one of our yeast two hybrid- indentified interacting proteins with WNK1 is dynein light chain- LC8. Though LC8 is associated with several different complexes in addition to dynein motor complexes, we are able to co-immunoprecipitate endogenous dynein motor complexes with WNK1. One of the most important regulatory proteins for dynein motor complexes is dynactin. Loss of dynactin could phenocopy loss of WNK1 in the spindle defects and abscission. Furthermore, loss of dynactin also affects the formation of several dynein motor complexes-dependent organells- including Golgi and lysosomes. When we examined loss of WNK1 cells, we do observe defects of Golgi and lysosomes as well. Currently, we are investigating the mechanism by which WNK1 may affect dynactin and dynein motor complexes through its catalytic or non-catalytic activity.

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Mitotic kinases and the Set1 methyl transferase complex act in concert to regulate mitosis and cell growth.

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In *Aspergillus nidulans*, the mitotic kinases Cdk1 and NIMA are required for the G2-M transition. To gain insight into the mechanism of NIMA function, a synthetic lethal screen was carried out utilizing the deletion of the non-essential *nimA* orthologue, *KIN3*, in *Saccharomyces cerevisiae*. Using the *nimA7* temperature-sensitive allele, four of the synthetic genetic interactions were found to be conserved in *A. nidulans*, including synthetic lethality between *nimA7* and deletion of the ortholog of a subunit of the Set1 methyl transferase complex (*An-swd1*). Importantly a similar synthetic lethality was subsequently found to exist between partial loss of Cdk1 activity (*nimT23*^{ts} allele) and *ΔAn-swd1*. Moreover, since the deletion of *An-set1*, which encodes the catalytic protein, also causes synthetic lethality with partial loss of mitotic kinase phosphorylation, these genetic interactions are caused by the absence of Set1 complex mediated methyl transferase activity.

Analysis of the *ΔAn-swd1* + *nimT23*^{ts} double mutant indicates a role for Set1 complex mediated methylation in the G2-M transition. Cells with partially active Cdk1 exhibit a mild delay in the G2-

M transition but no marked mitotic defects consistent with Cdk1 activation being a biphasic switch. Importantly, in the *ΔAn-swd1 + nimT23^ts* double mutant an enhanced defect in mitotic initiation was observed. This indicates that both Set1 complex mediated methylation and Cdk1 phosphorylation act in conjunction to regulate the G2-M transition.
Unlike cells with partially active Cdk1 activity, cells with partially active NIMA do not exhibit a G2-M delay but instead show lack of coordination between mitotic events. For instance, low NIMA function prevents the normal process of partial nuclear pore complex disassembly while allowing other mitotic events like spindle formation to occur (although often defectively), suggesting that NIMA activation is not a biphasic switch. Furthermore, lack of Set1 complex methylation modifies the phenotype of cells that lack NIMA function resulting in growth defects and a lack of coordination between septation and mitosis. Our results indicate that the synthetic lethality in cells lacking Set1 complex function combined with partial NIMA function is due to a combination of defects in mitosis and septation causing growth defects.
Taken together, these results indicate that Set1 complex methylation and mitotic kinase phosphorylation act in concert in the regulation of mitosis and septation.

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Regulation of the Chromosomal Passenger Complex.

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The *Xenopus* chromosome passenger complex (CPC), composed of Aurora B and its regulatory subunits INCENP, Survivin and Dasra, ensures correct chromosome alignment and segregation during mitosis. The CPC regulates each chromosome autonomously, however, the mechanism is not well understood. Positive feedback can explain how a checkpoint signal generated from a single misaligned chromosome can be amplified to cause mitotic arrest. Our aim is to develop an understanding of the regulation of AuroraB kinase activity and the role of co-factors involved in this process. We have investigated the kinetics of recombinant CPC in vitro. In this study we have also included a few known activators of Aurora B kinase such as microtubules, DNA and Monopolar spindle 1(MPS1). Microtubules have been shown to stimulate AuroraB kinase activity. MPS1, a mitotic dual-specificity kinase has been shown to phosphorylate Dasra to regulate AuroraB activity. Lambda-phosphatase treated CPC lacks critical activation marks such as phosphorylation of the Aurora B T-loop and lacks kinase activity. When a small amount of active CPC is added, the phosphatase-treated CPC is activated, indicating requirement of intermolecular phosphorylation. The kinetics of activation suggests positive feedback. Additionally, we have seen that the catalytic domain of CPC has higher affinity for microtubules under low activity state. Kinase activity of the whole complex is higher than the catalytic domain (AI+TSS) alone, although when AI+TSS is pre-incubated with microtubules it shows comparably high activity as CPC. This suggests that microtubules might act as a platform for inactive CPC to concentrate, where it is activated by auto-phosphorylation and is then released. MPS1 immuno-precipitated from *Xenopus* egg extracts can also activate lambda-phosphatase treated CPC in vitro. In conclusion, our study so far reveals that the activation of the CPC involves a 'trans' phosphorylation event and generates a positive feedback loop, which could be greatly amplified by cofactors like microtubules and MPS1 to generate a robust checkpoint signal from a single unaligned chromosome.

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Inhibitory phosphorylation of CDC2A is required to release CSF arrest in mouse oocytes.

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Entry into and exit from the M-phase in the cell cycle require activation and inactivation of maturation-promoting factor (MPF), a complex of CDC2A and cyclin B. The activity of MPF is regulated by phosphorylation of two highly conserved residues of CDC2A, T14 and Y15. In mouse oocytes, the inhibitory phosphorylations are catalyzed by the WEE kinases, whereas dephosphorylation of these residues is catalyzed by the CDC25 phosphatases. Although regulation of MPF activity by CDC2A phosphorylation is well established during entry into meiosis or mitosis, it remains unclear whether this regulation functions later during the exit from M-phase. Here, we show that MPF activity is regulated by the state of CDC2A phosphorylation as well as cyclin B degradation during meiotic exit. When WEE1B was down-regulated, oocytes were not able to exit from cytostatic factor (CSF)-mediated arrest in response to Ca2+ signals. Conversely, knockdown of CDC25 phosphatases induced release from CSF arrest. Surprisingly, MPF inactivation, mainly regulated by cyclin B degradation during anaphase onset, was associated with an increase in the inhibitory phosphorylation of CDC2A in CDC25 knockdown oocytes. Conversely, this phosphorylation was significantly decreased after WEE1B down-regulation. Furthermore, WEE1B kinase and CDC25 phosphatases were regulated by CaMKII and CDC14 phosphatase during the release from the CSF arrest, respectively. These findings suggest that MPF activity during CSF arrest is maintained not only by cyclin dynamics but also by CDC2A phosphorylation, which is regulated by the balance between CDC2A inhibitory kinases, WEE1, and the counteracting phosphatases, CDC25. Therefore, the concerted action of phosphorylation and dephosphorylation of CDC2A and synthesis and degradation of cyclin B cooperates in fine-tuning the activity of MPF during CSF arrest and MII release in mouse oocytes.

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Casein Kinase 1 Dependent Control of Wee1 Degradation Regulates Mitotic Entry and Cerebellar Granule Cell Proliferation.

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Eukaryotic mitotic entry is controlled by Cdk1, which is activated by the Cdc25 phosphatase and inhibited by Wee1 tyrosine kinase, a target of the ubiquitin proteasome pathway. Here we use a reporter of Wee1 degradation, K328M-Wee1-luciferase, and a kinase directed chemical library to demonstrate that CK1d/e kinase activities are essential for Wee1 degradation and mitotic entry in cerebellar granule cell progenitors (GCPs). Novel small molecule CK1d/e inhibitors (FKL135 and RJR159) specifically disrupted Wee1 destruction and arrested GCP proliferation and development in vitro and ex-vivo (Figure 1 below). Pharmacological inhibition or siRNA knockdown of CK1d/e also reduced Wee1 turnover and mitotic entry of GCPs, underscoring the importance of Wee1 regulation in controlling proliferation during brain development. Since defects in GCP proliferation are associated with medulloblastoma, these findings suggest that small molecule inhibitors of CK1d/e kinase may provide novel cancer therapy.

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Pin1 acts as a negative regulator in G2/M transition.

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Pin1 is the first identified and essential prolyl isomerase (PPIase) for cell division in both yeasts and mammalian cells. Structurally and functionally, Pin1 can be divided into N-terminus WW domain and C-terminus PPIase domain. The WW domain works as a phosphoserine/threonine-proline (pSer/Thr-Pro) binding module that helps Pin1 to target phosphoproteins. The PPIase domain has shown to specifically isomerize pSer/Thr-Pro peptide bond in a defined subset of phosphoproteins including transcription factors and cell cycle regulator. Pin1 therefore provides a novel post-phosphorylation regulatory mechanism that induces conformational changes following phosphorylation to control protein functions. This post-phosphorylation regulatory mechanism is pivotal for controlling many physiological processes and normal cell cycle progression. Deregulation of Pin1 can lead to some human diseases such as Alzheimer's disease and cancer. However, the functional regulation of Pin1 in cells remains unclear. In previous studies, the phenotype of Pin1 depletion is characterized by premature entry into mitosis, followed by mitotic arrest, while overexpression Pin1 inhibits the G2 to M progression, these results indicate that Pin1 is essential for regulation of mitosis at G2 to M transition and may act as a negative regulator for mitotic activity in G2 to prevent lethal premature entry into mitosis. However, very little is known about the mechanism. In this study, we demonstrated that Pin1 can be negatively regulated by Aurora A and involved in controlling G2 to M transition whereas during mitosis, Pin1 will feedback to regulate Aurora A function by promoting the degradation of hBora and the formation of Aurora A/TPX2 complex. Our results showed that Aurora A phosphorylates Pin1 at Ser16 and Ser16 phsophorylation levels consist with Aurora A activity during the cell cycle; Aurora A colocalizes and coimmunuoprecipitates with Pin1; overexpress Aurora A can increase Pin1-S16 phsophorylation and silence Aurora A expression or inhibit its activity can reduce Pin1-S16 phsophorylation. Moreover, we also find that Pin1 interacts with Aurora A cofactor-hBora in a phosphorylation-dependent manner; in Pin1-/- MEF cells, hBora steady-state expression is elevated compare to Pin1 wt MEF cells and re-express Pin1 in Pin1-/- MEF cells, hBora expression is reduced. Pin1 overexpression in cells overrides the effect of Aurora A-mediated Pin1 inactivation and thereby delays mitotic entry through reducing hBora steady-state level by β-TrCP-mediated proteasomal degradation pathway.

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Proper ER morphology and partitioning during mitosis require phosphorylation-driven dissociation of STIM1 from EB1.

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STIM1 is a trans-endoplasmic reticulum (ER) membrane protein that functions as an ER Ca^{2+} sensor in the process of store-operated Ca^{2+} entry (SOCE). In addition, STIM1 functions in ER morphogenesis through its interaction with the microtubule plus-end tracking protein EB1. Via this interaction, STIM1 mediates the extension of ER tubules along growing microtubules. How these distinct STIM1 functions are regulated under physiological conditions is a topic of intense investigation. To this end, we previously showed that STIM1 is phosphorylated during mitosis at sites recognized by the phospho-specific MPM-2 antibody, resulting in suppression of SOCE during cell division. We now test the hypothesis that STIM1 phosphorylation also regulates its interaction with EB1 during mitosis. In fixed interphase cells, eYFP-tagged STIM1 (eYFP-S1) colocalized with the ER and with endogenous EB1. However, during metaphase eYFP-S1 was found in concentric ER sheets surrounding the spindle but was nearly completely segregated from EB1, which localized to spindle microtubules. This suggests that the interaction of STIM1

with EB1 is disrupted during mitosis. To determine whether phosphorylation is responsible, we expressed eYFP-S1 with all 10 putative MPM-2 phosphorylation sites mutated to alanine (eYFP-10A). Remarkably, eYFP-10A exhibited extensive co-localization with EB1 in the metaphase spindle, strongly supporting the hypothesis that phosphorylation negatively regulates the interaction of STIM1 with EB1 during mitosis. Further, concomitant mutation of the EB1 interaction site reversed the colocalization of eYFP-10A with EB1, verifying that EB1 interaction is responsible for this striking effect of mutation of STIM1 phosphorylation sites. In live-cell imaging, eYFP-10A exhibited microtubule tip tracking behavior within the metaphase spindle, similar to that seen with a tagged EB1 construct, whereas tip tracking was not evident with eYFP-S1. Further, when eYFP-10A was co-expressed with ER-targeted dsRed (ER-dsRed), the ER was seen to extend extensively within the spindle. This was in stark contrast to cells co-expressing eYFP-S1 and ER-dsRed or ER-dsRed alone, in which the bulk of the spindle was completely devoid of ER-dsRed with only a small amount localized at the spindle poles. Abnormal localization of ER-dsRed in cells expressing eYFP-10A remained through telophase and abscission, during which ER-dsRed associated with mid-body microtubules. Thus, STIM1 phosphorylation is critical for maintenance of proper ER morphology and partitioning during mitosis.

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Cracking the Eggshell: Protective Barrier Assembly Following Fertilization of the *C. elegans* Embryo.

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Metazoan oocytes have an extracellular coating that governs fertilization. Following fertilization, this covering is altered to prevent polyspermy and protect the developing embryo. In *C. elegans*, a vitelline layer covers oocytes prior to fertilization. Fertilization initiates conversion of the vitelline layer into a trilaminar eggshell consisting of an outer vitelline layer, a middle chitin-containing layer, and an inner layer proposed to serve as a permeability barrier. Here, we characterize CPG-1 and CPG-2, functionally redundant chondroitin proteoglycans that are the first described protein eggshell components. We show that CPG-1 and CPG-2 are delivered to the extracellular space after formation of the chitin layer by cortical granule exocytosis during meiosis I. Although they contain multiple chitin binding domains, CPG-1 and CPG-2 localize to the inner eggshell layer, whereas chitin is confined to the middle layer. We show that the inner eggshell layer is not the permeability barrier for small molecular weight solutes. Instead, this function resides in a previously undescribed layer that assembles between the eggshell and the plasma membrane following meiosis II. Disruption of the permeability barrier leads to solute permeability and osmotic stress. Disruption of the inner CPG-1/2 eggshell layer causes these phenotypes, as well as adhesion of the embryonic plasma membrane to the eggshell and cytokinesis failure. Interfering with chitin layer assembly results in the inner layer phenotypes, plus polyspermy and catastrophic eggshell rupture. We conclude that the eggshell layers and permeability barrier are laid down in a step-wise and cell cycle-dependent fashion, with later assembly events requiring successful completion of previous ones. To build on this work, we conducted an RNAi screen to identify additional genes that regulate eggshell and permeability barrier assembly. Several screen hits rendered the eggshell permeable, with minimal deleterious effects on early embryonic development. We therefore developed a reliable method to permeabilize and immobilize embryos to allow acute drug/inhibitor treatment to study early embryonic processes with live imaging.

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Polar body size is minimized by the centralspindlin complex.

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Polar body formation involves completion of a highly asymmetric cytokinesis that results in a large egg and 2 small polar bodies. Unlike mitotic contractile rings, polar body contractile rings assemble over one spindle pole so that the spindle must move through the contractile ring before cytokinesis. During time-lapse imaging of *C. elegans* meiosis, the contractile ring moved downward along the length of the spindle and completed scission at the midpoint of the spindle, even when spindle length or rate of ring movement was increased. Patches of myosin heavy chain and dynamic furrowing of the plasma membrane over the entire embryo suggested that global cortical contraction forces the meiotic spindle and overlying membrane out through the contractile ring center. Consistent with this model, depletion of myosin phosphatase increased the velocity of ring movement along the length of the spindle. Global dynamic furrowing, which was restricted to anaphase I and II, was dependent on myosin II, the anaphase promoting complex and separase, but did not require cortical contact by the spindle. Large cortical patches of myosin during metaphase I and II indicated that myosin was already in the active form before activation of separase. To identify the signal at the midpoint of the anaphase spindle that induces scission, we depleted two proteins that mark the exact midpoint of the spindle during late anaphase, CYK-4 and ZEN-4. Depletion of either protein resulted in the unexpected phenotype of initial ingression of a polar body ring with twice the diameter of wild type. This phenotype revealed a novel mechanism for minimizing polar body size. Proteins at the spindle midpoint are required for initial ring ingression to occur close to the membrane-proximal spindle pole.

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Regulation of cell wall separation and osmotic stability by the *Saccharomyces cerevisiae* nuclear membrane proteins Brl1 and Brr6.

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The ability of yeast to replicate and divide properly through mitosis is crucial for the maintenance of species stability. Previous work in the budding yeast *Saccharomyces cerevisiae* has suggested that the peripheral nuclear membrane proteins Brr6 and Brl1 may play a role in the regulation of membrane fluidity in response to both environmental change and cell cycle progression (Hodge *et al.*, 2010). To assess the influence of Brl1 and Brr6 on the cell division process, we explored the cell growth and division properties of the temperature-sensitive conditional mutants *brl1-1* and *brl1-2*, as well as the cold-sensitive mutant *brr6-1*. When grown at their restrictive temperatures, all conditional mutant strains exhibited cell separation failures, with several yeast cells chained together or in clusters. In addition, the *brl1* strains showed an increased tendency to grow hypha-like structures and other abnormal shapes, indicating a failure to properly regulate daughter cell development during cell division. Zymolyase digestion analysis was performed to see if such treatment would be sufficient to break up cell chains and clusters. Treatment of the *brr6-1* strain grown at the restrictive temperature resulted in only single and budded cells, suggesting that the previously observed chains and clusters of cells were the result of a failure in cell wall remodeling following mitosis. The *brl1* strains showed a similar tendency following zymolyase treatment but more significantly became mostly desiccated in this same process, suggesting that Brl1 may play a significant role in osmotic regulation. This connection was further established in growth experiments with the osmotic stabilizer sorbitol. Use of 1M sorbitol completely rescued the growth defect of *brl1* mutant cells

grown at the restrictive temperature. Together, these findings strongly link Brl1 and Brr6 to the cell wall remodeling necessary to permit cell separation following mitosis and further implicate Brl1 in osmotic regulation and cell shape determination during the cell division process.

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The Greatwall Substrate Arpp19 Controls Mitotic Entry By Inhibiting PP2A.

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Initiation and maintenance of mitosis requires the activation of protein kinase cyclin B-Cdk1 and the inhibition of protein phosphatase 2A (PP2A), which respectively phosphorylate and dephosphorylate mitotic substrates. The protein kinase Greatwall (Gwl) is required to maintain mitosis through PP2A inhibition however this inhibition appears to be indirect. The objective of this work was the identification of the Gwl substrates that mediate PP2A inhibition and mitotic entry. To that Xenopus egg extracts were biochemically fractionated and submitted to *in vitro* phosphorylation with an ectopic Gwl kinase. The identity of the phosphorylated proteins was subsequently determined by mass spectrometry. We identified cyclic adenosine monophosphate-regulated phosphoprotein 19 (Arpp19) and α-Endosulfine as two substrates of Gwl that, when phosphorylated by this kinase, associate with and inhibit PP2A, thus promoting mitotic entry. Conversely, in the absence of Gwl activity, Arpp19 and α-Endosulfine are dephosphorylated and loose their capacity to bind and inhibit PP2A. Although both proteins can inhibit PP2A, endogenous Arpp19, but not α-Endosulfine, is responsible for PP2A inhibition at mitotic entry in Xenopus egg extracts. These results describe how Gwl activation results in PP2A inhibition. Thus, at mitotic entry Arpp19 phosphorylation by Gwl promotes the binding of Arpp19 to PP2A and the inhibition of this phosphatase allowing the phosphorylation of cyclin B-Cdk1 substrates and promoting mitotic entry.

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APC/C-mediated multiple monoubiquitination provides an alternative degradation signal for cyclin B1.

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The Anaphase-Promoting Complex/Cyclosome (APC/C) regulates progression through mitosis by orchestrating the ubiquitination of cell-cycle regulators such as cyclin B1 and securin. Although Lys48-linked ubiquitin chains represent a canonical signal targeting proteins for degradation by the proteasome, they are not required for the degradation of cyclin B1 in a reconstituted system. Recently, Lys11-linked ubiquitin chains have been implicated in degradation of APC/C substrates, but the Lys11-chain forming E2 UBE2S is not essential for mitotic exit. Together these findings raise important questions about the nature of the ubiquitin signal that targets APC/C substrates for degradation. Here, using a reconstituted system and Xenopus egg extracts, we demonstrate that multiple monoubiquitination of cyclin B1, catalyzed by UBCH10 or UBC4/5, is sufficient to target cyclin B1 for destruction by the proteasome. However, Lys11-linked ubiquitin polymers elaborated by UBE2S become increasingly important when the number of ubiquitinatable lysines in cyclin B1 is restricted. We therefore define a novel proteolytic signal in the ubiquitin-proteasome pathway that confers flexibility in the requirement for particular E2 enzymes in modulating the rate of ubiquitin-dependent proteolysis.

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The Budding Yeast Kinesin Kar3/Cik1 Corrals Interpolar Microtubule Plus Ends During Mitosis.

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During mitosis, spindle pole separation is maintained by interpolar microtubules (ipMTs) that emanate from each pole and that are bundled in the spindle midzone in antiparallel fashion. The budding yeast kinesin-14 Kar3/Cik1, a minus-end directed microtubule motor, is involved in microtubule bundling and localizes to ipMTs' plus ends. However, its exact role in organizing these microtubules is unclear.
To better understand the regulation and organization of spindle microtubules during mitosis, we performed a synthetic lethal screen against a mutant whose spindle microtubules adopt a random length. This screen produced mutant alleles of Kar3 and Cik1, among other genes. Analysis of the spindle localization of these proteins in various backgrounds using still and time-lapse fluorescence microscopy has shed light on how and when this motor is important for proper spindle function. Unlike many spindle components, Kar3/Cik1 foci do not concentrate at a particular point along the spindle axis; instead they are highly mobile, possibly tracking with polymerizing ipMT plus ends. Mutating Cik1 results in poorly bundled ipMTs in anaphase, while mutating the Kar3 motor domain results in a doubling of Cik1 fluorescence intensity, suggesting that motility is required for proper clearance of Kar3/Cik1. In addition, this Kar3 mutant displays defects in metaphase pole separation that appear to be a result of failure to properly create and maintain ipMT bundles. These results confirm that Kar3/Cik1 is necessary for proper ipMT organization and suggest that its role may be to track with polymerizing ipMT plus ends to keep them in the vicinity of the ipMT bundle.

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Dual role of separase during the metaphase-to-anaphase transition ensures chromosome segregation in mammalian cells.

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Separation of sister chromatids has long been recognized as a dynamic and irreversible event in the cell cycle. A fascinate feature of this process in mammalian cells is that the separation occurs abruptly in a highly synchronous manner at the onset of anaphase. Dissociation of sister chromatid cohesion is triggered by the cleavage of cohesin complexes, which is mediated by a cysteine endopeptidase separase. Activation of separase depends on proteolysis of separase inhibitors such as securin and cyclin B1 in metaphase, but the exact timing of activation and the relative importance of these inhibitors are not well understood. Here we developed a fluorescence-based sensor for separase activity, and found that separase is tightly regulated until the end of metaphase when the activity was abruptly detected on entire chromosome lengths. This switch-like activation is achieved by the relative abundance of securin over separase that prevents precocious activation and by securin's ability to target separase to chromosomes, whose function can be largely if not completely substituted by cyclin B1. Significantly, we found that the binding of separase to cyclin B1 is facilitated in anaphase and this binding is required to silence cdk1 activity and to promote sister chromatid separation. We propose that the switch-like activation of separase protease activity and subsequent inhibition of cdk1 is an ideal mechanism to ensure synchronous removal of sister chromatid cohesion and poleward movement of unpaired chromatids in mammalian cells.

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The anaphase B switch: the role of kinesin-13.

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Accurate chromosome segregation in mitosis involves chromosome-to-pole movement in anaphase A and spindle elongation in anaphase B. Several studies suggest that anaphase B spindle elongation is driven by a “sliding filament” mechanism that depends on the action of kinesin-5, a bipolar, MT crosslinking motor which slides apart interpolar (ip) MTs. During metaphase and anaphase A (referred to as pre-anaphase B) in Drosophila embryonic mitosis, MT minus-end depolymerization at the poles balances the outward ipMT sliding, producing MT “poleward flux”, the movement of tubulin subunits from the equator to the poles and maintaining the spindle length constant. We propose that this depolymerization is due to KLP10A, a Drosophila kinesin-13, which is concentrated at spindles poles throughout embryonic mitosis. At anaphase B onset, cyclin B degradation induces suppression of MT depolymerization at the poles, and thus outward ipMT sliding drives spindle elongation.

To test if suppression of KLP10A activity leads to anaphase B spindle elongation, we experimentally reduced KLP10A activity in pre-anaphase B arrested spindles that had reached their steady spindle length. Specifically, we first injected embryos with non-degradable GST-cyclin B (cyc B) to arrest mitosis before anaphase B. Then an anti-KLP10A antibody was injected into embryos that had started anaphase A chromosome segregation, but had no anaphase B spindle elongation. While embryos injected with only cyc B reproducibly showed no evident spindle elongation from metaphase on and arrested in pre-anaphase B for 10-30 min, a subsequent anti-KLP10A injection into the cyc B treated embryos caused abrupt spindle elongation of those pre-anaphase B arrested spindles, at rates similar to wild-type anaphase B elongation rates. Fluorescent speckle microscopy further showed that cyc B injection perturbed the anaphase B switch by allowing MT poleward flux to persist, however, the subsequent KLP10A inhibition converted MT poleward flux to spindle elongation, which mimicked the anaphase B switch. These findings strongly support the hypothesis that inhibition of KLP10A-mediated ipMT depolymerization at the poles allows kinesin-5 ipMT sliding to drive anaphase B spindle elongation in wild type Drosophila embryos.

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Molecular analysis and mathematical modeling of the ‘switch’-like behavior of the metaphase-to-anaphase transition.

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The transition from metaphase to anaphase is one of the crucial steps in the cell cycle, at which the events of sister chromatid separation and mitotic exit need to be precisely coordinated. Central to the transition is the anaphase promoting complex/cyclosome (APC/C), which targets cyclin B and securin for proteasomal destruction. Cyclin B degradation inactivates the cyclin-dependent kinase CDK1 and hence promotes mitotic exit. Securin degradation releases its binding partner separase from the inhibitory association. Subsequently, free separase proteolytically cleaves a cohesin subunit and thereby promotes the loss of chromosomal cohesion and the irreversible separation of sister chromatids.
Surprisingly, sister chromatid separation is highly synchronous and about 20 times faster than the underlying securin degradation. We want to understand how slow securin degradation translates into abrupt sister chromatid separation and how this contributes to the robust

coordination of the events during mitotic exit. To this end, we employ a combination of live cell imaging, quantitative proteomics and computational modeling.
We have established two imaging assays with which we follow securin degradation and sister chromatid separation at high temporal resolution in *Schizosacharomyces pombe*. Consistent with findings in other organisms, we show that in *S. pombe* securin degradation is about a magnitude slower than sister chromatid separation. Furthermore, sister chromatid separation occurs invariantly when 25 % of securin have been degraded. In order to approach the underlying mechanism of this 'switch'-like behavior, we systematically changed abundances or activities of key proteins at the transition and observed the system's response to the alterations. First results suggest that APC/C but not CDK1 activity influences the threshold level at which sister chromatid separation takes place as well as the 'sharpness' of sister chromatid separation. We have subsequently tested several models of the transition, e.g. inhibitor-sequestration or positive feedback models, *in silico* for their ability to reconcile with our experimental data. Currently, we are in the process of testing experimentally the predictive power of the different models by further system changes.

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Mechanism of inhibition of the Anaphase-Promoting Complex by a small molecule antagonist of the IR tail of Cdc20.

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The Anaphase-Promoting Complex/Cyclosome (APC) is a multi-subunit ubiquitin E3 ligase that drives anaphase onset by targeting securin and cyclin B1 for proteolysis by the 26S proteasome. APC activity in mitosis requires the activator Cdc20. Because of the APC¡¯s essential function in mitosis, it is important to understand its catalytic mechanism and how its activity is regulated by the mitotic machinery. Pharmacologically, the APC is a potential target for anti-mitotic cancer drugs.
We previously identified Tosyl Arginine Methyl Ester (TAME) as the first small molecule inhibitor of APC-dependent proteolysis and developed a cell permeable prodrug (proTAME) that induces mitotic arrest in cells. We then showed that TAME inhibits APC activation by competing with the C-terminal IR tail of Cdc20 for APC binding. Here we show that in the absence of APC substrates, TAME ejects Cdc20 from the APC by promoting Cdc20 auto-ubiquitination in its N-terminal region. Ubiquitination in N-terminal region reduces the binding affinity of Cdc20 for the APC. Cyclin B1 antagonizes TAME¡¯s effect by promoting the binding of free Cdc20 to the APC and suppressing Cdc20 auto-ubiquitination. However, TAME causes cyclin B1 ubiquitination to terminate before reaching the threshold necessary for proteolysis, because partially ubiquitinated cyclin B1 has reduced binding affinity for Cdc20 and loses its ability to promote Cdc20 binding to the APC in the presence of TAME. Furthermore, TAME reduces the kcat of the APC/Cdc20/substrate complex without affecting the Km, suggesting that docking of the IR tail of Cdc20 is critical for APC activity beyond recruiting Cdc20. Together these results provide a definitive answer to TAME¡¯s mechanism of action and shed light on the catalytic mechanism of the APC.

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Is Cdk1 phosphorylation of the APC essential?

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Cdk1 promotes the metaphase to anaphase transition by phosphorylating the anaphase promoting complex (APC), a multisubunit ubiquitin ligase that targets specific mitotic regulators

for degradation by the proteasome. The degradation of these proteins is critical for accurate sister chromatid segregation and mitotic exit.
Here we show that overexpression of the kinase Swe1 (the budding yeast homolog of Wee1), which inhibits Cdk1, stabilizes mitotic cyclins and Pds1 (securin), and impairs sister chromatid segregation. These effects are not due to activation of the DNA damage or spindle checkpoint, which both act to inhibit the metaphase to anaphase transition. Furthermore, if Swe1 is overexpressed in anaphase, after degradation of Pds1, we see that Pds1 is re-stabilized, showing that Swe1 can block APC-mediated proteolysis throughout mitosis. Swe1 overexpression also reduces APC phosphorylation in vivo, which results in a drop in the in vitro ubiquitination activity of the APC. In vitro Cdk1 kinase treatment causes an increase in APC phosphorylation, which reactivates the activity of APC isolated under conditions of Swe1 overexpression. We believe the effects of Swe1 are therefore caused by inhibition of Cdk1-dependent phosphorylation of APC subunits, and suggest that Cdk1-dependent phosphorylation of the APC may be essential for mitotic APC activity.
Despite the results of the Swe1 overexpression studies, blocking phosphorylation of Cdk1 consensus sites on two different subunits of the APC (Cdc16 and Cdc27) does not lead to reduced activity of the APC in an in vitro ubiquitination assay. Preliminary genetic analysis, however, suggests that the presence of these phosphorylation sites is essential for viability when a number of the non-essential subunits of the APC have been deleted. In vitro Cdk1/Clb2 kinase treatment of the APC also shows that one of the non-essential subunits, Cdc26, is phosphorylated, indicating the presence of additional Cdk1 phosphorylation sites on the APC. We are currently furthering our investigation of the role of the non-essential subunits of the APC.

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New Roles For The Budding Yeast Wee1 Kinase and PP2A Phosphatase In Regulating Anaphase Onset.

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Wee1 kinase plays a critical role in linking cell growth to cell division. Wee1 phosphorylates and inhibits Cdk1. This inhibition is relieved by the phosphatase Cdc25. Deletion of Wee1 causes cells to divide at a reduced cell size and deletion of Cdc25 promotes increased cell growth and is lethal. Cdk1 inhibition by Wee1 has been shown to delay mitotic onset. The budding yeast Wee1, Swe1, is also important in regulating mitotic onset in response to cues regarding cell morphogenesis. Mutations and drug treatments that inhibit bud growth cause a Swe1-dependent cell cycle delay. This delay is also dependent on Cdc55, a regulatory subunit of the PP2A phosphatase, however the target of PP2A in this response is unknown.

Interestingly, overexpression of Swe1 or treatment with the actin depolymerizing drug Latrunculin A delays cell cycle progression with separated spindle pole bodies, suggesting a role for Swe1 inhibiting anaphase onset. The metaphase to anaphase transition is triggered by Cdk1-dependent phosphorylation and activation of the Anaphase Promoting Complex (APC) associated with its activating subunit Cdc20. Here we show that Swe1 inhibition blocks Cdk1-dependent activation of the APC and that this inhibition plays a role in the timing of anaphase in every cell cycle. Additionally, our preliminary results suggest that PP2A-Cdc55 delays anaphase onset by dephosphorylating Cdk1 sites on the APC.

Although Swe1-dependent inhibition of Cdk1 blocks APC activity, stabilization of the APC substrate Pds1 (securin) does not fully explain the block to anaphase initiation caused by Swe1 activity. We therefore believe the effects of Swe1 are caused by inhibition of both Cdk1-

dependent phosphorylation of APC subunits, as well as Cdk1-dependent inhibition of a second substrate that promotes anaphase in concert with the APC. We are currently trying to identify this critical second Cdk1 substrate (or substrates) whose activation is needed to promote anaphase.

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Dynamics of kinesin-5 and kinesin-14 throughout the anaphase B switch in *Drosophila* syncytial embryos.

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The mitotic spindle is a dynamic protein machine. In *Drosophila* syncytial embryos, Fluorescence Recovery after Photobleaching (FRAP) experiments have shown that tubulin exhibits turnover on the order of a few seconds during all stages of mitosis. However, the extent of recovery changes at anaphase B onset: before anaphase B, recovery is complete all along the spindle, whereas during anaphase B, recovery near the poles is lower. The bipolar kinesin-5 is required during mitosis. Kinesin-14, although not required, acts antagonistic to kinesin-5. What are the dynamics of these two kinesins? We performed FRAP experiments in *Drosophila* embryos expressing either KLP61F-GFP in a *klp61f* mutant background, or GFP-Ncd in an *ncd* mutant background, in each case the transgene rescued the corresponding mutant phenotype. Both motor proteins have turnover rates of 3-4 seconds and recover 80 to 90% of fluorescence intensity. KLP61F-GFP exhibits a decrease in recovery near the poles during anaphase B similar to that seen in tubulin FRAP. Fluorescence Speckle Microscopy (FSM) of these proteins shows that a fraction of KLP61F-GFP motors are stationary on the spindle, while interpolar microtubules (MTs) are slid apart. The other fraction of KLP61F-GFP and most of GFP-Ncd appears to move in both directions. We have developed a computational model that describes the dynamics of the MTs, and the two mitotic kinesins' binding and detachment from the MTs. We are varying the biochemical properties of the motors in *in silico* FSM and FRAP and comparing simulation results with our experimental observations to further study the motors' mode of function and interdependence. KLP61F data conform to a reaction-diffusion model in which most KLP61F is bound to spindle MTs, with the remainder diffusing freely. KLP61F appears to transiently bind MTs, moving short distances along them before detaching.

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Optical traps can stop movements of Mesostoma spermatocyte kinetochores.

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Mesostoma spermatocytes have 3 bivalents, each bipolarly oriented. Each bivalent's kinetochore moves to and away from its spindle pole at speeds of 6-12µm/m in excursions of 6-7µm, reversing its direction at 1-2 minute intervals. Kinetochore oscillations in *Mesostoma* spermatocytes continue for approximately 2 hours prior to the onset of anaphase, making *Mesostoma* spermatocytes ideal for determining the force required for chromosome movement. To study the force required for chromosome movement, we focused a Nd:YVO4 continuous wave 1064 nm wavelength laser using a 63x oil immersion objective (na 1.4) to a single point [Shi *et al Microsc Res Tech* **69** (2006) 894] in order to trap kinetochores that moved to or away from their poles. We were able to stop movement as the kinetochore moved in either direction using laser power of 300mW or greater in the focused spot but kinetochore movement did not recover when the trap was released. Consequently we lowered the laser power and were able to stop chromosome movements with powers as little as 26mW in the focused spot. Movements

recovered when the kinetochores were released from traps with these lower powers. When individual kinetochores were stopped by the traps the oscillatory movements of their partner kinetochores were not affected. From the power in the trap we calculated that the minimum force required to stop chromosomes moving to the pole or away from the pole is of the order of 10^{-6}dynes (10^{-11}Newtons).

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Investigating the role of SPD-3 in homolog pairing and synapsis of meiotic chromosomes in *C. elegans.*

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Faithful segregation of chromosomes at the first meiotic division is dependent on proper homologous pairing and synapsis. In *C. elegans*, specific regions on each chromosome called Pairing Centers are essential for linking chromosomes to the microtubule cytoskeleton, thereby facilitating chromosome motion required for the proper coordination of pairing and synapsis. Previous work from our lab has shown that specific nuclear envelope proteins aggregate at sites of Pairing Centers and are important for the establishment of chromosome-MT bridges across the nuclear envelope. In the absence of the inner nuclear membrane protein SUN-1 and KASH-domain bearing protein ZYG-12, chromosomes fail to pair and undergo inappropriate synapsis with random, nonhomologous partners. How these components execute their function to facilitate proper pairing and synapsis remains to be determined.

A candidate screen for new factors involved in meiotic chromosome dynamics revealed a potential role for SPD-3 in pairing and synapsis. A temperature-sensitive allele, *oj35*, results in variable meiotic defects when shifted to the restrictive temperature (25°C) at the L4 stage. To deplete SPD-3 more completely, I performed RNAi in *spd-3(oj35)* mutants at the restrictive temperature. *spd-3(oj35, RNAi)* mutants undergo extensive nonhomologous synapsis in pachytene, similar to that seen in *sun-1* loss of function mutants, indicating that SPD-3 may play a similar role in coordinating pairing and synapsis.

Using SPD-3 as bait in a yeast two-hybrid screen revealed potential interactions with a putative ubiquitin ligase RFP-1 and a small, uncharacterized protein, K12H4.2, among other candidates. Both *rfp-1* and *K12H4.2* exhibit a synthetic genetic interaction with *spd-3*. Knockdown of either RFP-1 or K12H4.2 by RNAi shows no meiotic defects. However, knockdown of either protein in the *spd-3(oj35)* background results in nonhomologous synapsis even at the permissive temperature. These observations suggest that SPD-3 is a bona fide interactor of both RFP-1 and K12H4.2. I am continuing to investigate the function and relationship of these proteins and how they contribute to early events of meiosis in C. elegans.

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CDK16/PCTAIRE kinase 1 is activated by Cyclin Y and is essential for spermatogenesis.

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Cyclin dependent kinase 16 (CDK16, PCTK1) is a poorly characterised protein kinase highly expressed in the brain and in the testis. We found that membrane-associated cyclin Y (CCNY) binds to and activates CDK16. The PKA activator forskolin blocked, while kinase inhibition promoted, CCNY-dependent targeting of CDK16-GFP to the cell membrane, implying a kinase-

controlled mode of cyclin-CDK interaction. CCNY binding to CDK16 required a region upstream of the kinase domain and was found to be inhibited by phosphorylation of serine 153, a potential PKA phosphorylation site. Thus, unlike other CDKs, CDK16 is regulated by phosphorylation controlled cyclin binding. In human cell lines, endogenous CDK16 was phosphorylated at S153 and inactive. In contrast, CDK16 isolated from murine testis was less phosphorylated, interacted with CCNY and exhibited kinase activity. Mice lacking CDK16 developed normally but male mice were infertile. Spermatozoa isolated from the epididymis of *Cdk16* knockout mice displayed thinning and elongation of the annulus region, adopted a bent shape and showed impaired motility. Moreover, CDK16-deficient spermatozoa had malformed heads and excess residual cytoplasm suggesting a role of CDK16 in spermiation. Thus, CDK16 is a membrane-targeted CDK essential for spermatogenesis.

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Protein Phosphatase 1 is Required Downstream from Cdk1 Inactivation in Exit from Mitosis but Cdc14 Phosphatase is not.

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We show that inactivation of Cdk1/Cyclin B (Cdc28/Clb2 in the budding yeast, Saccharomyces cerevisiae) induces cells to leave mitosis and return to interphase. S. cerevisiae strains carrying the mutation cdc28-as1, which makes Cdc28 (Cdk1) uniquely sensitive to inhibition by a specific ATP analog, were arrested at metaphase and then treated with the analog. These cells did not undergo cytokinesis but did exit mitosis based on the following observations: Rebudding was initiated, MAT a cells underwent “shmooing” when treated with alpha-factor, Sic1-GFP was stabilized in the nuclei, and the mitotic cyclin Clb2 and the securin Pds1 were degraded. These results show that specific inhibition of Cdk1 (Cdc28) in mitotic cells is sufficient to induce mitotic exit. They also provide a chemical genetic system in which to test whether particular gene products are required downstream from Cdk1 inactivation in the transition from mitosis to G1-phase. Using this approach and various conditional mutations in protein phosphatase genes, we demonstrate that Protein Phosphatase 1 (Glc7 in S. cerevisiae) is required downstream from Cdk1 inactivation during exit from mitosis, but the Cdc14 Phosphatase is not.

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Dynamic mobility of Chk2/Mnk is required for DNA damage responses in early Drosophila embryos.

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DNA damage that persists in cells can cause severe problems including cancer and genetic diseases. A tumor-suppressor Chk2 (CHeckpoint Kinase 2) is one of the major signal transducers that receive the DNA damage signal from upstream kinases such as ATM (Ataxia Telangiectasia-Mutated) or ATR (AT and Rad3-related), amplify the signal, and target cellular effectors to cause a wide variety of DNA damage responses. The *Drosophila* homolog of Chk2, which is known as Mnk or Loki, is essential for DNA damage responses in early embryos. The responses include a block of mitotic progression (centrosome/sindle disruption and spindle checkpoint activation), nuclear dropping from the embryo cortex during the syncytial blastoderm stage, and blocks of transcriptional activation of zygotic genes and termination of a rapid cleavage cell cycle at the midblastula transition. We have shown that Chk2/Mnk dynamically changes its localization to the nucleus and the centrosomes during interphase, and to the

centrosomes (spindle poles), kinetochores, midbody, and pseudo-cleavage furrows during mitosis, by observing transgenic embryos that express EGFP-Mnk under the control of UAS/GAL4. To understand the mechanisms of embryonic DNA damage responses, we have further created a variety of transgenic strains that are designed to express deletions or point mutants of Mnk conjugated with EGFP. We have shown that the FHA (Fork-Head Associated) domain of Mnk plays essential role to recruit the Mnk to the key sub-cellular locations such as the centrosomes and kinetochores, by means of its phosphopeptide binding property. The DNA damage responses described above do not occur in *mnk* null mutant embryos. We investigated functional competency of the each Mnk mutant construct (conjugated with EGFP) to rescue *mnk* null mutant phenotype. We found that the SQ/TQ motif, function of the FHA domain, and the kinase activity were all required for the Mnk function to cause the mitotic disruption and the nuclear dropping in response to DNA damage. We also disrupted interphase nuclear localization of Mnk by deleting a putative NLS motif, and the Mnk mobility that was required for targeting the Mnk to the sub-cellular locations other than the nucleus by tethering the Mnk onto chromatin. We found that both the nuclear recruitment and the mobility of the Mnk were essential to the embryonic DNA damage responses.

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The role of mRNA export factor RAE1 in leukemogenesis.

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Chromosomal translocations involving chimeric fusions of the nucleoporin NUP98 protein have often been described in acute myelogenous leukemia (AML). All the fusion proteins have an identical NUP98 N-terminus, which contains the GLEBS motif for interaction with the mRNA export factor RAE1, and FG repeats that associate with the transcription factors HDAC1 and p300. It is virtually unknown whether these interactions affect leukemogenesis. Many human cancers have irregular chromosome content, a condition known as aneuploidy, and we previously showed that RAE1 depletion caused aneuploidy. Therefore we speculated that RAE1 may also be directly involved in NUP98 fusion-mediated leukemogenesis. We show here that knockdown of NUP98 by RNA interference method caused severe chromosome segregation defects and disrupted RAE1 but not HDAC1 expression and localization. Next, we performed rescue experiments to confirm that the RAE1-NUP98 complex orchestrates proper chromosome segregation. Interestingly, we found diverse behaviors of NUP98 and the leukemogenic fusion protein NUP98-HOXA9 throughout the cell cycle. Strikingly, in NUP98-HOXA9-transfected cells, RAE1 protein were reduced and mislocalized. Our cellular interpretations were further confirmed by NUP98-HOXA9 transgenic mice and the NUP98-HOXA9 AML patient. These data suggest that RAE1 orchestrates NUP98-mediated leukemogenesis and raise the possibility that targeting this system may provide a new strategy for the therapy of aggressive leukemias.

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SPIM imaging of live cell division dynamics in 3D in large spheroid tumor models.

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Multicellular tumor spheroids are models of increasing interest for cancer biology studies; however, 3D imaging in living spheroids is difficult and has never been reported with large spheroids. Here, we have engineered spheroids expressing a fluorescent reporter protein, and specifically designed sample holders to image cell division dynamics in large spheroids in 3D

with single-plane illumination microscopy, paving the way for studies of tumor cell proliferation in this model.

G1, G1-S, and S Phase Regulation

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Regulation of DNA Polymerase ζ by Cdc7/Dbf4 kinase.

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Translesion DNA synthesis (TLS) is a tolerance mechanism in response to DNA damage. In *Saccharomyces cerevisiae* there are three TLS polymerases; Rev1, polymerase eta (Polη/Rad 30), and polymerase zeta (Polζ), composed of the Rev3 and Rev7 proteins. These specialized low-fidelity polymerases, through a polymerase-switching mechanism, allow the DNA replication machinery to replicate past DNA lesions which escape repair prior to replication. It is known that Cdc7, the catalytic subunit of Dbf4-dependent kinase (DDK), is required for UV-induced mutagenesis and our lab has previously shown that *CDC7* and *REV3* are epistatic in response to UV exposure, suggesting that Cdc7 has a direct role in Polζ-mediated TLS (Pessoa-Brandão and Sclafani, 2004). Here, we identify Rev7 as a potential substrate of Cdc7 and have shown that the putative DDK phosphorylation sites of Rev7 are required for UV-induced mutagenesis. These findings are the first demonstration of Rev7 regulation by direct phosphorylation and provide a new model for Polζ function in TLS.

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Chk1 Phosphorylation by p90 Ribosomal S6 Kinase (p90 RSK).

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In response to DNA damage, ATR phosphorylates Chk1 at Ser317 and Ser345, which then induces Chk1-Ser296 autophosphorylation. These Chk1 phosphorylation events are required for the transmission of checkpoint signals to arrest cell cycle until the repair of damaged DNA. However, less is known about Chk1 phosphorylation in other cellular events. In this study, we found that Chk1 was phosphorylated specifically at Ser280 and translocated from cytoplasm to nucleus in response to serum (growth factor) stimulation. Chk1 mutation at Ser280 to Ala attenuated nuclear Chk1 accumulation although the mutation to Glu had a reverse effect. The treatment with p90 RSK inhibitor impaired not only Chk1-Ser280 phosphorylation but also nuclear Chk1 accumulation. The *in vitro* analysis supported that p90 RSK directly phosphorylated Chk1 at Ser280. These results suggest that p90 RSK controls nuclear Chk1 accumulation through Chk1 phosphorylation. We consider that this signaling pathway may play an important role in the preparation to monitor genetic stability during cell proliferation.

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Chk1-S is a ubiquitously expressed splice variant and endogenous inhibitor of Chk1 that regulates cell cycle and DNA damage checkpoints.

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Checkpoint Kinase 1 (Chk1) is essential for normal cell cycle progression and a critical regulator of cellular DNA damage response. Chk1 activation during the unperturbed S-phase and during

DNA damage response prevents mitotic entry till DNA is properly replicated or repaired. Presently, it is unclear how Chk1 activity is controlled during normal cell cycle progression. After DNA damage, Chk1 is activated by ATR-mediated phosphorylation; however it is not entirely clear how the phosphorylation results in Chk1 activation. Here we report an N-terminally truncated alternative splice variant of Chk1, Chk1-S that is ubiquitously expressed in most mammalian cells and tissues. Importantly, we show that Chk1-S is an endogenous repressor and regulator of Chk1. In unperturbed cell cycle, Chk1-S interacts with and antagonizes Chk1 to promote S to G2/M phase transition. During DNA damage, Chk1 is phosphorylated and the phosphorylation disrupts the Chk1/Chk1-S interaction, resulting in activation of Chk1 and cell cycle arrest. We propose that Chk1-S temporally regulates Chk1 activity and is thus essential for cellular proliferation.Chk1-S is mainly expressed during the G2/M phase and its expression is high in proliferating normal human fetal and cancer tissues. Interestingly, Chk1-S over-expression results in untimely inhibition of Chk1 activity resulting in premature mitotic entry and mitotic catastrophe. In tumor xenografts, overexpression of Chk1-S induces mitotic catastrophe and reduces tumor growth. Identification of the widely expressed Chk1-S as a novel splice variant and key regulator of Chk1 provides new insights into cell cycle regulation, DNA damage response, and cancer therapy.

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Determining Functional Conservation of β-Finger Hairpin Structures Between Archaeal MCM Proteins and MCM Proteins of Saccharomyces cerevisiae.

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Objective: The objective of our research is to characterize the in vivo function of conserved beta–finger structures identified in the archaeal MCM atomic structures. Mini-chromosome maintenance (MCM) proteins form complexes that are required for DNA replication and are highly conserved throughout evolution. The replicative helicase of eukaryotic organisms is composed of the six paralogs MCM2-7, which form a heterohexameric ring structure. In contrast, the structure of the archaean replicative MCM helicase is a single MCM protein that forms a homohexameric complex. Atomic structures of archaeal MCMs have identified multiple beta-finger structures in MCM proteins whose in-vivo function is unknown[1,2].

Methods and Results: In vitro assays using archaeal MCM complexes have demonstrated the importance of the N-terminal beta-finger for DNA binding and the PS1H beta-finger for helicase activity[3]. Utilizing structural information from the archaeal MCM atomic structure, our lab has previously shown that the eukaryotic Mcm5 N-terminal beta-finger is important for replication initiation because it promotes the binding of the Mcm2-7 complex to origins of DNA replication in *Saccharomyces cerevisiae*[4]. In the present study, we have investigated the in vivo role of the PS1H beta-finger of Saccharomyces cerevisiae Mcm4 and Mcm5 in replication initiation and elongation. The PS1H beta-finger mutant of Mcm5 (mcm5 K506A) has a growth defect at 22˚ C and 37˚C by serial dilution analysis. Mutation of the MCM4 PS1H beta-finger (mcm4 K658A) does not have a growth defect by serial dilution analysis, indicating a differential importance of the PS1H beta-finger structures of different MCM helicase subunits. PS1H beta-finger mutants form hexameric MCM helicase complexes by co-immunoprecipitation experiments. PS1H beta-finger mutants have a plasmid loss phenotype that is suppressible by origin dosage, which indicates a defect in replication initiation. ChIP analysis will be used to determine the binding of PS1H-beta finger mutants to origins of DNA replication and BrdU incorporation analysis of replication will be presented.

Conclusions: Characterization of highly conserved beta-finger hairpin structures of MCM proteins in vivo indicates the importance of the PS1H beta-finger function in DNA replication. (Supported by PHS grant GM35078 to R. Sclafani).

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Pds5 Viability is Rescued by Deletion of Elg1 and Exhibit Cell Cycle Progression Defect.

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Cells that progress through the cell cycle must properly replicate each chromosome to produce sister chromatids, tether together chromatids to identify sisters through mitosis, and finally segregate each sister pair into resulting daughter cells. To maintain that identity from early S phase until anaphase onset, sister chromatids are tethered together by cohesin complexes. In budding yeast, cohesins are loaded onto chromatin in G1/early S phase, but sister chromatid pairing requires that chromatid-bound cohesins become activated (“established”) during S phase by an establishment factor termed Ctf7/Eco1 (Skibbens et al. 1999; Toth et al. 1999). Pds5 is an essential cohesion regulatory protein that binds Ctf7 and functions in concert with Ctf7 to establish sister chromatid pairing and then maintain that pairing into M phase (Hartman et al. 2000; Panizza et al. 2000; Noble et al. 2006). Numerous studies reveal that Pds5 also exhibits anti-establishment activity which antagonizes Ctf7 function(Tanaka et al. 2001; Sutani et al. 2009; Rowland et al. 2009). Several models suggest that Ctf7-establishment function and the process of replication are linked, yet it is not known whether Pds5 also functions in concert with the replication fork (Kenna et al. 2003; Terret et al. 2009). Evidence from this lab identified an alternative replication factor C large subunit, Elg1, as an anti-establishment factor that rescues viability in both *ctf7* and *pds5* mutant cells(Maradeo and Skibbens 2009; Maradeo and Skibbens 2010). Here, we explore the link between Pds5 and Elg1. Our results reveal that *pds5-1 elg1* double mutant strains exhibit both increased viability as well as decreased cohesion defects, compared to *pds5-1* mutant strains. We also show that the double mutant strain has a unique G1/early S phase delay phenotype that is DNA damage independent, suggesting that S phase progression is dependent on the function of Pds5. Here, we discuss possible roles of Pds5 outside of cohesion establishment and maintenance.

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Tau-Dependent Cell Cycle Re-Entry of Post-Mitotic Neurons Induced by β-amyloid Oligomers.

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One of the most enigmatic features of Alzheimer’s disease (AD) neurons is their tendency to express markers of cell cycle re-entry. For example, compared to healthy age-matched brains AD brains contain a pronounced increase in hyperploid neurons and the levels of multiple cell cycle regulatory proteins, including PCNA, and cyclins D, E and A. In mouse models of AD the presence of such cell cycle markers can precede the onset of behavioral symptoms by 3-6 months and propagate through brain in a pattern that mimics the spread of AD in human brain. Moreover, studies of cultured primary neurons have indicated that this putative, ectopic cell cycle re-entry can be induced by extracellular exposure to oligomers of β-amyloid (Aβ). To investigate the molecular mechanisms that underlie this process, we compared the responses

of primary neurons derived from wild type (WT) versus tau knockout (KO) mice to Aβ42 oligomers using incorporation of BrdU into nuclear DNA as an indicator of apparent cell cycle re-entry. Neurons derived from WT, but not tau KO mice frequently contained BrdU-positive nuclei within 24 hours of exposure to Aβ42 oligomers. BrdU uptake was accompanied by tau phosphorylation at Y18, S409 and S416, which are catalyzed respectively by the src-family kinase, fyn, the cyclic-AMP dependent protein kinase A (PKA) and calcium/calmodulin dependent kinase-II (CaMKII). Pharmacologic inhibition of fyn, PKA or CamKII individually prevented BrdU incorporation by WT neurons exposed to the Aβ42 oligomers, but did not block activation of the other two kinases in either WT or tau KO neurons. In addition, CaMKII-catalyzed phosphorylation of tau at S416 potentiated PKA-catalyzed phosphorylation of tau at S409 in both primary neurons and *in vitro* reconstituted sytems using purified tau and kinases. Collectively, these data indicate that Aβ42 oligomers induce activation of neuronal fyn, PKA and CamKII independently of tau expression, and that in the presence of tau the three kinases function in parallel pathways, without cross-activating each other, to induce cell cycle re-entry.

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Rad53 protein kinase regulates DNA replication by interacting with histones.

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Objective: The objective of our research is to further characterize the novel function of Rad53 in replication initiation in budding yeast *S. cerevisiae*. Protein kinase Rad53/Chk2 is central to activation of DNA checkpoints. The *rad53Δ* allele is synthetically lethal with *cdc7* mutations and suppresses *mcm5-bob1* bypass of DDK function (Cdc7/Dbf4 kinase)[1]. Rad53 also maintains cellular histone levels[2], and the functions of Rad53 in DNA replication and in histone regulation are independent of Rad53-mediated regulation of DNA checkpoints[1,2]. We hypothesized that Rad53 plays a role in DNA replication initiation independently of its roles in checkpoints and that Rad53's role in replication initiation is related to its role in regulating cellular histone levels.

Methods and Results: We isolated the *rad53-rep* separation of function allele[3], which is checkpoint proficient but is synthetically lethal with *cdc7*. The H426R mutation in the Rad53 kinase domain confers the *rad53-rep* phenotype, but the mutant protein has kinase activity similar to wild-type Rad53 during the DNA checkpoint response. In contrast, the checkpoint defective *rad53 fha1 fha2* mutant is normal in replication functions. Unlike wild-type or kinase dead Rad53, Rad53-rep protein cannot interact with origins of replication in the ARS-lacZ one-hybrid assay and the *rad53-rep* mutant displays a chromosome loss phenotype suppressible by origin dosage. The *rad53-rep* mutant is also sensitive to overexpression of histone H3/H4 similar to rad53Δ[2]. In contrast, a *rad53 fha1 fha2* checkpoint mutant is normal in these assays. Thus, loss of checkpoint function is not needed for histone sensitivity. *rad53-rep-cdc7* synthetic lethality is suppressed by deletion of the major H3/H4 gene pair. Furthermore, the *rad53-rep* mutant accumulates excess soluble histones, suggesting Rad53's functions in replication initiation and in histone homeostasis are related.

Conclusions: Identification and characterization of *rad53-rep* supports our hypothesis that Rad53 functions in DNA replication initiation independently of its checkpoint role. We propose that during DNA replication initiation, Rad53 acts as a "nucleosome buffer" to prevent histones from associating with replication origins and interfering in the origin activation process that is regulated by CDK and DDK (Supported by PHS grant GM35078 to R. Sclafani).

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Lipid droplets buffer histone availability during early Drosophila embryogenesis.

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Histones are essential for proper chromatin packing and gene expression, but cells face a tremendous challenge to manage their overall supply: sufficient histones need to be available to package newly replicated DNA, yet free histones not incorporated into chromatin are toxic. In most cells, multiple regulatory mechanisms ensure that new histone synthesis is tightly coupled with DNA replication. Here we report that Drosophila embryos transiently sequester histones on lipid droplets to buffer histone availability; without such sequestration, these embryos are very sensitive to altered histone expression, leading to abnormal cell cycles, mitotic defects and lethality. A buffering role for lipid droplets may be very general since transient droplet association of proteins from other cellular compartments, including but not limited to histones, has been reported from yeast to mammals.

We had previously found that early Drosophila embryos contain a thousand-fold excess of certain histones relative to DNA content and that these extra histones are bound to lipid droplets. Transplantation and photo-activation experiments demonstrate that droplet-bound histones can be transferred to nuclei. We now report that the novel droplet protein Jabba acts as a histone receptor: Jabba co-immunoprecipitates with histones; histone levels on droplets are decreased in parallel with Jabba levels; and without Jabba, histones are entirely absent from droplets. In cultured cells that do not usually exhibit histone recruitment to droplets, Jabba expression is sufficient to induce droplet association.

In *Jabba* mutants, overall embryonic histone levels are greatly reduced; extra histones not bound to lipid droplets apparently cannot be stably maintained. These embryos are also hypersensitive to changes in histone expression: both reduced dosage of classical histones and overexpression of the variant H2Av cause a dramatic increase in defective mitoses and disruption of early development. We propose that in wild-type embryos lipid droplets help maintain a balanced pool of extra histones that supports the rapid cell cycles of early development.

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γ-tubulin plays an important role in inactivating APC/C^{Cdh1} in interphase.

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A cold-sensitive γ-tubulin mutation of *Aspergillus nidulans*, *mipA*D159, causes a nuclear-autonomous failure of inactivation of the anaphase promoting complex/cyclosome (APC/C) in interphase at restrictive temperatures, resulting in constitutive destruction of cyclin B and removal of nuclei from the cell cycle (Nayak et al., 2010, J. Cell Biol., 190, 317-30). γ-tubulin, thus, has an important role in regulating the APC/C in interphase. The APC/C is activated by two proteins, Cdc20 in metaphase and Cdh1 in anaphase through G_1. Inactivation of APC/C^{Cdh1} at the end of G_1 is normally required for accumulation of S-phase cyclins. In *A. nidulans*, cyclin B is both the S-phase cyclin and M-phase cyclin. We identified, GFP-tagged, and deleted the *A. nidulans* homologs of *cdc20* and *cdh1. Ancdc20* is essential but *Ancdh1* is not. GFP-AnCdc20 is excluded from interphase nuclei, but enters the nucleoplasm when the nuclear pore complex disassembles at mitotic entry. It moves back into the cytoplasm in late mitosis. APC/C activity in the nucleoplasm is, thus, regulated in part by the localization of AnCdc20. AnCdh1-GFP

localizes to the nucleoplasm and spindle pole body in late G_2, disappears in mitosis, returns at the beginning of G_1 and disappears again a few minutes later. Deletion of *Ancdh1* significantly shortens the period of time in which cyclin B is absent from interphase nuclei, consistent with APC/C^{Cdh1} targeting cyclin B for destruction in G_1. Deletion of *Ancdh1* also eliminates the constitutive destruction of cyclin B caused by *mipA*D159. Since inactivation of APC/C^{Cdh1} is required for S-phase cyclin accumulation, these data indicate that *mipA*D159 causes a failure of inactivation of APC/C^{AnCdh1} in interphase and, thus, that γ-tubulin is important for APC/C^{Cdh1} regulation. Additional data indicate that constitutively active APC/C^{AnCdh1} causes destruction of BubR1, resulting in abrogation of the spindle assembly checkpoint. Supported by NIH grant GM031837.

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Nitrilase 1, a New Player in Plant Cell Cycle and Programmed Cell Death.

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Nitrilase-1 (Nit1), member of C-N hydrolases superfamily, was previously described as an enzyme involved in glucosinolate catabolism in Brassicaceae family. We identified Nit1 among proteins copurified with γ-tubulin from Arabidopsis extracts. Both endogenous Nit1 and Nit1-GFP protein expressed from native promoter localized in Arabidopsis cells in cytoplasm and a weaker signal was observed in the nuclei. The protein was enriched at the nuclear periphery, and on microtubular arrays throughout the cell division. Nit1 protein was present in plant extracts in high molecular forms; that when examined by EM were shown to be linear polymers. Arabidopsis cell culture overexpressing Nit1 showed lower mitotic index and slower growth rate compared to the wild type cells. In vertebrates, homologue of plant and bacterial Nit1 was demonstrated as a possible tumor suppressor gene involved in induction of apoptosis. Correspondingly, we found that level of programmed cell death was higher in cells overexpressing Nit1 in comparison to the controls. Severe defects of shoot apical meristem development were observed in plants with Nit1 downregulated by RNAi. Differentiation of the first true leaves was remarkably impaired and instead, ectopic cell division occurred. Furthermore, disruption of final symmetric division of guard cells in the stomata complex indicated cell division defects. Our data showed that plant Nit1 belongs to the growing number of metabolic enzymes with ability to assemble polymers and suggested for Nit1 function in cell division and programmed cell death.

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Mck1p, a yeast homologue of GSK-3 kinase, targets Cdc6p at C-terminus for its degradation to inhibit DNA re-replication.

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Orc6p is a part of the pre-replicative complex (pre-RC) which binds to origin of DNA replication to promote initiation of DNA replication. DNA replication takes place only once per cell cycle. After the initiation of DNA replication, pre-RC components are phosphorylated by Cyclin/CDK in order to inhibit DNA re-replication. In addition to this mechanism, S-phase cyclin (Clb5p) directly binds to Orc6p to inhibit Cdt1/Mcm2-7 loading to ORC. To better understand the DNA re-replication control in *S. cerevisiae*, synthetic genetic array (SGA analysis) was performed using *ORC6* mutant, *ORC6-rxl*. *ORC6* is an essential gene. The *ORC6-rxl* mutation eliminates Clb5-Orc6 binding, but these strains are viable. We looked for haploid gene deletion strains in which

ORC6-rxl was synthetically lethal; in other words, haploid deletion strains which require Clb5p-Orc6p protein binding for their survival. Interestingly, we found that *mck1* deletion cells showed synthetic lethality with the cells containing the *ORC6-rxl* mutation. The *mck1* deletion genetically interacted with *ORC6* or *ORC2* phosphorylation mutant as well. To study the genetic interaction between *MCK1* (a yeast homologue of GSK-3 kinase) and *ORC6* (origin binding protein), temperature sensitive mutant of *mck1* was generated by PCR mutagenesis. The temperature sensitive mutant, *mck1-16*, in the combination of *ORC6-rxl* induced moderate DNA re-replication followed by DNA damage checkpoint activation at 37 degree. Combination of *mck1-16* with *MCM7-NLS*, *ORC6-ps* and *ORC2-ps* induced extensive DNA re-replication suggesting that mechanism on DNA re-replication inhibition by Mck1p is additive. However *mck1* deletion did not genetically interact with *CDC6* mutant such as *CDC6ΔNT*. Therefore Mck1p and Cdc6p could act in the single pathway.

It has been shown that Cdc6p at N-terminus are phosphorylated by Cyclin Dependent Kinase (CDK) for its degradation. Here we show that Mck1p, a yeast homologue of Gsk-3 kinase, promotes Cdc6p degradation. Cdc6p was stabilized in the *mck1* deletion cells during late S phase and mitosis, and overexpression of Mck1p promoted rapid Cdc6p degradation. Mck1p interacted with Cdc6p through GSK-3 consensus site at C-terminus. The protein interaction between Mck1p and Cdc6p was abolished in the Cdc6 mutant which contains an amino acid substitution (from threonine to alanine) at GSK-3 consensus site. These results suggest that Mck1 kinase and CDK target Cdc6p for its degradation to inhibit DNA re-replication. We will discuss the molecular mechanism how Mck1p and CDK corporate to trigger complete degradation of Cdc6p.

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Changes in intracellular signaling and cell growth rate accompany regulation of cell size at S-phase entry.

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The mechanisms coordinating mammalian cell growth with cell cycle have not yet been elucidated. Here, we use quantitative fluorescence microscopy to simultaneously measure cell cycle position, cell size, and the level of cell growth regulators such as phosphorylated rpS6 in very large numbers (>10^6) of fixed HeLa cells. Cell cycle is monitored by expression of stage-specific fluorescent proteins, cell size is measured by staining all proteins with a dye-conjugated succinimidyl ester, and protein phosphorylation levels are measured by immunofluorescence. By combining these measures, we obtain profiles of cell growth and growth-related signaling over the course of the cell cycle. We find that cell growth slows significantly in late G1, concurrent with a transient decrease in cell-to-cell size variation prior to S-phase entry. This tightening of the cell size distribution is accompanied by several signaling events, most notably a shift to high levels of translation component phospho-rpS6 and a decrease in Akt phosphorylation. Interestingly, we also find that the inactivating phosphorylation of translational repressor 4EBP1 is cell-size dependent, with smaller cells maintaining higher phospho-4EBP1 concentrations than large cells. Increased homogeneity of phospho-S6 levels, an overall slowing of cell growth due to decreased phospho-Akt, and an increased growth rate in small cells due to differential 4EBP1 phosphorylation could all contribute to the observed decrease in cell size variance. To further explore these findings, we use growth-inhibiting drugs to perturb cell size regulation. We find that under low doses of rapamycin or cycloheximide, cells can proliferate normally, but shift to an average size 10% smaller than control cells. These cells display the same growth dynamics as undrugged cells throughout the cell cycle, including the late-G1-slowdown described above. With rapamycin treatment, the cell-cycle-dependent dynamics of both rpS6 and Akt phosphorylation are lost, and the variance in cell size increases

by 18%. These results implicate late-G1 rpS6 and Akt phosphorylation dynamics in cell size control. In contrast, with cycloheximide treatment, these dynamics are more closely conserved, and cell size variance does not increase. We are currently using the methods described here to extend these results and dissect the molecular mechanisms used to maintain cell size uniformity.

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How do cells coordinate growth and division?

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Any process that requires sustained cellular proliferation over many generations relies on the coordination of cell growth and division. In the budding yeast *S. cerevisiae*, proliferation rates are limited by the rate of cell growth; growth rate, in turn, can be limited by the cells' nutrient environment. While it is well established that the cell cycle contains homeostatic mechanisms to abolish size differences between mother and daughter cells, it is unclear how it accommodates environmentally-driven depressions in growth rate. Here, we explore this question in the context of glucose withdrawal. We demonstrate that cell cycle progression is dependent on the presence of glucose: withdrawal induces immediate growth arrest, irrespective of cell cycle position. In the arrested state, cell size remains constant for hours; new budding and cytokinesis events are not observed.

Ongoing work aims to understand these observations in light of glucose's dual role as a hormone-like initiator of signal transduction and the preferred raw material for fermentative metabolism. Exchanging glucose for 2-deoxyglucose, a metabolically inert analog, produces an equivalent growth arrest, suggesting that active glycolytic metabolism is a pre-requisite for cell cycle progression in glucose-grown cells. Additionally, modulating glucose-responsive signal transduction can 1) bypass growth arrest upon glucose withdrawal, and 2) increase the probability that arrested cells will escape to resume the cell cycle at their present position. Together, these data suggest that glucose withdrawal elicits an unidentified, glycolysis-dependent intracellular signal that inhibits both growth and cell cycle progression.

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Cell cycle checkpoint acquisition in early embryogenesis.

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Cell cycle checkpoints are crucial for the maintenance of genomic integrity and the fidelity of cell divisions. Interestingly, many metazoan embryos undergo a brief developmental stage characterized by synchronous, rapid cell divisions that lack gap phases of the cell cycle and the mitotic and DNA damage response checkpoints. When cells reach a critical nuclear to cytoplasmic (N:C) ratio, the mid-blastula transition (MBT) is triggered, zygotic transcription is activated, and cell cycle remodeling occurs: cell cycles lengthen and acquire gap phases, and checkpoints become functional. It is unclear how checkpoint function is acquired at the MBT. In this work, we use zebrafish embryos to address this question, focusing specifically on the effects of transcriptional activation, cell cycle lengthening and the N:C ratio. By inhibiting RNA Polymerase II at the MBT, we show that checkpoint acquisition is independent of zygotic transcription. This result demonstrates that components of the checkpoint signaling pathway are present prior to the MBT. However, we find that pre-MBT embryos do not phosphorylate histone H2AX upon DNA damage, which indicates that DNA damage signaling is blocked at an early step. We also use a Cdc25a morpholino and Cdk inhibitor to determine the effect of longer cell

cycle lengths on checkpoint function in pre-MBT embryos. We are developing an integrated model of how the N:C ratio, cell cycle lengthening, zygotic transcriptional activation and checkpoints influence one another at the MBT.

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The circadian factor Period2 modulates p53 stability in unstressed cells.

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Circadian rhythms are mechanisms that measure time on a scale of about 24 h and that adjusts our body to external environmental signals. Core circadian clock genes are defined as genes whose protein products are necessary components for the generation and regulation of circadian rhythms. Circadian proteins also regulate genes involved in either cell division or death; and a perturbation of the balance among these processes leads to cancer development and progression.
A key aspect of cancer research is identifying new regulatory pathways involved in proliferation and differentiation of cell. Disruption of circadian rhythm has recently emerged as a new potential risk factor in the development of cancer, pointing to the core gene period 2 (per2) as a tumor suppressor. However, it remains unclear how the circadian network regulates tumor suppression, nor which, if any, of its components is either the ultimate effector that influences the fate of the cell.
Initial experiments were devoted to identify new interacting partners for Per2 using a two-hybrid system. Interestingly, among the positive clones analyzed was the tumor suppressor protein p53. This result was validated by immunoprecipitation of recombinant and endogenous Per2/p53 complexes from unstressed cells. Pull-down assays using tagged-expressed proteins fragments and labeled proteins were later used to map the interacting regions between Per2 and p53. Our results show Per2 binds to the C-terminus of p53 in a region that includes the DNA binding and tetramerization domains and that includes the ubiquitination sites. Thus, we hypothesized that binding of Per2 to p53 might act by blocking Mdm2-mediated ubiquitination of p53 target residues in the C-terminus and therefore altering its stability. We next examined the formation of the Mdm2/p53/Per2 complex by immunoprecipitation. Our data show anti-p53 antibody is able to co-immunoprecipitate Per2 and Mdm2. Moreover, in vitro and in vivo ubiquitination assays show that binding of Per2 to p53 prevented ubiquitination of p53 by Mdm2 without altering their binding. Immunofluorescence studies using H1299 cells (p53-) confirmed Per2 role in p53 stabilization and for localization. Overall our results suggest that Per2 modulates the stability of p53 in unstressed cells, and might be responsible for the oscillatory levels of p53 observed in a 24 h cycle.

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Polyploidizing cell cycles yield unstable genomes and aneuploidy in *Drosophila.*

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Cell cycle checkpoints ensure alteration between complete DNA replication and mitosis, and evasion of these checkpoints promotes a host of pre-cancerous genome stability defects. Programmed endo-cell cycles represent a tolerated form of checkpoint evasion, as in these cell cycles mitosis is truncated, yielding polyploid cells. Understanding whether endo-cell cycles promote genome instability has proven challenging, as normal polyploid cells typically do not divide further, while in contrast polyploidy is one of numerous potential drivers of early stage cancer. My previous work[1] established a *Drosophila* model to elucidate connections between endo-cell cycles, polyploidy, and genome instability. During *Drosophila* hindgut development, endo-cell cycles generate polyploid cells that then re-enter mitotic cell cycles. In these wild-type

polyploid cells, chromosome segregation in mitosis is highly error-prone. In further characterization of these polyploid divisions, I have identified two principal causes of polyploid genome instability. By conducting time-lapse imaging of polyploid divisions, I observed polyploid hindgut cells to elaborate numbers of centrosomes. These cells frequently yield multipolar spindles and aneuploid daughter cells. Following progression of polyploid hindgut cells through development revealed a non-canonical origin of centriole elaboration involving *de novo* centriole synthesis. However, many polyploid cells with normal centrosome number also exhibit error-prone division, and prior to dividing polyploid chromosomes exhibit “fragile” sites indicative of defects in DNA replication. By taking advantage of a developmental sequence of polyploidization followed by error-prone mitotic divisions, I have uncovered *in vivo* roles for centriole and DNA replication as causes of genome stability in variant cell cycles. These same mechanisms are likely contributors to early cancer progression.

1. Fox DT, Gall JG, and Spradling AC. (2010). Error-prone polyploid mitosis during normal Drosophila development. *Genes and Development* 24: 2294-2302.

Kinases and Phosphatases

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Cadherin regulation in fibroblast and fibrosarcoma cells: influence of culture density and MAPK activity.

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Cadherins are integral proteins that play an important role in cellular adhesion. If E-cadherin is cleaved, releasing an 80 kDa fragment, it is no longer active. Interestingly, this 80 kDa product has been found to be increased in several types of cancers. In this work, the presence of an 80 kDa protein reactive with E-cadherin antibody, consistent with the inactive cleavage product of E-cadherin, has been detected in normal fibroblasts (BJ cells) and fibrosarcoma cells (HT-1080), and is expressed at a slightly higher level in subconfluent cells than confluent cells. Similarly, matrix metalloproteinase-9 (MMP-9), which is capable of cleaving cadherins, is also expressed more in subconfluent cells. Levels of MMP-2 were found not to be altered with increased culture density. Therefore, obtaining a confluent state is correlated with decreased MMP-9 expression, and loss of the 80 kDa fragment. ERK is upregulated in proliferating cells, and may also upregulate expression of E-cadherin. To investigate the potential role in the regulation of the 80 kDa fragment, dexamethasone treatment was used. Dexamethasone treatment resulted in lower expression of the 80 kDa fragment, as well as decreased expression of phosphorylated ERK. These results suggest a relationship between increased culture density, ERK activity, and regulation of cadherins.

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Modulation of STAT3 activation and prostate cancer proliferation by cyclin-dependent kinase 5.

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STAT3 (signal transducer and activator of transcription 3) is a major transcription factor in prostate cancer. Phosphorylation is an important translational modification for transactivation. Initially, the protein biochemical interaction between Cdk5 and STAT3 was identified and this

interaction relied on Cdk5 activity. Besides, Ser727 phosphorylation of STAT3 was positively regulated by Cdk5 in LNCaP cells and xenografted tumors. The S727A mutant declined the protein interaction between Cdk5 and STAT3. The distribution of phospho-Ser727-STAT3 proteins and the expression of STAT3-regulated gene are regulated by Cdk5 activity. By using a Cdk5 inhibitor roscovitine (Rv, also named Seliciclib or CYC202, a clinical trial drug on non-small cell lung cancer and leukemia), we subsequently found that STAT3 was a Cdk5-regulated survival factor through Ser727 phosphorylation in prostate cancer cells. Finally, our clinical evidence demonstrated the significant correlations of phospho-Ser727-STAT3 with Cdk5 and with Gleason score in prostate cancer patients. These clinical data also revealed that the protein level of phospho-Ser727-STAT3 was relatively higher in tumor tissue as compared to individual normal one. In conclusion, our data indicates that Cdk5 is a positive modulator of STAT3 activation and prostate cancer cell proliferation through Ser727 phosphorylation.

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JNK-1 Promotes Survival or Programmed Death of Cardiac Myocytes by Differential/ATP-dependent Regulation of Akt and ERK Survival Pathways.

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INTRODUCTION The survival kinases PI3K-Akt and Raf-MEK1/2-Erk1/2 are required to protect the heart during ischemia/reperfusion and are key components of the protection afforded by ischemic preconditioning. Our group recently reported that JNK-1 activity is essential for the activation of Akt when reperfusion followed brief but not extended ischemia (Wei et al., J Biol Chem. 286:13995-4006, 2011). We show here that JNK-1 is also essential for the activation of ERK under the same conditions of simulated brief ischemia. HYPOTHESIS JNK-1 mediates differential regulation of both Akt and ERK in a manner that is dependent on the time of ischemia and [ATP] at the time of reoxygenation/reperfusion. METHODS Cardiac myocytes were cultured in physiological glucose ((PG) 5mM glucose) or low glucose ((LG), 0.5mM glucose) and subjected to 8hrs of hypoxia followed by reoxygenation in the presence or absence of constitutively active JNK-1 (caJNK) or JNK inhibitors (Ad-dnJNK or SP600125). Proteins were analyzed by western blots using phosphor antibodies. RESULTS When myocytes were subjected to hypoxia with LG, glucose was depleted within 4hrs and intracellular ATP decreased to 18±7% at 8hrs. Glucose and ATP remained high in the PG condition. Myocyte death after reoxygenation was greater in the LG condition (31± 5% vs. 12± 4%; $p<0.05$). JNK inhibition conferred massive cell death in the PG condition (38±12 vs. 12± 4%), but was protective in the LG condition (not shown). Death in the PG condition was paralleled by increased caspase 3 cleavage (7.5±2.9-fold), enhanced release of cytochrome c, and opening of the mPTP. As indicated in Fig 1, P-ERK was activated by ReOx but inclusion of dnJNK-1 reduced the levels of P-ERK2, P-MEK and P-Raf1 by >80% (n=3). Ad-dnJNK did not reduce the P-ERK cascade under the LG condition and caJNK significantly activated P-ERK only in the PG condition (not shown). CONCLUSIONS: JNK-1 is essential for activation of ERK as well as Akt during ReOx with sustained ATP.

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Anti-allergic Effects of compounds isolated from wheat bran extract on mast cell-mediated allergic model.

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In order to determine anti-allergic activity, this study investigated whether 5-*n*-nonadecylresorcinol and 5-*n*-heneicosylresorcinol isolated from wheat bran extract suppress immunoglobulin E (IgE)-mediated allergic response and expression of inflammatory cytokines in mast cells. We observed that two compounds significantly inhibited release of β-Hexosaminidase, as a marker of degranulation, and expression of proinflammatory cytokines such as TNF-α, IL-4, IL-10 and COX-2 a dose-dependent manner in antigen-stimulated RBL-2H3 mast cells. Moreover, two compounds blocked phosphorylation of AKT, p38MAPK, JNK and ERK1/2 and activity of nuclear factor- κB (NF-κB) in mast cells. We also showed two compounds exhibited 5-lipoxygenase inhibitory activity with EC_{50} values of 233.06 and 184.56 μM, respectively. Our findings suggest that 5-*n*-nonadecylresorcinol and 5-n-heneicosylresorcinol isolated from wheat bran extract suppress mast cell-derived allergic response and expression of inflammatory cytokines and that these effects were related by inhibition of AKT, p38MAPK, JNK and ERK1/2 phosphorylations and NF-κB.
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Lucidenic acids-rich extract from antlered form of *Ganoderma lucidum* enhances TNFα induction in THP-1 monocytic cells possibly via its modulation of MAP kinases p38 and JNK.

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The *Ganoderma lucidum (G. lucidum)* is one of the oriental fungi that has been reported to have immunomodulatory properties. Although effect of β-glucans from *G. lucidum* has been well documented, little is known about how other major bioactive components, the triterpenes, contribute to the immunomodulatory function of *G. lucidum*. Here, we showed that triterpenes-rich extract of antlered form of *G. lucidum* (*G. lucidum* AF) induces TNFα production in monocytic THP-1 cells. Furthermore, the extract also synergized with lipopolysaccharide (LPS) to induce TNFα production in THP-1 cells, suggesting an immunostimulatory role of triterpenes-rich extract of *G. lucidum* AF. Notably, the extract enhanced LPS-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK), while it suppressed LPS-induced phosphorylation of c-Jun N-terminal kinase (JNK) MAPK. p38 Inhibitor suppressed TNFα production, while JNK inhibitor enhanced TNFα production, implying that synergistic effect of the extract may work by modulating p38 and JNK MAPKs. Moreover, we found that the triterpenes-rich extract of *G. lucidum* AF contains high amounts of lucidenic acids. Lucidenic acid-A, -F and -D_2, which seem to dominantly exist in the extract, were purified from the triterpenes-rich extract. We also identified Lucidenic acid-A and -F as modulators of JNK and p38, respectively. Thus, our data demonstrate that lucidenic acids-rich extract from *G. lucidum* AF enhances LPS-induced immune responses in monocytic THP-1 cells possibly via the modulation of p38 and JNK MAPKs activation.

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The role of the Akt substrate APE (Girdin, GIV) in insulin signaling.

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Akt kinases are important mediators of the insulin signal. Several Akt substrates are directly involved in glucose homeostasis, e.g. Glycogen synthase kinase 3 (GSK3) and Akt substrate of 160 kDa (AS160). Recently, Akt phosphorylation enhancer (APE) has been described as a new Akt substrate. It is expressed ubiquitously in mammals and highly in a variety of cancer cell lines. Based on its actin and microtubule binding domains it is involved in remodelling of actin cytoskeleton and cell motility. In addition, APE is also a Gαi–interacting protein and controls the Gαi triggered cell migration. However, not much is known about its role in insulin signalling.
To investigate the function of APE we knocked down APE with siRNA and found a reduced Akt and Insulin receptor substrate 1 (IRS-1) phosphorylation. We then established C2C12 cell lines which permanently overexpress APE. With this approach we found that Akt was constitutively active, as determined by its phosphorylation at T308 and S473. Consequently, GSK3 phosphorylation was also increased after insulin stimulation. One possible reason for the activation are changes in the expression of several proteins. The insulin receptor was upregulated and tyrosine phosphorylation of IRS-1 enhanced even though the protein itself was down regulated. Additionally, the tyrosine kinase Abelson murine leukemia viral oncogene homolog 1 (c-ABL) and its substrates e.g. Avian sarcoma virus CT10 oncogene homolog (CrkII) and Src homology 2 domain containing transforming protein 1 (p66shc) were constitutively phosphorylated. To investigate medium-term insulin responses we measured the C14-glucose incorporation into glycogen. The APE overexpressing cells showed a higher basal glycogen synthesis without stimulation. In addition, the insulin sensitivity was increased with glycogen synthesis peaking at 1 nM of insulin compared to wild type cells (100 nM). Moreover, the internalisation of the insulin receptor is slowed down in the APE overexpressing cell line compared to C2C12 cells which permanently overexpress the insulin receptor to an equal extent. Of note, these effects were independent of APE phosphorylation by Akt. An Akt phosphorylation site mutant, in which serine and threonine (S1417/T1419) are mutated to alanine behaved similar to wild type APE.
We conclude that changes in the APE level have strong effects in C2C12 cells indicating that APE is an important mediator of the insulin signal in muscle cells.

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PI3K and Akt play an essential role for quiescent myogenic cell activation.

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Muscle satellite cells (MSCs), which are stem cells in skeletal muscle, have a role in skeletal muscle growth and repair. MSCs are normally quiescent but are activated in response to various stimuli such as injury and overload. Activated MSCs enter the cell cycle to produce a large number of myogenic progenitor cells. In regenerating skeletal muscle fibers, the activation of MSCs must be tightly regulated by certain mechanisms. However, the molecular mechanisms of MSCs activation are not understood.
Reserve cells, a model of quiescent MSCs in vitro, are activated by fetal bovine serum in medium (FBS) but are not activated by serum free medium. We found that treatment with epidermal growth factor (EGF) alone in serum free medium was not sufficient to activate reserve cells. However, treatment with a combination of insulin and EGF (i+E) could activate the reserve cells almost as efficient as FBS. We also found that the reserve cell activation treated with i+E was dependent on extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK).

In this study, we focused on the involvement of PI3K/Akt pathway in the mechanism of quiescent myogenic cell activation. We also sought inducible factors of JNK phosphorylation.
We investigated the involvement of PI3K/Akt pathway during reserve cell activation with i+E by detecting incorporation of BrdU in the presence of an inhibitor for PI3K or Akt. PI3K inhibitors (LY294002 or wortmannin) or an Akt inhibitor (triciribine) prevented incorporation of BrdU into reserve cells. Furthermore, triciribine also prevented it into MSCs on single myofibers isolated from adult C57BL/10 mice.
To elucidate factors involving JNK induction, we analyzed the expression and phosphorylation of JNK in reserve cells treated with insulin, EGF or i+E by Western Blot. We found that JNK phosphorylation increased with EGF and i+E, but did not change with insulin. These results suggested that JNK phosphorylation during the reserve cells activation is induced with EGF.
We conclude that PI3K and Akt are involved in the reserve cell activation induced in i+E. In addition, JNK phosphorylation is induced with EGF. Therefore, we found that quiescent myogenic cell activation is induced with not only activation of ERK but also with activation of PI3K, Akt and JNK. We conclude these pathways play an essential role for quiescent myogenic cell activation.

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DYRK3 couples mTORC1 signaling to mRNA granule turnover.

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Mammalian cells dynamically regulate the activation and repression of messenger ribonucleoprotein complexes by growth factor signaling and the formation of cytoplasmic stress granules. However, little is known about the molecular mechanisms that connect these activities. Using an unbiased phospho-proteomic and protein microarray approach, we here report that the dual- specificity tyrosine-phosphorylation regulated kinase DYRK3 is essential for basal as well as growth factor-induced mTORC1 activity by directly phosphorylating PRAS40, a negative regulator of mTORC1. We show that small compound inhibitors are able to induce a kinase-inactive state of DYRK3, and that kinase-active and -inactive DYRK3 have distinct cellular effects. In the kinase-active state, DYRK3 dynamically associates with P-bodies and activates mTORC1 signaling, while in the kinase-inactive state, DYRK3 is stably associated with stress granules to prevent stress granule disassembly and blocks mTORC1 signaling. Based on our findings, we propose that DYRK3 couples mRNA granule turnover to the regulation of protein translation during normal and stressful conditions.

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Influence of the PI3K/Akt signaling pathway on the regulation of HIV-1 alternative RNA splicing.

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In contrast to constitutive splicing, alternative splicing describes the differential removal of specific exonic sequences from mature mRNA or inclusion of intronic ones and is therefore a major contributor to proteomic diversity and an important mechanism for the regulation of gene expression.
Beside the importance of alternative splicing for the cellular gene expression, the replication of many viruses (e.g. HIV-1) depends on alternative splicing to generate mRNAs encoding viral proteins essential for viral replication. More than 40 different alternatively spliced HIV-1 mRNAs

are produced through the differential combination of four 5` splice donor sites and eight 3`splice acceptor sites.
Like most biological processes the splicing process is tightly regulated. This regulation depends on the strength of splice sites and regulatory cis acting elements within the exons and introns, which function by recruitment of sequence specific trans- acting factors that either activate (like SR proteins) or repress (hnRNPs) the use of adjacent splice sites.
Until now, little is known about the influence of transduction pathways on the regulation of alternative splicing. Nevertheless, in the last years it has been shown that the PI3K/Akt pathway affects the activity of the SR proteins SRSF1, SRSF5 and SRSF7 in vitro and thereby influences the alternative splicing of the Protein kinase C and Fribonectin mRNA.
Using the PI3K/Akt signaling pathway inhibitor LY294002, we have analysed the importance of this pathway on the HIV-1 splicing pattern and the viral replication.
By performing RT- and real time- PCRs we first carried out experiments using HIV-1 based splicing reporter constructs. Here the inhibition of the PI3K/Akt signaling pathway resulted in a shift from spliced mRNA species to intron retaining RNA species indicating that the inhibition of the PI3K/Akt pathway interferes with the splicing process. Next we conducted experiments with cells infected with HIV-1. Beside the accumulation of intron containing mRNAs, we found that the PI3K/Akt inhibition resulted in the generation of alternative tat mRNAs and the use of a novel HIV-1 splice donor site. Further, the inhibition of the PI3K/Akt pathway interfered with the viral replication shown by diminished p24 detection in the presence of the inhibitor.
These results clearly demonstrate that there is an influence of PI3K/Akt on the HIV-1 splicing pattern and the viral replication and that the inhibition of this pathway could be a new approach for antiviral therapy.

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PDK1 mediates rephosphorylation of the activation domain of atypical PKC during the keratin intermediate filament dependent rescue from degradation.

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Phosphorylation of the activation domain of PKC isoforms is essential to start a conformational change that results in an active catalytic domain. While there is consensus that PDK1 is the activating kinase that phosphorylates this domain in newly synthesized molecules, it is unclear what kinase performs that function in molecules that were previously de-phosphorylated and refolded by Hsp70 chaperones. The latter, considered a “rescue” mechanism is responsible for the maintenance of the steady-state levels of atypical PKC and other isoforms. Even in the presence of normal levels of transcription and translation, atypical PKC levels fall by one order of magnitude if the rescue from degradation is abrogated. To identify the activating kinase during the rescue mechanism, we inhibited protein synthesis and analyzed the stability of the remaining atypical PKC pool. Two different PDK1 inhibitors, BX-912 and a specific pseudosubstrate peptide, destabilized that pool. PDK1 was shown to co-immunoprecipitate with PKC iota in cells without protein synthesis. We confirmed that PDK1 rephosphorylates atypical PKC during the rescue mechanism by in vitro rephosphorylation assays, where rephosphorylation was abrogated by immunodepleation of PDK1 and rescued with recombinant PDK1. Importantly, we demonstrate that the keratin scaffold that sustains atypical PKC rescue contains all the components necessary for the mechanism except PDK1. We also found that in Caco-2 epithelial cells PDK1 distributes to an apical membrane compartment comprising plasma membrane and endosomes, which, in turn, colocalized with intermediate filaments. These results shed light on a novel function of keratin intermediate filaments sustaining normal intracellular signaling.

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Identification of proteins that interact with tyrosine-phosphorylated catalytic subunits of cyclic-AMP-dependent protein kinase A.

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Growth factors and their cognate receptor tyrosine kinases mediate a host of cellular behaviors including proliferation, differentiation and cellular migration. cAMP-dependent protein kinase A (PKA) is one protein that is activated downstream of growth factor receptors. Recent results from our laboratory demonstrated that 1) the catalytic subunit of PKA (PKA-C) is phosphorylated on tyrosine 330 (Y330) *in vitro* by growth factor receptor tyrosine kinases 2) PKA-C subunits isolated from growth factor-stimulated cells are phosphorylated on Y330 and 3) the kinetic activity of PKA-C is enhanced upon phosphorylation (Caldwell et al., J Cell Biochem. 2011 Aug 22. doi: 10.1002/jcb.23325. [Epub ahead of print]). Phosphorylation of tyrosine residues often induces a new protein interaction, therefore, we hypothesized that phosphorylation of PKA-C on Y330 also alters its binding properties. An SH2 domain array was employed to screen for candidate proteins that interact specifically with PKA-C phosphorylated on Y330. Positive interactions were observed for six Src family kinase members (c-Src, Fyn, Fgr, Yes, Hck and Lck), phospholipase C- γ, RasGap and the p85 regulatory subunit of PI3 kinase. Interestingly, c-Src was also found to phosphorylate PKA-C on Y330 *in vitro*. Since PKA-C is phosphorylated on Y330 in response to growth factors, our results identify several proteins that may interact with PKA to coordinate growth factor-mediated signaling events.

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Regulation of Pigment Granule Aggregation by Protein Kinase A in Fish Retinal Pigment Epithelial (RPE) Cells.

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In the eyes of fish and amphibians, light flux into the retina is controlled by the bidirectional movement of pigment granules within retinal pigment epithelial (RPE) cells. Pigment granules disperse from the cell body into apical projections in response to light and aggregate back into the cell body in the dark. Both aggregation and dispersion require actin filaments. RPE pigment granule aggregation *in vitro* is stimulated by cAMP, however, it has not been shown that cAMP, the canonical activator of protein kinase A (PKA), indeed activates PKA in the aggregation process. Using an *in vitro* kinase assay, PKA activity was demonstrated in RPE lysates. RPE cells isolated from sunfish, *Lepomis spp.*, were treated with the PKA inhibitors H89 and myristoylated PKI, a cell-permeable inhibitor of PKA. H89 blocked pigment granule aggregation, however, H89 is not specific to PKA, but is also known to inhibit rho kinase (ROCK). PKI concentrations of 10-100 µM had no effect on pigment granule aggregation. Efficacy of PKI was tested by treating rod inner and outer segments (RIS-ROS), which normally shorten myoids in response to PKA, with either H89 or PKI. H89 blocked myoid shortening in RIS-ROS, however PKI did not. It is possible that PKI cannot enter cells, despite myristoylation, which is expected to enhance membrane permeability. To directly assess whether cAMP stimulation of PKA activates aggregation, isolated RPE cells were microinjected with purified PKA catalytic subunit. Injected cells aggregated pigment granules, demonstrating that PKA elicits pigment granule aggregation in RPE cells.

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Analyzing the role of a Golgi transport-vesicle coat protein in PKC-epsilon-dependent signaling.

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Protein Kinase C (PKC) epsilon has been implicated in a variety of human diseases, including cell transformation and cardioprotective signal transduction associated with heart disease.
The localization and function of PKC-epsilon has been shown to involve the beta'-COP subunit of coatomer, a coat protein of intracellular transport vesicles. Our objective has been to use NIH3T3 fibroblast cell line, cultured cardiomyocytes, and cell-free Golgi-binding assays as model systems to understand the role of coatomer in PKC-epsilon-dependent signaling.
We confirm that activating PKC-epsilon with phorbol esters causes PKC-epsilon to translocate from the Golgi apparatus to the cell surface in fibroblasts. Disrupting the Golgi apparatus with the drug brefeldin A, disrupts the steady state localization of PKC-epsilon at the Golgi apparatus but does not interfere with PKC-epsilon translocation to the cell surface. Mutations that are known to affect the binding interaction between PKC-epsilon and beta'-COP have only subtle effects on the translocation of PKC-epsilon. The majority of beta'-COP does not appear to translocate with PKC-epsilon to the cell surface upon treatment with phorbol ester. We find that multiple coatomer subunits colocalize with PKC-epsilon at steady state suggesting that the entire coatomer complex could contribute to PKC-epsilon function. We have confirmed that phosphorylation of PKC-epsilon at residue 729 stimulates PKC-epsilon localization to the Golgi apparatus. The oncogenic constitutively active mutant, PKC-epsilon A159E, also localizes predominantly to the Golgi apparatus. The intact-cell experiments support a role for coatomer in both the steady-state localization of PKC-epsilon and the activation-induced translocation. We have used a cell-free Golgi-binding assay to begin defining the molecular basis for the regulated PKC-epsilon binding interaction with the Golgi apparatus. We confirm that the ARF1 GTPase increases the binding of both coatomer and PKC-epsilon to Golgi membranes and that binding of PKC-epsilon to isolated Golgi membranes is stimulated by phorbol esters. Our results indicate that protein phosphorylation and ARF1/coatomer recruitment play connected regulatory roles in PKC-epsilon binding to the membrane.
Both the intact-cell and cell-free binding studies support a model wherein the beta'-COP subunit of coatomer helps specify the steady-state localization of PKC-epsilon at the Golgi apparatus. In this way, coatomer would influence human-disease-related signaling by directing PKC-epsilon subcellular distribution.

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Localization and Binding Partners of Obscurin Kinases in Striated Muscle Cells.

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Obscurins are giant muscle proteins that have been implicated in myofibrillogenesis and linked to hypertrophic cardiomyopathy. They are composed of tandem arrays of structural and signaling motifs, including an isoleucine-glutamine (IQ) repeat, a Src homology 3 (SH3) domain, a pleckstrin-homology (PH) domain, a Rho-guanine nucleotide exchange factor (Rho-GEF) motif, and two serine/threonine kinase domains, namely SK1 and SK2. To understand the cellular pathway(s) that the obscurin kinases are involved, we sought to identify their interacting partners using the yeast-two-hybrid system. Our screening demonstrated that the β-subunit of Na+/K+-ATPase (NKA) and N-cadherin are potential interacting partners of SK1 and SK2, respectively. Immunofluorescence and Duolink® immuno-histological staining demonstrated that the identified SK1/NKA and SK2/N-cadherin interacting pairs are localized in close

proximity (<40 nm) at the level of the sarcolemma and the intercalated disc, respectively. Detailed deletion analyses revealed that the extracellular domain of the β-subunit of NKA is sufficient to interact with the catalytic portion of SK1, whereas both the extracellular and intracellular regions of N-cadherin are required to interact with the catalytic portion of SK2. Immunostaining of non-permeabilized primary cultures of adult flexor digitorum brevis (FDB) muscle with antibodies recognizing the obscurin-kinase isoforms suggested that at least some parts are localized extracellularly. Consistent with this, select obscurin-kinase isoforms are efficiently and specifically retained by lectin columns, but not if pre-treated with Peptide: N-Glycosidase F (PNGase F). Thus, our results demonstrate that at least some portions of the obscurin-kinase isoform(s) are localized extracellularly, potentially functioning as ectokinases, where they may undergo glycosylation.

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PP4 is required for NHEJ-mediated repair of DNA double strand breaks.

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Reversible phosphorylation is an essential post-translational modification to turn on/off a protein function, regulating many cellular activities, including DNA repair. DNA double-strand break (DSB) is the most lethal form of DNA damage and is mainly fixed by the high-fidelity homology-directed repair and the error-prone non-homologous end joining (NHEJ) repair. In this report, we performed a genomewide serine/threonine phosphatase siRNA screen using a reporter system of NHEJ-mediated DSB repair and identified PP4c, when depleted by siRNA, compromised NHEJ-mediated DSB repair. Both PP4C and its regulatory subunit PP4R2 physcially interacted with the chromatin condensation factor KAP1 (KRAB-associated protein 1). Depletion of PP4c led to sustain phosphorylation of KAP1. Conversely, overexpression of PP4c resulted in a decrease of KAP1 phosphorylation. PP4 dephosphorylated pKAP1 in vitro. Depletion of both PP4c and KAP1 did not have any significant synergistic effect on NHEJ-mediated DSB repair. Taken together, our results suggest that PP4 is required for NHEJ-mediated DSB repair through, at least partially, regulating phosphorylation status of KAP1.

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Receptor-type protein tyrosine phosphatase sigma a (ptprsa) expression in the central nervous system of adult zebrafish and brainstem-derived primary neuron cultures.

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In the mammalian central nervous system (CNS), the transmembrane protein tyrosine phosphatase PTPsigma was recently identified as a receptor for chondroitin sulfate proteoglycans (CSPGs), such as neurocan, which inhibits axon regeneration following an injury. The goal of our project is to elucidate the role of PTPsigma in zebrafish axon regeneration following spinal cord injury (SCI). Unlike mammals, in adult zebrafish damaged axons regenerate across and beyond a SCI site. We hypothesize that the zebrafish homolog of PTPsigma, protein tyrosine phosphatase sigma a (ptprsa), like its mammalian counterpart, is a receptor for CSPGs. Furthermore, we suspect that axon regeneration in the zebrafish CNS is due in part to reduced PTPsigma activity following injury. To qualitatively investigate ptprsa expression following CNS injury in the zebrafish, we are currently using Reverse Transcriptase Polymerase Chain Reactions (RT-PCR). We are also investigating possible ptprsa interactions with CSPGs through the evaluation of ptprsa expression in adult zebrafish brainstem-derived

primary neuron cultures. We observed ptprsa mRNA expression in uninjured brain and spinal cord tissue as well as in injured brain tissues. We have also detected ptprsa mRNA expression in brainstem neuron cultures grown on a growth-permissive laminin substrate, as well as on a CSPG-containing/laminin substrate. Our qualitative RT-PCR data suggest that overall ptprsa gene expression may not be governed by injury (*in-vivo*) or substrate (*in-vitro*). We are currently determining whether ptprsa activity is differentially regulated in the presence versus absence of injury through a post-transcriptional mechanism. Using morpholino knockdown, *in-vitro* experiments are also underway to determine whether ptprsa functionally interacts with zebrafish specific neurocan. Funded by U.S. Dept. of Defense W81XWH-10-1-0617 to JP.

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Dual function of the Bub1 kinase in the spindle checkpoint and the DNA damage response.

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The DNA damage response (DDR) and the spindle assembly checkpoint (SAC) are two critical mechanisms by which mammalian cells maintain genome stability. There is a growing body of evidence that DDR elements and SAC components crosstalk. Here we report that Bub1 (Budding Uninhibited by Benzimidazoles 1), one of the critical kinetochore proteins essential for SAC, is required for optimal DDRs. We found that knocking-down Bub1 resulted in prolonged H2AX foci and comet tail formation as well as hypersensitivity in response to ionizing radiation (IR). Further, we found that Bub1-mediated Histone H2A Threonine 121 phosphorylation was induced after IR in an ATM-dependent manner. We demonstrated in vitro and in vivo that the ATM kinase, being activated in mitosis and in response to DNA damage, phosphorylated Bub1 on Serine 314. We further showed that ATM-mediated Bub1 Serine 314 phosphorylation was required for Bub1 activation during mitosis, for initiation of SAC and for the optimal DDR. Together, we elucidate the molecular mechanism of Bub1 activation and highlight dual functions of the Bub1 kinase in DDR and SAC.
Key words: ATM, Bub1, DNA Damage Response, Spindle Assembly Checkpoint

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The Nek8 protein kinase, mutated in the human cystic kidney disease nephronophthisis, is both activated and degraded during ciliogenesis.

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Mutations in the NIMA-related kinase, Nek8, are associated with cystic kidney disease in both humans and mice, with Nek8 being the NPHP9 gene in the human juvenile cystic kidney disease, nephronophthisis. Previous localisation studies have shown that Nek8 localises to centrosomes and cilia in dividing and ciliated cells, respectively, strengthening the ciliary hypothesis of cystic kidney disease. However, the role of Nek8 in ciliogenesis remains to be defined and no substrates for this kinase have yet been identified. Here, by establishing Nek8 kinase assays, we first confirmed that localization of Nek8 to centrosomes and cilia is dependent upon both its kinase activity and its C-terminal non-catalytic RCC1 domain. The kinase domain alone is active, but does not localize correctly, while the RCC1 domain does localize correctly and can be phosphorylated by Nek8. We propose that centrosome recruitment is mediated by the RCC1 domain, but requires a conformational change in the full-length protein that is promoted by autophosphorylation. Interestingly, three NPHP9-associated mutants retain

full kinase activity. However, only two of these, L330F and A497P, localize correctly, suggesting that the third mutant, H425Y, disrupts the centrosome-targeting motif in the RCC1 domain. Importantly, we also found that serum starvation induces proteasomal degradation of Nek8, specifically in cell lines in which serum starvation induces quiescence and ciliogenesis. Strikingly, serum starvation also induces activation of the Nek8 kinase, whilst overexpression of Nek8 leads to microtubule hyperacetylation and suppression of ciliogenesis. Taken together, these findings reveal important insights into the mechanisms through which Nek8 activity and localization are regulated, and suggest that Nek8 is an inhibitor of ciliogenesis.

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A novel role for Ack1 in the regulation of CTP synthase.

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Ack1 (activated cdc42-associated kinase 1) is an evolutionarily conserved effector of the Rho GTPase Cdc42 although little is known regarding the biological functions of this non-receptor tyrosine kinase. To investigate the role of Ack1 in vivo, we studied the homologous protein DAck in the genetically tractable organism *Drosophila melanogaster*. *DACK* null female flies displayed reduced fertility and defects in oogenesis, suggesting a possible function for DAck in germ cell development. In female germ cells, DAck localized to unusual 20 μm long filamentous structures distinct from conventional cytoskeletal elements. Co-localization studies revealed that these filaments correspond to recently described novel filaments of the enzyme cytidine triphosphate synthase (CTPS). CTPS plays an essential role in CTP synthesis and in the production of phospholipids. The inactive form of CTPS forms filaments in bacteria and *Saccharomyces cerevisiae*, suggesting that filament formation controls CTPS activity and supporting the notion that these structures serve an important evolutionary function. We find that CTPS filament number, morphology, and distribution are altered in *DACK* null or *DACK* overexpressing flies, implicating DAck as a critical regulator of CTPS during oogenesis. Furthermore, in mammalian cells, chemical inhibition of CTPS results in the formation of morphologically identical filaments that contain both CTPS and activated Ack1. Our results identify Ack as a second component of CTPS filaments and implicate Ack in the regulation of CTPS activity in both flies and mammals.

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Simple Informatics Approach to Correct Arginine-Proline Conversion in SILAC Data.

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Stable isotope labeling by amino acids in cell culture (SILAC) is widely used to monitor the change in the abundance of proteins in cells. In this method, a natural amino acid (most commonly arginine and/or lysine) in one of two cell cultures is replaced with heavy labeled amino acid, and the culture is treated with the biologically active sample. The other cell culture is cultured with light (natural) amino acid and treated with a control sample, and finally the findings are validated by swapping the labels. However, it has become known that heavy labeled arginine is catabolically converted to heavy labeled proline inside the cells, which could cause a miscalculation of the ratios of proline-containing light and heavy peptides. Accurate quantification of protein, especially phosphorylations is a key step to understanding many cellular events so we developed a correction method of calculating arginine–proline conversion easily.

To this end, we analyzed 1:1 mixtures of 4 lines of cultured cells grown in light or heavy media using an LTQ Orbitrap mass spectrometer. The histograms, light vs heavy and heavy vs light, showed a large shift in the distribution of peptide ratios. This shift became greater as the

number of prolines per peptide increased but did not occur with other amino acids. When we looked at the subset of peptides in which prolines increased, we found that the median of this subset increased linearly. By using this correlation, the large shift in the ratio distribution was completely overlapped and centered at zero. We tested 4 lines of cultured cells, and found that the shift in the ratio distribution varied according to the cell line and also to the culture condition (i.e. duration of labeling, treatment with a drug). We applied this method to see the effect of PD0325901 (a pure MEK inhibitor) on cellular phosphorylation of proteins in COLO205, DLD1, HCT15, and HCT116 cells.
In conclusion, we established a new simple calibration method for SILAC data which does not rely on special software to solve the arginine–proline conversion. We hope this finding will facilitate the quest to understand cell mechanism more precisely than before.

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Signal transduction network mediated homeostatic photobiomodulation on high glucose induced dysfunctions of C2C12 myoblasts.

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Background and Objective: Photobiomodulation promotes the wound healing of diabetes. Summer sunlight improves diabetes. These phenomena were simulated with the modulation of red light (640±15 nm) at 0. 35 W/m^2 from light emitting diode array for 15 min (RLED) once a day on high glucose induced dysfunctional C2C12 myoblasts and their signal transduction pathways were studied in this paper. **Study Design/Materials and Methods:** The C2C12 myoblasts were cultivated in 10% fetal bovine serum and the glucose at **22.5** (CON) and 90 (HG) **mmol/L**, respectively. The RLED irradiated HG groups were with or without LY294002 or SP600125, the inhibitor of phosphatidylinositol 3-kinase (*PI3K*) or Jun NH2-terminal kinase (JNK). All the parameters were routinely assessed. **Results: (1) There were no effects of RLED on the proliferation in the CON group or** HG +SP600125(10 μmol/L) group, of SP600125 at 1, 2 or 5 μmol/L on the HG+RLED proliferation, and of SP600125(10 μmol/L) on HG proliferation**. (2)** Compared with the CON group at the 72nd h from HG-cultivated C2C12 myoblasts on**, HG** induced apoptosis ($P < 0.05$), inhibited proliferation ($P < 0.01$) and ATP activity ($P < 0.01$), and increased caspase-3/7 activities ($P < 0.05$). According to Western blotting, HG reduced AKT Threonine 473 phosphorylation and increased JNK phosphorylation, respectively. **HG further** increased the mRNA level of forkhead transcription factor (FOXO) 3a ($P < 0.01$) and Bim ($P < 0.05$). HG increased the reactive oxygen species level ($P < 0.05$) at the 18th h, extracellular activities in lactate dehydrogenase ($P < 0.01$) at the 24th h, loss of mitochondrial membrane potential at the 48th h ($P <0.05$) and at the 72nd h ($P < 0.01$). **HG also increased** at the 4th h but decreased at the 72nd h $NAD^+/NADH$ ($P < 0.01$). RLED inhibited all those HG effects, but LY294002 inhibited all those RLED effects. **(3) At** the 72nd h**, HG decreased** manganese superoxide dismutase (MnSOD) mRNA level ($P <0.05$) and sirtuin 1 (SIRT1) protein level, but increased p27 mRNA level ($P < 0.05$). RLED inhibited all those HG effects, but there were no effects of LY294002 on all those RLED effects. (4) **At** the 72nd h**, HG increased** insulin-like growth factor (IGF) 1 mRNA level ($P < 0.05$) and decreased SIRT1 mRNA level ($P < 0.05$), but the mRNA level of the two genes was increased with RLED ($P < 0.05$) and further increased with LY294002 ($P < 0.01$). (5) **At** the 72nd h**,** HG proliferation was significantly promoted by SP600125 at 1, 2 or 5 μmol/L ($P<0.05$), which was further significantly promoted by RLED ($P < 0.01$). **Conclusions:** RLED might self-adaptively promote the establishment of proliferation-specific homeostasis of C2C12 myoblasts by inhibiting HG-induced apoptosis, which might be coordinatedly mediated by PI3K, JNK and IGF-1 signal transduction pathways.

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Temporal evaluation of differential expressive proteins during the progression of balloon angioplasty-induced neointimal hyperplasia.

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Background: Restenosis is still a limiting factor for clinical applications of percutaneous transluminal coronary angioplasty (PTCA) with stent deposition. Elucidation of the molecular mechanisms involved in the progression of restenosis remains a pivotal issue.
Objectives: We attempted to explore the candidate proteins with temporal differential expression in the balloon-injured artery thus identifying the potential targeting molecules for preventing neointimal formation.
Methods: Neointimal hyperplasia was induced in rat model of balloon angioplasty, and the candidate proteins with temporal differential expression were screened by the method of two-dimensional gel electrophoresis (2-DE) coupling with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Additionally, the small interfering RNA (siRNA) oligonucleotides were applied to clarify the possible effect of the candidate proteins in the cell growth and migration of vascular smooth muscle cells (VSMCs).
Results: Four candidate proteins, glutamate dehydrogenase 1 (GLUD1), glutathione S-transferase pi (GSTp), protein disulfide isomerase family A member 3 (PDIA3) and phosphoglycerate kinase 1 (PGK1), have been identified with temporal difference in protein expression after balloon angioplasty. Our current results showed that silencing PDIA3 or PGK1 reduced VSMCs proliferation, and PGK silence was found to be associated with activation of AMP-activated protein kinase (AMPK) and deactivation of extracellular signal-regulated kinase 1/2 (ERK1/2). Furthermore, migration of VSMCs can be suppressed by silencing GSTp, PDIA3 or PGK1. Of these, PDIA3 or PGK1 silence was resulted in a decreased expression in both matrix metalloproteinase (MMP)-2 and MMP-9 proteins.

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Mild electrical stimulation with heat shock ameliorates diabetic nephropathy.

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Diabetic nephropathy is a serious complication of diabetes with only limited treatment. Glomerular visceral epithelial cells (podocytes) play a critical role in maintaining the structure and function of the glomerular filtration barrier. It is known that podocyte foot process effacement in glomeruli is observed in early stage of diabetic nephropathy and is caused by apoptosis and disruption of cytoskeleton of podocytes, leading to progression of chronic renal injury. Previous studies have shown that specific deletion of the insulin receptor from podocytes in mice resulted in kidney diseases, implying that podocyte insulin signaling is important for normal kidney function. We previously showed that mild electrical stimulation and heat shock (MET) activated insulin signaling in various cell lines. Results of our investigation in healthy human subjects indicated that MET tended to improve serum creatinine level and blood urea nitrogen. These results suggested that MET could have protective effects against podocyte apoptosis and might induce reorganization of the cytoskeleton by activating the insulin signaling, leading to the improvement of diabetic nephropathy. Here, we investigated whether MET could affects podocyte insulin signaling. MET treatment for 10 min induced Akt phosphorylation in immortalized mouse podocytes (MPC5). We also assessed the effect of MET on high glucose (25mM)-induced apoptosis of MPC5. Pre-treatment of MET reduced high glucose-induced apoptosis of MPC5. To determine the effect of MET on podocyte cytoskeleton, we performed

phalloidin staining in MPC5. MET reorganized the cytoskeleton, similar to insulin. Finally, we investigated the effect of MET on in vivo model of diabetic nephropathy. It is known that db/db mice (leptin receptor-deficient mice, resulting in diabetes) have features of diabetic nephropathy. Db/db mice were treated with MET for 10 min twice a week for 5-20 weeks. MET prevented albuminuria and serum creatinine. To determine renal injury, we assessed mRNA in mice kidney. MET tended to decrease mRNA of renal injury markers and pro-inflammatory cytokines and pro-fibrotic cytokines. Our results indicated that MET could improve diabetic nephropathy by preventing apoptosis and cytoskeleton destruction of podocytes through activation of insulin signaling.

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Zinc protoporphyrin up-regulates heme oxygenase-1 in PC-3 cells via the PI3-kinase-ERK-Nrf2 signaling pathway.

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Objectives. Zinc protoporphyrin (ZnPP), a naturally occurring molecule formed in iron deficiency or lead poisoning, is a potent competitive inhibitor of heme oxygenase-1 (HO-1). It also regulates the expression of HO-1 at the transcriptional level. However, the effect of ZnPP on HO-1 expression is controversial. It was shown to induce HO-1 expression in some cells, but suppress it in the others. In fact, it even suppressed the induction of HO-1 by inducers, such as statins and lipopolysaccharide. The objective of this study is to investigate the mechanism of action of ZnPP on the induction of HO-1 in human prostate cancer PC-3 cells. Methods. PC-3 cell line was used as a model to study the mechanism of action of ZnPP. Cell proliferation was determined using CellTiter assay. Activation of HO-1 promoter by ZnPP was determined by enhancer-luciferase reporter assays. Induction of HO-1 protein was studied by Western blot analyses. Results. ZnPP induced significant cell proliferation in PC-3 cells at a concentration of 0.6-10 μM, but suppressed cell growth above 20 μM. Therefore, 10 μM ZnPP was used for all subsequent experiments. Basal level of HO-1 protein in PC-3 was undetectable. Incubation of the cells with 10 μM ZnPP for 4 h showed only a slight induction of HO-1 protein, but the induction was high after 16 h and was maintained through 48 h of incubation. Of all the known responsive elements in the HO-1 promoter, ZnPP activated mainly the stress response elements (StREs). StRE3 showed the highest (6.6-fold) induction level by ZnPP, although these elements had different levels of relative luciferase activities due to different copy number of the response elements present in the luciferase-reporter constructs. ZnPP did not activate the HSE, SREBP and SP1 elements. To investigate the effect of various protein kinase inhibitors and antioxidant on the activation of StRE by ZnPP, cells transfected with StRE3-pGL3 were pretreated with SB203580 (p38-MAPK inhibitor), LY294002 (PI3-kinase inhibitor), U0126 (MEK inhibitor), SP600125 (JNK inhibitor), IPA-3 (p21-activated Kinase Inhibitor III) or N-acetyl cysteine (NAC, antioxidant) prior to treatment with ZnPP. SP600125 had little effect. SB203580, IPA-3, and NAC reduced the activation by less than 50% of the control. However, LY294002 and U0126 suppressed the activation to 28.3 and 22.8%, respectively, of the control, suggesting the involvement of PI3-kinase and ERK. Conclusions. ZnPP up-regulated HO-1 expression in PC-3 cells via PI3-kinase-ERK-Nrf2 signaling pathway.

Signaling Scaffolds and Microdomains

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Molecular characterization of a multiprotein complex that links membrane depolarization with gene expression in skeletal muscle.

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Electrical activity regulates the expression of skeletal muscle genes by a process known as “excitation-transcription” (E-T) coupling. We have previously demonstrated that release of ATP after depolarization activates membrane P2X/P2Y receptors, being a fundamental mediator between electrical stimulation, slow calcium transients and gene expression. We propose that this pathway would require the proper coordination between the voltage sensor (dihydropyridine receptor, DHPR), pannexin hemichanel (ATP release conduit), nucleotide receptors, and several signal molecules. The goal of this study was to assess protein-protein interactions within the E-T machinery in skeletal muscle, in order to unveil a putative signaling complex.

Co-immunoprecipitation of the selected proteins was achieved in extracts of newborn rat derived myotubes, and in rat and mouse adult muscle isolated triads. Components of multiprotein complexes were also resolved using blue-native SDS/PAGE, and selected proteins detected by immunoblot. Immunofluorescence assays were performed in isolated fibers from mice adult muscles. We constructed cDNA for fused $P2Y_2$- or PnX1-proteins with the One-STrEP-tag and transfected into L6-rat skeletal muscle cells to allow one-step purification of protein complexes on Strep-tactin Superflow.

DHPR, $P2Y_2$ receptor, Panexin 1, phospholipase Cγ1 and dystrophin, all co-immunoprecipitated in a crossed manner in the different preparations assessed. DHPR, pannexin-1, $P2Y_1$, $P2Y_2$ and $P2X_7$ did show a striated pattern of distribution by immunofluorescence, possibly representing location at the T-tubules in adult skeletal fibers. Blue-native SDS/PAGE for adult muscle triads showed that DHPR is an integral part of large multiprotein complexes of different sizes, some of them also containing $P2Y_2$ and Pannexin-1 protein. Novel interactors within these complexes using MALDI-TOF mass spectrometry are being explored. We have also expressed $P2Y_2$- and PnX1- proteins with the One-STrEP-tag into L6-rat skeletal muscle cell line in order to isolate and characterize the functional multiprotein complex in different physiological conditions.

Results strongly suggest that there is a multiprotein complex located in skeletal muscle T-tubules that is relevant to link membrane depolarization with gene expression. Unveiling general mechanisms that lead muscle plasticity could help to understand and treat a variety of skeletal muscle disorders associated with muscle homeostasis.

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Stimulus-specific activation and actin dependency of distinct, spatially separated ERK1/2 fractions in A7r5 smooth muscle cells.

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A proliferative response of smooth muscle cells to activation of extracellular signal regulated kinases 1 and 2 (ERK1/2) has been linked to cardiovascular disease. In fully differentiated smooth muscle, however, ERK1/2 activation can also regulate contraction. Here, we use A7r5 smooth muscle cells, stimulated with 12-deoxyphorbol 13-isobutylate 20-acetate (DPBA) to induce cytoskeletal remodeling or fetal calf serum (FCS) to induce proliferation, to identify factors that determine the outcomes of ERK1/2 activation in smooth muscle. Knock down experiments, immunoprecipitation and proximity ligation assays show that the ERK1/2 scaffold caveolin-1 mediates ERK1/2 activation in response to DPBA, but not FCS, and that ERK1/2 is released from caveolin-1 upon DPBA, but not FCS, stimulation. Conversely, ERK1/2 associated with the actin cytoskeleton is significantly reduced after FCS, but not DPBA stimulation, as determined by Triton X fractionation. Furthermore, treatment with the inhibitor of actin polymerization, cytochalasin D, inhibited DPBA, but not FCS induced ERK1/2 phosphorylation, indicating that the actin cytoskeleton is not only a target but also is required for ERK1/2 activation. Our results show that (1) at least two ERK1/2 fractions (caveolar and actin-associated) are regulated separately by specific stimuli, and that (2) the association of ERK1/2 with the actin cytoskeleton regulates the outcome of ERK1/2 signaling. Our findings help explain how in smooth muscle, ERK1/2 signaling can be directed either towards proliferation, as associated with atherosclerosis, or towards contractility associated with hypertension. Support: HL80003, HL86655.

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Identification of a novel Bcl10 domain that contributes to NF-κB activation.

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The NF-κB transcription factor is a centrally important mediator of T cell functional responses. Activation of the NF-κB signaling pathway via T cell receptor (TCR) stimulation requires the adaptor protein Bcl10. How Bcl10 transmits NF-κB -activating signals remains incompletely understood. To better understand the function of the Bcl10 protein, we have performed deletion and site-directed mutagenesis of the 12-amino acid Bcl10 N-terminal peptide, a domain which has not yet been studied in-depth. Our results show the Bcl10 N-terminal peptide is functionally important for TCR-mediated activation of NF-κB. Deletion of this peptide or replacement of amino acids 2-12 with alanines abolishes Bcl10-mediated NF-κB activation, and mutation of residues 5 and 10-12 also substantially reduces Bcl10 signaling to NF-κB. Further analysis of these N-terminal peptide mutants by co-immunoprecipitation has shown that mutation at specific sites, such as 10-12, causes a pronounced decline in binding to partner proteins, including CARMA1. Imaging of these mutants in stimulated T cells shows absent or aberrant Bcl10 translocation to known cytoplasmic NF-κB signaling clusters called POLKADOTS. Altogether these results demonstrate that the Bcl10 N-terminal peptide is required for Bcl10 association with signaling partners, Bcl10 cellular translocation, and for signal transmission to NF-κB. These data establish the N-terminal peptide of Bcl10 as a novel determinant of Bcl10 function.

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Co-recruitment with the GM-CSF Receptor Beta Chain to Intersectin/Clathrin-Coated Pit is Essential for JAK2V617F-mediated Autonomous Cell Growth.

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The V617F mutation of JAK2 has been frequently identified in neoplastic myeloproliferative disorders. JAK2V617F-mediated autonomous cell growth requires the interaction of JAK2V617F with un-liganded cytokine receptors. However, the underlying mechanism remains elusive. Here we demonstrate that the GM-CSF receptor β chain (GMR β) constantly undergo ligand-independent endocytosis, albeit such process can be enhanced by the presence of ligand and the GM-CSF receptor α chain. Recruitment of the GMR complex onto intersectin/clathrin-coated pit (CCP) is essential for GM-CSF signaling. A WxxxI motif in the membrane proximal region of GMRβ is required for the GMR complex to be recruited to intersectin/CCP where wild-type JAK2 is activated in a ligand-dependent manner. Interestingly, blocking GMRβ recruitment to intersectin/CCP via mutation of the WxxxI motif or knockdown the expression of intersectin or clathrin abolished ligand-independent activation of JAK2V617F. This study reveals that un-liganded GMR promotes JAK2V617F-mediated autonomous cell growth via co-recruitment of this oncoprotein to intersectin/CCP for activation.

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Role of IQGAP1 in S6K-Akt1 Feedback.

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Defining the mechanisms that orchestrate cell growth and division to regulate cell proliferation is crucial to understanding tumorigenesis. The mTOR pathway, the center of cell growth control, is commonly activated in human cancers but proved ineffective as a clinical target due to incomplete understanding of its mechanisms in cell growth inhibition. IQGAP1, a widely conserved effector/regulator of CDC42GTPase is a putative oncogene that control cell proliferation, however, its mechanism in tumorigenesis is unknown. Using complementary studies in yeast and mammalian cells, here we report that IQGAP1 has a key role in regulating the negative feedback loop (NFL) of the target of rapamycin complex1 (mTORC1) that control cell growth. A Two-hybrid screen identified yeast TORC1-specific subunit, Tco89p, as an Iqg1p-binding partner sharing roles in rapamycin-sensitive growth, axial bud-site selection and cytokinesis, thus coupling cell growth and division. Mammalian IQGAP1 binds mTORC1 and Akt1 and in response to epidermal growth factor (EGF), cells expressing the mTORC1/Akt1-binding region, IQGAP1^{IR-WW}, contained attenuated *p*ERK1/2 activities and inactive glycogen synthase kinase 3α/β (*p*GSK3α/β), which control apoptosis. Interestingly, these cells displayed high *p*Akt1^{S473} activity but attenuated activity of the mTORC1-dependent kinase, *p*S6k1^{T389} and induced mTORC1/Akt1-depnedent transformed phenotypes. Moreover, IQGAP1 influences cell abscission and its activity is elevated in carcinoma cell lines. These findings support the model that IQGAP1 acts upstream on the mTORC1/S6K1→Akt1 NFL and downstream of it to couple cell growth and division and regulate cell homeostasis, dysregulation of which leads to tumorigenesis. These results could have implications on developing the next generation of anticancer therapeutics.

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Oxidative stress enhances pro- inflammatory signal cascade by increasing association of TLR4 with it's adaptor TIRAP (MAL), enhancing of TIRAP-MyD88 signaling pathway.

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Resuscitated hemorrhagic shock following trauma is known to contribute to the development of late organ dysfunction in patients who survive the initial trauma insult and thus contributes to morbidity and mortality in this patient population. There is ample evidence to suggest that hemorrhagic shock followed by resuscitation fluids causes excessive immune response via enhanced reactive oxygen species (ROS). TLR4 is a trans- membrane receptor on immune system cells that transduces Gram negative bacterial ligand's signal into the cell, culminating in synthesis of pro- inflammatory cytokines. Two sets of TLR4- related adaptor proteins exist: MyD88-TIRAP (MAL) and TRAM-TRIF that relay the signals into the cell. The mechanism of ROS stimulation of the innate immune response is incompletely understood. To gain insight into the mechanism by which ROS enhances the innate immune response, we created a murine macrophage cell line (RAW 264.7) that constitutively produce mouse TLR4 tagged with HA. This cells were transiently co- transfected with TIRAP (MAL) -flag. Using immunoprecipitation with HA antibody and immunobloting with flag antibody, we show that Hydrogen Peroxide (H2O2) treatment results in increased association of TLR4 with the adaptor protein TIRAP (MAL) in temporal and dose- related fashion. Furthermore, H2O2 enhanced signaling through the MyD88-TIRAP (MAL) pathway (p-p38); and decreased signaling through the TRAM-TRIF pathway (p-IRF3). These findings suggest that H2O2 disrupts the immune response homeostasis by increasing the MyD88-TIRAP (MAL) pathway that may culminate in production of pro- inflammatory cytokines; and decreasing the TRAM-TRIF pathway that may result in production of anti- viral agents like INFβ.

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Phase Transitions of Multivalent Signaling Proteins Regulate Actin Assembly.

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The actin cytoskeleton plays a key role in various biological processes such as endocytosis, differentiation and motility. However, how actin assembly is spatially and temporally controlled in cells remains elusive. In cells, the Arp2/3 complex plays a central role in controlling actin dynamics by catalyzing nucleation of new actin filaments. The Arp2/3 complex, in turn, is regulated by various members of the neuronal Wiskott Aldrich Syndrome Protein (N-WASP) family.

In podocytes, epithelial cells in the kidney, the cytosolic portion of the transmembrane protein Nephrin recruits Nck to the membrane, which in turn recruits N-WASP. Nck has three SH3 domains that interact with the six proline-rich motifs of N-WASP, and one SH2 domain that interacts with the three phosphotyrosine motifs of Nephrin. Cooperative interactions between Nephrin, Nck and N-WASP oligomerize N-WASP and enhance its activation of the Arp2/3 complex.

We have discovered that the multi-valent interactions between N-WASP, Nck and Nephrin lead to a sharp transition between small complexes and large polymer. This transition coincides with a macroscopic phase transition that produces micron-size droplets of a second liquid-like phase in equilibrium with bulk solution, which we term the "droplet phase." This phase transition occurs as a function of the number of phosphotyrosines in Nephrin, suggesting that this phenomenon

could be regulated by kinases in cells. The phase transition also corresponds to a sharp change in activity toward the Arp2/3 complex, indicating that a switch-like transition from small oligomers to large assemblies of N-WASP could be an important mechanism to spatially and temporally control actin assembly.

In cells, the interaction between Nephrin, Nck and N-WASP occurs at the membrane, and therefore the “droplet phase” will be the 2-dimensional corollary to the observed 3-dimensional droplets. To understand this behavior in the context of the membrane, we have reconstituted interactions between N-WASP, Nck and Nephrin on supported lipid bilayers. We have discovered that the multi-valent interactions between these players drive them to form micron-sized dynamic puncta, and now aim to study the physical properties of the puncta and the parameters that control their formation.

Multi-valent molecules are present everywhere in biology - both at the membrane and in the cytoplasm. This kind of phase transition, resulting from multi-valent interactions, could be a general phenomenon that nature uses to regulate biological functions.

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Detergent-resistant membranes are essential along the signalling pathways of prostate-specific membrane antigen.

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Prostate-specific membrane antigen (PSMA) is a surface protein overexpressed in prostate cancer and neovasculature of further tumours. It is therefore one of the most promising biomarkers in diagnosis and treatment of prostate cancer.
PSMA is associated with detergent-resistant membranes (DRMs). The mature form of PSMA is mainly insoluble in Lubrol WX, but does not associate with Triton X-100 DRMs.
Recently we could demonstrate that cross-linking of cell surface PSMA with specific antibodies activates different signalling cascades like the MAPK-pathway. Here we show that internalization of PSMA is increased after antibody binding and this leads to redistribution of PSMA to Triton X-100 DRMs in a time-dependent manner and to alterations in the expression levels of several proteins in these DRMs. One of these proteins is laminin receptor-1 which directly interacts with PSMA in co-immunoprecipitation experiments.
Our results propose an essential role for different DRM types in the signalling pathways involving PSMA.

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Hedgehog signaling is dependent on ciliary trafficking proteins in the sea urchin embryo.

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A recent frontier in Hedgehog (Hh) signaling research is the requirement of the primary cilium and intra-flageller transport (IFT) proteins for signal transduction. Studies in vertebrates have shown that proper trafficking of Hh pathway components within the primary cilium is essential for Hh signaling. This machinery is not necessary, however, for Hh signal transduction in protostomes such as Drosophila. As a basal deuterostome, the sea urchin occupies a unique phylogenetic position and can provide crucial insight into the evolution of hedgehog signaling. Here we provide evidence that, as in vertebrates, Hh signaling in the sea urchin relies on the presence of cilia. It has been previously shown by Robert Morris and John Scholey that Kinesin II is essential for cilia assembly. Indeed, knockdown of Kinesin II using an antibody phenocopies Hh morphants. Likewise, knocking down the microtubule associated protein, Costal2, also

produces a phenotype consistent with inhibition of Hh signaling. These findings lead to a model of sea urchin Hh signaling that closely resembles the pathway as it functions in vertebrates. This indicates the necessity of cilia for Hh signal transduction is not vertebrate specific as previously speculated but is in fact deuterostome specific.

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PSD-95 binding capacity of GPR30, an estrogen receptor that can be identified in CA1 dendritic spines in vivo.

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Dendritic spines are often the sites for excitatory synapse formation, and the steroid hormone estrogen stimulates an increase in spine formation and subsequent synaptogenesis in the hippocampus. Recent studies have demonstrated that the estrogen receptor can be immunolocalized to non-nuclear sites in hippocampal neurons, and specifically, estrogen receptors have been identified at CA1 pyramidal neuron dendritic spines. Such spine-targeting of estrogen receptors potentially allows for local signaling from hormonal stimuli in order to modify dendritic spine structure or to downstream neuronal responses.
To determine how the estrogen receptor is localized to the dendritic spine, and to identify specific protein binding partners that might selectively direct a rapid, estrogen-stimulated non-genomic response, we are biochemically investigating the estrogen receptor interaction with dendritic spine proteins. One potential membrane-localized estrogen receptor that has described is GPR30. Our preliminary studies demonstrate that GPR30 is expressed in the stratum radiatum of the rat hippocampus in vivo, and can be immuno-detected in hipppocampal synaptosome lysates in vitro. Therefore, it is important to understand how receptors such as GPR30 might interact with spine-specific proteins. GPR30 target partners relevant to our studies include the spine scaffolding protein PSD-95, and GPR30 localization to the dendritic spine avails the receptor to interact with and potentially to modulate other post-synaptic receptors.

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ErbB receptors exist in distinct signaling domains in primary cilia.

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The epidermal growth factor receptor (EGFR) signaling axis exacerbates autosomal dominant polycystic kidney disease (ADPKD) caused by the expression of mutant polycystin-1 (PC1) or polycystin-2 (PC2) proteins. PC1 and PC2 are localized to primary cilia where they are central to ciliary calcium signaling and tyrosine kinase cascades. Our objective was to elucidate the mechanism by which the ErbB signaling proteins interface with polycystins in the cilia by analyzing their localization to and organization within the primary cilium and how this is disrupted in ADPKD. Our results show that EGFR, ErbB2, and PC2 localize to the primary cilium of renal epithelial cells. Ciliary localization of all three proteins is perturbed in PKD cells. Based on co-immunoprecipitation experiments, EGFR is in a complex with PC1 and PC2. Our previous work demonstrated that the polycystins associate with specialized membrane domains organized by cholesterol binding flotillin proteins. Here we show that the flotillin-1 and -2 proteins also localize to cilia and interact with the polycystins and EGFR. The data suggest that the cholesterol binding flotillins may serve a major function in trafficking signaling proteins to the primary cilium and subsequent organization of ciliary signaling hubs. Furthermore, we have identified a conserved VxPx motif common to the polycystins, EGFR, and ErbB2 which serves as primary binding sites for a multimeric GTPase complex with known function in exocytosis.

The VxPx motif has been shown by our lab to be essential for Arf4 GTPase mediated ciliary trafficking of PC1. Purified, active Arf4 interacts with EGFR. In conclusion, we have shown for the first time that EGFR and ErbB2 localize to the primary cilium of renal epithelia and interact with domain organizing flotillins in addition to polycystins which, when mutant, cause ADPKD.

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The Role of CD82 in Hematopoietic Stem Cell Niche Adhesion.

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The spatial organization and dynamics of proteins and lipids within the cell membrane is important for the regulation of cell signaling, adhesion, and cell communication. Within the bone marrow niche, communication between hematopoietic stem/progenitor cells (HSPCs) and niche cells is essential for regulating their proliferation, differentiation, and survival. Previous work from our laboratory has ascertained that HSPCs utilize a polarized domain on the plasma membrane that serves as the contact site with osteoblasts, which are important members of the bone marrow niche. Using primary CD34+ cells and the progenitor-like KG1a cell line, this domain was found to be enriched in the specific tetraspanin proteins, CD63, CD81, and CD82. Tetraspanins are multi-spanning membrane proteins that act as scaffolds for the organization of membrane domains important for regulating adhesion and signaling. In particular, our characterization of CD82 interactions using CD82 blocking antibodies revealed a significant decrease in adhesion of HSPCs to niche cells as well as in the *in vivo* homing and engraftment capabilities of these cells. In order to determine the molecular mechanisms of CD82's role in adhesion, we cloned CD82 into the mCherry expression vector and transfected this vector along with the mCherry control vector into KG1a cells. Stable cell lines were selected and isolated by fluorescence cell sorting. Expression of the CD82-mCherry and mCherry control constructs were verified by fluorescence imaging, and the overexpression of CD82 was confirmed by Western blot. We next performed adhesion assays to assess the effects of CD82 overexpression on cell adherence. Our data indicate that CD82 overexpression leads to at least a two-fold increase in adhesion to fibronectin, in addition to increased adhesion to an osteoblastic monolayer. These data suggest that CD82 may have a role in altering the affinity or avidity of integrins, and our primary focus has been on the $\alpha4\beta1$ integrin, also known as VLA4. Future studies will be directed towards evaluating these cell lines in proliferation and VLA4 affinity assays.

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LRP1 mediates the Shh-induced endocytosis of the GPC3-Shh complex.

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Glypicans are a family of heparan sulfate (HS) proteoglycans whose members are bound to the cell membrane through a glycosylphosphatidylinositol (GPI) link. Glypicans regulate the activity of several growth factors including Hedgehogs (Hh), Wnts and bone morphogenetic proteins. We have recently reported that Glypican-3 (GPC3), one of the 6 mammalian glypicans, regulates embryonic growth by inhibiting the hedgehog (Hh) signaling pathway. GPC3 binds Hh and competes with Patched (Ptc), the Hh receptor, for Hh binding. The interaction of Hh with GPC3 triggers the endocytosis and degradation of the GPC3/Hh complex with the consequent reduction of Hh available for binding to Ptc. Currently, the molecular mechanism by which the GPC3/Hh complex is internalized remains unknown. Here we demonstrate that the low-density-lipoprotein receptor-related protein-1 (LRP1) mediates the Hh-induced endocytosis of the

GPC3/Hh complex by showing that internalization is inhibited by the chaperone RAP that blocks the interaction of ligands to LRP1. Furthermore, we also demonstrate that the Hh-induced endocytosis of the GPC3/Hh complex is inhibited by LRP1 siRNA treatment. In addition, we show that internalization of this complex is required for the Hh-inhibitory activity of GPC3. Co-immunoprecitation, pull-down and cell binding assays demonstrate that GPC3 binds through its HS chains to LRP1. Finally, we show that GPC3 is mainly localized outside of lipid rafts/detergent-insoluble membrane microdomains, something unexpected for a GPI-anchored protein, and that the HS-mediated interaction with LRP1 is what causes the removal of GPC3 from the lipid raft domains.

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Dynasore- an inhibitor of clathrin- mediated endocytosis (CME) is a potent activator of NFkB independent of its effect on CME.

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Clathrin- mediated endocytosis (CME) is a process whereby cargo is recruited into clathrin-coated pits, which then form clathrin- coated vesicles that internalize into the cell. It is involved in a variety of cellular processes e.g. internalization of signal complexes and protein and nucleic acid uptake. The GTPase dynamin is essential for CME. The role of CME in activation of downstream signaling pathways was studied using dynasore, a reversible specific inhibitor of dynamin GTPase. Here we show that dynasore is a potent stimulus for NF-κB activation. Specifically, dynasore treatment led to degradation of IκBα, phosphorylation of p65, translocation of phospho-p65 to the nucleus and activation of NF-κB - luciferase construct. Dynasore also augmented phosphorylation of MAPKs, especially SAPK/JNK. These effects were not due to LPS contamination. Further, pretreatment with the antioxidant N-Acetylcysteine (NAC) decreased dynasore- induced activation of NFκB, suggesting that dynasore might exerts its action through a pathway involving Reactive Oxygen Species. Interestingly, the effect was not recapitulated by other CME inhibitors including chlorpromazine or myristyl trimethyl ammonium bromide suggesting that dynasore's effect on downstream signaling is not mediated through inhibition of CME. Data presented underscore the notion that caution should be taken in interpretation of experiments utilizing dynasore. Effects on other GTPase known to be sensitive to dynasore may be responsible for its downstream signaling effects.

Rho-Family GTPases

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ROCK1 & 2 perform essential non-compensatory roles in angiogenesis and angiosarcoma tumor progression.

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The serine/threonine protein kinase paralogs ROCK1 & ROCK2 have been heavily implicated as essential modulators controlling multiple aspects of physiological and tumor angiogenesis; however the majority of published studies have utilized general pharmacological inhibitors of ROCK proteins which exhibit no paralog specificity. To determine the paralog specific roles of ROCK proteins in angiogenesis and vascular tumor formation, we utilized stable shRNA knockdown technology to ablate the expression of ROCK1 or 2 in endothelial and angiosarcoma

cells. Knockdown of ROCK1 & 2 expression results in a non-compensatory disruption of in vitro capillary network formation, cytoskeletal dynamics, and cell migration, while the expression of ROCK2, but not ROCK1, is essential for phosphorylation of myosin light chain phosphatase. Knockdown of ROCK1 or 2 results in significant survival advantages over control cells following serum starvation. Whole genome microarray analysis reveals that ROCK1 & 2 share largely overlapping roles in their ability to counteract VEGF-induced regulation of gene expression in endothelial cells, suggesting that alterations in cytoskeletal dynamics are capable of overriding the effects of mitogenic signaling. Finally, shRNA knockdown of ROCK proteins results in a significant reduction of angiosarcoma solid tumor size. Our data reveal that ROCK1 & 2 exhibit unique and essential roles in endothelial and angiosarcoma cells. Moreover, disruption of ROCK expression is capable of blocking VEGF-induced gene transcription, suggesting that therapeutic targeting of this pathway may have a profound impact on diseases reliant on aberrant angiogenesis.

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Involvement of Rac1 in Ethanol-Induced Endothelial Barrier Dysfunction.

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The microvascular barrier is critical for normal fluid homeostasis, and can be disrupted by concentrations of ethanol achieved after moderate to severe drinking. RhoA and Rac1 are two GTPases involved in endothelial barrier function. RhoA disrupts and Rac1 enhances barrier integrity. We tested the hypothesis that ethanol disrupts the endothelial barrier by increasing RhoA activity or decreasing Rac1 activity. Barrier function of human umbilical vein endothelial cell (HUVEC) monolayers was assessed by determining transendothelial electrical resistance (TER) before and after ethanol treatment (5-100 mM). RhoA and Rac1 activity was also determined in HUVEC treated with 100 mM ethanol for 1, 5, 10, 30, 60, 90, and 120 min. with an ELISA kit. To determine if the ethanol-induced decrease in TER could be inhibited by activation of Rac1, the selective Epac1 activator, 8-CPT-2'-O-Me-cAMP (8-CPT) was applied. The results show that ethanol disrupts TER in a concentration-dependent manner. Ethanol also significantly decreased Rac1 activity at 1, 5, 10, 30, and 60 min. (53% ± 5.8, 55% ± 5.1, 60% ± 5.3, 77% ± 8.9, and 73% ± 4.8 vs. control, $p<0.01$). RhoA activity remained unchanged compared with the baseline activity of vehicle-treated controls. Pretreatment with 8-CPT increased TER and attenuated the overall ethanol-induced decrease in TER, although this was mainly due to the elevated TER after 8-CPT treatment. Interestingly, when added 5 or 10 min after ethanol treatment, 8-CPT shortened the time to recover to baseline TER. The results suggest that ethanol disrupts endothelial barrier function by inactivating Rac1 and not increasing RhoA activity. In addition, Epac1-mediated Rac1 activation may be a useful target to resolve ethanol-induced hyperpermeability. Supported by NIH P20 RR018766, the American Heart Association and the ABMRF/Foundation for Alcohol Research.

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The N-terminal DH-PH domain of Trio induces cell spreading and migration by regulating lamellipodial dynamics in a Rac1-dependent manner.

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The guanine-nucleotide exchange factor Trio encodes two DH-PH domains that catalyze nucleotide exchange on Rac1, RhoG and RhoA. The N-terminal DH-PH domain is known to activate Rac1 and RhoG, whereas the C-terminal DH-PH domain can activate RhoA. Our study shows that the N-terminal DH-PH domain activates Rac1 and RhoG independently from each

other in HeLa cells. In addition, we show that the flanking SH3 domain binds to the proline-rich region of the C-terminus of Rac1, but not of RhoG. However, this SH3 domain is not required for Rac1 or RhoG GDP-GTP exchange. Rescue experiments in Trio-shRNA expressing cells showed that the N-terminal DH-PH domain of Trio, but not the C-terminal DH-PH domain, restored defects in fibronectin-mediated cell spreading and migration that are observed in Trio-silenced cells. Kymograph analysis revealed that the N-terminal DH-PH domain, independent of its SH3 domain, controls the dynamics of lamellipodia. Using siRNA against Rac1 or RhoG, we found that Trio-D1-induced lamellipodia formation required Rac1 but not RhoG. Together, we conclude that the GEF Trio induces lamellipodia formation through its N-terminal DH-PH domain, thus promoting fibronectin-mediated spreading and migration in a Rac1-dependent fashion.

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Crucial role of the small GTPase Rac1 in insulin-stimulated translocation of glucose transporter 4 to the mouse skeletal muscle sarcolemma.

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The Rho family GTPase Rac1 has been implicated in the regulation of glucose uptake in myoblast cell lines. However, no evidence for the role of Rac1 has been provided by a mouse model. The purpose of this study is to test the involvement of Rac1 in insulin action in mouse skeletal muscle. Intravenous administration of insulin indeed elicited Rac1 activation in gastrocnemius muscle, suggesting the involvement of Rac1 in this signaling pathway. We then examined whether insulin-stimulated translocation of the facilitative glucose transporter GLUT4 from its storage sites to the skeletal muscle sarcolemma depends on Rac1. We show that ectopic expression of constitutively activated Rac1, as well as intravenous administration of insulin, caused translocation of GLUT4 to the gastrocnemius muscle sarcolemma, as revealed by immunofluorescent staining of a transiently expressed exofacial epitope-tagged GLUT4 reporter. Of particular note, insulin-dependent, but not constitutively activated Rac1-induced, GLUT4 translocation was markedly suppressed in skeletal muscle-specific rac1-knockout mice compared to control mice. Immunogold electron microscopic analysis of endogenous GLUT4 gave similar results. Collectively, we propose a critical role of Rac1 in insulin-dependent GLUT4 translocation to the skeletal muscle sarcolemma, which has heretofore been predicted solely by cell culture studies.

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Analysis of RhoA and Rho GEF activity in the cell nucleus.

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We have recently demonstrated that a fraction of the total cellular pool of the small GTPase RhoA resides in the nucleus, and that the nuclear guanine nucleotide exchange factor (GEF) Net1 plays a role in the regulation of its activity. In this protocol we describe a method to measure both the activities of the nuclear pools of RhoA and Rho GEFs. This process required the development of a nuclear isolation protocol that was both fast and virtually free of cytosolic

and membrane contaminants, as well as redesigning the existing RhoA and Rho GEF activity assays so they work in nuclear samples. This protocol can be also used for other RhoGTPases and Rho GEFs that have also been found in the nucleus. Completion of the procedure, including nuclear isolation and RhoA or Rho GEF activity assay, takes 1h 40 min.

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Computational analysis of Rho GTPase cycling: extraction of parameters and inference of pathways.

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The Rho family of GTPases control actin organization during diverse cellular responses such as directed migration, cytokinesis and endocytosis. Although the primary members of this family, RhoA, Rac and Cdc42 all have different downstream effects on actin, they share a basic mechanism that involves targeting to the plasma membrane and activation by GTP binding. Our hypothesis is that the details of GTPase cycling between membrane and cytosol are key to the differential upstream regulation of these biochemical switches.
We developed a computational modeling framework to analyze experimental data on GTPase cycling, including binding to the plasma membrane and to GDI. We use published in vivo experimental data to extract kinetic and equilibrium constants for Rac and GDI cycling. The complete model is reformulated as a compact non-dimensional steady state system. The cell surface to volume ratio and GDI concentration are sensitive parameters for the overall system behavior. To explore the effect of regulatory signals, we calculated the membrane fraction of GTPase over a wide range of relevant parameters. This analysis was applied to Rac re-distribution in pancreatic β-cells upon glucose stimulus.
Analysis of experimental data for Rac membrane cycling reveals that the lower apparent affinity of GDI for RacGTP compared to RacGDP can be fully explained by the faster dissociation of the latter from the membrane. While Rac membrane fraction is sensitive to the total GDI expressed in the cell, Rac dissociation rate from the membrane is independent. In contrast, based on published in vitro experimental data, Cdc42 membrane dissociation rate is dependent on GDI and effector concentrations. By increasing the GEF/GAP ratio, a larger fraction of Rac is distributed to the membrane. In β-cells, the model reveals that glucose stimulated Rac re-distribution cannot be completely attributed to modification of the GDI phosphorylation state alone. There must be another signal that decreases Rac dissociation constant with the plasma and granular membranes. PLD activation has been previously proposed as such a signaling molecule.
In conclusion, systematic experiments addressing dissociation rates and membrane fractions of Cdc42 and RhoA in vivo could be used to complete the model parameters for these GTPases. The methodology for pathway inference used for Rac redistribution in β-cell can be extrapolated to guide how specific modifications may compensate for altered GTPase-GDI balance in disease scenarios. (Supported by NIH grant TR01DK087650).

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Mechanical force on ICAM1 leads to RhoA-mediated cytoskeletal changes in endothelial cells.

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RhoA mediated cytoskeletal rearrangements in endothelial cells (ECs) play an active role in transendothelial cell migration (TEM), a normal physiological process in which leukocytes are

recruited from the blood circulation into tissues. While much has been learned about RhoA signaling pathways downstream from ICAM-1 in ECs, little is known about the consequences of the tractional forces that leukocytes generate on ECs as they migrate over the surface before TEM. We have found that after applying mechanical forces to ICAM-1 clusters, there is enhanced RhoA signaling and cellular reinforcement compared to ICAM-1 clustering alone. Using the RhoA activity assay, we have determined ICAM-1 clustering alone leads to ~1.75 fold increase in RhoA activity over untreated cells, whereas the addition of force leads to ~2.5 fold higher RhoA activation over control. There is a ~3.5 fold increase in myosin light chain phosphorylation with force over control compared with a ~2.5 fold increase over control with clustering alone. In addition, we observe a 26% increase in RhoA-dependent cellular stiffening measured by pulling on ICAM-1 clusters with magnetic tweezers. We also have confirmed the cytoplasmic tail of ICAM-1 is required for the observed findings. Taken together these findings indicate that the tractional forces leukocyte generate on ECs leadings to changes in the ECs cytoskeleton. These changes may assist in downstream leukocyte TEM. Further exploring how ECs respond to forces generated by leukocytes on ICAM-1 will contribute to our understanding of inflammatory diseases where leukocyte TEM correlates with disease progression, such as atherosclerosis, ischemic cardiomyopathy, and ischemia reperfusion injury.

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Negative Regulation of RhoA Translation and Signaling by hnRNP-Q1 Affects Cellular Morphogenesis.

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The small GTPase RhoA has critical functions in regulating actin dynamics which affects cellular morphogenesis through the RhoA/ROCK signaling cascade. RhoA signaling controls stress fiber and focal adhesion formation and cell motility. In the central nervous system, RhoA signaling is involved in several aspects of neuronal development which include neuronal migration, growth cone collapse, dendrite branching and spine growth. Altered RhoA GTPase signaling is implicated in cancer and neurodegenerative disease and is linked to forms of inherited intellectual disabilities. While much is known about factors regulating RhoA activity and/or degradation, little is known about molecular mechanisms regulating RhoA expression and their implications in regulating RhoA signaling. We hypothesized that the posttranscriptional control of RhoA translation may provide a mechanism to regulate RhoA signaling and the downstream effects on cellular morphogenesis. Here, we uncovered a cellular function for the mRNA-binding protein hnRNP-Q1 to control neuronal and non-neuronal cell morphogenesis which involves the negative regulation of RhoA synthesis and signaling. We show that hnRNP-Q1 directly interacts with the 3'UTR of RhoA mRNA and represses RhoA translation. Additionally, hnRNP-Q1 knockdown lead to upregulated endogenous RhoA expression and induced morphologic and molecular phenotypes associated with elevated RhoA protein levels and RhoA/ROCK signaling. These morphologic changes were rescued by treatment with the ROCK inhibitor Y-27632 and/or RhoA knockdown. These findings reveal a new functional connection between an mRNA binding protein and RhoA signaling, whereby hnRNP-Q1 plays a key role to negatively modulate RhoA expression and signaling and affect cellular morphogenesis.

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The role of RhoB in the response to kidney ischemia.

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The small GTPase RhoB is an important regulator of cellular response to stress. It is an early response gene to UV damage and low levels of RhoB in cancer cells correlates with resistance to chemotherapy. In these situations, RhoB is pro-apoptotic. However, loss of RhoB expression in an epithelial to mesenchymal transition induced by TGFβ, results in an increase in apoptosis. It thus appears that RhoB signaling may be either anti-apoptotic or pro-apoptotic depending on context and the nature of the stress. RhoB expression is also up regulated in response to hypoxia and ischemia. To investigate the role of RhoB signaling in ischemic injury, we used substrate depletion and antimycin A treatment to deplete cells of ATP in a cell culture model of ischemia using a mouse proximal tubule S3 segment cell line. We found that RhoB activity is increased sixty minutes after fifteen minutes of ATP depletion. Additionally, TNFAIP1 (tumor necrosis factor inducible protein 1) is found in the GTP-bound fraction of a RhoB activity assay following depletion at the same time as RhoB activity reaches its peak. TNFAIP1 also co-immunoprecipitated with total (active+inactive) RhoB following 15 minutes of depletion, but not under normal growth conditions. Cytochrome C release from mitochondria also peaked after depletion. When we isolated a mitochondria-rich fraction following depletion we found RhoB and TNFAIP1 were transiently associated with this fraction. Based on these data we suggest that increased RhoB activity following depletion or ischemic injury may facilitate delivery of pro-apoptotic molecules (such as TNFAIP1) to the mitochondria and initiation of cytochrome c release.

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Activation of RhoC in lamellipodia regulates mDia1-mediated actin polymerization, membrane protrusion and directional cell migration independently of RhoA-ROCK signaling.

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Eukaryotic cell movement requires the dynamic reorganization of the actin cytoskeleton, which is controlled by the Rho family of small GTPases. RhoA and RhoC share a high degree of sequence similarity with identical effector binding domains and have, therefore, been presumed to have redundant biological functions. However, recent studies suggest that RhoA and RhoC regulate different aspects of cellular migration, with RhoC emerging as a pro-invasion gene in a variety of cancers, although the molecular basis of RhoC-mediated cellular migration and invasion is currently not known. We now show that RhoC, and not RhoA, engages mDia1 to promote directional cell migration. Using kymography analysis, we further demonstrate that RhoA and RhoC differentially regulate membrane protrusion of the leading edge, with RhoA serving to restrict membrane dynamics, and RhoC acting to promote protrusion of the leading edge. Examining the organization of the actin cytoskeleton within lamellipodia, we find that the loss of RhoC or mDia1 causes overstabilization of actin-containing dorsal arcs, whereas depletion of RhoA or inhibition of Rho-associated kinase causes the disassembly of dorsal arcs. Furthermore, live-cell microscopy reveals that dorsal arcs that are overstabilized impair lamellipodium protrusion and cause cells to change their direction of migration, consistent with the loss-of-persistence phenotype observed in cells depleted of RhoC and mDia1. Furthermore, we have designed novel reporters of RhoA and RhoC activation utilizing bifluorescence complementation analysis imaging (BiFC) to observe the spatial activation of these Rho GTPases in lamellipodia. Whereas BiFC-RhoA is activated uniformly throughout lamella and

lamellipodium as previously seen by FRET analysis, BiFC-RhoC is activated in the protruding areas of lamellipodia colocalizing to the dendritic actin network and becomes inactivated as actin filaments assemble. Therefore, we report for the first time that RhoC is specifically activated in lamellipodia to promote mDia1-induced actin polymerization and membrane protrusion. Finally, we have identified the Rho-specific guanine nucleotide exchange factor, TEM4, as the upstream activator of RhoC required for actin polymerization and membrane protrusion. Depletion of TEM4 impairs the directionality of cell migration and the dynamics of membrane protrusion, abolishes the activation of RhoC during cell spreading and extinguishes RhoC activation in the lamellipodia as visualized by BiFC assay. In summary, we identify a TEM4-RhoC-mDia1 signaling nexus that controls directional cell migration by regulating dynamic actin polymerization in lamellipodia.

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Investigating the Roles of RhoGAP1 and RhoGAP8 in Regulating Rho GTPase Activity During Single-Cell Wound Healing.

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The ability to respond to physical damage is an essential and evolutionarily conserved process in single cells and tissues. The Rho family of GTPases has been shown to coordinate the cytoskeletal rearrangements needed to reestablish the integrity of the plasma membrane and underlying cortex in response to cell damage. The precision with which Rho and Cdc42 are activated and maintained in distinct activity zones throughout the healing process suggests the involvement of multiple Rho GTPase regulators at the wound. To date, a candidate screen for guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) involved in regulating Rho GTPase activity at the wound has identified a single wound regulator, Abr. By continuing the candidate screen, we have identified two additional GAPs, RhoGAP1 and RhoGAP8, as potential wound regulators. eGFP-tagged RhoGAP1 localizes between the zones of active Rho and Cdc42 in wounded *Xenopus laevis* oocytes where it may be acting as enzymatic barrier to prevent Rho GTPase activity zone mixing during wound healing. Targeting is mediated by its C-terminal region which contains both a GAP domain and a proline-rich region. Further, overexpression of RhoGAP1 results in a reduction in active Rho at wounds. We have also discovered that RhoGAP1 and RhoGAP8 colocalize at wounds which, in addition to previously reported evidence of their interaction, suggests they may form a complex to regulate Rho GTPase activity.

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Doublecortin orchestrates microtubule and actin dynamics to promote neuronal progenitor cell migration in a manner dependent on phosphorylation by PKA.

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Doublecortin (DCX), which is specifically expressed in neuronal cells, is a microtubule-associated protein. Genetic mutation of DCX causes the lissencephaly disease. Although the abnormal cortical lamination observed in lissencephaly patients is thought to be attributed to neuronal cell migration defects, how DCX is involved in the migration of neuronal progenitor cells (NPCs) remains unclear. In this study, we found that pituitary adenylate cyclase-activating polypeptide (PACAP) accelerated the migration of NPCs in a manner dependent on protein kinase A (PKA) activation. We demonstrated that PKA directly phosphorylated DCX at Ser47 in

vitro, and the phosphorylation-mimicked DCX mutant (S47E) promotes the cell migration. Furthermore, DCX (S47E) exhibited the lower binding affinity for microtubule, compared with wild type DCX. Interestingly, the activation of PKA and DCX (S47E) decreased the microtubule bundling, and induced the formation of lamellipodia mediated by Rac. PACAP and DCX (S47E) induced the activation of Rac, and DCX (S47E) interacted with Asef2, a guanine nucleotide exchange factor (GEF) for Rac1. We propose the novel mechanism how the phosphorylation of DCX by PKA regulates neuronal migration, and this signaling pathway is indispensable to proper brain lamination.

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The role of Calcium-Independent Phospholipase A2 in homeostasis of aqueous humor drainage: Ca+2 sensitization of trabecular meshwork contraction.

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Objective: To explore the role of calcium-independent phospholipase A_2 ($iPLA_2$), which is known to control eicosanoid synthesis and tissue contractile properties, in regulation of aqueous humor (AH) outflow drainage.
Methods: Distribution of $iPLA_2$ isoforms in the outflow pathway tissues was evaluated by immunohistochemical analysis. Using irreversible, chiral, mechanism-based isoform specific inhibitors of $iPLA_2$, R-Bromoenol lactone (R-BEL, $iPLA_{2\gamma}$ specific) and S-Bromoenol lactone (S-BEL, $iPLA_2\beta$ specific), we determined the specific role(s) of $iPLA_2$ isoforms in regulation of TM cell contractile properties, Rho GTPase activity, myosin phosphatase activity and actin cytoskeletal dynamics by biochemical analyses, and evaluated for changes in AH outflow facility in enucleated porcine eyes.
Results: Both human and porcine TM cells were confirmed to express $iPLA_2\beta$ and $_\gamma$ by RT-PCR and immunoblot analyses. Immunohistochemical analysis of $iPLA_2\beta$ and $_\gamma$ showed an intense staining of both isoforms distributing throughout the outflow pathway including TM, juxtacanalicular tissue and Schlemm's canal. Inhibition of $iPLA_{2\gamma}$ by R-BEL or silencing of its expression by siRNA induced dramatic changes in TM cell morphology, and decreased actin stress fibers, focal adhesions and myosin light chain phosphorylation (MLCp). AH outflow facility in the enucleated porcine eyes increased progressively and significantly over the sham-treated control eyes, following R-BEL perfusion. This response was associated with a significant decrease in MLCp in the drug perfused TM tissue and increased formation of giant vacuoles in the innerwall of Schlemm's canal. Contrarily, S-BEL did not influence either aqueous outflow facility or TM cell contractile properties. Moreover we confirmed that the induction of cellular relaxation induced by inhibition of $iPLA_{2\gamma}$ was associated with significant decrease in the levels of Rho-GTP, Phospho-CPI-17 and phospho-MYPT1. Inhibition of $iPLA_{2\gamma}$ also significantly decreased the levels of arachidonic acid, a major free fatty acid product of $iPLA_2$.
Conclusions: This is the first study to demonstrate a critical and isoform specific involvement of $iPLA_2$ in regulation of AH outflow through the conventional pathway. Importantly, pharmacological inhibition of $iPLA_{2\gamma}$ increases AH outflow facility through the trabecular pathway by affecting the tissue contractile characteristics and Rho GTPase activity.

Ubiquitin and Proteasome Function

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Involvement of HslV pore motif in the control of interaction between HslV and HslU and the proteolytic activity of ATP-dependent HslVU protease.

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HslVU is an ATP-dependent protease in bacteria consisting of hexameric HslU ATPase and dodecameric HslV protease. In the HslVU complex, the HslU and HslV central pores are aligned, so that HslU transfers peptides and protein substrates through the pores into the inner proteolytic chamber of HslV. Here we show that the Leu88 residue in RMLR pore motif of HslV is critically involved in the control of HslVU complex formation and thereby its proteolytic activity. Replacement of Leu88 by Ala or Gly led to a marked increase in the binding affinity of HslV to HslU and the proteolytic activity of HslVU, whereas that by Ser or deletion of the RMLR sequence abrogated them. The L88A and L88G mutants, but not the others, also dramatically activated the HslU ATPase. Surprisingly, L88A and L88G by themselves were capable of cleaving small peptides as well as by HslV in complex with HslU and ATP, and their peptidase activities could be further activated by HslU in the absence of ATP nearly as well as in its presence in a manner that requires the C-terminal tails of HslU. L88A and L88G by themselves could also hydrolyze certain unfolded proteins, such as α-casein, although they were unable to degrade native proteins, including SulA and Arc. Remarkably, under normal growth conditions overexpression of L88A in *hslVU* null *E. coli* cells inhibited cell growth whether HslU was coexpressed or not, despite the fact that HslVU is not essential for it, suggesting that the Leu-to-Aa and Leu-to-Gly mutations deregulate HslV activity by relaxing the control mechanisms that normally safeguard essential cellular proteins from random degradation by HslV. Collectively, our results suggest that Leu88 in the pore motif plays critical role as a regulatory gate in the interaction between HslV and HslU and thereby the proteolytic function of HslVU.

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Functional isolation of a novel PAP for proteasome activation.

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Various neurodegenerative diseases share a common feature, accumulation of aggregation-prone proteins which include mutant Huntingtin and Tau. One of the major roles or effects of these proteins is inhibition of the proteasome activity and aggregation of these proteins is results from the inhibition of proteasomal activity. However, a signal responsible for proteasome dysfunction is not known. Thus, discovery of novel proteasome regulators is important to understand aggregation disease and further to provide effective treatment of neurodegenerative disease. To identify new modulators regulating proteasome activity, a cell-based functional screening was established using Degron-GFP. From screening thousands of cDNAs for gain-of-function assay, 5 putative positive genes were isolated. Among them, the PAP (Proteasome-Activating Protein) was investigated in detail. Over-expression of PAP enhanced GFP^{U} degradation in a dose- and time- dependent manner in HEK 293T cells and increased proteasome activity as assayed with fluorogenic substrate. On the contrary, down-regulation of PAP expression with shRNA markedly increased accumulation of degron and inhibited proteasome assembly on glycerol gradient fractionation. Ectopic expression of PAP enhanced proteasome assembly on native gel. In addition, the expression of PAP and proteasome activity was regulated by treatment with insulin/IGF-1 in SH-SY5Y and HT22 cells. Further, ectopic

expression of PAP reduced the occurrence of mutant Huntingtin protein aggregation by approximately 50% in SH-SY5Y cells. Taken together, these results indicate that PAP acts as a potential proteasome activator and may serve as a therapeutic activator for aggregation disease, such as Huntington disease.

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Examining the structural basis of ubiquitin ligase self-assembly and its implication as a molecular rheostat.

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Ubiquitylation is among the most widespread forms of cellular post-translational modification and is capable of mediating and eliciting an array of functional outcomes. Central to the mechanism of ubiquitylation is the E3 ubiquitin ligase, which serves to recognize the target protein and recruit it into complex with the E2 ubiquitin-conjugating enzyme. Recent insights into the mechanism of ubiquitylation demonstrate that E3 ubiquitin ligases can possess active regulatory properties beyond those of a simple assembly scaffold. Prominent among these properties is the ability of many E3 ligases to self-assemble into large complexes both in vitro and in vivo. Moreover, it has been well documented that disruption of these assemblies through mutagenesis significantly compromises their ubiquitylation activity. However, the structural bases for these self-assemblies and the mechanisms for activity enhancement remain poorly characterized. Here we present our biophysical, X-ray crystallographic and functional studies into the structure and activity of a multi-subunit BTB-cullin E3 ligase, SPOP-Cul3. Interestingly, we find that this E3 ligase possesses tandem oligomeric domains that function in concert to generate a dynamic, expandable complex with potent ubiquitylation activity. We propose that self-assembly serves to enhance ubiquitin transfer by enhancing avidity for the substrate and creating a critical mass of the catalytic machinery—thus allowing multiple substrates to be acted upon simultaneously by a large number of E2 conjugating enzymes. Additionally, we have gone on to characterize a SPOP-Cul3 homolog that is specifically deficient in high-order self-assembly and that, through heteromeric complex formation, is capable of restricting SPOP-Cul3 self-assembly. This two-component system indicates a powerful cellular mechanism by which self-assembly can be used to create a tunable means of regulating E3 ligase activity.

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FAT10 binds to polyglutamine proteins and modulates their solubility.

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Expansion of polyglutamine (pQ) chain by expanded CAG repeat causes dominantly inherited neurodegeneration such as Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA) and numbers of other spinocerebellar ataxias. Expanded pQ disrupts stability of the pQ harboring protein and increases its susceptibility to aggregation. Aggregated pQ protein is recognized by ubiquitin proteasome system and the enzyme ubiquitin ligase covalently attach ubiquitin, which serves as a degradation signal by the proteasome. However, accumulation of the aggregated proteins in the diseased brain suggests insufficient degradation machinery. Ubiquitin has several functionally related proteins that are similarly attached to target proteins through its carboxy (C) terminus glycine residue. They are called ubiquitin like molecules and some of them are similarly related to protein degradation pathway. One of the ubiquitin like molecule FAT10 is known to accelerate protein degradation through ubiquitin independent manner but its role in pQ aggregate degradation was completely unknown. Thus we investigated its role in HD cellular model and found that FAT10 were covalently attached to

huntingtin through its C terminus glycine. FAT10 binds preferably to huntingtin with short pQ chain and completely aggregated huntingtin was FAT10 negative. Knock down of FAT10 enhanced cellular toxicity of huntingtins. In addition, ataxin-1,3 and DRPLA protein were all positive for FAT10 and aggregation enhancement were observed upon FAT10 knockdown, which was similar to the findings with huntingtin. Our new finding will provide a new role of FAT10 in the pathogenesis of polyglutamine diseases.

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The Ubiquitin Ligase Wwp1 Mediates Lysosomal Degradation of Connexin 43 through the Formation of K29-linked Polyubiquitin Chains.

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Connexin 43 (Cx43) is a component of gap junctions (GJ). GJ are channels which facilitate intercellular communication between adjacent cells by allowing the passage of small molecules such as ions and metabolites. *In vivo*, Cx43 is rapidly turned over with a half-life that ranges between 1 and 5 hours. However, the precise molecular mechanisms regulating Cx43 turnover are not well understood. Previous studies have shown that phosphorylation and ubiquitylation of Cx43 play a role in its internalization and degradation. Although Nedd4 (a member of the HECT family of E3 ubiquitin ligases) has been shown to bind and ubiquitylate Cx43, the overexpression of this ligase in cells does not result in Cx43 degradation. Therefore, the objective of this study was to identify the molecular signaling events that mediate the degradation of Cx43. We hypothesized that Wwp1, a Nedd4-like family member known to localize to the endosome, could bind and ubiquitylate internalized Cx43 in the early endosome, resulting in the sorting of Cx43 to and degradation in the lysosome. We found that Wwp1 co-immunoprecipitated with and ubiquitylated Cx43 in HEK293T cells and that these processes were enhanced upon pretreatment of cells with phorbol 12-myristate 13-acetate. Interestingly, the utilization of a series of ubiquitin mutants that were either incapable of making a polyubiquitin chain or only capable of extending a polyubiquitin chain from one of the seven lysine residues of the ubiquitin molecule revealed that Wwp1 mediated K29-linked polyubiquitylation of Cx43. Laser scanning confocal microscopy analysis of immunofluorescently labeled HeLa cells stably transfected with Cx43 demonstrated that the ubiquitin ligase activity of Wwp1 was required to traffic Cx43 from early endosomes to CD63-positive late endosomes. In addition, lysosomal inhibition prevented the degradation of Cx43 in 293T cells over-expressing Cx43 and Wwp1. Taken together, these data support the assertion that Wwp1 polyubiquitylates Cx43 via a K29 linkage and this post-translational modification is an important signal which routes the Cx43 substrate from the early endosome to the late endosome and subsequently to the lysosome for degradation. Our findings offer insights on the mechanisms of Cx43 turnover and the regulation of GJ intercellular communication.

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CAND1 Governs SCF Complex Assembly.

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Attachment of a polyubiquitin chain with at least four ubiquitins linked together through their lysine 48 residue targets proteins to the proteasome for degradation. A cascade of three enzymes carries out the synthesis of polyubiquitin chains: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3). Cullin-RING ubiquitin ligases (CRLs) are the largest family of E3s and are typified by the SCF complexes, which in humans are composed of four proteins: the scaffold Cul1, the RING containing Rbx1, the adaptor Skp1, and a substrate binding protein that contains the Fbox motif. 69 proteins in the human genome have Fbox motifs, and 42 have been shown to form SCF complexes. Although this modular

design of SCF complexes allows for recognition of a diverse set of target proteins, how SCF complex formation is regulated remains unclear.

Cullin-associated and neddylation-dissociated protein 1 (CAND1) was originally isolated as a Cul1 associated protein whose binding was mutually exclusive with the Fbox/Skp1 sub-complex. CAND1 appeared to inhibit SCF mediated ubiquitylation through the dissociation of Fbox/Skp1. Furthermore, CAND1's dissociation from Cul1/Rbx1 was coupled to the attachment of the ubiquitin like protein Nedd8, an activator of CRLs, to Cul1. For these reasons, CAND1 was recognized as a negative regulator of SCF complex assembly.

To characterize the assembly properties of SCF complexes, we developed a real time assay based on FRET that monitors the binding dynamics between the subcomplexes of Fbox/Skp1 and Cul1/Rbx1. Surprisingly, we found that CAND1 is able to actively remove Fbxw7/Skp1 from Cul1/Rbx1 by changing the dissociation rate of the complex 10 million fold. Our kinetic assays reveal the existence of a transient complex of all four SCF components with CAND1 before dissociation into sub-complexes. Amazingly, CAND1 does not affect the assembly rate of SCF Fbxw7. Nedd8ylation of Cul1 abolishes the affect of CAND1 on SCF assembly.

We establish the first kinetic framework for the dynamic assembly of SCF complexes using direct real time biophysical assays. We conclude that CAND1 is directly involved in the assembly of SCF complexes and serves as an Fbox/Skp1 exchange factor. Our kinetic framework serves as a starting point to re-evaluate the function of CAND1 in vivo.

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Mechanistic basis of host-cell remodeling by the HIV-1 protein Vpu.

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Many viral genomes encode proteins that selectively and potently target certain host cell proteins for degradation. HIV-1 also uses this general strategy to selectively downregulate CD4, an essential receptor for HIV-1 entry into cells. This downregulation is important for preventing superinfection, maximizing virion production, and achieving full infectivity of the released virons. Previous studies have established that Vpu interacts with newly synthesized CD4 at the endoplasmic reticulum and recruits an E3 ligase, which is supposed to be SCF-βTrCP complex, to mediate CD4 ubiquitination. The ubiquitinated CD4 then is extracted from ER and degraded by the proteasome in cytosol.

While this basic pathway has been defined on the basis of cell culture studies, the molecular mechanisms that mediate this ubiquitination or the downstream degradation are not well understood. Here we found that in vitro translated CD4 on the microsome from Vpu stable cell line can be ubiquitinated probably by the SCF-βTrCP complex pre-associated with the microsome membrane. The ubiquitinated CD4 can be dislocated from microsome in a cytosol dependent manner.

In order to dissect the mechanism, we recapitulated this process using minimum system containing recombinant Vpu and SCF-βTrCP complex, radiolabeled CD4 and the synthetic liposome. With both in vitro assays, future studies are expected to provide mechanistic insight into how Vpu escapes from direct ubiquitination by SCF-βTrCP complex and which factor channels the extraction of CD4 from ER membrane to cytosol.

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Fluorescence polarization (FP) and chemiluminescent based substrate alternatives for analyzing DUB activity.

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Deubiquitinating enzymes (DUBs) play an important role in a multitude of cellular processes as well as being implicated in a number of human diseases. Despite this important role, the substrate specificity and kinetic parameters of many DUBs are poorly defined. This is due to the lack of appropriate reagents where DUB activity can be monitored in real time while utilizing small amounts of the DUBs themselves which are often challenging to purify. Ub-AMC has been a workhorse substrate, but the linkage between fluorophore and Ub doesn't mimic the isopeptide bond between Ub and conjugate and many DUBs do not process Ub-AMC rapidly enough to get even useful qualitative data.
Here we describe the development of two new substrates for analyzing DUB activity that separately address these issues. First, Ub modified by a 5-TAMRA-Lys-Gly (Ub-KTG) contains an isopeptide bond between ubiquitin and the flurophore, mimicking Ub-conjugation to *in vivo* substrates. This makes the Ub-KTG substrate more relevant to understanding how DUBs act during physiological processes. The extremely photostable fluorophore provides a strong FP signal over extended time periods. Release of the fluorophore by DUB activity provides a change in FP signal that can be utilized to determine kinetic parameters. As the FP signal is concentration independent, low amounts of substrate (100 nM) can be used to obtain kinetic parameters. Ub-KTG substrate provides similar results to Ub-AMC when tested against a panel of DUBs. The second substrate developed, Ub-aminoluciferin (Ub-AML), addresses the issue of sensitivity. Rather than fluorescence as the indicator of DUB activity, DUB liberated luciferin is processed by luciferase to give a luminescence signal. Ub-AML not only produces a stronger signal, but also has an excellent signal to noise ratio over traditional fluorophores. The Ub-AML substrate was used for reliable detection of DUB activity using an order of magnitude less enzyme than needed for equivalent results when using Ub-AMC. This makes it possible to rapidly assess the activity of DUBs that poorly utilize Ub-AMC while using much lower levels of the DUBs themselves. Nedd8-AML and SUMO-AML have also been synthesized via the same method and behave very similarly in terms of DUB amount used and having a very high signal to noise ratio.

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Parkin functions as a surveillance E3 ligase in cytoprotection against misfolded protein-induced toxicity.

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Protein misfolding and aggregation underlie the pathogenesis of many neurodegenerative disorders, including Parkinson disease (PD) and Alzheimer disease. Recent evidence indicates that, under the conditions of proteotoxic stress, misfolded proteins are actively sequestered in a pericentriolar structure called an aggresome and subsequently degraded via macroautophagy (hereafter referred to as autophagy). The aggresome-autophagy pathway has emerged as a crucial cellular defense system against toxic buildup of misfolded proteins, but the molecular machinery and regulatory mechanisms that control this pathway remain mostly unknown. We have recently shown that parkin, an E3 ubiquitin-protein ligase which is mutated in early-onset PD, selectively binds and K63-polyubiquitinates misfolded DJ-1 and this ubiquitination serves as a targeting signal for degradation by the aggresome-autophagy pathway. We have identified another misfolded protein (MFP) that is specifically recognized by parkin. We found that parkin selectively polyubiquitinates MFP but not its wild-type counterpart. Moreover, parkin

overexpression protects cells against MFP-induced toxicity. Our results support a function of parkin as a surveillance E3 ligase for regulating misfolded protein processing by the aggresome-autophagy pathway and have important implications for understanding and treating PD and other neurodegenerative diseases.

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The competition roles of proteasomes and autophagy in the degradation of ubiquitylated soluble cytosolic proteins.

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The ubiquitin-proteasome (UPS) system is a highly conserved protein degradation pathway in eukaryotes that relies on ubiquitylation, the modification of proteins with ubiquitin moieties, to mediate proteasomal degradation of target proteins. Autophagy, which is another protein turnover process in eukaryotes, is processed by the sequestration of cytoplasm or organelles into double membrane vesicles termed autophagosomes that eventually fuse with the vacuole/lysosome for massive breakdown of proteins or organelles. In mammalian cells, ubiquitylated proteins are not only substrates for proteasomal degradation but also form aggregates in cytosol that were recently shown to be specific substrates for autophagic degradation. However, in this study, we showed that ubiquitylated EGFP did not form protein aggregates in *Saccharomyces cerevisiae*. Moreover, we found that ubiquitylation, which mediates protein degradation in proteasomes, interferes with autophagic degradation of cytosolic soluble proteins. We propose that the interactions between ubiquitin moieties and unknown ubiquitin-interacting factors restrict ubiquitylated EGFP from autophagic degradation. In addition, lack of homologues to the metazoan adaptor proteins, such as p62 and NBR1, responsible for autophagic degradation of ubiquitylated cytosolic substrates in yeast maybe partially responsible to our observation.

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Regulation of endoplasmic reticulum-mitochondria interaction, mitochondrial fission and mitophagy by the gp78 ubiquitin ligase.

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Gp78 is a critical E3 ubiquitin ligase in endoplasmic reticulum-associated degradation (READ). Gp78 is localized to mitochondria-associated smooth endoplasmic reticulum (ER) and increased ER-mitochondria association upon gp78 overexpression significantly reduces mitochondrial mobility and induces mitochondrial fragmentation. Mitochondrial depolarization with CCCP induces mitophagy in gp78 overexpressing cells independently of Parkin expression. CCCP treatment of gp78 overexpressing cells recruits the autophagic marker LC3-GFP to the gp78 and calnexin labeled ER closely associated to mitochondria defining a role for the ER in gp78-induced mitophagy. Gp78 regulation of mitochondrial mobility, fission and mitophagy is dependent on gp78 ubiquitin ligase activity and mitofusin2 (Mfn2), the mitochondrial fusion factor, was identified as a gp78 substrate. However, gp78 appears to also function independently of Mfn2 as it still stabilizes ER-mitochondria association and enhances mitochondrial fission and mitophagy in Mfn2 knockdown cells. Furthermore, in HT-1080 fibrosarcoma cells, gp78 knockdown disrupts ER-mitochondria contacts and prevents mitophagy in Mfn2 knockdown cells. This study therefore describes a novel mechanism linking gp78 ubiquitin ligase activity and polyubiquitylation to the regulation of ER-mitochondria contacts, mitochondrial dynamics and mitophagy.

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Establishment and Maintenance of Polarity I

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Apical Polarization of Na+,K+-ATPase in Retina Pigment Epithelium (RPE); role of β2-isoform.

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Na+,K+-ATPase also known as the sodium pump is an ion transporter that plays a central role in maintaining the sodium gradient responsible for transport of virtually all nutrients and metabolites. In epithelial monolayers Na+,K+-ATPase is enriched to a cell membrane-specific domain. Pump polarity is achieved by a complex sequence of events involving both targeting to and selective retention at the lateral cell membrane. In contrast to the majority of epithelial cell models, the sodium pump is enriched in the apical membrane domain of retina pigment epithelium RPE cells. This 'reverse polarity' of the RPE was brought to our attention given that the polarization mechanism of the sodium pump is still not clear. Our working hypothesis is that the pump polarity in RPE cells is regulated by its β−subunit and more precisely by the type of isoform that is expressed. Although it is well documented that both in situ and in vitro the sodium pump is localized to the apical membrane there is no clear information about the β - subunit isoform/s that accompany the α-subunit in the apical domain. In this study we have examined and compared the expression patterns of the three known isoforms of Na+,K+-ATPase β-subunits (β1, β2 and β3) in the human cell line ARPE-19 and in thin sections of pig and human eyes. Western blot (WB) and immunofluorescence (IF) studies were carried using ARPE-19 cells that have been cultured for 8 weeks (re-morphogenesis) on laminin covered clear inserts. The expression of the different isoforms was time and domain dependent. All three isoforms (β1, β2 and β3) were detected. Nevertheless, while β1 subunit is detected during all 8 weeks the expression of β2 and β3 subunits is up-regulated during re-morphogenesis; both are absent during the first three weeks and appear around the 4th week and thereafter as detected by WB and IF. While β1 and β3 are expressed mainly on basolateral membrane β2 is observed on apical membrane. Moreover, in thin sections obtained from RPE of pig and human eyes, IF staining of α1, β1- and β2-subunits show that the β isoform expressed on the apical domain is β2. RT-qPCR analysis shows a similar kinetics for β1 and β2 mRNAs during re-morphogenesis. This is the first expression study of all three β subunit isoforms in ARPE-19 cells. Our results indicate that the apical localization of the sodium pump in RPE is related to the expression of β2 subunit which carries an apical sorting information.

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Polarized expression of human riboflavin transporters -1, -2, and -3 in human intestinal epithelial cells.

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Riboflavin (RF) is essential for normal cellular function. Humans obtain RF from exogenous sources via intestinal absorption, a process that involves transport of RF across the brush border membrane (BBM) and basolateral membrane (BLM) of polarized enterocytes. Both these transport events are carrier-mediated. All human riboflavin transporters (hRFT-1, hRFT-2 and hRFT-3) are expressed in the intestine but little is known about the membrane domain(s) at which they are expressed. Here, we used live cell confocal imaging of intestinal epithelial Caco-

2 (and renal MDCK) cells to show that hRFT-1 is expressed at the BLM; hRFT-2 is exclusively expressed at the apical membrane, while hRFT-3 is mostly localized inside intracellular vesicular structures with some expression at the BLM. We also found that the level of hRFT-2 expression in Caco-2 cells and in native human small intestine is significantly higher than that of hRFT-1 and -3; also hRFT-2 was more efficient in transporting 3H-RF than hRFT-1 and -3. These findings suggest an important role for hRFT-2 in normal intestinal RF uptake, a conclusion that was further supported by hRFT-2 gene-specific siRNA silencing approach. (DVA, DK 56061).

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Tyrosine 156 regulates basolateral trafficking of epiregulin and mutation to alanine induces transformation.

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Objective: The EGF receptor ligand epiregulin (EREG) is overexpressed in a number of cancers where its levels directly correlate with metastasis and responsiveness to cetuximab. However, it is unknown whether EREG contributes to tumor initiation.

Methods: MDCK cells stably expressing various EREG constructs were allowed to polarize on Transwell™ filters forming distinct apical and basolateral surfaces. Localization and trafficking of EREG constructs were analyzed by confocal immunofluorescence microscopy, domain-selective cell surface biotinylation and metabolic labeling pulse-chase experiments. MDCK cells stably expressing wild-type EREG and various cytoplasmic tail mutants were observed for their ability to form cysts when grown in Matrigel™ and to form tumors in nude mice upon subcutaneous injection.

Results: Wild-type EREG localizes exclusively to the basolateral surface in polarized MDCK cells. Using sequential truncations and single amino acid substitutions in the cytoplasmic tail of EREG, we identified that a single tyrosine controls its basolateral trafficking. Mutation of this tyrosine to alanine (Y156A) completely relocalizes EREG to the apical surface. Of interest, phosphorylation of this tyrosine is required for basolateral localization of EREG. When grown in Matrigel™, MDCK cells expressing wild-type EREG constructs formed cysts with cleared lumens, like parental MDCK cells. However, MDCK cells expressing apically misdirected (Y156A)EREG formed cysts with uncleared lumens, a phenotype that correlates with transformation. In addition, (Y156A)EREG-expressing cells formed much larger and highly invasive tumors in nude mice compared to their wild-type counterparts.

Conclusion: Redirecting EREG from the basolateral to apical surface in polarized MDCK cells results in a transformed phenotype.

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Palmitoylation of Ankyrin-G Promotes Its Polarized Membrane Targeting in Epithelial Cells.

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Ankyrins (ankyrin-R, -B, -G) are a family of membrane associated adaptor proteins, which coordinate the localizations of proteins to diverse membrane domains including axon initial segment of neurons and lateral membrane of epithelial cells. However, the mechanism for targeting ankyrin-G to membrane domains remains unclear. Using a calcium switch assay, we found that in non-polarized MDCK cells, ankyrin-G persists on the plasma membrane even though its known partners including β2-spectrin and E-cadherin redistribute to intracellular sites. Previous research revealed that ankyrin purified from erythrocytes preserve hydrophobicity and is potentially lipid-modified. Here we demonstrate that ankyrin-G is S-acylated at cysteine 70

using biotin-switch assays and mass spectrometry. Protein S-acylation is a common lipid modification on cysteines via thioester bonds, and plays significant roles in protein targeting, transportation and cell signaling. Our further radiolabeling experiment showed that ankyrin-G cysteine 70 can be S-acylated by palmitic acid. In order to characterize the biological functions of ankyrin-G palmitoylation, we generated stable MDCK cell lines based on wild type and C70A ankyrin-G. A calcium switch assay was used to measure the membrane association capacity of wild type and C70A ankyrin-G. Our results demonstrated that palmitoylation of ankyrin-G contributes to its membrane association, and restricts its lateral motility in plasma membrane.

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Polarizing yeast cells with electric fields.

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Endogenous electrical signals surround tissues and cells and are thought to participate in the regulation of many polarization events such as wound healing, metastasis and development. Application of exogenous electric fields (EFs) can orient polarity in many cell types ranging from bacteria, to neutrophils and neurons. Different cell types polarize to different directions, some orienting to the anode and other to the cathode or even perpendicular to the EF. EFs are thought to signal through the membrane by locally altering membrane potential or ion fluxes distribution around the cell; however the mechanisms for how cells sense and orient to EFs remain poorly characterized. Here, we introduce the Budding yeast Saccharomyces cerevisiae as a rigorous model for cell polarity to dissect mechanisms underlying these effects. These cells polarize by locally selecting sites of bud emergence or by growing shmoo projection for mating. We apply EFs by immobilizing yeast cells in microfluidic chambers, which allow for heat control, straight EF lines and parallelization of the assay. While, the budding pattern was generally resistant to the EF in haploid WT cells, mutants in the bud site selection pathway that normally bud in random direction, such as rsr1 (Bud1), a Ras-like GTPase, were polarized by the EF towards the cathode. In the presence of pheromones (a-factor), the EF directed polarized growth of the shmoo, but interestingly in the opposite direction, towards the anode. This shmoo orientation in the EFs depended on the formin Bni1. To understand the effect of EFs at the cell surface, we screened a set of characterized mutants in ion transporters and drugs that perturb ion transport. These experiments suggested that proper membrane potential regulation was key to mediate these effects. For instance, mutants lacking the Na+ releasing systems that display depolarized membrane potential or hyperpolarized mutants lacking K+ import directed shmoo polarization to the cathode in the EF, reverting the WT phenotype. This work begins to identify conserved factors and a process involved in EF response and brings novel insight into general mechanisms of cell polarity.

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Negative feedback enhances robustness in the yeast polarity establishment circuit.

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Many cells undergo symmetry-breaking polarization towards a randomly-oriented “front” in the absence of spatial cues. In budding yeast, such polarization involves a positive feedback loop that enables amplification of stochastically-arising clusters of polarity factors. Mathematical modeling suggested that if more than one cluster were amplified, the clusters would compete for limiting resources and the largest would “win”, explaining why yeast cells always make one and only one bud. Imaging with improved spatiotemporal resolution, we now demonstrate the transient co-existence of multiple clusters during polarity establishment, as predicted by the

model. Unexpectedly, we also found that initial polarity factor clustering was oscillatory, revealing the presence of a negative feedback loop that disperses the factors. Mathematical modeling predicted that negative feedback would confer robustness to the polarity circuit, and make the kinetics of competition between polarity factor clusters relatively insensitive to polarity factor concentration. These predictions were confirmed experimentally.

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An actin-dependent phosphoinositide-bis-phosphate gradient.

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Candida albicans, similar to a range of human fungal pathogens, grows in different forms in response to environmental cues. In particular, its ability to switch from yeast to a filamentous form is important for pathogenicity and requires reorganization of the actin cytoskeleton.

Membrane phospholipids, such as phosphoinositide phosphates, have been shown to play important roles in a range of signaling pathways despite being relatively minor component of membranes. For example phosphoinositide phosphates are required for cytoskeleton organization, G-protein signaling, cell polarity and morphogenesis. In *Candida albicans*, neither PI(3,4,5)P_3 nor PI-3-kinase homologs have been found, raising the possibility that the PI(4,5)P_2 fulfills some functions of PIP_3. In this organism there is a single PI(4)P-5-kinase (encoded by *MSS4*) and three PI-4-kinases (encoded by *LSB6*, *STT4* and *PIK1*). In the yeast *S. cerevisiae*, both Mss4 and Stt4 kinases are required for viability, for organization of the actin cytoskeleton and are localized to the plasma membrane.

We have examined whether PI(4,5)P_2 is required for *Candida albicans* filamentous growth. We have generated strains in which the level of the Stt4 or Mss4 phosphoinositide kinases can be manipulated using the Tetracycline repressible promoter system. In repressive conditions, the *stt4* and *mss4* mutants are viable, however they are defective in filamentous growth in both liquid and solid media. Using a fluorescent lipid associated reporter (FLARE), we have observed a striking PI(4,5)P_2 asymmetry in budding cells and a steep gradient which occurs concomitant with emergence of the hyphal filament. Both sufficient PI(4)P synthesis and an intact actin cytoskeleton are necessary for this steep PI(4,5)P_2 gradient. In contrast, neither microtubules nor asymmetrically localized mRNAs are critical for this gradient. Furthermore, the Mss4 protein is localized to the tip of the bud and hyphal filament.

Our results indicate that a gradient of PI(4,5)P_2, generated in part by the sole filament tip localized PI(4)P-5-kinase Mss4 and the slow diffusion of PI(4,5)P_2 in the plasma memberane, is crucial for the yeast to filamentous growth transition.

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The formin FHOD1 interacts with the TAN line component, nesprin-2Giant, and is required for actin-dependent nuclear movement during centrosome orientation in migrating fibroblasts.

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In migrating fibroblasts, actin-dependent movement of the nucleus behind a stationary centrosome establishes the initial anterior orientation of the centrosome between the nucleus

and the leading edge (Gomes et al., Cell, 2005). Rearward nuclear movement is mediated by retrograde flow of dorsal actin cables that are coupled directly to the nuclear envelope through trans-membrane actin-associated nuclear (TAN) lines, a novel structure composed of the outer nuclear membrane protein, nesprin-2Giant (nesprin-2G) and the inner nuclear membrane protein, SUN2 (Luxton et al., Science, 2010). To better understand TAN line formation and function, we searched for novel nesprin-2G interacting proteins and identified the diaphanous-related formin (DRF), FH1/FH2 domain-containing protein 1 (FHOD1). GST-fusions of residues 1340-1678 of nesprin-2G pulled down over-expressed HA-tagged wild type FHOD1 (HA-FHOD1-WT) or an N-terminal fragment of FHOD1 (HA-FHOD1-1-339) from cell lysates. This interaction was also detected using a yeast-two-hybrid assay; however, no direct biochemical interaction was observed. Depletion of FHOD1 by siRNA inhibited nuclear movement and centrosome orientation in wound edge fibroblasts and these defects were rescued by expression of either wild type FHOD1 (GFP-FHOD1-WT) or constitutively active FHOD1 (GFP-FHOD1-ΔC). Rescue by either construct required the FH1, FH2, and coiled-coil domains indicating that actin nucleation and dimerization of FHOD1 were critical for this process. Surprisingly, expression of GFP-FHOD1-ΔC, but not GFP-FHOD1-WT in serum-starved fibroblasts induced rearward nuclear movement in the absence of external stimuli such as lysophosphatidic acid or serum. However, nuclear movement induced by GFP-FHOD1-ΔC did not result in centrosome orientation as the centrosome failed to remain at the cell centroid. Over-expression of HA-FHOD1-ΔC induced robust actin cables that contained FHOD1 and co-localized with GFP-mini-nesprin-2G on the nuclear envelope, while over-expression of another constitutively active DRF, mDia1, did not recruit GFP-mini-nesprin-2G to the actin cables generated by mDia1 activation. The co-localization of HA-FHOD1-ΔC with GFP-mini-nesprin-2G required the actin-binding calponin homology domains of nesprin2G. Interestingly, TAN lines still formed in FHOD1-depleted fibroblasts suggesting that FHOD1 may contribute to nuclear movement by controlling the movement and/or organization of the dorsal actin cables required for nuclear movement. Our results identify FHOD1 as a new factor involved in TAN line-dependent nuclear movement in fibroblasts polarizing for cell migration.

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Distinctive Cell Polarization Mechanisms Depend on Cell Shape and Cell-Cell Contacts.

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In several migratory cells, the microtubule organizing center (MTOC) is repositioned between the leading edge and nucleus, creating a polarized morphology. While our understanding of polarization in collective cell migration has progressed with the use of the scratch-wound assay, the mechanisms governing single-cell polarization are unclear. Here, we use protein micropatterning to control the shape of single cells and compare the pathways regulating MTOC and nuclear positions to those in confluent cells. We find that MTOC and nuclear positioning are critically and independently affected by extracellular guidance cues, including cell shape and confluence; MTOC off-centering correlates with the polarization of single cells; and microtubule dynamics and acto-myosin contractility and assembly are required for single-cell polarization. Additionally, we demonstrate that Par3, LIC1, LIC2, and EB1 are essential for proper MTOC positioning in confluent cells, while only LIC1 and EB1 are required in single cells. These results suggest that polarity pathways diverge based on cellular shape and the presence of cell-cell contacts.

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Front-Rear Polarization of the Cytoskeleton develops in Cell migration from Soft to Stiff Matrix.

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It has become increasingly clear that cytoskeletal polarity is strikingly different in cells functioning in an in vivo environment compared to cells studied in vitro. Reasons for this discrepancy are unknown and whether matrix rigidity plays a role in affecting cell polarity is has yet to be studied. Tissues have been measured to have varying stiffnesses or deformabilities, and examples of matrix stiffening include scarring in the heart after a myocardial infarction or scarring of the liver in cirrhosis – which lead to rigidification of tissue through extensive collagen crosslinking, and it has been reported that adherent cells, including mesenchymal stem cells (MSCs) accumulate or 'home' to such scarred tissue. It is unknown how MSCs localize and, in particular, whether cell polarity plays a role in directing their migration from soft to stiff matrix. Our data show that the position of the microtubule organizing center (MTOC) with respect to the nucleus is influenced by matrix rigidity and thus microtubule density within the cell also depends on surface stiffness. Because myosin is responsible for cell contractility which deforms and probes matrix stiffness, we investigated the role non-muscle myosin (both MIIA and MIIB isoforms) may have in cell polarity and directed cell migration to stiff matrix. Using siRNA knockdown we determined that directed migration toward stiff matrix is more sensitive to MIIB and less so to MIIA, even though mass spectrometry determines there is ~10-fold more expressed MIIA in MSCs than MIIB on the protein level. Reports have shown that MIIB localizes to the cell rear in polarized migrating cells, but as we alter the matrix stiffness we demonstrate that this polarization diminishes when matrix stiffness becomes softer than 7 kPa. Our studies also show that the MTOC begins to polarize to the cell front at matrix stiffness above 7 kPa, suggesting a similar mechanism for stiffness induced polarization of MTOC and MIIB. Scratch wound assays induced polarization of MTOC toward the wound edge on stiff substrates but failed to do so when performed on soft matrix. Interestingly, we also found the same stiffness dependent rearward polarization in MIIB in anuclear cell fragments. We find that the polarity of the cytoskeleton is not required for cell migration but for directing cells toward stiffer matrix. Based on our data, we believe cytoskeletal polarization thus acts as an elasticity-sensitive compass in directing migration toward stiff matrix.

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Multiple pathways function together to polarize the Drosophila oocyte.

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The establishment and maintenance of polarity is a hallmark feature of most eukaryotic cells. The Drosophila oocyte is one of the most widely used models for examining mechanisms of cell polarity establishment. Elegant experiments over the past two decades have shown that mRNA localization is key to establishing polarity in the Drosophila oocyte. These mRNAs are localized using microtubules and microtubule based motors. However, very little is known regarding the organization of the microtubules within the oocyte. We have addressed this question by examining the localization of microtubule plus end binding proteins EB1 and CLIP-190 in the Drosophila oocyte. EB1 and CLIP-190 were both enriched at the posterior of the oocyte, indicating that microtubules within the oocyte are in fact polarized, with their growing plus ends preferentially localized to the posterior pole. Surprisingly, however, this polarity was not required for mRNA localization, but rather functioned downstream of a localized mRNA called oskar. We found that proper localization of oskar mRNA and subsequent expression of Oskar protein was required for recruiting growing microtubule plus ends to the posterior of the oocyte. The

posterior region of the oocyte has been shown to be very active in endocytosis in a manner that is dependent on Oskar protein. We provide evidence that recruitment of microtubule plus ends to the oocyte posterior by Oskar protein enables efficient and high levels of endocytosis in this region of the cell. Thus, our results demonstrate a functional link between mRNA localization, microtubule plus end recruitment and endocytosis.

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Control of Cell Growth and Polarity in Fission Yeast by Cytokinesis.

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For cells to function properly, they must polarize in response to external stimuli and cell cycle cues. The fission yeast, *Schizosaccharomyces pombe,* is a useful model organism for studying how cell polarity is controlled, for this organism is rod-shaped and grows from its tips in a cell cycle-dependent manner. After each cell division, *S. pombe* initially grows from one end, termed the old end because it previously served as an end for the mother cell. Following an event in G2 known as new end take-off, the new end, which is created by the preceding cell division, also undergoes extension to establish bipolarity. As this growth pattern suggests, signaling factors enriched at cell tips are thought to drive this process. However, we have uncovered evidence that the process of cytokinesis, which occurs at a medial division site, also impacts the establishment of bipolar growth in subsequent cell cycles. Notably, loss of *S. pombe* Fic1, a non-essential protein previously only implicated in cytokinesis, results in a higher than normal incidence of monopolar growth in asynchronous and G2-arrested cultures. Consistent with this defect, several polarity factors, including the Rho-GEF, Rgf1, and actin patch-associated proteins, localize mainly to one tip of *fic1Δ* cells. Mutations and truncations of Fic1 that abolish its anchoring at cell tips do not affect its polarity function. However, a truncation of Fic1 that fails to localize to the division site phenocopies the cell polarity defect of *fic1Δ* cells. This indicates that medial targeting of Fic1 likely confers its polarity function and suggests that the cytokinesis machinery of which Fic1 is a part influences ensuing cell growth. Indeed, time-lapse DIC imaging showed that tip growth of *fic1Δ* cells fails only at new ends formed by cell division, and a point mutant of Fic1 that does not bind the essential cytokinesis scaffold, Cdc15, also exhibits similar polarity defects. Although *fic1Δ* cells do not exhibit defective assembly or constriction of the cytokinetic ring, genetic analyses suggest a role for Fic1 in later stages of cytokinesis and cell separation. Intriguingly, disruption of other proteins known to mediate these terminal stages in cell division exhibit similar polarity and growth defects. Collectively, our data highlight a previously unappreciated role for the process of cytokinesis in modulating bipolar cell growth in *S. pombe*, and these findings predict a mechanism wherein the cellular division machinery delays polarization at new ends until cytokinesis is effectively completed.

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How does Cdc42p polarize actin cables?

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The prevailing model for how yeast cells generate polarized actin cables pointing towards the bud is that GTP-Cdc42p directly recruits and activates the formin Bni1p to the incipient bud site, promoting local nucleation of actin cables. This model is based on the presence of a GTPase-binding domain (GBD) in Bni1p. However, the GBD can interact with several Rho proteins, and redundant cable-orientation pathways exist that render Bni1p itself dispensable. Thus, the direct Cdc42p-Bni1p link remains speculative and its importance remains untested. We recently engineered a strain in which polarity establishment was synthetically rewired to be an actin-

dependent process. Unlike in wild-type cells where polarity establishment precedes actin polarization, actin cables and Bni1p become polarized coincident with polarity regulators in the rewired cells. However, the other formin, Bnr1p, is not recruited to the polarization site until several minutes later. This time interval between Bni1p and Bnr1p recruitment suggests a unique role for Bni1p to initiate actin cable polarization in the rewired cells. Indeed, *BNI1* is absolutely essential for viability of the rewired cells, and the unregulated Bni1p catalytic domain cannot support polarization. Fusing a heterologous Cdc42p-binding domain to the formin catalytic domain restores growth to the rewired cells, indicating that directly linking formin activity to Cdc42p bypasses the otherwise necessary regulations. Contrary to the prevailing view, deleting the endogenous GBD neither abolishes Bni1p function nor prevents it from localizing to the incipient bud site. Additional truncations showed that formin interactions with the “polarisome” components Spa2p and Bud6p are also dispensable, and that uncharacterized N-terminal domains can mediate an essential crosstalk between Cdc42p and Bni1p.

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The yeast cell polarity protein Bem3 requires and affects vesicle trafficking.

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Cell polarity is essential for a wide array of cellular functions including cell migration and cell fate determination. Although both RhoGTPase signaling and vesicle trafficking pathways play central roles in cell polarity establishment, the mechanisms by which they are coordinated are still unclear. In order to address this topic, we used S. cerevisiae as a model organism for studying the interplay between the cell polarity protein Bem3 (Bud Emergence protein 3) and vesicle trafficking.

Bem3 is a peripherally-associated membrane protein that functions as a Cdc42 RhoGAP (RhoGTPase Activating Protein). Here, we demonstrate that its targeting to sites of polarized growth depends on the integrity of the endocytic and recycling/secretory pathways. Specifically, knock-out of SLA2 and RCY1 led to Bem3 accumulation in depolarized puncta and in intracellular compartments, respectively. Indeed, we observed that the membrane fraction of Bem3 was partitioned between the plasma membrane and intracellular membrane-bound compartments. These Bem3-positive compartments were polarized towards the site of bud emergence and appeared to be present only during the pre-mitotic phase of apical growth. In addition, electron micrographs clearly showed accumulation of Bem3-positive vesicles at sites of polarized growth. Cell biological and biochemical approaches identified these intracellular Bem3 structures as belonging to the endosomal and secretory systems.

Strikingly, we found that Bem3 is not just a passive cargo of vesicle trafficking pathways. Specifically, expression of this protein from a GAL1 inducible promoter led to an increase in size of the Bem3-positive compartments, and this was proportional to the intracellular concentration of Bem3. Importantly, the secretory pathway marker, Sec4, was recruited to these compartments and in the absence of Bem3, was unable to efficiently reach sites of polarized growth. Additionally, we demonstrated that these Bem3 effects on the secretory pathway were independent of its GAP activity.

This work unveils unsuspected and important details of the relationship between vesicle trafficking and elements of the cell polarity machinery: 1. A peripherally associated protein relies on vesicle trafficking for maintaining its proper localization. 2. Bem3 requires and in turn, controls vesicle trafficking. 3. It establishes the first GAP-independent function of the Cdc42 regulator Bem3.

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Spatial control of Cdc42 GEF Gef1, a TUBA/DNMBP homolog by conserved NDR kinase Orb6.

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Conserved NDR kinases regulate cell morphology in eukaryotes. We have previously reported that fission yeast NDR kinase Orb6 maintains cell polarity by spatially regulating Cdc42 GTPase, a key morphology control factor. Loss of Orb6 kinase activity leads to ectopic localization of the Cdc42 GEF Gef1, normally found at the cell tips, to the cell sides. Gef1 is an ortholog of the mammalian Cdc42 GEF DNMBP/TUBA, which is implicated in the control of kidney cell polarization. Like TUBA, Gef1 contains a Dbl homology domain followed by a Bin/Amphiphysin/Rvs (BAR) domain instead of a PH domain.

By mass spectrometry analysis, we determined that Orb6 kinase phosphorylates a single site in the Gef1 N-terminus, serine S112. The N-terminus is necessary and sufficient for Gef1 localization to the cell cortex. Since S112 is part of a RXXS amino acid sequence, we speculated that it mediates the interaction of Gef1 with a 14-3-3 protein. Consistent with this idea, we found that Gef1 co-purifies with 14-3-3 protein Rad24 and that rad24Δ; mutant cells display ectopic localization of Gef1, similarly to orb6 mutants. Finally, we found that the rounded phenotype of rad24Δ; cells is suppressed by deletion of gef1, indicating that Gef1 mediates the morphology control function of Rad24.

To address the effect of blocking Gef1 phosphorylation by Orb6 kinase, we engineered a non-phosphorylatable gef1S112A mutant. We found that this mutation leads to increased cortical localization of Gef1, similar to orb6 and rad24 mutants. Thus these results suggest that Orb6 kinase maintains cell polarity by restricting the amount of Gef1 that can localize to the cell tips. This function is mediated by the interaction of Gef1 with 14-3-3 protein Rad24, that sequesters it in the cytoplasm. Our findings provide a molecular mechanism for the morphology control function of Orb6 kinase. Further, they indicate that NDR kinases can also utilize 14-3-3 proteins as mediators of their functions, as previously shown for the related conserved LATS kinase family.

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Tea5p, a Pseudokinase that Functions in Cell Polarization.

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Cell polarization introduces asymmetry. This enables cells to acquire and maintain a given shape and localize proteins and cellular processes to specific sites. The fission yeast *Schizosaccharomyces pombe* shows a highly regulated pattern of polarized growth. After division, the rod-shaped cells initiate growth at only one cell end. Later the second cell end starts to grow during new end take off (NETO). A pathway defined by microtubule +TIP proteins tea1p and tea4p has been shown to regulate NETO and cell morphogenesis. Here in a genetic screen for new NETO mutants, we identify a new factor tea5p. Tea5p is likely a pseudokinase as it lacks key residues required for kinase activity. Tea5p functions downstream of tea1p in localizing active cdc42p GTPase and the formin for3p to the second cell tip, but significantly, is only required for a subset of tea1p functions. Genetic studies suggest that tea5p functions in cdc42p activation via a signaling pathway consisting of the NDR kinase orb6p and the cdc42p activator gef1p. Thus, tea5p constitutes a novel connection between microtubule +TIP proteins and activation of cdc42p at cell ends. These studies begin to define distinct pathways in the establishment of cell polarization by microtubules.

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Optical control of MAPK signaling and polarized growth in budding yeast.

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The polarized assembly of protein complexes is fundamental to the establishment of cellular morphology. During mating, yeast orient polarized growth along a gradient of peptide pheromone secreted by a potential mate. Detection of the pheromone activates a mitogen-activated protein kinase (MAPK) cascade and the small GTPase Cdc42 by triggering the assembly of multi-protein complexes at the plasma membrane. In order to experimentally manipulate the assembly of cellular signaling complexes, we have created tunable, light-controlled interacting protein tags (TULIPs) based on dual genetic fusion with an engineered LOV2 domain from *A. sativa* phototropin 1 and a non-native binding partner. The affinity profile and dissociation kinetics of TULIPs are highly tunable, facilitating use in signaling pathways with varying sensitivities and response times. We used TULIPs to confer novel light sensitivity to several functionally distinct components of the yeast mating MAPK and Cdc42 pathways, thereby eliciting light-triggered cell cycle arrest and light-directed polarized growth. Current efforts are aimed at identifying factors that are sufficient to drive polarization, along with temporal requirements for their activity.

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A Model of Fission-Yeast Cell Shape Driven by Membrane-Bound Growth Factors.

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Fission yeast serves as a model for how cellular polarization machinery is used to regulate cell growth. Recent studies identify active Cdc42, found in a cap at the inner membrane of growing tips, as a master growth regulator, likely through control of exocyst tethering and formin-based nucleation of actin cables. To investigate how biochemistry might control shape, we propose a simple model based on the hypotheses that (i) the delivery and internalization rate of wall or membrane components limits cell expansion and (ii) a growth factor, such as Cdc42, signals for delivery of these components. We numerically simulate cell growth according to an axisymmetric, finite-element computational model that couples growth-factor-directed orthogonal expansion of the cell membrane and cell-wall remodeling to reaction and diffusion of the growth factor on that membrane. We explore limiting conditions for polarized growth and consider the additional effects of membrane elasticity and flow. We find a relationship between cap size and diameter, and motivate future experiments on the link between cell signaling and shape. Fission-yeast Cdc42 is regulated by a number of proteins whose absence lead to defects in shape or polarized growth, such as cells of varying diameter, round cells, and branched cells. Among these proteins, Gef1 and Scd1 assist the activation of Cdc42 at the tips and Rga4 restricts the location of its activation. We compare model results to cell morphologies of mutants of Cdc42 regulators and suggest possible mechanistic roles for these regulators. An extension to three dimensions adds the capacity to reproduce bent-cell morphology and to investigate the stability of axisymmetric solutions.

Germ Cells, Gametogenesis, and Fertilization

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Obesity Impairs Leydig Cells Function in Adults Rats Due to Down Regulation of Androgen Receptor and 17β-HSD.

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Obesity changes the hypothalamic-pituitary-gonad axis and affects negatively different aspects of reproductive function. However, little is known about the response of Leydig cells to the obesogenic condition. Therefore, this investigation aimed to evaluate the effects of high-fat diet-induced obesity on Leydig cell steroidogenic capacity. Adult male Wistar rats were fed for 15 weeks with balanced (Control, 4% fat) or high-fat (Obese, 20% fat) diet. Various biometric and metabolic parameters were monitored, such as body weight, adiposity index, determination of testosterone and insulin plasma levels and insulin tolerance test (ipITT). Testis were processed for histological and morphometric analysis, immunohistochemistry for androgen receptor (AR), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD), and western blotting for AR and 17β-HSD. The daily sperm production (DSP) was also determined. Obese rats presented an increase of 16 % in body weight, 50% in adiposity index and a remarkable diminution of 70% in testosterone. In addition, obesity led to hyperinsulinemia (C: 3.4 ± 1.2; O: 5.1 ± 0.8 mg/ml), causing insulin resistance that was detected by ipITT. The histological and morphometric analysis showed that the cytoplasmic (C: 165 ± 10.5; O: 126 ± 9.5 μm^2) and nuclear (C: 42 ± 2.5; O: 33 ± 1.8 μm^2) area of Leydig cells decrease, while the nuclear/cytoplasmic ratio remains the same. This diminution indicates that there was a reduction of secretory activity in these cells. Immunohistochemistry showed no significant reduction in the frequency of AR-, 3β-HSD- and 17β-HSD- positive Leydig cells in obese animals, but the content of AR and 17β-HSD decreased in crude testis samples of these animals. Furthermore, obesity reduced about 22% the daily sperm production. Our data corroborate previous studies which report that obesity is associated with impairment of steroidogenesis and spermatogenesis, and demonstrate that it is associated with reduction in AR and 17β-HSD expression. Financial Support: FAPESP.

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Direct Differentiation of Human Pluripotent Stem Cells into Haploid Spermatogenic Cells.

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Male prepubescent and adult patients rendered sterile by cancer therapies, immunosuppressants or injury prior to delivering a sperm sample are permanently sterile and are unable to produce offspring. Unfortunately there are no proven treatments to re-establish fertility in these patients. Consequently, patient-specific stem cell treatments represent one potential future cure to restore fertility. Here we show that human induced pluripotent stem cells (hiPSCs) and embryonic stem cells (hESCs) differentiate directly into advanced male germ cell lineages such as PLZF-positive spermatogonial stem-like cells (SSCs) and post-meiotic, round spermatid-like cells in vitro without genetic manipulation. Furthermore, our differentiation procedure mirrors spermatogenesis in vivo by replicating an SSC-like lineage followed by a small fraction of cells becoming haploid expressing both acrosin and protamine 1, found

uniquely on both spermatids and sperm. These results demonstrate that male pluripotent stem cells have the capability to differentiate into advanced germ cell lineages, including post-meiotic round spermatid-like cells. These results also represent a potential technique for both studying spermatogenesis in vitro as well as further consideration for restoring fertility in sterile male patients including developing models for discovering the mechanisms of the remaining causes of idiopathic male infertilty.

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Smt3 Posttranslational Modifications Mediate Critical Regulatory Events during Drosophila Spermiogenesis and Spermatogenesis.

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Introduction: *Drosophila* spermatogenesis is a dramatic, temporally-orchestrated developmental stage-specific process. Sperm production includes marked changes in mitosis and meiosis, chromosomes, transcription, translation, and posttranslational modifications, with striking nuclear remodeling during spermiogenesis. The posttranslational small ubiquitin-like modifier (SUMO) protein has been shown to play diverse roles in many highly conserved cellular processes such as spermatogenesis in various species including man and rodents. The purpose of this study was to define the precise stage-specific timing of fly Smt3 (*Drosophila* SUMO)-mediated events during germ cell development, determine whether Smt3-deficiency affects sperm production, and initially identify Smt3-modified proteins for comparison with those during mammalian spermatogenesis.

Methods: For bioimaging, unconjugated Smt3 and Smt3-modified proteins were detected by immunofluorescence using both whole mounts and squash preparations of testis from wild-type and heterozygous Smt3-deficient mutant stocks, avoiding homozygous larval lethality. Smt3-SUMOylated proteins were determined by immunoprecipitation (IP) and Western blot analyses. Protein extracts prepared from human and rodent testes were used for comparison. Male fertility of fly mutants was assessed by matings with wild-type females.

Results: In wild-type flies, Smt3-SUMOylated proteins show strikingly different patterns in most stages of spermatogenesis including spermatogonia undergoing mitosis, resting and meiotically active spermatocytes, and round and elongating spermatids in various stages of nuclear condensation during spermiogenesis. Smt3 was not detected in hub cells. The testes of heterozygotes showed reduced levels of Smt3 and an altered SUMOylated protein profile compared to wild-type. Interestingly, the reduction of Smt3 signals was readily observed in meiotic spermatocytes; no change for mitotic spermatogonia was apparent. Heterozygote males exhibited a reduced fertility and their testes show a marked defect in sperm transfer to the seminal vesicles. Smt3-modification of an essential cell cycle kinase was observed only during anaphase-to-telophase transition. SUMO-modifications were confirmed using human and rodent testis with normal spermatogenesis.

Conclusion: Our data are suggestive that 1) precise timing of SUMOylation events in developing fly germ cells is required for normal spermatogenesis; 2) Smt3-deficiency can result in failure of spermatids to properly undergo spermiogenesis and sperm transfer, findings consistent with marked reduction in fertility. Taken together, our results indicate important roles for Smt3 and SUMOylation during and after meiosis in *Drosophila* testis.

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Is altered epidermal growth factor signalling responsible for decreased epididymal connexin 43 in azoospermic men?

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Gap junctions enable direct communication between adjacent cells. Gap junctional pores are formed by connexons comprised of connexins (Cxs). Cx43 expression by Sertoli cells is essential for spermatogenesis in the testis and is regulated by thyroid hormones and androgens in both testis and epididymis. There is no information on the expression or regulation of Cx43 in the human epididymis. The objectives of this study were to determine if Cx43, Cx32 and Cx26 are present in the human epididymis and to determine if these are altered in infertile azoospermic men. Epididymides were obtained from fertile, non-obstructive and obstructive azoospermic patients and subdivided into caput, corpus and cauda. Real-time RT-PCR analysis revealed that Cx43, Cx32 and Cx26 mRNA levels were present throughout the epididymis of fertile men. In both non-obstructive and obstructive azoospermic patients, Cx43 and Cx32 were significantly decreased in the cauda epididymis. No significant effect was noted for Cx26. Previous microarray data suggested that EGFR mRNA levels were lower in the epididymis of infertile men. EGFR mRNA levels were significantly decreased in the cauda epididymidis of azoospermic patients. Cx43 and EGFR were localized between basal and principal cells and between principal cells. In the obstructive azoospermic patient, Cx43 was predominantly in the cytoplasm of basal cells as compared to fertile patients where Cx43 was along the plasma membrane. To determine whether or not EGFR regulated Cx43, a human caput epididymal cell line was used to assess the regulation of Cx43 by EGFR. Using EGF stimulation and pathway-specific inhibitors we showed that EGF could induce both the ERK1/2 and PI3K/AKT pathways. EGF increased the expression of Cx43 over 24 hrs. In the short-term, Cx43 phosphorylation was increased by EGF via ERK1/2, and not via the PI3K/AKT signalling pathway. However, the EGF-induced increase in total Cx43 protein levels was inhibited by the PI3K inhibitor LY294402, indicating that expression of Cx43 can be regulated by the PI3K/AKT pathway. These results suggest that EGFR is down-regulated in the epididymis of azoospermic men and that this decrease may contribute to lower levels of Cx43 in the epididymis. Such effects may be responsible for altered coordinated epididymal function in the human epididymis of infertile men. Supported by NSERC and CIHR.

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Effects of Dehydroepiandrosterone on Androgen Receptor Protein Expression in Testes and Epididymides in Two Strains of Mice.

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Strain differences in *in vitro* fertilizability still constitute a serious problem in mouse reproduction. To improve the *in vitro* fertilizability of mouse sperm, we examined the effects of implanting time-release pellets of dehydroepiandrosterone (DHEA), a testosterone precursor, on androgen receptor protein (AR) expression in testes and epididymides in two strains of mice. DHEA pellets (5 mg/pellet, 21-day release form, Innovative Research of America) or placebo pellets were implanted subcutaneously in 9-week-old male mice from two strains: C57BL/6CrNSlc (B6) and 129X1/SvJJmsSlc (129X1). After 21 days, testes and epididymides were collected from each male. Proteins from these tissues were extracted using ReadyPrep protein extraction kit for Total Proteins (Bio-Rad). Quantitative Western blotting using testicular protein extracts with glyceraldehyde-3-phosphate dehydrogenase as an internal control showed that the expression of androgen receptor protein was significantly higher in B6 than in 129X1 males ($p < 0.05$ by

two-way ANOVA), while there was no significant effect of DHEA on the amount of AR in either strain (DHEA vs placebo: 0.028±0.006 vs. 0.021±0.005 in B6 and 0.012±0.002 vs. 0.010±0.002 in 129X1, arbitrary unit, mean±SEM, n=3). Comparison of epididymal AR expression by quantitative Western blotting indicated no significant difference between strains or between DHEA- and placebo-treated males (DHEA vs placebo: 0.023±0.007 vs. 0.032±0.001 in B6 and 0.030±0.003 vs. 0.025±0.008 in 129X1, n=3). Interestingly, in B6 testes, two-dimensional electrophoresis indicated that one protein spot was denser in DHEA-treated males than in placebo-treated males. In contrast, DHEA did not change the density of the corresponding spot in 129X1 testes. Thus, 21-day-release pellets of 5 mg DHEA had no significant effect on AR expression in two organs in two strains, but the DHEA pellets induced a strain-dependent change in the testicular protein expression, which might be due to the differential AR expression between two strains. DHEA treatment of male mice has the potential to modulate steroid receptor expression, which might alter *in vitro* fertilizability in mice.

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Characterization of the pannexin 1 promoter.

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Pannexins (Panxs) are transmembrane proteins that form hemichannels and are implicated in cellular communication through the secretion of biomolecules. We have previously shown that Panx1 and Panx3 are expressed in the male rat reproductive tract and are regulated by androgens in the epididymis. We also reported the presence of a splice variant for Panx1 in which exon 3 is missing. In the present study, our objectives were to characterize the promoter of Panx1 in order to better understand its gene regulation in the rat epididymis. Total RNA was extracted from the epididymis of an adult rat and RACE (Rapid Amplification of cDNA Ends) experiments were performed. Multiple transcription start sites (16) were identified, encompassing the region between the positions -481 to -369 relative to the ATG. Using a SMARTer RACE kit, 14 sites for the transcription initiation were first identified. The use of a RLM (RNA Ligase mediated)-RACE kit, which allow cDNA amplification only from full-length mRNAs, resulted in the identification of only 3 transcription start sites, at positions -443, -429, and -393. Computational analysis revealed that the more distal transcription start site (position -481) was part of an initiator (Inr) consensus motif and was located immediately 5’ to a cAMP responsive element and a binding site for PEA3 (Polyomavirus enhancer activator 3). Several SP1 binding sites were also present in the region where the transcription initiation sites were identified. Genomic sequence analysis revealed the presence of a CpG island at the same location (position -563 to -56). To determine importance of this region in gene transactivation, 2 kb of the Panx1 promoter was amplified and cloned into a vector containing a luciferase reporter gene. High levels of luciferase activity were detected in transfected rat caput epididymal (RCE) cell line. Deletion constructs indicated that the highest transactivation levels were achieved with shorter constructs (-973 to -346 and -550 to -346). Similar results were obtained using CV-1 cells which do not express Panx1. These results suggest that Panx1 gene expression may be regulated via changes in promoter methylation and by specific transcription factors. Elucidation of such mechanisms should allow the understanding of the role of Panx1 in the epididymis. Supported by NSERC.

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The High-Fat Diet Leads to Premature Detachment of Germ Cells and Reduces Daily Sperm Production in Rats.

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The present study evaluated the consequences of obesogenic environment during different phases of development on the testicular structure and sperm production of rats at adulthood. Adult male Wistar rats were assigned in the following groups: 7 month old animals previously fed for 15 weeks with balanced (CT1) or high-fat diet (O/A); 4 month old animals subjected to normal feeding (CT2) or to obesogenic environment during gestation, lactation up to sexual maturity (O/E-A). Both O/A and O /E-A rats showed an increase in the body weight, waist circumference, epididymal and retroperitoneal fat weights and glicemia but only the later were diabetic (CT1: 106 ± 3.6; O/A: 129 ± 10.9; CT2: 109 ± 3.0; O/E-A: 179 ± 11.7 mg of glucose/dl). Obesity resulted in insulin resistance and decreased serum levels of testosterone (CT1: 340 ± 59; O/A: 103 ± 21; CT2: 382 ± 40; O/E-A: 112 ± 25 ng/dl) independently of the developmental period. The diameter of the seminiferous tubules increased in O/A (CT1: 211±2.5; O/A: 219±2.3 µm) and decreased in O/E-A (CT2: 364 ± 6.13; O/E-A: 343 ± 5.15 µm). The histophatological and quantitative analysis demonstrated that there was a greater detachment of cells from the seminiferous epithelium in the obese (O/A: 35 ± 3.2%; O/E-A: 41 ± 15.5%) than in controls (CT1: 3.0 ± 1%; CT2: 9 ± 1.4%) groups. Regardless of developmental period, the obesogenic environment reduced the number of final spermatids in the testis, daily sperm production and sperm count in the epididymis, but did not alter the sperm transit time through the caput/corpus and cauda of this organ. Ours findings show that regardless the developmental period the high-fat diet ingestion reduces the sperm production, due premature germ cell detachment and testosterone withdrawal. These data also indicate as longer the period of exposure to obesogenic environment higher is the impairment in daily sperm production. Financial Support: FAPESP.

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The calcium-binding protein, Dysferlin, differentially localizes in cells during early development of the sea star, *Patiria miniata*.

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Mass spectrometry analysis was performed on egg plasma membrane-enriched fractions subjected to SDS-PAGE and candidate proteins were identified from the sea star, *Patiria miniata* (*P. miniata*), oocyte transcriptome. One interesting candidate protein identified was Dysferlin, a member of the ferlin family of proteins involved in calcium-regulated membrane fusion. Fer-1, a homologue of Dysferlin, is involved in calcium-mediated membrane fusion during spermatogenesis in *C. elegans*, and in vertebrates, it is mutated in distrophic skeletal muscle cells. In sea urchin embryos, Dysferlin has been implicated in calcium-mediated intracellular signaling in response to membrane wounding. We raised an affinity-purified polyclonal antibody against a peptide from the predicted dysferlin domain and immunofluorescence was utilized to characterize dysferlin expression during early development in *P. miniata*. Dysferlin is localized to the cortex of immature oocytes and eggs. Interestingly, Dysferlin was detected in the germinal vesicles of young immature oocytes. Next, we investigated Dysferlin localization during sperm-egg binding. Immature oocytes and eggs were incubated with sperm for 10 minutes, fixed and immunolabeled. Dysferlin appears enriched in

areas of the plasma membrane where egg-sperm interaction is occurring. Finally, we aimed to characterize Dysferlin localization after fertilization. In addition to the periphery of the cell, Dysferlin localized in nuclear regions of blastomeres from an 8-cell embryo. In swimming gastrulae, mesenchymal cells contained low levels of Dysferlin, while ectodermal cells expressed higher levels of dysferlin localized in apical regions. Collectively, these preliminary results indicate Dysferlin in differentially localized in cells during early development of *P. miniata*.

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Maintaining the Integrity of Sea Urchin Eggs Using Liposomal Glutathione.

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This study examined the potential protective effect of liposomal glutathione (L-GSH) on L. variegatus sea urchin eggs. GSH is an antioxidant believed to protect against cell damage. Preliminary results showed that at a concentration of 1.08 mg/mL, L-GSH was capable of maintaining the integrity of sea urchin eggs for 6 days. This is significant because most studies have reported that the shelf life of an unfertilized sea urchin egg is less than 1 day. Following exposure to L-GSH, eggs were monitored by direct microscopic observation and counted. In a control experiment, eggs kept in sea water with no L-GSH began to disintegrate after 24 hours and were heavily infested with protists. In contrast, eggs exposed to L-GSH maintained intact cell membranes for up to six days. Moreover, exposure to L-GSH appeared to prevent infestation by protists up to day 6. Future studies will focus on examining the viability of sea urchin eggs in the presence of L-GSH and determining whether these eggs can be fertilized to produce embryos. In addition, studies will be done in which sea urchin embryos will be exposed to cell damaging agents, such as UV radiation, in the presence and absence of L-GSH. The results of these experiments will provide insight into whether there is a protective effect of L-GSH on animal cell membranes and developing embryos.

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The Role of Lipids in the Induction of Apoptosis in *Xenopus* Oocytes.

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Xenopus oocytes can survive for extended periods (a few days to two weeks) in culture. However, once matured to eggs by the action of progesterone, death will occur within 24 hours. This striking contrast in viability provides an excellent model for studying a variety of factors that impinge on the balance of life and death. In particular, sphingolipid pathways have been shown to alter the balance of life and death in a variety of cell types, with ceramide tipping the balance towards apoptosis. The objective of our studies on lipids that regulate apoptosis is to shift the balance away from apoptosis in order to increase oocyte life span. This will enhance our ability to use oocytes for translation and expression of exogenous mRNA and proteins, and for in vitro fertilization. Using a microinjected near-infrared dye caspase substrate (LI-COR Biosciences), we have demonstrated an increase in caspase activity in individual Xenopus oocytes during spontaneous death, after peroxide addition, or in response to ceramide (200 nM). Both natural (porcine brain) and synthetic (C-6) ceramide, when microinjected or added to the medium result in a significant increase in caspase activity within 90 minutes of exposure. Over a longer time course, morphological indications of apoptosis become evident. These results demonstrate that ceramide can trigger apoptosis in Xenopus oocytes, and that ceramide may play a role in spontaneous death in cultured oocytes. We are currently investigating the kinetics of ceramide-induced apoptosis during both progesterone and insulin-stimulated oocyte maturation, along with various strategies to inhibit apoptosis in these cells.

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Biphasic traveling calcium wave during fertilization is generated independently of a sperm-supplied egg activation factor SPE-11 in *Caenorhabditis elegans*.

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A mature egg transforms into a totipotent embryo in a process known as egg activation. The egg activation in most animals is initiated by a change of intracellular calcium concentration in a pattern characteristic to each species. However, there are no genetic analyses for the calcium response and the molecular mechanisms for egg activation are still poorly understood. Here we report a detailed spatiotemporal pattern of the fertilization calcium response in *Caenorhabditis elegans* and that the calcium response is independent of SPE-11, a sperm-supplied factor required for egg activation.
Time-lapse movies of the calcium dynamics during fertilization were obtained by using a fluorescent calcium indicator and by spinning disk confocal microscopy. The calcium dynamics of the oocyte region was quantified by using computational image-processing methods. The quantification revealed that the fertilization calcium response is a biphasic traveling wave. The wave composed of a fast local wave that arises from the sperm entry point seemingly at the time of the sperm entry, followed by a slow global wave that propagates throughout the oocyte. No response was observed after the oocyte exits from the spermatheca where it is fertilized. The biphasic waveform could be explained by a well-known mathematical model, the bistable reaction-diffusion equation, under initial conditions that assume a transient calcium elevation at the one end of the oocyte. These results are consistent with the view that fertilization triggers a transient calcium elevation and the calcium-induced calcium release machinery propagates the calcium wave.
To gain insights into the molecular mechanism for the generation of the calcium wave and its role for egg activation, we observed the fertilization calcium response of mutants defective in sperm function: *spe-9*, *spe-41* and *spe-11*. Neither *spe-9* nor *spe-41* mutant sperm can fuse with the oocyte probably due to deficits in oocyte recognition and/or fusion, whereas *spe-11* sperm fuses with the oocyte but fails to activate it. We found that calcium fluorescence hardly changes in *spe-9* or *spe-41* mutants as the oocyte passes through the spermatheca, whereas a wild type-like wave is observed in *spe-11* mutants although they fail to activate eggs. These results suggest that fertilization calcium wave was generated by a process that requires SPE-9 or SPE-41 function and that a factor(s) other than SPE-11 triggers the calcium wave.

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Insulin Treatment of Diabetes Mellitus Improves Sperm Quality and Fertility Rate.

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Diabetes mellitus (DM) is one of the most widespread diseases that threaten human health in the modern world, affecting over 171 million people. In recent studies, the prevalence of infertility was higher in diabetic compared to non-diabetic patients. Sexual dysfunctions related to the diabetic state have been described, but their pathophysiological pathways are not yet to be clearly demonstrated. The goal of the current paper was to elucidate if treatment with insulin can improve fertility in diabetic sperm. This study determined that sperm quality, fertilization capacity, and subsequent embryo development in 3 experimental groups: control, diabetic, and diabetic treated with insulin for 7 days. The functional analysis of male reproduction was determined by measuring sperm motility using computer-assisted sperm analysis (CASA). In the diabetic group, the percentage of motile sperm was significantly lower than in control. Sperm samples were stained with Annexin-V to detect apoptosis. Samples were also stained with

Propidium Iodide (PI) to distinguish live cells from dead cells. Diabetic group showed a higher number of Annexin-V-positive sperm than control. Comet assay revealed increased DNA fragmentation in diabetic sperm when compared to control. These parameters normalized in diabetic sperm treated with insulin. Furthermore, to examine the fertility of the diabetic sperm, in-vitro fertilization (IVF) were performed. Treatment with insulin therapy increased significantly the fertility rate of sperm. The embryo developmental rates to the blastocyst stage were also higher in diabetic sperm treated with insulin than diabetic sperm. We conclude that the lack of insulin was directly related to the diabetes-induced sexual dysfunction, and Insulin supplementation restored these parameters.

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Phospholipase D Inhibitors FIPI and 1-Butanol Inhibit Calcium Release at Fertilization.

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Fertilization in *Xenopus* involves sperm activation of Src which in turn, activates phospholipase Cγ to increase IP3 and release calcium (Sato, Fukami and Stith, Sem Cell Dev Bio 285-92, 2006). Our large scale quantification of lipids at fertilization (Petcoff et al. J Lipid Res 2365-78, 2008), and prior work showing an increase in choline and 1,2 DAG during fertilization (Stith et al., Mol Bio Cell 8:755, 1997), suggests that phospholipase D1b production of Phosphatidic Acid (PA) plays a role in fertilization. Furthermore, as measured by HPLC/evaporative light scattering mass detection, PA mass increased at fertilization, and synthetic PA addition will stimulate Src tyrosine kinase to increase phospholipase Cγ tyrosine phosphorylation, IP3 mass and calcium release.

We have now perfected the whole cell measurement of intracellular calcium during *Xenopus* fertilization through prior injection of calcium indicator dye Fluo-4 into whole eggs. After examining various methods, we have perfected injection of eggs without artificial activation.

We can visualize transient sperm-egg interaction by recording the intensity, duration and diameter of “puffs” of calcium release. After one to two puffs, there is a subsequent larger calcium wave generated. PLD inhibitor 1-butanol (0.25%) reduced the PA increase at fertilization and blocked calcium release whereas 2-butanol (which is unable to inhibit PLD) did not. FIPI, a potent and specific inhibitor of phospholipase D, did not sperm egg interaction as noted by no change in the average number or size of the “puffs” of calcium release. However, FIPI inhibited Src activation, induction of the fertilization calcium wave and subsequent fertilization events (videos will be shown). This suggests that PLD and PA production do not play a role in the initial calcium release at the sperm binding site but PA may play a role in the large calcium release (wave).

Furthermore, addition of synthetic PA (but not anionic control lipid PS) to *Xenopus* oocytes or eggs also produced a large calcium release. Synthetic PA induction of calcium release was inhibited by IP3 receptor blocker 2APB, or a Tyrosine Kinase Inhibitor (Herbamycin A). Calcium ionophore ionomycin causes a much faster calcium release (2 vs. 11 min) that is not blocked by 2APB or herbamycin A. PA also increased IP3 mass to a level seen by sperm addition and this is in contrast to a much smaller increase induced by ionomycin.

As compared with other lipids (PIP2, LPA, PS and 11 other major phospholipids), PA specifically bound Xenopus Src but not PLCγ (Echelon PIP Strips).

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Evidence that mechanisms of nuclear fusion are conserved from yeast to the green alga, Chlamydomonas.

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Zygote formation in many eukaryotes is completed when the nuclei of the two merged gametes fuse to form the diploid nucleus, yet the cellular and molecular mechanisms that underlie nuclear fusion remain poorly understood. During fertilization in the unicellular, biflagellated alga Chlamydomonas, haploid plus and minus gametes adhere to each other by their flagella, thereby activating a cAMP-dependent signaling pathway that prepares the gametes for cell-cell fusion. Upon fusion, the cytoplasms of the two gametes coalesce, and soon thereafter their nuclei fuse (karyogamy). After an obligatory period in the dark, the zygotes undergo meiosis and germinate to produce 4 haploid progeny. Recently, we identified a Chlamydomonas homolog of the Saccharomyces cerevisiae nuclear fusion protein, KAR5. Based on RT-PCR and other transcript analysis we found that CrKAR5 is expressed only in gametes, and not asexual vegetative cells. Moreover, CrKAR5 transcripts are not detectable in asexual cells of either mating type and are expressed at low levels in gametes of both mating types. Unexpectedly, transcripts undergo a nearly 20-fold increase during gamete fusion and during gamete activation induced either by flagellar adhesion or by incubation of gametes in db-cAMP, but only in minus gametes. Preliminary results with an epitope-tagged form of CrKAR5 suggest that the protein is nuclear. In cells depleted of CrKAR5 transcripts by artificial micro RNA (amiRNA), zygote germination is substantially reduced. Our results indicate that basic mechanisms of nuclear fusion are broadly conserved in eukaryotes. Experiments are in progress to generate cells with a disrupted CrKAR5 gene. Supported by NIH GM56778 to W. J. S.

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Purification of Yeast Zygotes: Interrogation of the Haploid-to-Diploid Transition.

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The zygote, formed by fusion of two haploid cells, serves as the transition from haploid to diploid states. To generate *S. cerevisiae* zygotes for purification, haploid mating pairs of cells in the S288C background were transformed to cause expression of cytoplasmic green fluorescent protein (GFP) or mCherry-tagged polysomes. Cells of opposite mating type (mCherry MATa x GFP MATalpha) were co-incubated on an agar surface to allow zygote formation, harvested at various times (1.75 or 2.5 hours), followed by a two-step flow cytometry-based sorting protocol to isolate zygotes (cells exhibiting red-green double positivity). This bi-color technique (red vs. green) provides a convenient visual assessment of zygotic purity and maturation. Fluorescent deconvolution microscopy distinguishes pre-zygotes (fused haploid cells with discontinuous red and green cytoplasmic compartments) vs. more mature zygotes (that show cytoplasmic continuity). The final yield of zygotes is approximately 10% of input cells, and the average purity is greater than 95%. According to the duration of the cross, unbudded or budded zygotes can be recovered. We have applied this purification protocol for our ongoing studies investigating the biological significance of transcripts that are regulated at early stages vs. later stages of zygote maturation. The current zygote purification technique thus provides a convenient model system for “-omic” studies of fertilization, for recovery of initial progeny, and for systematic investigation of morphogenesis.

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Integrin adhesion drives the emergent polarization of active cytoskeletal stresses to pattern cell delamination.

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Cells in epithelia respond to and integrate a variety of biochemical and mechanical cues to generate stereotypic patterns during development and homeostasis. Here we investigate how cells of the amnioserosa, an active participant of Drosophila dorsal closure respond to a local subcellular mechanical stimulus. We show that a subcellular laser perturbation (that ensures plasma membrane integrity) triggers cell delamination/extrusion. An initial phase of cell expansion precedes extrusion enabling us to estimate cellular prestress (tension). We characterize the spatial reorganization of the cytoskeleton and the temporal hierarchy of their deployment in the perturbed cell and its nearest neighbours during cell delamination. We find an emergent polarization of actin (lamellipodial protrusions and cortical enrichment), myosin (streaming movement of medial myosin and cortical enrichment) and microtubules (polarized rearrangement) at the interface of the perturbed cell and its nearest neighbours in response to the perturbation. We identify novel roles for microtubules- in the streaming movement of myosin and timely completion of delamination in response to the subcellular perturbation. We further demonstrate that the reorganization of actin and myosin is dependent on integrin adhesion and precede changes associated with microtubules. Our results indicate that cell delamination is patterned locally through differential contributions of adhesion, cytoskeleton and local mechanical cues.

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Regulation of planar cell polarity and Vangl2 trafficking by Tmem14a.

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Planar cell polarity (PCP) coordinates the uniform structure, orientation, and movement of cells within a tissue. In zebrafish, PCP is also required for the directed re-intercalation of neuroepithelial cells following asymmetric cell division in the neural tube. PCP is regulated by a conserved core group of signalling proteins that include Frizzled, Dishevelled, Vangl, Prickle, Flamingo, and Diego. Mutations in human VANGL1 and VANGL2 have been linked to neural tube defects. However, little is known about the intracellular trafficking pathways that target Vangl to the cell membrane. Tmem14a is a 99-amino acid tetraspanin protein with unknown function that was identified in our lab as a potential interactor of Vangl2 in a membrane yeast-two hybrid screen. Here, we show that GFP-tagged Tmem14a is localized to a polarized trans-Golgi compartment of zebrafish neuroepithelial cells. Morpholino knockdown of Tmem14a activity results in PCP phenotypes such as convergent extension defects at gastrulation and disrupted neural tube morphogenesis. Morphant embryos also exhibit curved body axes at 1-2 dpf, suggesting additional roles for Tmem14a in regulating apical/basal polarity and ciliogenesis. Preliminary analyses reveal that motile cilia lining the floorplate of Tmem14a morphant embryos are disorganized, a phenotype similar to maternal-zygotic *vangl2* mutants. Preliminary analysis

of GFP-Vangl2 expression in Tmem14a morphants also revealed defects in GFP-Vangl2 membrane localization. Our results suggest that Tmem14a acts as a regulator of PCP signalling through its potential role in trafficking Vangl2 to polarized compartments of the cell membrane.

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Septin controls polarized actomyosin distribution during PCP-dependent collective cell movements.

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The Planar Cell Polarity (PCP) pathway is a critical regulator for cell behaviors during development, and convergent extension (CE) is an essential collective cell movement regulated by the PCP pathway. During this process, cells align and interdigitate to narrow and lengthen a tissue. PCP signaling is necessary for cell polarity during CE, but little is known about how PCP signaling controls the machinery that executes cell behaviors, for example actomyosin. Fritz is one of the PCP effector proteins that acts downstream of core PCP proteins (such as Dishevelled and Vangl2) to control specific processes in Drosophila. We show that vertebrate Fritz is necessary for CE and physically interacts with septins, cytoskeletal elements that provide cortical rigidity. Knockdown of Fritz or septin7 leads to abnormal cell membrane dynamics (rapid undulations) and defects in both cell shape and cell-cell contact. We have also found that Fritz controls septin7 and actin localization at the medio-lateral cell cortex. Fritz or septin7 KD disrupts polarized actin localization, and ectopic actin accumulation is observed instead. From these results, we conclude that polarized actin distribution is regulated by Fritz and septins downstream of PCP signaling to control membrane stability and cell shape during collective cell movement.

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Contact mediated transport of signaling proteins over long distance by Drosophila cytonemes.

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Cytonemes are types of filopodia that have been proposed to be involved in long distance signaling between producing and target cells. The present work characterizes cytonemes emanating from a group of collectively migrating tracheal cells forming air sac primordium that associate with the wing imaginal disc. These tracheal cells are dependent upon FGF and Dpp produced in the wing disc and they have at least two types of cytonemes - one that is responsive to FGF to which they segregate FGFR, and another to which they segregate the Dpp receptor, Thickveins (Tkv). Both FGFR and Tkv have punctal distributions that localize specifically in responding cytonemes, and the ligands colocalize with their respective receptors. These puncta are motile. Puncta containing both ligand and receptor are in cytonemes that appeared to touch ligand-expressing cells. Extracellular GFP reconstitution between cytonemes of recipient cells and ligand-expressing cells provided evidence for such direct contacts for signal reception. These findings confirm cytoneme-based movement of signaling proteins and are consistent with the idea that cytoneme-based transport of signaling proteins is the mechanism for cell-cell communication in which controlled amounts of signaling protein can be targeted to a specific recipient cell.

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A novel role for members of the PAR polarity complex in tracheal terminal cell development in Drosophila.

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Cell shape is key to cell function, but the processes that generate elaborate cellular morphologies are not well understood. To investigate this problem, we use *Drosophila* tracheal terminal cells, a component of the insect respiratory system. These cells undergo two distinct morphogenetic processes: subcellular branching morphogenesis, and subcellular apical lumen formation. Wild-type terminal cells initiate branching from a central branch, containing the cell body. Side branches then grow out and bifurcate, with a general reduction in cell diameter. Terminal cells also contain a single continuous, air-filled lumen, which extends through the central branch and into all subsequent side branches. Interestingly, despite their apical characteristic, these lumens form without cellular junctions but instead may arise by a process of vesicle trafficking and fusion.

We have found that both branching and lumen morphogenesis in terminal cells are regulated by components of the PAR-polarity complex. This complex is composed of the proteins Par-6, Bazooka (Par-3), aPKC, and Cdc42 and is best known for roles in establishing asymmetry during cell division and in initiation and maintenance of apical/basal polarity in epithelia. We find Par-6, Bazooka, and aPKC, as well as known interactions between them, are required for subcellular branch initiation, but not for branch outgrowth. We also find that these polarity proteins are downstream of an FGF signaling pathway that is known to regulate terminal cell branching and outgrowth. We have isolated two novel alleles of Par-6, one of which specifically truncates the Par-6 PDZ domain and analyzed single and double mutants of *par-6* and other PAR-polarity proteins. From this analysis we conclude that dynamic interactions between PAR-complex members controls the branching pattern of terminal cells. In contrast, we find that while Par-6 and aPKC are both required for subcellular lumen formation, neither Bazooka, nor a direct interaction between Par-6 and aPKC is needed for this process. Thus a novel, non-canonical role for the polarity proteins Par-6 and aPKC is used in formation of this subcellular apical compartment. Our results demonstrate that proteins from the PAR complex can be deployed independently within a single cell to control two different morphogenetic processes.

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Using Drosophila to dissect the role of eIF2alpha phosphorylation in development.

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In response to multiple stresses, phosphorylation of the translation initiation factor eIF2alpha attenuates new protein synthesis. In mammals, four stress-activated eIF2alpha kinases have been identified (GCN2, PERK, HRI and PKR) and are antagonized by two eIF2alpha phosphatases, constitutively expressed CReP (PPP1R15b) and ER stress-inducible GADD34 (PPP1R15a). Although all eukaryotes posses at least one eIF2alpha kinase, the cognate phosphatases are less common. Importantly, they appear to be absent in yeast and nematodes. We noted that Drosophila CG3825 shares homology with the mammalian PPP1R15 proteins. We confirm functional homology using biochemical and genetic strategies. It has been shown previously that Ppp1R15a/b double knockout is embryonic lethal in mice at an early stage, but the precise requirement for selective eIF2alpha dephosphorylation during development remains unexplained. We show that silencing of CG3825 in Drosophila by RNAi similarly results in

developmental delay and use this model to dissect further the role of eIF2alpha phosphorylation in development.

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Rfx2 is required for the intercalation and ciliogenesis of epidermal multi-ciliated cells in *Xenopus*.

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Specialized mucociliary epithelia are essential for many major organs, such as the respiratory system. The embryonic epidermis of *Xenopus* embryo has similarities with mammalian mucociliary epithelia. Therefore, the epidermis of *Xenopus* provides a good model to study the morphogenesis of mucociliary epithelia. The embryonic skin of *Xenopus* embryo contains specialized multi-ciliated cells that are distributed in a spacing pattern. The ciliated cell precursors first differentiate from the inner or sensorial layer of the ectoderm and then intercalate radially into the outer epithelial cell layer to form multi-ciliated cells. However, the mechanisms of controlling the multi-ciliated cells intercalation remain unclear. We observed that a transcription factor, Rfx2, is expressed from early multi-ciliated cell precursors to mature multi-ciliated cells in the epidermis, suggesting that Rfx2 might be essential for different steps regulating the formation of epidermal multi-ciliated cells. When we targeted delivery antisense oligonucleotides to knock down Rfx2, we observed the similar number of α-acetylated tubulin-expressing cells in both control and Rfx2 knockdown embryos, indicating that multi-ciliated cell specification is unaffected by loss of Rfx2. However, most multi-ciliated cell precursors fail to intercalate properly into the outer epithelial cell layer in Rfx2 knockdown embryos. In addition, some multi-ciliated cells with mild intercalation phenotypes in Rfx2 knockdown embryos have only a few short axonemes, as compared to control epidermis, where dozens of long cilia are assembled on multi-ciliated cells. We also consistently observed the shorter cilia phenotypes in the neural tube and gastrocoel roof plate in Rfx2 knockdown embryos. Together, these data show that the transcription factor Rfx2 is essential for the multi-ciliated cells intercalation and ciliogenesis.

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Differential remodeling of peripheral glial subtypes during the larval to adult transition in Drosophila.

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INTRODUCTION: In the Drosophila larva, 8 pairs of abdominal nerves, A1-A8, extend from the ventral ganglion to innervate muscles in the body wall. This pattern is re-organized during metamorphosis to generate the adult pattern- three pairs of anterior abdominal nerves directly exit the abdominal ganglion, and the remaining five pairs of posterior nerves (A4-A8) form a fused Terminal Nerve Trunk (TNT). Segmental nerves defasciculate from the TNT at multiple levels to innervate the body wall muscles. The OBJECTIVE of this study is to understand how glia, which wrap around each peripheral nerve are rearranged during the transition to allow five pairs of nerves to form a fused TNT. Peripheral nerves in the Drosophila larva are surrounded by four layers- the most external is the neural lamella, a deposition of extracellular matrix, followed by 3 glial layers - the perineurial layer just below the lamella, subperineurial glia which form septate junctions and wrapping glia which are the innermost. METHODS: Glia specific antibodies and layer specific markers (Stork et al, 2008; J. Neurosci.) were used for labeling, followed by visualization with confocal microscopy. RESULTS: A 3-fold increase in glial nuclei is

seen at the end of the first day of metamorphosis (24h APF; n=15). At this time, the outermost layer (the lamella) and the innermost layer (wrapping glia) are absent (n= 8 and 10 respectively). Interestingly, cells of the perineurial layer are the most abundant (80% of total glia; n=14); the rest are sub-perineurial (n= 12). Persistence of this layer is not surprising given that it forms the auto-cellular septate junctions that serve as the blood brain barrier. These data allow us to CONCLUDE that glial layers undergo differential remodeling (retraction of some layers and expansion of others) in the early pupa, which is initiated before nerve fusion. FUTURE DIRECTIONS: Each glial class will be examined during and after TNT formation. BrDU labeling will be used to follow proliferation, single cell labeling will be used to examine cellular extents, and ultrastructural studies will examine relative organization of glia and axons in peripheral nerves during the transition. These studies will lay the groundwork for manipulating individual glial layers to better understand their contribution to TNT formation.

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A dual role for the NaKATPase during brain ventricle development.

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We demonstrate that the NaKATPase plays a dual function during vertebrate brain ventricle development, using the zebrafish as a model. The first is regulation of neuroepithelial polarity and permeability, which can be modulated by RhoA; while the second function is not sensitive to RhoA, and likely includes cerebrospinal fluid (CSF) production. Both the alpha subunit, (*atp1a1*) and the *FXYD1* subunit, which we newly isolated, set up neuroepithelial polarity and continuous apical junctions, leading to a cohesive neuroepithelium. Partial loss of *atp1a1* function prevents brain ventricle inflation, however, while neuroepithelial polarity appears normal, neuroepithelial permeability is strikingly increased relative to controls, as measured by a novel dye retention assay. Conversely, neuroepithelial permeability is reduced by expression of activated RhoA or overexpression of Atp1a1, in a dose-dependent fashion. Expression of activated RhoA restores continuous junctions after severe loss of Atp1a1 or FXYD1, and leads to normal inflation in FXYD1 loss of function embryos. However, inflation is not rescued after Atp1a1 loss of function + RhoA, indicating that a RhoA-independent activity, likely CSF production, is required for inflation. In a wild type embryo, formation of both a cohesive neuroepithelium, as well as brain ventricle inflation require the pump function of the Atp1a1 subunit, and intracellular Na^+ concentration ($[Na^+]_i$) increases after pump inhibition. High $[Na^+]_i$, is associated with failure to form a cohesive neuroepithelium, whereas after a more modest increase in $[Na^+]_i$, the neuroepithelium appears intact but has increased permeability. These data suggest that the NaKATPase directs brain ventricle development through two pathways, one of which promotes formation of a neuroepithelium capable of retaining fluid, and the other that leads to production of CSF.

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EXC Proteins Regulate Early Endosome Trafficking to Maintain Apical Surface in Narrow Tubules of C. elegans.

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Single-celled tubules must continually regulate their narrow apical diameter in order to maintain normal fluid flow, and in the case of Schwann cells, to maintain close contact for electrical insulation of neurons. The nematode excretory canals provide a genetically manipulable model of such tubules. In *C. elegans*, loss-of-function mutations in any of a set of *exc* genes cause the

excretory canal lumen to swell into large fluid-filled cysts, as a result of mechanical failure of the canal apical surface.

Three of the EXC proteins show genetic interactions with each other, and interfere with the ability of early endosomes at the apical surface to progress to recycling endosomes. *exc-5* encodes a guanine exchange factor homologous to the mammalian FGD proteins that activate CDC42; defects in human FGD4 cause Schwann Cell malformation in Charcot-Marie-Tooth disease type 4H. *exc-9* encodes the small LIM-domain protein CRIP, and *exc-1* contains two Ras domains homologous to those of human IIGP (interferon-inducible GTPase). While the three genes are expressed in various tissues in the nematode (*exc-5* in the pharynx, *exc-9* in the uterine seam, and *exc-1* in glial cells), all three are expressed in the excretory canals. Overexpression of *exc-5* rescues mutants in the other two genes, and overexpression of *exc-1* rescues *exc-9* mutants. Yeast-2-hybrid experiments show that constitutively active EXC-1 binds to itself and to EXC-9.

Constructs expressing subcellular organelle markers within the excretory canals showed that loss of EXC function caused a large accumulation of labeled EEA-1 (early endosome antigen) in areas of the apical surface where the tubule lumen collapsed, and loss of RME-1 (human EHD1) expression in those same areas. Most importantly, the buildup of EEA-1 was found to precede loss of structural integrity in embryonic canals. These results strongly suggest a model in which the function of EXC proteins is integral for vesicle progression from early endosomes to recycling endosomes at the apical surface of the excretory canals, and that EXC proteins are essential for maintenance of tubule integrity at the luminal surface of narrow single-celled tubules.

Marker constructs modified from constructs provided by Barth Grant, John White, and Brian Ackley. Financial support from NINDS R03-NS067323.

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Basal constriction, a novel cell shape change regulated by Wnt5b, Src kinase and FAK signaling.

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Basal constriction occurs during formation of the zebrafish midbrain-hindbrain boundary constriction (MHBC), and this appears to be a widespread, but largely uncharacterized, epithelial shape change during invertebrate and vertebrate organogenesis. At the MHBC, basal constriction requires Laminin in the basement membrane (Gutzman J. et al., Mech Dev. 2008) and we have recently identified Wnt5b and Focal adhesion kinase (FAK) as crucial for basal constriction, acting independently of Laminin. Wnt5b RNA is concentrated at the MHB and, in transient transgenics, RNAi against Wnt5b expressed from a CNS-specific promoter prevented basal constriction. Frizzled2 and Ryk receptors are required for basal constriction and their activity is strongly synergistic, while the Src kinases, Fyn and Yes, are also required for basal constriction, putatively acting upstream of FAK. Autophosphorylated FAK (on Y397) is enriched at the MHBC of 24 hpf embryos and transplant assays show that FAK is required cell autonomously for basal constriction. Using a photoactivatable FAK antisense construct, we showed that FAK is required at the time of basal constriction in MHBC cells. Expression of human FAK or phosphomimetic FAK (Y397E) rescues basal constriction after Wnt5b loss of function, indicating that Wntb5b and FAK function in a linear pathway to direct basal

constriction. Together our data demonstrate a role for a non-canonical Wnt pathway in basal constriction during brain morphogenesis.

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Hydraulic stress induced bubble nucleation and growth during pupal metamorphosis.

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Here I describe the role of physical fluid stress during pupal metamorphosis in flies. During early stages of pupation of larvae into adult flies, a physical gas bubble nucleates at a precise spatial and temporal location, as part of the normal development program in Diptera. Although its existence has been known for the last 50 years, the origin and control of this "gas nucleation" event has remained completely mysterious. Where does the driving negative pressure for bubble nucleation come from? How is the location of the bubble nucleation site encoded in the pupae? How do molecular processes control such a physical event? What is the role of this bubble during fly development? By developing imaging techniques including X-ray microscopy and bio-physical measurements for live insect pupal structures, here I elucidate the physical mechanism for the appearance and disappearance of this bubble. Via growth rate measurements of this bubble in a developing pupae subjected to variable fluid stress environments for three different species (Drosophila melangastor, Musca domestic, Sarcophaga bercaea), I directly measure the evaporative stress and the resulting negative pressure in the pupal cavity. The sharp increase in this negative pressure specifically encodes the exact timing of the nucleation event. Furthermore, controlled buckling of the main tracheal conduits breaks symmetry and thus govern the physical location of the nucleation site. Gaining physical insights into this hydraulic mechanism also allows us to finally predict the mechanics and inherent design of pupal shell architecture in various species.

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Map1b Is Involved In Neural Convergence During Neurulation In Zebrafish.

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The neural tube (the precursor of the brain and spinal chord) is shaped during neurulation from a flat sheet of cells on the dorsal surface of the embryo known as the neural plate. Neural convergence, an early stage of neurulation that is conserved across vertebrates, results in the narrowing and elongation of the neural plate. In zebrafish, this morphogenetic process is brought about by polarized migration towards the dorsal midline within the neural plate. Preliminary data from our laboratory suggests that microtubule (MT) stabilization is important for this process. First, the stability of MTs increases as neurulation proceeds, as visualized using a marker for detyrosinated MTs. In addition, MTs appear destabilized in *linguni* mutants, which are defective in neural convergence, but not convergence of the underlying mesodermal layer. These observations suggests that the MT destabilization phenotype is specific to neural convergence, as opposed to being a general developmental defect. Based on these observations, we predict that microtubule associated proteins (MAPs) are likely implicated in inducing MT stability during neural convergence.
We report here on the role of Map1B during neurulation. Map1B is a developmentally regulated phosphoprotein predominantly expressed in nerve cells. It is one of the first structural MAPs to be expressed at high levels in growing axons and growth cones. *MAP1B* mouse knockout studies have revealed a significant role for this protein during central nervous system development, but it has not yet been implicated in neurulation. To address whether Map1b plays a role in neural convergence, we examined its expression using *in situ* hybridization. *Map1b* is ubiquitously expressed during early stages of neural tube development and becomes more

concentrated in the hindbrain region at later stages. To address whether Map1b is required for neural convergence, we treated embryos with morpholinos to knockdown Map1B protein and analyzed expression of Dlx3, a marker for the edge of the neural plate. This assay revealed that the width of the neural plate was 1.5 times wider in the *Map1b* morpholino-injected embryos as compared to uninjected embryos. Interestingly, a similar phenotype has been observed in embryos depleted of the receptor Neogenin and its ligand RGMa, both of which have been implicated in neural tube formation in previous studies. We are currently investigating whether Neogenin/RGMa signaling induces activation of Map1b to influence MT stability during neural convergence. These studies reveal a novel role for Map1b in mediating the morphogenetic movements that shape the vertebrate nervous system.

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Identification of Mist, a GPCR Involved in Cellular Contraction and Epithelial Morphogenesis in the *Drosophila* Folded gastrulation Pathway.

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The ability to perceive and respond to information from the environment is a fundamental property of living cells. Morphogenesis of the early *Drosophila* embryo offers a powerful system to study the mechanisms and principles of signal transduction and we have been using the Folded gastrulation (Fog)-concertina (cta) signaling pathway as a model. During gastrulation, the secreted protein Fog causes epithelial sheet remodeling by inducing the presumptive mesodermal cells to constrict apically and internalize. The same pathway is used reiteratively throughout *Drosophila* development to induce actin and myosin based cell shape changes. Our lab has developed a cell culture based assay to study the components of this pathway. S2R+ cells, but not S2 cells, respond to exogenously added Fog protein by forming an acto-myosin contractile ring. RNAi-mediated depletion any of the known Fog-cta pathway components blocks the ability of S2R+ cells to contract in response to Fog. We conducted a targeted RNAi screen to identify any *Drosophila* G-protein coupled receptors (GPCRs) involved in this pathway and identified a single orphan GPCR which is necessary for Fog-induced contractility in S2R+ cells. We have named this receptor Mesoderm-invagination signal transducer (Mist). Moreover, we found that this receptor is sufficient to confer Fog-responsiveness to normally unresponsive S2 cells. Mist is expressed in a stripe along the ventral side of the embryo prior to gastrulation corresponding to the ventral furrow primordium and is enriched apically during cellular constriction. Mist is also concentrated in epithelial furrows in larval imaginal discs. RNAi and overexpression of Mist in these tissues phenocopy similar genetic disruptions of known Fog pathway components. Further, reduction of Mist by RNAi rescues the embryonic lethality of ubiquitous Fog overexpression. We are in the process of creating a Mist genetic mutant for analysis. Our data suggest that Mist is a key signaling molecule that participates in epithelial remodeling events throughout *Drosophila* development.

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Exploring fc20, a Drosophila Eggshell Protein That Exhibits Prion-Like Behavior.

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Extracellular matrices are highly organized networks that not only provide mechanical strength to tissues but also serve as repositories for secreted molecules that regulate a variety of cellular processes. We are studying a highly specialized extracellular matrix, the Drosophila eggshell, to gain insight into how the assembly of such complex molecular networks is controlled *in vivo*. Largely proteinaceous, the eggshell consists of morphologically distinct layers that include an oocyte proximal vitelline membrane (VM) and an outer tripartite endochorion. Vitelline

membrane proteins are secreted during the early stages of eggshell formation (stages 9-10) and several possess hallmarks of the 38 amino acid signature VM domain. Endochorion proteins, characterized by their high glycine content, are secreted during the mid (stages 12-13) and late (stage 14) phases of eggshell formation. Located within a VM gene cluster, fc20 is expressed during stage 10, contains a VM domain, and consists largely of the repeating pentapeptide FGGPG. To investigate fc20, a His-tagged fc20 transgene was created. Western blot analyses revealed that fc20 undergoes N-terminal cleavage during stage 12. Interestingly, the Western signal from the C-terminal derivative is much more intense than the fc20 proprotein. Using immunofluorescence confocal microscopy and anti-histidine antibodies, a faint fc20 signal was detected surrounding the oocyte. A transient, but much more pronounced signal was observed in the anterior of stage 12 egg chambers, the region where endochorionic respiratory appendages form. This localization pattern is distinct from sV23, a major VM protein, but similar to s36, an early endochorion protein. These results suggest that despite possessing the signature VM domain, fc20 likely functions in endochorion morphogenesis. Taken together, we hypothesize that while made during the period of VM formation, fc20 is sequestered in an aggregated or largely insoluble form until the early phases of chorion formation when it is cleaved to its soluble C-terminal derivative. The effect of premature removal of the fc20 N-terminus on eggshell assembly, fc20 solubility, and fc20 localization is currently being investigated using a his-tagged fc20 transgene in which the region encoding the putative N-terminal prodomain has been deleted.

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RapGAP9 regulation of morphogenesis and development in *Dictyostelium.*

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The small GTPase Rap1 is involved in the control of diverse cellular processes, including integrin-mediated cell adhesion, cadherin-based cell-cell adhesions, cell polarity formation, and cell migration. Recent reports have demonstrated the importance of Rap1-specific GTPase-activating proteins (GAPs) in the spatial and temporal regulation of Rap1 activity during cell migration and development in Dictyostelium. Here, we identified another Rap1 GAP-domain containing protein by bioinformatic search of Dictyostelium database and temporarily referred to as RapGAP9. The putative Rap1 GAP domain of RapGAP9 showed high sequence homology with those of other GAP-domain containing proteins in other organisms, 45.8% and 41.1% sequence identities with those of human Rap1GAP and Dictyostelium RapGAP3, respectively, which mediates the deactivation of Rap1 at the late mound stage of development. To further investigate the functions of RapGAP9, we prepared rapGAP9 null cells by homologous recombination and found that RapGAP9 is required for proper development and morphogenesis processes. Loss of RapGAP9 resulted in a slightly delayed development and an altered morphology of fruiting body with a shorter length of stalk and spore. In the vegetative stage, rapGAP9 null cells are more flattened and spread than wild-type cells and displayed a small increase of cell-substratum attachment, compared to wild-type cells. These data suggest that RapGAP9 is involved in cell adhesion and multicellular development. Localization assay showed that RapGAP9 localizes to the cell cortex and the protruding region in moving cells. Upon chemoattractant stimulation, RapGAP9 transiently and rapidly translocated to the cell cortex from the cytosol.

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ADAM13-regulated Wnt activity is required for Xenopus eye development.

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Pericellular proteolysis by ADAM family metalloproteinases has been widely implicated in cell signaling and development. We recently found that *Xenopus* ADAM13, an ADAM metalloproteinase, plays an essential role in activation of canonical Wnt signaling during cranial neural crest (CNC) induction by regulating a novel crosstalk between Wnt and ephrin B (EfnB) signaling pathways. In the present study we show that the metalloproteinase activity of ADAM13 is required for normal eye development in *X. tropicalis*. Knockdown of ADAM13 results in reduced expression of eye field markers *pax6* and *rx1*, as well as that of the pan-neural marker *sox2*. Activation of canonical Wnt signaling or inhibition of forward EfnB signaling rescues the eye defects caused by loss of ADAM13, suggesting that ADAM13 functions through regulation of the EfnB-Wnt pathway interaction. Downstream of Wnt, the head inducer Cerberus was identified as an effector that mediates ADAM13 function in early eye field formation. Furthermore, ectopic expression of the Wnt target gene *snail2* restores *cerberus* expression and rescues the eye defects caused by ADAM13 knockdown. Together these data suggest an important role of ADAM13-regulated Wnt activity in eye development in *Xenopus*.

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Reddish-violet coloration owing to novel chromatophores in the teleost fish, *Pseudochromis diadema*.

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In teleost, chromatophores had been classified into six categories, melanophores, xanthophores, erythrophores, cyanophores, leucophores and iridophores. Here, we report that the reddish-violet coloration on the diadema pseudochromis *Pseudochromis diadema* is displayed by novel chromatophores with a reddish pigment and reflecting platelets. We named the novel cells ‘erythro-iridophores’. On optical microscope, erythro-iridophores displayed two hues, red and dark violet, when viewed under ordinary transmission light and epi-illumination optics, respectively. Our electron microscope observations revealed that the erythro-iridophores constitute a single layer that is lined by the dermis of the scale. The cytoplasm of the eythro-iridophores contained stacked, very thin reflecting platelets, resembling the damsel fish-type iridophores, but no typical pigmentary chromatosomes, e.g., erythrosomes. However, our high-performance thin-layer chromatography (HPTLC) analysis of the pigment eluted from the erythro-iridophores indicated that carotenoid is the main pigment generating the reddish color. These observations and analysis suggest that there are two colorations in the erythro-iridophores, i.e., a pigmentary red coloration and an interference phenomenon of a non-ideal type on the stacked reflecting platelets. Furthermore, our physiological examinations showed that the coloration of the erythro-iridophores under epi-illumination optics changed to blue in response to a K^+-rich saline solution, but the red coloration remained under transmission light. The motile activities of the erythro-iridophores may participate in the changes in the reddish-violet shades of the pseudochromis fish. We suggest that the erythro-iridophore belongs to a new category of chromatophore.

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Using zebrafish to model and characterize human fetal lethal congenital contracture syndrome 1 (LCCS1).

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Mutations in the gene encoding the mRNA transport factor Gle1 have been causally linked to a devastating motoneuron disease, lethal congenital contracture syndrome 1 (LCCS1). This autosomal recessive disorder is characterized by fetal immobility, multiple joint contractures, small jaws, underdeveloped lungs, and degeneration of anterior horn motoneurons. Using zebrafish as a model system, we investigated the link between Gle1 function and LCCS1 pathology. Through Ensembl database searches, we identified gle1 in the zebrafish genome as the sole orthologue of human *GLE1*. Zebrafish *gle1* and human *GLE1* share similar gene structures; both produce two isoforms through alternative splicing. RT-PCR and in situ hybridization showed that *gle1* transcripts are maternally inherited and widely expressed during zebrafish early development. To elucidate the function of Gle1 during development, we studied the consequences of Gle1 loss of function using a *gle1* insertional mutant line and the antisense morpholino knockdown strategy. Disrupting Gle1 function led to cell death in the head and spinal cord, small eyes, curved body axis, edema, underdeveloped intestine, and diminished pharyngeal arches. The mutant fish also became increasingly immotile with time and died around 5 days post fertilization. These phenotypes, characterized by pleiotropic abnormalities and immobility, parallel several key human LCCS1 syndromes. Gle1 depletion also results in motoneuron reduction and aberrant motor axon arborization. Unexpectedly, the motoneuron deficiency is caused by apoptosis of neural precursors, rather than by acute neuronal death. Mosaic analyses further indicate that Gle1 activity is required extrinsically in the environment for normal motor axon arborization. Together, our studies provide the first functional characterization of Gle1 in vertebrate development. We propose that actively dividing cells demand robust Gle1 activity to support the high metabolic rate during proliferation. Such selective vulnerability of highly proliferating cells to impaired Gle1 function is likely the common pathogenic mechanism that leads to the pleiotropic phenotypes in human LCCS1 fetuses.

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A Gal4 enhancer trap line useful to study morphogenesis of craniofacial perichondrial cells, floor plate and dorsal midline radial gila in the zebrafish larva.

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Zebrafish are good models to study vertebrate development because of transparency during embryogenesis, rapid development, and availability of powerful genetic tools. Enhancer trap screening has been performed intensely, and has been used to understand zebrafish development. Here, we analyzed expression pattern of one Gal4 enhancer trap line, *SAGFF(LF)134A*, in detail, by combining with *Tg(UAS:GFP)* line. First, on craniofacial cartilages, strong GFP signals were detected in not only chondrocytes but also perichondrial cells, enveloping chondrocytes to form single-layered structure, in *SAGFF(LF)134A*, while only chondrocytes were visualized in the *Tg(-4.9sox10:GFP)* line. Lineage tracing by using a local gene activation technique (IR-LEGO system) suggested that perichondrial cells were derived from the mesenchyme in pharyngeal arches adjacent to the precursor of the chondrocytes. Secondly in the spinal cord, strong GFP signal was found in the floor plate, and later in the dorsal midline radial glia. In order to observe the changes in morphology of floor plate cells, we generated *SAGFF(LF)134A /UAS:KikGR* double transgenic fish and photoconverted a single

floor plate cells by UV irradiation. The time laps imaging revealed that the floor plate cell changed its shape from cuboidal/trapezoidal to cyathiform in 48 hours. These results indicate that the line would be useful to study morphogenesis of tissues that had not been well characterized including perichondrial cells, floor plate cells at later stages and dorsal midline radial glia in the spinal cord.

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Turnover of signals that activate and delimit the epidermal wound reporters during *Drosophila* development.

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A localized wound response promotes efficient repair mechanisms after a break in the epidermal barrier. Failure to properly coordinate an epidermal wound response impedes reepithelialization and contributes to decreased survival after injury. The single-cell epidermal layer of the *Drosophila* embryo provides an excellent system with molecular and cellular tools to dissect the components that localize the epidermal wound response. We have defined a genetic pathway that depends on *Flotillin-2*, *Src42A*, *Duox*, and *Sec24CD* to balance the activation and inhibition of transcriptional wound response genes during epidermal wound healing in the *Drosophila* embryonic epidermis. *Flotillin-2* encodes a membrane-associated protein that promotes signal localization via lipid raft formation. *Src42A* encodes a member of the Src-Kinase family of protein tyrosine kinases and facilitates signal transduction. *Duox (Dual oxidase*) provides enzymatic activity to produce hydrogen peroxide. *Sec24CD* encodes a coat protein of COPII vesicles in the ER/Golgi trafficking pathway. A widespread epidermal wound reporter can be activated by a simultaneous puncture wound and injection of specific chemical compounds. Interestingly, treatments with chemicals that inhibit lipid raft formation or Src-Kinase activity have the same wound reporter phenotype as the loss-of function mutants in *Flotillin-2* or *Src42A*. Additional experiments have shown that injection of hydrogen peroxide is sufficient to activate a widespread epidermal wound reporter, and this result complements the phenotype observed in *Duox* mutant embryos. We are currently testing double mutant combinations of *Flotillin-2, Src42A, Duox, Sec24CD*, and the chemical wound response inhibitors to establish epistatic relationships in the pathway. To determine the mechanism of wound response signal turnover, we will monitor how vesicle transport contributes to the rapid spread of transcriptionally activated epidermal wound response genes during injury. Several trafficking markers are available to track transport during wound healing of loss-of-function or gain-of-function mutant alleles and chemical treated embryos. Using *Drosophila* to determine factors that coordinate the activation and inhibition of the epidermal wound response and the signaling that occurs between cells at the site of injury brings new understanding to a complex problem faced by all multi-cellular organisms.

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Ethanol-induced defects in zebrafish neural development.

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Fetal Alcohol Spectrum Disorder (FASD) is caused by prenatal alcohol exposure, producing a spectrum of defects including facial abnormalities, sensory (visual and auditory) deficits, impaired fine motor skills and learning deficits including mental retardation. Our laboratory and others have shown that fetal alcohol syndrome can be modeled in zebrafish by ethanol exposure during early development. We hypothesized that embryonic ethanol exposure may alter the gene transcriptional profile that produces the FAS associated defects. To test that idea,

we examined the ethanol induced gene expression changes in zebrafish using Affymetrix gene microarray analyses at early and mid-gastrulation (just prior to neurogenesis) stages. Expression of multiple genes involved in neurogenesis and patterning changed significantly after ethanol exposure, e.g., Delta, Hairy- and enhancer of split-related (Her) genes, Hoxb group of genes. Consistently, ethanol treated embryos showed defects in neural plate formation, neural patterning, neural differentiation, and cranial/lateral line ganglia formation, showing defects in a dose-dependent manner. However, the mechanisms by which ethanol affects normal neurogenesis is not yet clear. Ethanol induced changes in the temporal and spatial expression pattern of the transcription factor, neurogenin1 (ngn1), a basic Helix-Loop-Helix (bHLH) transcription factor that controls initial neurogenesis steps. Quantitative PCR experiments showed that ngn1 expression in the ethanol treated embryos was reduced at gastrulation stages and was increased during neurogenesis compared to that of the control embryos. We hypothesized that ethanol affects neural patterning by competing with retinaldehyde dehydrogenase, thus impairing retinoic acid synthesis. We observed that not only the phenotypic defects but many of the neural differentiation and patterning defects induced by ethanol exposure were rescued by a co-treatment with retinoic acid in the medium. Future work focuses on dissecting the molecular mechanisms behind ethanol defects and the retinoic acid rescue on neural development. Understanding neural development mechanisms disrupted by ethanol exposure will help identify potential therapeutic targets for neural defects in FAS.

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Effects of Ethanol and Folate on Peripubertal Mouse Mammary Gland.

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Alcohol consumption has been clearly linked with increased breast cancer risk in women, with risks incurred even at low levels of consumption. The proposed mechanisms whereby ethanol exerts its effects include decreased folate levels and diminished DNA repair capacity, coupled with acetaldehyde-generated DNA damage. Based on these proposed mechanisms, we hypothesized that ethanol would have increased deleterious effects during periods of rapid mammary gland epithelial proliferation, such as peripuberty. We also hypothesized that folate deficiency alone might mimic the effects of ethanol, and that folate deficiency would exacerbate the effects of ethanol. CD2/F1 female mice (N=8 per group) were pair-fed control and ethanol-containing liquid diets +/- 0.2g/L folate supplementation from 5 weeks until 9 weeks of age. Mice were sacrificed after this 4 week feeding regimen, and mammary glands were harvested for preparation of whole mounts and paraffin sections. Folate levels of liver and kidney were assessed to verify folate status. Analysis of mammary gland whole mounts revealed that mammary gland structure was significantly altered in folate-replete mice fed ethanol, compared to control-fed mice (no ethanol/ folate-replete). These ethanol/folate replete mice showed an increased total number of ductal branches, due to an increased number of terminal short branches; no effect was seen on the number of terminal end buds or primitive alveolar buds. Systematic histological analysis of paraffin sections using NIH Image J software revealed that diets containing ethanol in the presence of folate significantly increased the total number of ducts, ductal epithelial area, and number of ducts with epithelial multilayering. Serum estradiol was increased by ethanol feeding, only in mice that were folate replete. These results demonstrate that folate deficiency alone does not mimic the effects of ethanol, and that folate deficiency in the presence of ethanol blocks proliferative effects of ethanol on the mammary ductal tree. Future studies are examining the effects of peripubertal ethanol and folate exposure on gene expression in the mouse mammary gland.

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Beta-parvin mediates integrin-cadherin cross talk during *Xenopus* gastrulation.

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During *Xenopus* gastrulation cell rearrangements that drive axial extension require interactions between α5β1 integrin and fibronectin (FN). While FN ligation is essential one of the proposed roles for integrin adhesion is the modulation of cadherin activity. While this receptor cross talk has been well described the mechanisms underlying the modulation of cell adhesion remain elusive. We have characterized the integrin associated adapter protein beta-parvin in Xenopus. In early Xenopus embryos beta-parvin mRNA is expressed maternally and following zygotic expression is enriched in tissues that undergo integrin-mediated cell movements. Expression of dominant negative constructs indicate that beta-parvin is essential for both FN assembly as well as the cell rearrangements that characterize gastrulation. The N-terminal CH domain of beta-parvin (RP1) enables integrin-mediated adhesion and migration on FN in vitro. Significantly, when expressed in vivo this domain can rescue convergent extension in the absence of FN. Over-expression of RP1 results in an increase in Rac activity that is essential for cell intercalations. Conversely, the C-terminal CH domain (RP2) promotes cadherin mediated cell-cell adhesion, while negatively effecting integrin adhesion. We propose that beta-parvin is in a pathway upstream of both integrin and cadherin and mediates receptor cross-talk through the Rho family of GTPases.

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***Trim25-like* is required for epithelial development in zebrafish.**

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The establishment and maintenance of an epithelium is critical for its function as a barrier, yet relatively little is known about the genes that regulate epithelial development and cellular turnover. The epidermis of the developing zebrafish is a bilayer and serves as an excellent model to study many epithelial bilayers that coat mammalian organs. Here, we identify the *Trim25-like* gene in zebrafish as an important regulator of epidermal development. In humans, TRIM25 is over-expressed in many breast tumors and like other members of this tripartite motif (TRIM) family of proteins, it contains a RING domain with E3 ubiquitin ligase activity and is a putative transcription factor. Loss of *Trim25l* in developing zebrafish causes shedding of epithelial cells and leads to an early larval death. To determine how these epithelial cells are expelled, we examined the actin cytoskeleton and markers of cell death in the periderm and basal layer and find that many of the peridermal cells are shed alive and not through apoptotic cell extrusion. Moreover, many of the remaining cells are binucleate, suggesting that these cells have a problem progressing through cell division. Injection of full length RNA encoding *Trim25l* results in rescue of both the binucleate and cell shedding phenotype. Conversely, overexpression of *Trim25l* in wild-type animals leads to a disorganized epidermis and the formation of epithelial cell masses. To identify potential downstream targets regulated by *Trim25l*, we compared the expression profiles of wild-type and *Trim25l* mutant zebrafish. Our microarray analyses identify several genes that control both cell adhesion and the extracellular matrix. This data will help us uncover the role of *Trim25l* in the development of a functional epidermis and how over-expression of a related gene in mammals may contribute to epithelial cell transformation.

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The EXC-1 GTPase regulates endosomal trafficking to maintain tubule shape in the C. elegans excretory canal.

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Tubulogenesis involves the formation and regulation of tubule shape and diameter along both the apical (lumenal) and basal sides. Once the tubule shape is formed this structure then needs to be regulated and maintained. The *C. elegans* excretory canal provides a simple model to study these processes. The excretory canal is a single-cell tube that is located near the terminal bulb of the pharynx, which extends two hollow processes to the left and right lateral side of the worm, where they bifurcate and extend anteriorly and posteriorly to form an H-shaped structure. Our lab focuses on the set of *exc* genes that are involved in maintaining the structure of the apical surface of the canal. Mutations in the *exc* genes allow formation of fluid-filled cysts in the lumen of the canal.

The Exc-1 loss-of-function (lof) phenotype shows cysts in the canals that are often located at the ends of the canal. These cysts vary in size and number; from cysts not much wider than normal lumen up to cysts expanded to the entire diameter of the worm. We have cloned the *exc-1* gene, which encodes a homologue of the RAS GTPase family, specifically the family of Interferon Inducible GTPases (IIGP). This protein is expressed in the canals, and also in the *C. elegans* amphid sheath, a glial structure that ensheaths the amphid neuron sensory endings.

The Exc-1 gain-of-function (gof) phenotype, created by overexpression of *exc-1*, forms a tubule with a normal apical surface but is defective in the formation of the basal surface, a phenotype seen with other *exc* genes when overexpressed (*exc-5* and *exc-9*). EXC-1 shows genetic interactions with these other members of the *exc* family; genetic studies suggest that EXC-1 acts downstream of the EXC-9 LIM domain protein, and upstream of the EXC-5 guanine exchange factor. *exc-1* (lof) mutants also shows similar subcellular disruption of subcellular trafficking markers EEA-1 and RME-1 within the excretory canals. These results suggest that EXC-1 acts together with EXC-5 to allow efficient movement from early endosomes to recycling endosomes. We are using yeast two-hybrid assays to assess binding of EXC-1 to possible target proteins.

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Oncogenes and Tumor Supressors I

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Diameter and rigidity of multi-walled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis.

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Multi-walled carbon nanotubes (MWCNTs) have potential for widespread applications in engineering and materials science. However, due to their needle-like, nanoscale shape and

high durability, concerns have been raised that MWCNTs of certain characteristics may induce asbestos-like pathogenicity. Even though recent studies have demonstrated various types of reactivities induced by MWCNTs, the physicochemical features of MWCNTs that determine or trigger the cytotoxicity and carcinogenicity in mesothelial cells remain unclear. Here we show that the deleterious effects of non-functionalised MWCNTs on human mesothelial cells are associated with their diameter-dependent piercing of the cell membrane. Thin MWCNTs with high crystallinity (ƒÓ ~ 50 nm) showed mesothelial cell piercing and cytotoxicity in vitro and subsequent mesotheliomagenicity in vivo. In contrast, thick (ƒÓ ~ 150 nm) or tangled MWCNTs (ƒÓ ~ 2-20 nm) were much less toxic and carcinogenic, whereas all MWCNTs affected macrophages similarly. This work suggests that control of the diameter of MWCNTs may contribute to reduce the risks to human health.

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Comparative studies of matched normal and cancer lung cells reveal multiple events underlying tumor cell growth and hypoxia responses.

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Hypoxia is a common and often cure-limiting characteristic of malignant tumors. Understanding the responses of tumor and normal lung cells to hypoxia can provide important insights into the molecular mechanism underlying uncontrolled tumor cell growth and therapeutic resistance. Lung cancer remains the leading cause of cancer death in the US and worldwide. Studies of lung cancer cells to hypoxia are important for understanding and treatment of lung cancer. Hence, we decided to perform comparative studies of a matched pair of normal and lung cancer cell lines. These cell lines, the normal lung cell line HBEC30, and the cancer lung cell line HCC4017, were previously established by Drs. Minna and White's labs (Whitehurst et al., 2007). We found that cancer and normal cells respond to hypoxia differentially. Additionally, a genome-wide analysis of DNA methylation patterns shows that in response to hypoxia, a segment of DNA containing an array of genes such as FBXO25 and MYOM2 are preferentially methylated in cancer cells, but not in normal cells. Metabolomic and proteomic experiments are underway to characterize the molecular events underlying the differential responses of cancer and normal cells to hypoxia.

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Gene expression profile of human prostate cell lines (+/-CD82) through microarray analysis.

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KAI1/CD82, a member of tetraspanin super family, is a prostate tumor metastasis suppressor gene. During metastasis progression, CD82 expression is either decreased or completely lost. CD82 loss has also been identified in other cancers including cancers of the esophagus, colon, cervix, breast, skin, bladder, lung, pancreas, liver and thyroid. The exact mechanism by which CD82 regulates metastasis suppression is still unclear.

Previous studies performed in our lab, with Agilent Microarray technology on prostate cancer cell lines PC3 5V (- CD82) and PC3-29 (+CD82), have identified genes regulated by CD82. Alternatively, we performed microarrays on normal prostate cell lines (PEC 31) with or without CD82, along with another set of tumor cells PC3-5V (-CD82) and PC3-57 (+ CD82) to compare and further validate the results observed from our previous arrays. The top 100 most common

and significant genes from all three arrays were compared and the 20 most significant genes have been grouped based on the role they play in the cell. Out of these, the top ten genes identified to be involved in metastasis are currently being validated by qPCR and RT-PCR protocols. The results from these studies will allow us to identify the genes and/ proteins regulated by CD82. This will allow us to further identify the pathways and downstream signaling molecules involved and decipher a role for CD82 in metastasis tumor suppression.

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Functional validation of candidate colon cancer genes identified by a Sleeping Beauty transposon based forward genetic screen.

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Objective: Sleeping Beauty (SB) Transposon-based forward genetic screens identified Cnot1 and Cnot2, subunits of CCR4-NOT complex, as candidate colon cancer-causing genes. The principal objective of this study is to determine if alteration of these genes results in oncogenic phenotypes in vitro. CNOT1 and CNOT2 participate in multiple CCR4-Not complexes with multiple functions. One function implicated in cell cycle regulation is deadenylation leading to degradation of specific target mRNAs. So a second goal is to determine if the mechanism underlying CNOT1 and CNOT2's effects on cell viability is mediated via deadenylation. Methods: Human homologs of candidate cancer genes CNOT1 and CNOT2 and genes encoding potential interactors, CNOT6L, CNOT8 and ZFP36 were knocked-down by siRNA in SW480 colon cancer cell line. Knockdown was validated by qRT-PCR. Cell viability was measured by MTT assay. mRNA levels of two potential target genes, CDKN1B (P27KIP1) and CDX2, were determined by qRT-PCR. Results: siRNA transfection resulted in ~ 60% knockdown of CNOT1 and CNOT2 mRNA levels and depletion of each of these two genes led to ~70% reduction in SW480 cell viability, suggesting that genetic alteration may cause them to act as oncogenes. To begin to test whether CNOT1 and CNOT2's effects on cell viability are mediated by deadenylation, we examined mRNA levels of two deadenylation targets, CDKN1B and CDX2. CDKN1B and CDX2 mRNA levels are increased by 1.5 and 2.5-fold respectively in CNOT1-depleted but not CNOT2-depleted cells. As a further test of the role of deadenylation, CNOT1-interacting proteins known to contribute to deadenylation, ZFP36, CNOT6L and CNOT8, were evaluated to determine if they share a common phenotype with CNOT1. mRNAs encoding these proteins were knocked-down by siRNA. Cell viability, and CDKN1B and CDX2 mRNA expression were evaluated. Cell viability in ZFP36, CNOT6L and CNOT8-depleted cells was reduced by 40%, 60% and 70%, respectively. Depletion of ZFP36, CNOT6L and CNOT8 resulted in an increase of the relative levels of CDKN1B mRNAs by 1.5, 1.7 and 1.3-fold respectively. Interestingly, knockdown of ZFP 36 and CNOT8 resulted in 1.5 and 1.8-fold increases of CDX2 transcripts but a 40% decrease in CNOT6L depleted cells. Conclusion: These results suggest that CNOT1 and CNOT2 may contribute to oncogenesis in colon cancer and suggest a model in which CNOT1 works in complexes to regulate the mRNA level of some genes by deadenylation. For example, CNOT1, CNOT6L, CNOT8 and ZFP36 may work together to regulate the level of CDKN1B while CNOT1, CNOT8 and ZFP36 may regulate the level of CDX2. To test these models the deadenylation function of these complexes and physical interaction of subunits will be investigated in future work.

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Paclitaxel treatment is associated with reduced or inactive micro-RNAs in breast cancer MCF7 cells.

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Antimitotic agents, such as paclitaxel, that are commonly used in chemotherapy protocols, target β-tubulins and halt cell cycle progression in G2/M phase. Paclitaxel stabilizes microtubules in mitotic spindles and increases in β-tubulin class III have been associated with resistance to paclitaxel, possibly due to its ability to reduce microtubule stability. We found that 40nM and 400nM paclitaxel treatment induce an increase in both β-tubulin classes IIA and III mRNA. Because micro-RNAs are known to regulate mRNA levels, we investigated changes in micro-RNAs associated with paclitaxel treatment using micro-RNA arrays. Four micro-RNAs were upregulated (200c, 191, 92a and 203)and three were downregulated (100, 146a and 138). We have previously reported regulation of β-tubulin classes I, IIA, IIB and V by micro-RNA 100, one of the down-regulated micro-RNAs [Lobert et al., *Cytoskeleton* (2011). 68:355-362]. One of the upregulated micro-RNAs miR-200c is predicted to target β-tubulin classes I, IIA and III. We found that MCF7 cells transfected with miR-200c had significantly reduced levels of β-tubulin classes I, III and IVb. MiR-200c is regulated by the zinc finger repressor protein ZEB1. Furthermore, ZEB1 mRNA is a target of miR-200c. We found that ZEB1 increases with 40 nM and 400 nM paclitaxel treatment. These data together indicate that, although miR-200c increases 40-fold with paclitaxel treatment, this micro-RNA is inactive, possibly due to impaired export from the nucleus. β-tubulin isotypes are suggested to be biomarkers for cancer patient survival and clinical disease progression. These data suggest that changes in β-tubulin isotypes in response to treatment with paclitaxel may be surrogates for changes in activity of micro-RNA tumor suppressors.

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Validation of candidate gastrointestinal cancer genes with ion channel functions, identified from Sleeping Beauty transposon-mediated mutagenesis screens in mice.

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Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the US. To identify the genetic alterations contributing to CRC, our labs have used Sleeping Beauty (SB)-transposon-mediated mutagenesis screens in *Apc*$^{+/+}$ and *Apc*^{Min} mice. These screens have identified a set of common insertion site (CIS) associated candidate cancer genes, that when dysregulated, may contribute to CRC development. Depletion of several CIS genes identified from the SB screen in *Apc*^{Min} mice resulted in a significant decrease in human CRC cell viability. The SB screen in *Apc*$^{+/+}$ mice has generated a list of CIS genes including two ion-channel encoding genes: *Kcnq1* (potassium voltage-gated channel, KQT-like subfamily, member 1) and *Cftr* (cystic fibrosis transmembrane conductance regulator, ATP-binding cassette sub-family C, member 7) which act together to promote chloride ion secretion in the normal colon. We hypothesize that these candidate genes, when dysregulated, contribute to the development of CRC. The function of the candidate genes is being tested in a mouse model of *Kcnq1* haploinsufficiency that was introgressed into the *Apc*^{Min} model of GI cancer and in human CRC cell line DLD-1. In the mouse model, *Kcnq1* expression is abrogated by targeted germline mutagenesis, resulting in a null allele. In cell culture, expression of each candidate gene is

depleted by siRNA knockdown, confirmed by quantitative real time PCR and followed by measurements of cancer-related phenotypes which include cell viability measured by the MTT assay. We found that *haploinsufficiency* for *Kcnq1* significantly enhances *tumorigenesis* in Apc^{Min} mice. In support of this result, a 60% knockdown of *KCNQ1* in DLD-1 cells resulted in ~1.4x increase in cell viability, compared with a control siRNA treatment at day seven after transfection. The normal physiological functions of *KCNQ1* indicate it may work with *CFTR* to prevent inflammation in the GI tract, thus suggesting that loss of *KCNQ1* or *CFTR* may be oncogenic via an inflammatory mechanism. To begin testing this idea, colon tissues of $Kcnq1^{+/+}$ and $Kcnq1^{-/-}$ mice were compared for expression of the inflammatory mediator *Nfkb*. *Nfkb* mRNA level was increased by ~1.6x in $Kcnq1^{-/-}$ mice. To begin testing the potential connections between *KCNQ1* and *CFTR*, *CFTR* was successfully depleted by 40% in DLD-1 cells and the effect on cell viability and inflammatory mediators' expression is being determined. In summary, our results of both *in vivo* and *in vitro* studies confirm a tumor suppressor role for *KCNQ1* in the GI tract. Current work is focused on investigating the model that genetic alterations of *KCNQ1* and *CFTR* promote oncogenesis by a common pathway, possibly by an inflammatory mechanism.

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Understanding the mechanistic basis for genetic redundancy of the Retinoblastoma protein in C. elegans.

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Our laboratory has been largely focused on understanding the developmental and cellular functions of the Retinoblastoma protein (pRb) ortholog in C. *elegans*, LIN-35. In contrast to the mammalian and fly systems, loss of LIN-35 function in C. elegans leads to only subtle developmental defects and *lin-35* homozygous mutant worms are viable and fertile. We have exploited this lack of overt phenotype to screen for mutations that show strong phenotypic effects when combined with loss of *lin-35*. More specifically, we have used synthetic-lethal screening approaches to identify genes whose functions overlap with those of *lin-35*. In doing so, we have discovered a number of distinct functions for LIN-35/pRb within the context of C. *elegans* development. In addition, we have sought to mechanistically link LIN-35/pRb activities to those of the identified synthetic-lethal mutant protein products. This approach has led to the elucidation of several different regulatory networks in which LIN-35/pRb participates to control diverse aspects of development. Implicated functions for LIN-35/pRb include roles in cell fate decisions, morphogenesis, organ function, and cell proliferation. Our poster will summarize some our studies reported over the past ten years and discuss how our findings may have relevance to the roles of pRb as a tumor suppressor in humans.

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Characterization of the Role of L.4.1 in Growth Suppression in a Drosophila melanogaster model.

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Drosophila melanogaster is a powerful genetic model for studying of cancer cell pathways and tumor suppressor genes. The six hallmarks of cancer are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Genetic manipulation in *D. melanogaster* allows for the rapid identification and characterization of genes involved in these six key processes. Our group recently identified *L.4.1* as a novel homozygous recessive point mutation that produced overgrowth phenotypes by blocking apoptosis in the adult mosaic eye of

D. melanogaster. The studies utilize a conditional growth suppressor screen that used a block in cell death. Combined use of *D. melanogaster* and inactivation of the apoptosis pathway through mutations in the *dark[82]* gene makes it possible to observe phenotypes from novel homozygous recessive point mutations in flies that would otherwise induce apoptosis. The objective for the present study was to characterize the role of *L.4.1* in cell growth regulation, mitosis, and differentiation pathways through genetic mosaics. Specific protein markers were used to analyze differences in the described pathways between mutant and wild-type L.4.1 eye discs. Confocal microscopic analysis was used for illumination of specific protein levels between mutant and GFP marked homozygous wild type tissue within the mosaic eye. Compared to wild-type, *L.4.1* mutants had a significant decrease in DIAP staining, suggesting that mutant *L.4.1* overgrowth is conditional upon a block in apoptosis. Clones of *dark[82]*, *L.4.1*, and eye discs entirely mutant for *dark[82], L.4.1* demonstrated a significant delay in differentiation. This was confirmed by a corresponding decrease in the Elav protein; a marker for cell differentiation. This data suggests that the delay in differentiation has a correlation with prolonged cellular proliferation within the cell. The *D.melanogaster* genome contains many human orthologs which function in similar cellular pathways and processes. Genetic mapping of the L.4.1 gene could thus be a valuable tool in studying genes that play a role in the development of human cancer.

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Defining novel pathways that arrest genetically unstable tetraploid cells.

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Tetraploid cells, which are a common byproduct of cell division failures, are genetically unstable and have the capacity to facilitate tumorigenesis. Consequently, p53-dependent tumor suppression mechanisms exist to limit the continued proliferation of these cells by promoting G1 cell cycle arrest. However, unlike other common cellular insults that activate p53 and promote G1 arrest, such as the DNA damage response, the mechanisms governing G1 arrest in response to tetraploidy remain largely unexplored. To gain insight into these mechanisms, we have developed a novel genome-wide RNAi screening assay to comprehensively identify proteins that are necessary to activate or maintain G1 cell cycle arrest after the induction of tetraploidy. Results from this screen, in combination with complementary bioinformatic approaches, have begun to illuminate the nature of the stresses associated with tetraploidy. More importantly, this work sheds light on the adaptive mechanisms that near-tetraploid tumors have evolved in order to overcome these stresses. These findings have the potential to lead to the identification of new pathways that can be targeted by chemotherapeutics to specifically reinforce G1 arrest in grossly aneuploid cancer cells.

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Mir-301b modulates proliferation, invasion and cancer stem cell differentiation through the inhibition of multiple RNA targets.

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MicroRNAs modulate gene expression through their complementary binding to untranslated 3' UTRs of specific mRNAs, which leads to the reduction of their mRNA stability and translation. Due to the widespread action, microRNAs have been involved in all cellular processes,

including stem cell biology and cancer. Cancer stem cells (CSCs) are a subpopulation of cells found in most type of solid and circulating tumors. They express several embryonic stem cell properties (gene expression programs, low differentiation, self renewal ability, multipotency) in comparison to the rest of the tumor cells, being the only cells capable of initiate a tumor in mice xenografts. They have become a major clinical issue in cancer because they greatly increase the invasion and chemoresistance potential of the tumor.
We thought to investigate the role of microRNAs in prostate CSC differentiation. We used an in vitro model of prostatospheres (PS) from prostate cell lines (DU145, LNCaP) grown in stem cell medium and induced to differentiate by exposure to serum. Gene expression analysis and tumor xenograft data presented here support our model. In order to study microRNAs differentially expressed during differentiation we extracted ARN from cells prior and after differentiation and we performed competitive hybridization on microRNA arrays. We found out that mir-301b is downregulated upon differentiation. Indeed, its overexpression significantly inhibits in vitro differentiation. Mir-301b is a conserved microRNA located in an intronic region of PPIL2 gene at cr22q11.21. It belongs to the gene family of mir-130a, mir-130b and mir-301a. It is expressed in most of the established prostate cell lines, showing an increased expression which correlates with cell line aggressiveness.
We then investigated the influence of mir-301b in several CSCs properties. We found that mir-301b is overexpressed in the CSCs subpopulation, reduces cell proliferation and greatly increases cell invasion. In order to study the genes modulated by this microRNA we performed global gene expression analysis of cells overexpressing mir-301b. We discuss pathways and genes modulated by this microRNA. For the downregulated genes that have a predicted mir-301b pairing sequence, we analyzed RNA levels and protein expression in large prostate cancer studies (“mir-301b activity”). That allowed as selecting a set of clinically relevant candidate direct targets. We present and discuss the significance of the data.

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Brahma regulates malignant transformation through the modulation of stromal-epithelial interactions.

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Cell-extracellular matrix (ECM) binding through integrins activate signaling processes that control cell differentiation, proliferation and survival. Integrin expression and activity are key regutators of tumor progression. In particular, the α5β1 integrin and its ligand fibronectin are overexpressed in invasive cancers. However, the molecular basis for altered α5 integrin levels during malignant transformation remains an area of active investigation. We found that α5 integrin and fibronectin are upregulated following growth factor and Ras trasnsformation of mammary epithelial cells. In the same cells the Brahma (BRM) subunit of the SWI/SNF chromatin remodeling complex is downregulated. Given that studies have suggested that the SWI/SNF chromatin remodeling protein Brg1 alters the expression of α5 integrin, we investigated the relationship between oncogenic transformation, BRM and integrin α5. Using α5 integrin blocking antibodies, we found that oncogene-driven transformation in the non-malignant mammary epithelial MCF10A cell line depends upon increased expression of the α5 integrin subunit, as assessed by growth in three dimensional (3D) reconstituted basement membrane (rBM). Interestingly, expression levels of the Brahma (BRM) catalytic subunit of the chromatin remodeling complex were inversely correlated with α5 integrin expression. Provocatively, loss of BRM caused the rapid and specific upregulation of α5 integrin and the acquisition of a premalignant phenotype as observed by pronounced luminal filling of 3D organoids by

immunofluorescence staining. This effect was dependent on the ATPase activity of BRM as demonstrated by the lack of reversion of malignant properties by an ATPase defective BRM mutant. Decreased BRM expression was observed in aggressive breast cancer cell lines. Furthermore, the ectopic expression of BRM in the transformed MCF10A-ErbB2 and MCF10A-RasV12 cell lines partially reverts their malignant phenotype in 3D rBM growth through decreased α5 integrin expression. Taken together, our data suggest that BRM may act as a tumor suppressor by modulating ECM-epithelial interactions and tumor cell transformation through the expression of the α5 integrin subunit.

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A putative oncogene, SWAP-70, a Phosphatidylinositoltrisphosphate Binding Protein Involved in Cell Motility.

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SWAP-70, a phosphatidylinositol trisphosphate (PtdIns(3,4,5)P3) binding protein, has been suggested to be involved in transformation of mouse embryo fibroblasts (MEFs) as well as membrane ruffling after growth factor stimulation of the cells. A mutant, SWAP-70-374, was found to be able to bind to F-actin in vitro, whereas wild-type SWAP-70 failed to do so. This mutant was present at the plasma membrane without any stimulation while the wild-type protein was present only in the cytosol unless cells were stimulated with EGF. Expression of this mutant in MEFs resulted in morphologic transformation, fast growth, and loss of contact inhibition, suggesting that SWAP-70 with this mutation can transform the cells. ERK1/2 was activated in SWAP-70-374-transformed cells. Use of MEK inhibitors revealed that the ERK1/2 pathway does not affect the cell growth of MEFs but is responsible for loss of contact inhibition. To investigate the function of SWAP-70 further, drugs that can inhibit SWAP-70-dependent cell responses were screened. Among various drugs, sanguinarine was found to inhibit transformation of MEFs by SWAP-70-374. This drug was able to inhibit SWAP-70-mediated membrane ruffling as well, suggesting that its effect was closely related to the SWAP-70 signaling pathway. These results suggest that SWAP-70-374 can activate some signaling pathways, including the ERK1/2 pathway, to transform MEFs.
Recently, point mutations have been detected in the SWAP-70 gene of three tumors. Examination of contribution of these mutations to tumorigenesis is now underway.

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CD44: Relationship to RANKL expression and osteoclast differentiation.

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The advanced stage of prostatic carcinoma eventually metastasizes to the bones in 85–100% of cases. Recent preclinical evidence suggests bone metastasis, activation of osteoclasts and increased bone resorption resulting from androgen ablation in prostate cancer patients may increase the risk of bone loss. Therefore, we used prostate cancer cell lines (PC3) originally derived from advanced androgen independent bone metastasized prostate cancer. The expression levels of osteopontin (OPN) and CD44 has been associated with the metastatic potential of several tumors. Our previous observations have indicated that osteopontin and CD44 are possible molecular biomarkers for prostate cancer progression [Desai et al., Mol. Cancer (2007), 6, 18; J Cell Biochem (2009), 108; 272]. Studies by others have shown that CD44 signaling has a role in tissue damage and bone destruction in the course of chronic inflammatory process. In view of this, we examined the role of CD44 in Receptor Activator of NF-Kappa B Ligand (RANKL) expression, osteoclast differentiation, and bone resorption. A

decrease in RANKL expression was observed in PC3 cells transduced with a TAT- fused CD44 peptide comprising the Ser323/325 amino acids as compared with PC3 cells transduced with control TAT- or scrambled peptide. Conditioned media from PC3 cells transduced with Ser323/325 CD44 peptides failed to support osteoclast differentiation. A decrease in the surface levels of CD44 was observed in these cells. These observations nevertheless provide support for the possible role of CD44 signaling in RANKL expression. Since further studies are needed to elucidate the mechanism of RANKL expression, we have generated stable PC3 cells knockdown of CD44. CD44 knockdown reduced RANKL expression in PC3 cells. RUNX2 is linked to RANKL production. CD44 knockdown reduces RUNX2 levels and hence RANKL expression in PC3 cells. We suggest that CD44 signaling may serve as a mechanism to modulate RANKL expression through RUNX2. Taken together, our observations have shed a light on the mechanism by which CD44 may promote osteoclast differentiation and bone loss; thus pointing ‘CD44’ as a valuable prognostic biomarker as well as a potential target for cancer therapy (Supported by RO1 grant by NIH-NIAMS (MAC) and T32 training grant to AG).

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Synergistic Effects of Chitosan and Docosahexanoic Acid on Osteopontin Expression and Secretion in an Ovarian Cancer Cell Line, SKOV-3.

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Ovarian cancer has the highest death rate of all gynecological diseases in developed countries. Current treatment for this deadly disease is surgical dubulking followed by chemotherapy or radiotherapy, but reoccurrence is still high. Hence, there is a need for alternative treatments, and more specifically a natural therapeutic product. Recent studies have shown that natural products, such as Chitosan and Omega-3 fatty acids, inhibit the proliferation of cancer cells. Chitosan is an N-deacetylated analog chitin, the second most abundant polysaccharide. Docosahexanoic acid (DHA) is an Omega-3 fatty acid and is found most exclusively in aquatic animals. Recent reports have implicated osteopontin (OPN), a cell surface and secretory glycoprotein, as an important biomarker for ovarian cancer. Therefore, the objective of this study was to determine the effect of chitosan and DHA on OPN expression levels in an ovarian cancer cell line, SKOV-3 cells. We hypothesized that chitosan and DHA will decrease OPN expression in a dose-dependent manner. To test our hypothesis, SKOV-3 cells were treated with (a) Control, (b) 250 ng/mL of chitosan, (c) 100 µM of DHA, (d) 250 ng/mL of chitosan and 100 µM of DHA for 24 and 48 hours. Western Blot analysis

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Argininosuccinate Lyase is a Novel Cancer Therapeutic Target.

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Many studies have been carried out to study carbohydrate and lipid metabolism in past decades. Recently, the amino acid metabolism in human cancer was found to be different from that in normal cells too. Cancer cells often grow in a poor environment such as nutrient deprivation. In addition, the endoplasmic reticulum stress is often observed in human cancer. Until now, the signal link between endoplasmic reticulum stress (ER stress) and amino acid metabolism is largely unknown. Therefore we hypothesized that (1) Can ER stress regulate the expression of arginine anabolic enzyme, argininosuccinate lyase (ASL)? And (2) the alteration of the expression of ASL may serve as novel cancer therapeutic targets. The ASL is a cytosolic protein, which is the fourth enzyme of the urea cycle, catalyzes the cleavage of argininosuccinate into arginine and fumarate. In this report, we tested the hypothesis that ASL is

important for the progression of hepatocarcinoma. The expression of ASL was induced by ER stress both in Human and mouse liver cell lines and in mice livers. ASL over-expressed in Hepatocarcinoma (HCC) was also been observed. HuH-7, HepG2 and ML-1 cells were transfected with ASL shRNA and stable transfectants were established. Knockdown of ASL expression either in mouse or human liver cell lines inhibited proliferation rates and anchorage-independent growth. Cells with downregulated ASL grew slower in BALB/c and NOD/SCID mice. Examination of the expression of protein related with cell cycle or apoptosis revealed that only cyclin A2 was decreased. The cyclin A2 mRNA was unaltered by ASL shRNA and the proteasome inhibitor restored the expression of cyclin A2 in HuH-7 and ML-1 cells. Cyclin A2 protein expression was also decreased when 293 cells were transiently transfected with ASL shRNA. On the other hand, overexpression of ASL further enhanced cyclin A2 protein expression in HuH-7 cells or 293 cells. Here we found that attenuation of ASL expression could decrease the tumorigenecity both in vitro and in vivo. Therefore, we concluded that ASL is important for the growth of tumor cells and may represent a novel therapeutic target.

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Aromatic Hydrocarbon Receptor Down-regulates MYCN Expression and Predicts Favorable Clinical Outcome of Neuroblastoma.

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Neuroblastoma (NB) is a heterogeneous neoplasm derived from sympathoadreanal lineage of the neural crest progenitor cells and is the most common malignant disease of infancy. MYCN amplification is a prognostic factor of NB. It indicates a highly malignant disease and poor patient prognosis. However, how MYCN affects the NB cell behavior remains unclear. By tissue oligonucleotide microarray and Ingenuity Pathway Analysis (IPA), aromatic hydrocarbon receptor (AHR) was found to reversely correlate with the MYCN expression in NB tumors. AHR expression in 85 NB tumors correlated well with histological grade of differentiation but reversely correlated with advanced disease stages and MYCN amplification. In addition, positive AHR expression by immunostaining of NB tumors predicted a favorable prognosis. AHR was known as the mediator of the toxicity of dioxin-like compounds. However, recent studies demonstrated that AHR is important in regulating cell death, proliferation, and differentiation. We hypothesize that AHR could regulate MYCN expression in NB. AHR over-expression promotes SK-N-DZ cell neuronal differentiation. Furthermore, ectopic expression of AHR suppressed MYCN promoter activity resulting in down-regulation of MYCN in mRNA and protein expression level. By using AHR shRNA, MYCN mRNA expression level was up-regulated after knock-down of AHR in SK-N-SH cell line. Our results suggested that AHR not only relates to the MYCN expression but also plays an important role in regulating tumorigenesis of NB.

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The relationship between structure and function of oncogenic protein, HBx.

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Chronic infection with hepatitis B virus (HBV) is strongly associated with the development of hepatocellular carcinoma and the X protein (HBx), encoded by HBV genome, is thought to be important in carcinogenesis as well as in viral replication. HBx is a small peptide comprising of 154 amino acids and it is shown that HBx interacts with a variety of cellular binding partners including p53 tumor suppressor protein, playing as a multifunctional regulator in host cells. However, functional domains of HBx have been poorly identified. One noticeable feature on

HBx protein is cysteine residues which confer 4 intra-disulfide linkages. In addition, inter-disulfide linkages may lead to the dimerization/oligomerization of HBx protein. To probe functional roles of disulfide linkages in HBx protein, we established cystein mutant constructs which lack disulfide linkages. We found that steady-state levels of wild-type HBx, Cys- (all Cys residues changed to Ala), C69 (one Cys at 69 remained), C26,69 (two Cys residues at 26 and 69 remained) mutants greatly differ, suggesting that disulfide linkages of HBx are involved in the maintenance of HBx stability. Ectopic expression of wild-type of HBx was primarily localized in the nucleus then moved to the cytoplasm. In contrast, its Cys mutants were mainly found in the cytoplasm, showing different subcellular localization. We further found differences in transactivation abilities among these HBx mutants. Thus, our results provide the evidence that disulfide linkages of HBx are closely related to cellular function of HBx.

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Does Cdk6 Modulate Eya-2-Directed Transcription?

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Cyclin dependent kinase (cdk)6, is known to regulate G1 phase of the cell cycle by partnering with D-type cyclins to phosphorylate pRb. Recent evidence has shown an emerging role for cdk6 in differentiation, a function not shared with cdk4. In this study, a yeast two-hybrid screen of a human fetal brain library identified the Eya2 protein interacting with cdk6. Eya2, a developmentally important co-activator, partners with the Six4 protein to regulate transcription. The interaction of cdk6 and Eya2 was confirmed by GST pull-down assay, and by co-immunoprecipitation from both transfected and endogenous cell lysates. GST binding assays also demonstrated that Six4 competed with cdk6 for Eya2 binding. Functional outcomes of this interaction could include transcriptional effects. Initial results of luciferase assays indicate that cdk6 quenches Eya2/Six4-directed transcription from MEF-3 sites. Elevated levels of Eya2 have been shown to be associated with poor short overall survival in advanced ovarian cancer (Zhang et al. Cancer Research 65, 2005).

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A Human Telomerase Holoenzyme Assembly Occurs in Nucleoli in Human Cancer Cells.

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Human telomerase is a ribonucleoprotein (RNP) complex that adds telomeric DNA repeats at the end of human chromosomes in tissue progenitor cells and cancer cells. Active human telomerase is composed of at least three subunits, including the human telomerase reverse transcriptase (hTERT), the telomerase RNA (TERC), and dyskerin. Until now, it was not clear where these three subunits assemble each others. Here, we show the telomerase biogenesis mechanism where an active human telomerase holoenzyme assembly occurs. hTERT colocalizes with both TERC and dyskerin in nucleoli, which is the site for maturation of RNP complex, and these telomerase holoenzyme components localizes at cajal bodies in late S phase. Also, the amount of hTERT bound to TERC and dyskerin peaks during S phase, evidence for cell cycle-dependent regulation of telomerase holoenzyme. Depletion of TCAB1 by using RNA interference leads to a loss of the telomerase RNP accumulation in cajal bodies, accumulates catalytic active telomerase in nucleoli without affecting active telomerase assembly and catalytic activities. These findings indicate that telomerase holoenzyme assembly occcurs in nucleoli prior to cajal body-specific processing of telomerase RNP, suggesting new insight for telomerase biogenesis in nucleus.

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NRIP roles in prostate development and carcinogenesis.

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Nuclear receptor interaction protein (NRIP) is also named DCAF6 or IQWD which is a WD40 repeat-containing protein. Previously, we demonstrated that NRIP is a transcription coactivator to enhance androgen receptor (AR)-driven gene expression and protects AR protein stability (Tsai et al., 2005; Chen et al., 2008). Additionally, we found that DDB2 is AR-interacting protein and degrades AR via CUL4A-DDB1 E3 ligase complex, and NRIP stabilizes AR protein by displacing DDB2 from AR-DDB2 complex. NRIP is an androgen-targeted gene, and prostate is a classically androgen/AR dependent organ. In this study, we used NRIP knockout mice to investigate NRIP role in prostate development. Our results showed that anterior prostate (AP) of NRIP KO mice develops delay but not ventral lateral prostate (VLP) and dorsal lateral prostate (DLP) at 8th wk but becomes normal at 12th week. It is due to AR deficiency of AP in NRIP KO mice at 8th wk hence scarce of AR delays branch formation. AP contains lower AR expression than DP and VLP, the latter two develop normal as wild type due to the crucial amount AR enough regardless of NRIP. protection. The delayed development of AP contain more K5, K8, tunnel markers, high Ki 67 in NRIP KO mice at 8th wk, because AR in luminar cells reportedly induces branch differentiation such as high K8, low Ki67, and high tunnel activity, As to why at 12th week, AP development of NRIP KO mice becomes normal as wild type that may be another factor such as DDB2, p53 expression plays a role to affect AR protein increase at 12 wks in NRIP KO mice. Additionally, we also confirmed that NRIP protects AR from DDB2 degradation via Cul4A-DDB1 complex in primary culture cells from NRIP KO mice. Moreover, NRIP expression is a cancer marker. NRIP and AR protein expression are significantly correlated in prostate cancer patients. In sum, NRIP can stabilize AR protein and play a role in prostate development and carcinogenesis.

Muscle Structure, Function, and Disease

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Distinct defects in zebrafish heart development due to exposure of ethanol at different critical stages of cardiac morphogenesis.

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Prenatal alcohol exposure can produce range of abnormalities and developmental defects to the developing fetus, referred to as fetal alcohol syndrome (FAS). FAS is characterized by a particular pattern of facial anomalies, poor growth, neurodevelopmental abnormalities, and defects in several organ systems including heart, kidney, vision, and hearing. FAS babies often have atrial and ventricular septal defects, and other structural cardiovascular defects. The mechanisms by which ethanol disrupts heart morphogenesis is not yet elucidated. We are using zebrafish embryos to investigate ethanol-induced cardiac defects and to dissect mechanisms underlying these defects. To determine heart developmental stage(s) most susceptible to ethanol exposure, we treated embryos in ethanol (0.6% and 0.9% v/v) during different critical stages of embryonic heart development. 1) Ethanol exposure during mesodermal cell migration to anterior lateral plate mesoderm region showed subtle abnormalities in chamber shape and size. 2) In contrast, ethanol treatment during cardiac specification until midline fusion of cardiomyocytes significantly delayed fusion of the bilateral population of myocardial cells (either developmental time or somite stage matched embryos) as revealed by cardiac myosin light

chain 2 (*cmlc2*) *in situ* hydridization and by live imaging of *tg(cmlc2: gfp)* embryos. Treated embryos exhibited smaller heart chambers and frequent heart looping defects compared to control at 36 hours postfertilization (hpf). 3) Ethanol exposure during stages from heart cone rotation until chamber formation severely affected heart looping, chamber size, and morphology. At 32 hpf, ethanol induced embryos show narrower, more elongated, poorly looped heart tubes, as compared to normally looped and expanded heart chambers in control embryos. We hypothesized that ethanol competes with retinol and impairs RA synthesis, which interferes with cardiogenesis. In an attempt to rescue the ethanol-induced defects, embryos were co-treated with ethanol and 10^{-9} M retinoic acid (RA). Strikingly, supplementing all-*trans*-RA restored cardiomyocyte migration, fusion and rescued morphological defects. We are now examining molecular mechanisms of ethanol-induced heart development defects. Understanding these mechanisms will significantly help us understand heart defects in FAS and might provide potential therapeutic targets for patients with this regrettably frequent birth defect.

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Identification of Human Myofibril-inducing RNA.

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The Mexican axolotl, Ambystoma mexicanum, has a naturally-occurring lethal recessive mutation which affects cardiac development in embryos. Hearts in mutant embryos develop without actomyosin filaments forming into organized sarcomeric myofibrils, hence the hearts fail to beat. Normal hearts, however, start to beat at embryonic stage 35. Interestingly, endoderm from normal embryos can rescue the development of mutant hearts. When mutant hearts are co-organ-cultured with normal anterior endoderm, or in medium conditioned by normal anterior endoderm, the mutant hearts contract and form myofibrils of normal morphology. This finding led to the idea that secreted factors from the endoderm promote normal heart development. In further studies in this laboratory, it was found that an RNA from normal axolotl embryonic endoderm, termed myofibril-inducing RNA (MIR), also rescues mutant hearts. In the present study, it has been found that treatment of mutant axolotl hearts with RNA extracted from human adult heart or human fetal heart also restores expression of tropomyosin and promotes formation of normally-organized myofibrils. We have cloned 400 randomized RNAs from human fetal heart. To accomplish this, cDNAs were synthesized from a mixture of fetal heart RNAs with the CloneMiner™ II cDNA Library Construction Kit (Invitrogen) and inserted into a pDONR™ 222 vector. This vector with recombinant cDNAs was transfected by electroporation into E. coli bacteria and grown into transfected colonies on agar plates, using kanamycin resistance genes. We randomly selected colonies and grew them individually in LB medium and then extracted plasmids from the bacteria. PCR with M13 primers conjugated at T7 binding sites was performed. We then purified the PCR product (cDNAs) and synthesized cRNAs with a MAXIscript T7 Kit (Ambion). Mutant hearts were dissected from axolotl embryos and treated with the synthesized cRNAs. Clones #6 and #13 promoted cardiac contractions in the mutant hearts. These hearts then were stained with immunofluorescently-labeled monoclonal anti-tropomyosin CG3 antibodies; significant tropomyosin expression in the form of organized myofibrils was observed. We plan to sequence these rescuing RNA clones to determine the active human RNA sequence component that promotes myofibril formation and rescues the mutant hearts. Supported by NIH grants HL61246 and HL58435 and an American Heart Association Grant.

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Physiology, Vasculature and Morpohology of Murine Myocardium with Reduced Actin Capping Protein.

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Increasingly, hypertrophic cardiomyopathies (HCM) are linked to mutations of sarcomeric proteins. Capping protein (CP) is an actin binding protein, anchoring the barbed end of actin filaments to the Z-line of the sarcomere. We characterized the cardiac physiology, vasculature and morphology of transgenic CPβ2 mice. Using micro-computed tomography (micro-CT), transgenic mice showed an increased left ventricular chamber volume and increased cardiac vasculature compared to wildtype mice. Transgenic mice displayed normal vascular localization of PECAM and VEGF using fluorescent microscopy. Radiotelemetric pressure sensitive catheters were implanted into the aortic arch of 6 month transgenic and wildtype mice and blood pressure and activity monitored for 8 weeks. Transgenic mice had decreased systolic and diastolic pressure, increased heart rate (HR) and lowered activity levels. Finally, using Scanning Electron Microscopy (SEM), alterations in myofibril organization were characterized. Transgenic myocardium displayed disarray relative to wildtype counterparts.

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Cardiomyocyte Changes Induced by Hyperglycemia.

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Diabetes is a serious worldwide health concern with the majority of cases attributed to type 2 diabetes. A complication of type 2 diabetes that affects heart function, known as diabetic cardiomyopathy (DCM), contributes to the mortality of the diabetic population. High blood sugar (hyperglycemia) has been identified as a major contributor to DCM's development and previous studies have found that apoptosis is involved in hyperglycemia-induced heart cell damage. However, the specific cellular mechanisms regulating hyperglycemia-induced damage remain unclear. Apoptosis is coordinated by a number of different proteins and factors at the cellular and mitochondrial levels, including the BCL-2 family of proteins which control mitochondria changes during apoptosis. This family of proteins contains distinct pro- and anti-apoptotic members and has been implicated in both survival as well as apoptotic events in cardiomyocytes. Several studies have demonstrated biochemical markers of apoptosis in in vitro and in vivo hyperglycemia cardiomyocyte models. However, the role of BCL-2 proteins in cardiomyocyte apoptosis due to hyperglycemia has not been thoroughly examined. We hypothesize that hyperglycemia induces changes to BCL-2 proteins in cardiomyocytes which leads to apoptosis, thus contributing to the development of diabetic cardiomyopathy. To test this hypothesis, HL-1 cells, a well-characterized cardiomyocyte cell line derived from mouse atrial cardiac muscle, were studied under normoglycemic and hyperglycemic conditions. We found various pro- and anti-apoptotic proteins, such as BCL-2, MCL-1, BID and BAX, are expressed in HL-1 cardiomyocytes. Exposure to high glucose for 24 hours modifies protein expression levels revealing important changes that can potentially affect apoptosis. Hyperglycemia also clearly induces apoptosis as indicated by decreased cell viability as well as caspase-3 activation. By gaining insight into the contribution of BCL-2 protein changes to DCM the underlying disease pathophysiology as well as novel therapeutic targets for type 2 diabetes could be revealed.

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Effect of Hypoxia on Muscle Resident Stromal Cells: A Key Factor that Influences Muscle Regeneration.

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Tissue regeneration is influenced by progenitor cells whose efficiency depends on several factors: the composition of the extracellular matrix, soluble factors produced by neighboring cells or made available through the systemic environment, and oxygen. In severely damaged tissue, the destruction of blood vessels alters oxygen availability. Concomitant to this damage, the multipotent stromal cell population often exhibits phenotypic changes. However, it is not clear how reduced oxygen levels influence the cells. In this study, we examine the effects of oxygen levels on muscle resident stromal cells (mrSCs) isolated from resting and damaged muscles.

We performed colony-forming assays to assess proliferation in normoxic and hypoxic conditions by seeding mrSCs from resting muscle. Following 2 weeks of culture, we found that the number of colonies formed in hypoxic conditions were significantly higher as compared to normoxia (2.9-fold; $p<0.0001$). Moreover, the average colony area was 6.1 fold greater in hypoxia than normoxia ($p<0.0001$). We also tested whether mrSCs from damaged tissue displayed a greater capacity to form colonies than those from resting muscle. Our results show that the number of colonies was not significantly different between the two muscle types in normoxia. However, cells from damaged muscle were able to form significantly more colonies in hypoxia as compared to cells from resting muscle (1.5-fold; $p<0.006$), although they displayed the same average colony area.

These results show for the first time that oxygen levels influence mrSC behavior, and cells that are cultured under hypoxic conditions show increased capacity to proliferate. More interestingly, it demonstrates that mrSCs isolated from severely damaged tissue have a greater capacity to adhere and expand under hypoxic conditions. In summary, our data suggests that under hypoxic conditions, cells from damaged tissue display a functionally different phenotype than those from resting muscle.

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Structural functions of skeletal muscle-specific calpain in Ca^{2+} efflux from the sarcoplasmic reticulum.

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CAPN3 (also called p94 or calpain-3) is an intracellular cysteine protease predominantly expressed in skeletal muscles. *CAPN3*/*Capn3* are responsible genes for limb-girdle muscular dystrophy type 2A (LGMD2A). Our previous studies demonstrated that dysfunction of CAPN3 protease activity caused muscular dystrophy using “knock-in” (referred to as *Capn3*$^{CS/CS}$) mice, in which the endogenous CAPN3 was replaced with a mutant CAPN3:C129S, that was proteolytically inactive but structurally intact CAPN3 (*K. Ojima, et al.* (2010) *J. Clin. Invest.* 120, 2672-2683). Dystrophic symptoms of *Capn3*$^{CS/CS}$ mice, however, was milder than those of

Capn3 null (*Capn3*$^{-/-}$) mice. This suggests that CAPN3 also has a non-proteolytic function. Here, we showed that CAPN3 functions as a structural element rather than as a protease in the sarcoplasmic reticulum (SR). Immunoblot and pull-down assays revealed that CAPN3 was a component of the SR. Although CAPN3 interacted with the SR components, CAPN3 did not proteolyze typical SR components including ryanodine receptor. Furthermore, *Capn3*$^{CS/CS}$ mice showed that CAPN3 molecule(s) was required for proper Ca^{2+} efflux from the SR to cytosol during contraction of skeletal muscles. Taken together, our results indicate that CAPN3 plays a role of non-enzymatic element for the Ca^{2+} efflux machinery in the SR. Thus, defects of the non-enzymatic function of CAPN3 as well as protease dysfunction of CAPN3 lead to LGMD2A.
Reference: *K. Ojima, et al.* (2011) *J. Mol. Biol.* 407: 439-449.

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Arbekacin as a therapeutic readthrough inducer for treatment of nonsense mutation-mediated *Duchenne* muscular dystrophy.

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Translational readthrough of a premature termination codon is a promising therapeutic method in more than 2,400 distinctly inherited human diseases. We previously reported that negamycin, a dipeptide antibiotic, that binds to the ribosomal decoding site and alters translational accuracy, successfully restored dystrophin expression with less toxicity than gentamicin in *mdx* mouse, which carries a premature termination codon in the dystrophin gene. In order to measure translational readthrough activity with quantitative accuracy, we established a novel transgenic mouse strain, named READ (Readthrough Evaluation and Assessment by Dual reporter). We found that arbekacin induced the in vivo nonsense suppression in READ mice dose-dependently, and promotes the accumulation of dystrophin, reduction of serum creatine kinase activity and improvement of contractile function in *mdx* mice. Moreover, arbekacin exhibits restoration of dystrophin expression on muscle cell obtained by biopsies from *Duchenne* muscular dystrophy patients caused by nonsense mutations. We have validated the efficacy of arbekacin on dystrophin-deficient muscle that we ultimately wish to treat. Arbekacin is a brekthrough readthrough-inducing drug for muscular dystrophy patients harboring nonsense mutations. This work was supported in part by The Ichiro Kanehara Foundation (to MS), Fugaku Foundation (to MS), Health and Labour Sciences Research Grant for Research on Psychiatric and Neurological Diseases and Mental Health (19A-020; to RM), Comprehensive Research on Disability Health and Welfare (H22-ShinkeiKin-Ippan-016; to RM), Nervous and Mental Disorders (20B-13; to RM) from the Ministry of Health, Labour and Welfare, Japan.

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Effects of Dietary Phosphate on Ectopic Calcification and Muscle Function in mdx Mice.

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Duchenne muscular dystrophy (DMD) is a severe muscular disorder affected about one in 3,500 boys. Lacking of dystrophin, a large cytoskeletal protein playing as a stabilizer of muscle fiber membrane, is the main cause of this disease. Patients of DMD show significant muscle weakness and wasting beginning at early childhood. They then complicate cardiac or respiration failure causing the early death by age 30.
Ectopic calcification in skeletal muscle has been reported in dystrophin-deficient mdx mice. We previously reported that the calcification was a form of hydroxyapatite, a deposition of calcium phosphate. Also C2C12 cells cultured in a high-phosphate medium expressed osteogenic markers and down-regulated myogenic pathway. Mdx mice had significantly higher serum

phosphate levels than the control mice while no significant difference in serum calcium levels (Kikkawa et al., 2009). When the kidneys are either mechanically or functionally disordered, phosphate metabolism is imbalanced. Abnormalities of phosphate metabolism related to kidney malfunction may play a central role in the deposition of calcium and phosphate in extra-skeletal tissues.
The aim of this study was to clarify the effects of dietary phosphate (Pi) on calcium deposits and muscle function in mdx mice. Mdx mice and the control mice (B10) were divided into three different diet groups (low-Pi, mid-Pi, and high-Pi) from weaning. Severe muscle necrosis and calcinosis were observed in 90-day-old mdx mice fed a high-Pi diet and serum phosphate levels were significantly rose. Few cases of calcium deposits were also observed in kidneys and the heart. These results indicated that high-Pi fed mdx mice were associated with hyperphosphatemia and suspected kidney dysfunction. In contrast, ectopic calcification was rarely seen in mdx mice under low-Pi diet.
We also compared muscle forces of B10 and mdx mice. While B10 mice fed any of the three Pi diets did not have significant differences, mdx mice fed a high-Pi diet had significantly lower maximal twitch and tetanic forces than those of mdx mice fed a mid-Pi diet. In contrast, both of the forces were significantly greater in mdx mice fed a low-Pi diet compared with a mid-Pi diet.
Noninvasive whole-body images of mdx mice taken by a CT scanning revealed that mice fed a high-Pi diet displayed an increased volume of ectopic calcification from 60 to 90 days of age, whereas mdx mice fed a low¬-Pi diet had a reduced. Our results indicated that lowering phosphate intake can be a complementary therapy for muscular dystrophy.

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Alterations in NF-κB signaling in dystrophic muscle.

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An examination of the levels of expression for several NF-κB signaling components indicated enhanced nuclear activation of p65 in the presence of elevated levels of cytosolic IκB-α in untreated adult dystrophic costal diaphragm suggesting that both elevated IκB-α kinase (IKK) activity and elevated p65 expression contribute to enhanced nuclear p65 activation in dystrophic muscle (Singh et al., J. of Neurological Sciences, 285, 159-171, 2009). To assess the role of constitutive factors in enhancing NF-κB signaling in dystrophic muscle, primary cultures of wild type (C57Bl10SnJ) and dystrophic (C57Bl10-mdx) myotubes were obtained and the distribution and expression of p65 was determined using confocal immunofluorescence techniques. The myotubes were examined at approximately 10 to 15 days after plating at a time when protein expression is high and the myotubes are spontaneously active. Untreated wild type and mdx myotubes exhibited no differences in either the proportion of nuclear p65 or the concentration of cytosolic p65, suggesting that the alterations in p65 distribution and expression observed in adult dystrophic muscle are not constitutive. To examine whether alterations in the rate of p65 degradation may influence p65 expression in adult dystrophic muscle, adult nondystrophic and mdx costal diaphragms were exposed to the proteasomal inhibitor MG132 for a period of 6 hours. This treatment produced a significantly larger increase in whole cell p65 expression in nondystrophic costal diaphragms, suggesting that the basal rate of ubiquitination and proteasomal degradation of p65 may be reduced in adult mdx muscle. Consistent with this conclusion, mdx costal diaphragms exhibited a slight but significantly reduced expression of the p65 ubiquitin E3 ligase, PDLIM2 (Tanaka et al., Nature Immunol., 8, 584, 2007). Whole cell expression of p65 was increased by about 12% in the mdx fibers, a value lower than that previously observed (Singh et al., 2009). These results suggest that dystrophic alterations in p65 signaling initially arise from extracellular mediators, and that prolonged exposure to these

mediators may produce secondary reductions in p65 degradation and corresponding increases in whole cell p65 expression (supported by AFM#13980 and NIHR15AR055360-01A2 to CGC).

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Three-dimensional microcirculatory measurements and the protective effects of endurance exercise on its complications in non-obese type 2 diabetic muscle.

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Purpose: Diabetes is closely linked to the risk of microcirculatory complications in tissues. Previous studies were based on two-dimensional capillary staining of muscle cross-sections, and the three-dimensional (3-D) microcirculatory networks and their relationships have rarely been studied in skeletal muscle. We visualized 3-D microcirculatory networks because of the recognition of the complexity and functional importance of skeletal muscle with type 2 diabetes (T2DM). The aim of the present study was to explore metabolic and microcirculatory changes in the skeletal muscle following endurance exercise training.
Methods: Male Goto-Kakizaki (GK) spontaneously non-obese T2DM rats were used in this study. Three groups of rats were studied: sedentary diabetic GK, exercised diabetic (GK+Ex), and sedentary non-diabetic Wistar (Con). The rats in the GK+Ex run at a low-intensity (15 m/min, serum lactate < 2 mmol/l) on a treadmill for 3 wks (60 min/day, 5 times/wk). The microcirculatory network of the soleus muscle was visualized using confocal laser microscopy. Plasma glucose and insulin levels, fiber oxidative enzyme activity, and the expressions of angiogenic and metabolic factors in the soleus muscle were examined after endurance exercise training for 3 wks.
Results: The levels of plasma glucose in diabetic GK and GK+Ex were higher than that of age-matched Con. The levels of plasma insulin in GK and GK+Ex were lower than that of Con. Although the oxidative enzyme activity of muscle fiber in the GK was lower than in the Con, that in the GK+Ex was greater than that in the Con and GK. The 3-D microcirculatory network of the soleus muscle regressed in GK group, whereas that in GK+Ex increased. Additionally, the capillary volume in GK was lower than that in Con, and the exercised muscle microcirculatory network was attenuated toward to the level of Con. Thus, endurance training could bring about the protective effect on the microcirculatory network in T2DM muscle. The expressions of angiogenic and metabolic factors were lower in GK than in the Con muscle. However, those in GK+Ex muscle were attenuated.
Conclusion: These data suggest that endurance exercise training may be an effective countermeasure to the detrimental effects of non-obese T2DM on skeletal muscles.

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Analysis of Expression of WWP1 protein in Mouse Normal Tissues.

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The *WWP1* gene was recently identified as a candidate responsible for chicken muscular dystrophy by genetic linkage analysis. This gene encodes a HECT-type E3 ubiquitin protein ligase (922 amino acids) that is composed of three functional domains: an N-terminal C2 domain for calcium-dependent phospholipid binding, a central region containing four WW domains recognize substrates with PY motifs, and a C-terminal HECT catalytic domain for ubiquitin transfer from E2 to substrates. Since oncogenic role of WWP1 has been suggested in prostate and breast tumors, many studies have been carried out using primary tumor cells and tumor cell lines. However, especially at protein levels, little is known about WWP1 expression in

normal tissues and organs. Thus, in this study, we have newly generated polyclonal antibody against a recombinant WWP1 fragment and have examined the expression of WWP1 in mice. Immunoblot analysis of various mouse tissues, i.e., brain, lung, heart, skeletal muscle, liver, spleen, kidney, intestine and testis, has detected a major protein band at approximately 120 kDa in all the tissue homogenates, revealing widely expression of WWP1 in a variety of tissues. This signal has been relatively abundant in lung and brain, but the lowest in heart. In addition to this, a relative weak 110 kDa signal has been also detected in all tissues except for heart and skeletal muscle. However, in these striated muscle tissues, an 85 kDa protein band has been detected instead of the 110 kDa band. Several alternative splice isoforms were reported in WWP1 expression. These multiple bands detected in the analysis would be protein products of the splice isofroms.

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Three-dimensional visualization of capillary and prevention of capillary regression by antioxidant supplementation in disused skeletal muscle.

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Purpose: Exposure to reduced activity induces a disuse atrophy of postural muscles. Disuse results in a decrease in muscle volume, strength and capillary regression. Oxidative stress might contribute to muscle atrophy and capillary regression, and this form of muscle atrophy could be retarded by the delivery of antioxidant supplementation. This study was designed to test two hypotheses in rats. First, we visualized the 3-D capillary because of the recognition of the complexity and functional importance of skeletal muscle with disuse atrophy. Secondly, antioxidant supplementation, i.e., astaxanthin (ASX), would regulate genes involved in capillary regression.
Methods: Male Wistar rats were used in this study. Four groups of rats were studied: control (Con), control with ASX(Con+ASX), hindlimb unloading (HU), hindlimb unloading with astaxanthin (HU+ASX) groups. In the Con+ASX and HU+ASX groups, astaxanthin (Fuji chemical industry, Japan) was orally administered twice in a day for seven consecutive days. The dose of ASX was based on 50mg/kg body weight, and interval of six hours was kept between first and second administrations in a day. Hindlimb unloading was applied to animals by suspending their tails for seven days and induced muscle atrophy. The three-dimensional (3-D) capillary of the soleus muscle was visualized using confocal laser microscopy. The expression levels of superoxide dismutase (SOD-1), VEGF, its receptors, angiopoietins and metabolic factors were measured in soleus muscle.
Results: Hindlimb unloading resulted in a decrease of the number of capillaries, capillary volume, and capillary luminal diameter. The 3-D capillary network of the soleus muscle regressed in HU. Additionally, HU caused the overexpression of SOD-1 and decreased the expression level of VEGF. However, the 3-D visualized capillary network of the soleus muscle prevented disuse-induced capillary regression in HU+ASX. Furthermore, ASX supplementation abolished the unloading-induced increase in SOD-1 and attenuated decrease in VEGF, and other angiogenic factors. Additionally, the expression levels of PGC1α and HIF1α in HU+ASX were maintained at Con level although those in HU decreased.
Conclusion: These results revealed that astaxanthin is effective to attenuate the development of oxidative stress and maintain the architecture of capillary network in disused skeletal muscle.

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Using Xenopus laevis to elucidate the roles of thin filament regulatory proteins: Leiomodin 3 and Tropomodulin 4 are important for skeletal muscle thin filament assembly.

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To generate optimal force for efficient contraction, actin-thin filament lengths must be closely regulated. Capping proteins contribute to thin filament length uniformity by regulating both elongation and depolymerization of the filaments at their ends. The Leiomodins (Lmod1-3), are structurally related to Tropomodulin (Tmod) proteins and are reported to localize to the pointed end of actin filaments and to contribute to thin filament length regulation.

This study aims to determine the role of Tmod4 and Lmod3 during de novo skeletal myofibrillogenesis in Xenopus laevis embryos. Developmental in situ hybridization studies show that Lmod3 and Tmod4 transcripts are expressed at high levels from the earliest stages of skeletal muscle development, suggesting that both molecules contribute to myofibril assembly and maintenance. Reducing the protein levels of Tmod4 or Lmod3 via morpholino technology compromised sarcomere development, thin filament assembly and the embryo's ability to swim. Double knockdown of Tmod4 and Lmod3 resulted in a more severe sarcomere assembly phenotype compared with single protein knockdowns. Our results suggest that Lmod3 and Tmod4 cooperatively regulate thin filament dynamics. Furthermore, we show that mis-regulation of actin filament assembly results in disruption of myofibrillogenesis in Xenopus skeletal muscle.

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Desmoplakin and Talin2 Are Novel mRNA Targets of Fragile X Related Protein-1 in Cardiac Muscle.

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RNA binding proteins are important players in the post-transcriptional regulation of mRNA transcripts. In cardiac muscle, where the precise assembly and interactions of numerous cytoskeletal and regulatory proteins are necessary for proper contraction and force transmission, RNA binding proteins must also play a key role in the assembly and maintenance of these specialized cardiac structures. The objective of this study is to investigate the molecular mechanisms and targets of muscle-specific Fragile X mental retardation, autosomal homolog 1 (FXR1), whose loss leads to perinatal lethality in mice and cardiomyopathy in zebrafish. Through immunoprecipitation, we found that desmoplakin and talin2 mRNAs associate with FXR1 in a complex. In vitro assays indicate that FXR1 binds these mRNA targets directly and represses their translation. Fxr1 KO hearts exhibit an up-regulation of desmoplakin and talin2 proteins, which is accompanied by severe disruption of desmosome as well as costamere architecture and composition in the heart, as determined by electron microscopy and deconvolution immunofluorescence analysis. Fluorescent in situ hybridization in combination with immunofluorescence staining further shows that FXR1 does not exert localization control over desmoplakin and talin2 transcripts. Our findings reveal the first direct mRNA targets of FXR1 in striated muscle and support translational repression as a novel mechanism for regulating heart muscle development and function, in particular the assembly of specialized cytoskeletal structures.

Immune System

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Electron tomographic studies of HIV in the gut.

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The human digestive system is one of the first lines of defense against infection and is home to almost half of the body's immune cells. As such, in HIV-positive patients the gut harbors large populations of the virus, especially at early stages of infection. At later stages, following depletion of T-cells, the virus may remain in regions of the gut where it is inaccessible to anti-retroviral drug therapy. We are using high-resolution electron tomography and immuno-EM to study HIV-infected gut tissue from humanized mouse models and biopsies from HIV-positive human patients. Our goal is to study early and late events of HIV infection in situ, identify the cells that harbor the virus at specific locations within the gut and determine where and how the virus may "hide" from HAART therapy. Both mature and immature (budding/recently-budded) virions can be identified by distinct structural features and by immunolabeling with antibodies against specific HIV antigens. Pools of mature HIV are often found in the intercellular spaces of the Crypts of Lieberkühn and viral budding profiles are detected on a subset of mucosal and Crypt-associated cells. The specific cell type (T-cell, macrophage or dendritic cell) can be determined by immunoEM. The nature and location of these virus pools gives us insight to how the virus may evade HAART and may suggest new therapies directed to these specific structures.

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Estradiol Differentially Regulates Calreticulin: A Potential Link with Abnormal Calcium Signaling in Systemic Lupus Erythematosus T Cells?

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Systemic lupus erythematosus (SLE) is an autoimmune disease that affects women nine times more often than men. Calcium signal transduction is altered in response to estradiol in SLE T cells but not in normal T cells. Microarray analysis suggested a key player in the calcium signaling pathway, calreticulin, is differentially regulated by estradiol in SLE T cells. To gain insight into the differential control of calreticulin, human T cells were purified from blood samples obtained from control females (n = 15) and SLE patients (n = 12). The T cells were cultured for 18 h without and with estradiol 17-β (10^{-7} M). Some samples were activated further by addition of phorbol 12 myristate 13-acetate (PMA, 10 ng/ml) and ionomycin (0.5 μg/ml) for 4h. Calreticulin expression was quantified in resting and activated T cells by real time polymerase chain amplification. Plasma estradiol at the time of blood draw was measured by ELISA. To assess whether calreticulin could affect estrogen receptor location and turnover, calreticulin was immunoprecipitated from nuclear and cytosolic compartments of T47D breast cancer cells and human T cells. The immunoprecipitates were analyzed by Western blotting using an estrogen receptor-α antibody. Estradiol stimulated (p= 0.034) calreticulin expression in activated control T cells. Calreticulin expression was not significantly different in either resting or activated SLE T cell samples in response to estradiol. Mean plasma estradiol concentrations were similar among SLE patients and controls. Calreticulin associated with estrogen receptor-α in the nucleus of resting cells. The results indicate that estradiol controls calreticulin expression in normal T cells

but not in SLE T cells. The results suggest shuttling of estrogen receptors from nucleus to cytosol, via a calreticulin mediated mechanism, could alter estrogen receptor turnover, calreticulin expression and calcium signaling in SLE T cells.

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Indeterminate Third-Generation Hepatitis C Recombinant Immunoblot Assay and HCV RNA Analysis: c22p-4+ Pattern Associated with Viremia in Immunosupressed Patients.

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Background: the aim of our work was to study the meaning of Indeterminate Hepatitis C Virus (HCV) Third Generation Recombinant Assay (RIBA®3.0 Ortho Diagnostics Systems) in donor (D) and patient (P) populations.
Methods: we retrospectively looked for individuals with Indeterminate RIBA®HCV 3.0 Pattern (IRP) between November 2001 and June 2011. D and P were screened with Abbott Prism® HCV and/or Vitros Eci Anti-HCV. HCV RNA was detected by bDNA, PCR and/or a NAT test: Procleix® Ultrio™ Assay, being excluded HBV and HIV infections when this method was performed. We followed the manufacturer's instructions for the assay protocol and results interpretation. We determined the patient´s diagnosis. Individuals not tested for HCV RNA were excluded for the IRP study. Data statistical analysis was performed with SPSS program (version 16.0) and *P* value.< 0.05 was regarded significant.
Results: we found 334 individuals with IRP, 74 D and 260 P; HCV RNA was not determined (ND) in 58 patients. The RIBA -3 indeterminate donors showed a significantly lower S/CO ratio distribution than patients (2.28 vs. 9.96 p.=.000). None of donors had positive HCV RNA; 31% of P were immunosupressed patients (IP) and 71% of these were HIV-1 infected (57). IP showed a higher S/CO compared to P not IP (13.90 vs 8.31; p.=.000). We found a significant positive relationship between S/CO value and HCV RNA (p.=.000) with a positive predictive value for the HCV infection when S/CO>5 (p.=.000). ND patients presented an average S/CO of 8.66. The patterns c100p and NS5 were associated with no viremia and c22p-4+, c33c-4+ and c33c-3+ showed association with the presence of HCV RNA (p.=.000). The c33c-2+ was the most frequent IRP associated with no viremia and the higher relation between IRP and the presence of RNA HCV was verified with c22p-4+ pattern. c22p-4+ was the IRP more frequent in the IP and the c33c-2+ was the most prevalent pattern in donors (p.=.000).
Conclusions: The prevalence of the disease on the population, the S/CO value, the RIBA indeterminate pattern including the type and the strength of the band are predictive for the presence of HCV infection. We found a high prevalence of IRP in immunosuppressed patients, namely in HIV-1 co-infected. We found a significant relationship between the S/CO of the screening tests and the presence of viremia. S/CO superior to 5 is predictive of infection and we failed to diagnose HCV infection in those individuals without determination of HCV RNA with an average S/CO of 8.66. There is a significant relationship between the IRP and HCV viremia. The IRP are significantly different in donor and patient populations. In immunosupressed patients c22p-4+ associated with a high S/CO ratio is predictive for HCV infection. Immunosuppressed patients with high S/CO and IRP should have HCV RNA periodic determinations since a negative viremia has not the meaning of no infection considering the natural course of Hepatitis C disease.

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Differential regulation of IL-1 receptor antagonist in human fibroblasts and fibrocytes by thyrotropin: A heretofore unrecognized link between the thyroid axis and inflammation.

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Interleukin-1(IL-1) is an important mediator of inflammation that has been implicated in many autoimmune diseases. As an endogenous IL-1 inhibitor, IL-1 receptor antagonist (IL-1RA) competitively binds IL-1 receptor (IL-1R), blocking its downstream signaling. The balance between IL-1 and IL-1RA determines the characteristics of chronic inflammation. Graves' disease and its orbital manifestation, thyroid-associated opthalmopathy (TAO) represents a systemic autoimmune process characterized by high levels of agonist antibodies against thyrotropin receptor (TSHR). Orbital fibroblasts in TAO represent important targets of inflammatory cytokines. Here we examined the effects of IL-1â and TSH on IL-1RA expression in orbital fibroblasts and bone marrow derived fibrocytes, which we believe replace orbital fibroblasts in TAO. Using real-time RT-PCR, flow cytometry, ELISA, and Western blot, we demonstrate differential induction by TSH and IL-1â of icIL-1RA and sIL-1RA in these cell types. These effects are mediated through TSHR and IL-1R, respectively, . The up-regulation results from modest increases in the activities of the two gene promoters but dramatic enhancement of mRNA stability, both occurring in a cell-specific manner. These findings further our understanding of fibroblasts heterogeneity and shed light on the mechanisms by which the TSH/TSHR pathway might modulate inflammation in GD and TAO.

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PEDF promotes tumor cell death by inducing macrophage membrane trail.

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Pigment epithelial-derived factor (PEDF) is an intrinsic anti-angiogenic factor and a potential anti-tumor agent. The tumoricidal mechanism of PEDF, however, has not been fully elucidated. Here we report that PEDF induces the apoptosis of TC-1 and SK-Hep-1 tumor cells when they are cocultured with bone marrow-derived macrophages (BMDMs). This macrophage-mediated tumor killing is prevented by blockage of TNF-related apoptosis-inducing ligand (TRAIL) following treatment with the soluble TRAIL receptor. PEDF also increases the amount of membrane-bound TRAIL on cultured mouse BMDMs and on macrophages surrounding subcutaneous tumors. PEDF-induced tumor killing and TRAIL induction is abrogated by peroxisome proliferator-activated receptor gamma (PPARγ) antagonists or small interfering RNAs targeting PPARγ. PEDF also induces PPARγ in BMDMs. Furthermore, the activity of the *TRAIL* promoter in human macrophages is increased by PEDF stimulation. Chromatin immunoprecipitation and DNA pull-down assays confirmed that endogenous PPARγ binds to a functional PPAR-response element (PPRE) in the *TRAIL* promoter, and mutation of this PPRE abolishes the binding of the PPARγ/RXRα heterodimer. Also, PPARγ-dependent transactivation and PPARγ/RXRα binding to this PPRE are prevented by PPARγ antagonists. Our results provide a novel mechanism for the tumoricidal activity of PEDF, which involves tumor cell killing via PPARγ–mediated TRAIL induction in macrophages.

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***Tsukushi* differentially modulates TLR2- and TLR4-mediated signaling in murine macrophages.**

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Tsukushi (*Tsk*), a member of the secreted small leucine-rich repeat proteoglycan (SLRP) family, has been recognized as a key molecule during developmental stages. However, its biological role in the innate immune regulation has not been elucidated. Here, we identify *Tsk* as modulators of downstream signaling of pattern recognition receptors toll-like receptor-2 (TLR2) and TLR4. Primary peritoneal macrophages from *Tsk*-deficient mice overproduced TNFα and KC mRNA expression in response to bacterial cell wall component peptidoglycan (PGN), a TLR2 ligand, suggesting the role of *Tsk* in the negative regulation of PGN-TLR2 signaling. On the other hand, *Tsk* (-/-) peritoneal macrophages exhibited reduced expression of TNFα, IL-6, KC and IFNβ mRNA after challenge with lipopolysaccharide (LPS), a TLR4 ligand derived from Gram-negative bacteria. Both overexpression and knockdown studies in LPS-treated RAW264.7 macrophage cell line further suggested the role of *Tsk* in the positive regulation of LPS-TLR4 signaling. Since deletion of *Tsk* did not affect gene expression of hes-1/hey-1 and Id1/Id2, downstream markers of Notch- and BMP/TGFβ-mediated signaling, respectively, in peritoneal macrophages, mechanisms underlying *Tsk*-dependent modulation of TLR2/4 signaling may be different from those in described in previous studies on developmental stages. Finally, *in vivo* experiments with mice revealed that *Tsk*-deficient mice are more susceptible to PGN-induced lethality, while more resistant to LPS-induced lethality, compared to their wild-type littermate mice, which are consistent with *in vitro* findings. Overall, our data reveal differential roles of *Tsk* in regulating TLR2- and TLR4- mediated signaling in murine macrophages.

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The effect of sTLT-1 on bateriaactivated neutrophils.

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It is well known that platelets regulate blood coagulation, however, recently they have been shown to play a major role in immune regulation. TLT-1 (Triggering Receptor Expressed in Myeloid (TREM)–like transcript-(TLT)-1 is a receptor found in the a-granules of platelets. Characterization of the treml1-/-mouse has shown that they bleed after an immune challenge. These results suggest that TLT-1 may be an important link between the inflammatory and coagulatory compartments thru the regulation of innate immune function. Moreover, recent publications demonstrate that activated platelets release large amounts of the soluble form of TLT-1 (sTLT-1) into the blood, although sTLT-1's role in coagulation or inflammation is not understood. Determination of sTLT-1 function is critical for a better understanding of the link between hemostasis and inflammation. As a preliminary step to this understanding, we devised a in vitro system using flow cytometry to measure neutrophil activation. Neutrophils were isolated thru density gradient centrifugation and incubated with E.Coli in the presence or absence of sTLT-1. Neutrophils activation was measured at 2 hours using degranulation (CD66b and CD35) and adhesion (CD 11b) markers by flow cytometry. Our results demonstrate a down-regulation of the expression of CD66b and CD35 on activated neutrophils. There was no apparent regulation of any sort in the expression of CD11b. These results suggest that sTLT-1 has an inhibitory effect on the degranulation of bacteria-activated neutrophils, which may provide insight into the immune bleeding phenotype of the treml1-/- mouse. Our studies suggest that TLT-1 may preemptively regulate neutrophil function as a mechanism to control bleeding. Further studies of the interaction between the sTLT-1 and neutrophils will allow us a broader understating of the physiological processes occurring in the hemostatic and immunological systems of a patient during sepsis.

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How Does SHP-1 Inhibition Improve Effectiveness of DC-Based Prostate Cancer Vaccines?

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A relatively new approach to prostate cancer immunotherapy is the use of Dendritic Cell (DC) based vaccines. These vaccines manipulate the immune system to thwart off prostate cancer using DCs and prostate cancer specific antigen. The most important cells in initiating an immune response are DCs. These cells are antigen presenting cells that engulf and process antigen in the periphery. From the periphery they migrate to the lymph nodes and interact with cytotoxic T lymphocytes (CTLs). This interaction causes CTL proliferation and cytotoxicity responses to the presented antigen. Src homology domain 2-containing protein tyrosine phosphatase-1 (SHP-1) is a naturally occurring intrinsic regulator of DCs that inhibits DC activation, migration, survival, cytokine production, and moreover, inhibits DC stimulation of CTLs. It is known that inhibition of SHP-1 in DCs improves efficacy of DC based prostate cancer vaccines, although a proper understanding of how this occurs is relatively unknown. Our objective is to explore SHP-1's function and mechanism of action in DCs, as well as, observe SHP-1 knockdown DCs effect on immune system development. We hypothesize SHP-1 knockdown DCs will have a developmental effect on the immune system. In addition, we believe that SHP-1 interacts with Interleukin-1-receptor-associated kinase 4 (IRAK-4), an important kinase molecule found in the Toll Like Receptor-4 (TLR-4) pathway. Lastly, we identify novel binding target candidates for SHP-1 in the TLR-4 pathway in DCs. We show DC specific SHP-1 deficiency affects system development of the spleen, an important immune system organ. We show spleen enlargement or splenomegaly in DC specific SHP-1 deficient mice. Upon cellular examination of these spleens we observed a significant decrease in splenic T-cell and B-cells, but a significant increase in splenic DCs. Molecularly in our DCs we show SHP-1 interacts IRAK-4, an important kinase molecule found in the TLR-4 pathway. The TLR-4 pathway activates Nuclear Factor-KappaB (NfkB), which allows for transcription of immune response genes necessary for DC activation, migration, survival, cytokine production, and DC stimulation of CTLs. In addition, we discovered a number of novel binding target candidates for SHP-1 in the TLR-4 pathway in DCs. Elucidating SHP-1 and its mechanism of action in DCs potentially could lead to novel DC regulation approaches. This can translate to new and improved DC based immunotherapeutics for prostate cancer and countless other diseases.

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The Role of Microvesicle Encapsulated RNA in Autoimmune Disorders.

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Previous work performed in our lab causes us to suspect that Toll-Like Receptors (TLRÕs),Ða specific class of pattern recognition receptors that play a key role in the innate mammalian immune response, are indicated in various autoimmune disorders including systemic lupus erythmatosus-induced glomerular nephritis. In our study, live podocyte cells were stimulated in cell culture using Nephrotoxic Serum (NTS) and a solution containing an array of complement proteins. Upon binding, this immunoglobulin-complement complex underwent a series of recruitment steps to form the membrane attack complex (MAC), which disrupted the membrane of the podocytes via penetration allowing intracellular components to flow into the cellular medium.
The contents of this intracellular medium is of specific interest because podocytes have a large and highly developed vesicular network that include a special class of endosomes called

microvesicles. Some of these microvesicles are thought to contain RNA that activates Toll-Like Receptor 7 (TLR7), causing the release of cytokines that induce inflammation and cellular pathology within the kidney. We exposed the podocytes to various concentrations of NTS (2%, 1%, and 0.5% utilizing 0% as a Control) and complement (3%, 2%, and 1%- Heat inactivated serum control) and collected the medium at various time points (1, 2, and 3 days). Utilizing a Quant-ITª RNA Assay, we quantified the RNA in the various medium aliquots and found that significant RNA release did occur in the cells exposed to a NTS concentration above 0.5% compared to the control and that there was an inverse dose-dependent response for the concentration of complement in the 2% NTS samples.

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Identifiying the anti-TR antigen in paraneoplastic cerebellar degeneration.

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Paraneoplastic cerebellar degeneration (PCD) is characterized by subacute cerebellar ataxia which coincides with the presence of specific tumour types and antineuronal antibodies. One particular form of PCD is associated with Hodgkin Lymphoma (HL) and the presence of antibodies against cerebellar Purkinje cells in several of these patients' sera. These later called anti-Tr antibodies recognize a specific punctuate immunoreactivity in the large dendritic tree as well as the soma of the Purkinje cells, but not in the axons. Although this characteristic immunoreactive pattern is considered to be a good hallmark for the presence of anti-Tr antibodies, further diagnosis is elaborate. In order to aid this diagnosis, and to understand the pathogenic nature of the PCD we aimed to identify the antigen recognized by anti-Tr antibodies.
Objective:
We aimed to identify the long sought antigen recognized by anti-Tr antibodies.
Methods: To identify the antigen we performed immunoprecipitation using four anti-Tr positive sera on total rat brain extract followed by mass spectrometry. By Western blotting and cell-based assays we subsequently determined the region of the epitope recognized by the anti-Tr antibodies. Deletion and mutant constructs were generated to further map the antigenic region.
Results: We identified Delta/Notch-like epidermal growth factor (EGF)-related Receptor (DNER) as the Tr antigen. In a cell-based screening assay 191 control samples were negative, whereas all of the 12 anti-Tr positive sera stained HA-tagged-DNER expressing cells. Using deletion constructs we pinpointed the main epitope to the extracellular domain. Glycosylation inhibitor tunicamycin and N-glycosylation mutations in DNER abolished the anti-Tr staining, indicating that DNER must be glycosylated to be recognized by anti-Tr antibodies.
Conclusion: Anti-Tr positive sera recognize DNER. Using the cell based assay, presence of anti-Tr antibodies in patients with PCD and HL can now be screened both quickly and reliably.

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Anti-P Autoantibodies Enhance Cytosolic Calcium and Neurotransmission in Hippocampal Neurons by Cross-Reacting with Cell Surface NSPA.

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Autoantibodies against the carboxyterminal region of three ribosomal phosphorylated proteins (anti-P) have been associated with psychosis and depression in systemic lupus erythematosus (SLE). We recently showed that anti-P antibodies induce calcium influx followed by apoptosis in cortical neurons and gave evidence of cross-reaction with a high molecular mass protein of unknown function, called NSPA (Neuronal Surface P Antigen) (Matus et al. J Exp Med, 2007). To test more directly the role of NSPA as an anti-P target we cloned the NSPA cDNA linked to GFP at its N-terminal region to transfect HEK293 cells, and also produced an NSPA-null/LacZ knock-in mice. Cell surface immunocapture, and biotinylation assays in Hek293 cells expressing NSPA-GFP point to NSPA as anti-P cell surface target. Beta-Gal staining in NSPA-null/LacZ knock-in mice shows a expression pattern mostly colocalized with that previously reported by immunohistochemistry, though encompassing additional brain regions. Because hippocampal neurons display the highest expression levels, we tested the effects of anti-P on these neurons. Anti-P autoantibodies increase cytosolic calcium levels in hippocampal neurons in primary culture and neurotransmission assessed by field excitatory postsynaptic potential in hippocampus slices, but only from wild type and not from NSPA-null mice. These results provide evidence that NSPA, and not other antigens, display a P epitope at the cell surface and may mediate deleterious effects of anti-P in brain neurons (Financed by CONICYT grant# PFB12/2007, grant# AT24100147 and FONDECYT grant# 1110849).

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Elucidating formation of the catalytically-competent V(D)J recombinase complex.

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The adaptive immune system is driven by the ability of T cell receptors and immunoglobulins to recognize invading pathogens. The intact functional genes encoding these antigen receptors are assembled during lymphocyte development through a process known as V(D)J recombination. The V(D)J recombinase, consisting of the RAG1 and RAG2 proteins, is essential for the first DNA cleavage steps in this process. However, the characteristics of the complex formed between the RAG proteins, and how each protein contributes to the overall catalytic activity is not yet well understood. Using a limited proteolysis approach, we identified regions in RAG1 that appear to be sequestered in the interface of the RAG1-RAG2 complex. From these results, we developed a model for how proper assembly of the V(D)J recombinase leads to a catalytically-competent complex. To test this model, we used fluorescence microscopy to compare the cellular co-localization properties of RAG2 with wild type and with mutant forms of RAG1. The microscopy results are consistent with our above model in that mutations in the putative RAG2-interacting regions of RAG1 resulted in partial defects in cellular co-localization of the RAG proteins. Moreover, defective co-localization properties correlated with reductions in cellular V(D)J recombination activity. One of the RAG1 mutants studied, E767K, leads to an immunodeficiency disease known as Ommenn syndrome, which is characterized by only limited

production of mature lymphocytes. Based on our results from limited proteolysis and cellular co-localization studies, we propose that the E767K mutation results in a partial disruption of the interface in the V(D)J recombinase, thereby leading to a catalytically-incompetent complex. We conclude that although the V(D)J recombinase interface is extensive, single point mutations can lead to subtle defects in how the complex assembles, with corresponding reductions in catalytic activity. The combined methods used here illustrate that RAG2 acts as a scaffold to orient multiple regions of RAG1 in formation of the RAG1 DNA cleavage active site.

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Immune responses in alcohol-induced pancreatic injury are associated with CCN2 expression and action in acinar cells.

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Introduction: Alcoholic chronic pancreatitis (ACP) is characterized by pancreatic necrosis, inflammation, and scarring, the latter of which is due to excessive collagen deposition by activated pancreatic stellate cells (PSC). We have have previously shown that in a novel model of ACP in mice (a species that is usually resistant to the toxic effects of alcohol), PSC are principally responsible for the production of connective tissue growth factor (CCN2) which is correlated spatially and temporally with sites of collagen production (Charrier & Brigstock; Lab Invest, 2010). This is consistent with our earlier in vitro data showing that PSC produce collagen in response to ethanol via the production of CCN2 (Lawrencia et al; Growth Factors, 2009).

During the course of the in vivo studies, we observed that, prior to the fibrotic phase (>Day 20), the combination of ethanol and cerulein caused a transient increase in CCN2 production in acinar cells, even though these cells are not collagenic. Thus we adopted in vivo and in vitro approaches to investigate this finding further

Methods: C57/Bl 6 mice received ethanol injections for 6-16 days against a background of cerulein-induced acute pancreatitis. Pancreata from mice that were treated with cerulein plus 33% ethanol were examined at Days 6, 9, 13, and 16 for the presence of CCN2 and its relationship to inflammatory responses. Additionally, the rat acinar cell line, AR42J, was grown in culture and treated with ethanol and both CCN2 and Interleukin-1 beta (Il-1 beta) mRNA levels were evaluated by qRT-PCR. AR42J cells transfected with pcDNA3.1 plasmid containing a CMV promoter and the entire human CCN2 gene were analyzed by an RT2 Profiler Inflammatory Cytokines and Receptors Array for downstream effects of CTGF expression.

Results: In the in vivo model, there was strong immunofluorescent signal for CCN2 in acinar cells by Day 16 of treatment with ethanol + cerulein and this was not co-localized to á-SMA-postive smooth muscle cells of the vasculature. This pattern of CCN2 staining was not apparent in pancreata of mice receiving water + saline. F4/80 immunofluorescence showed some macrophage infiltration of pancreatic tissue at days 6 and 9, but this was substantially increased on Day 16 and thus correlated with CCN2 production by acinar cells. Immunofluorescence staining further demonstrated that the increase in acinar CCN2 by ethanol + cerulein was spatially and temporally correlated with that of IL-1 beta.
In vitro studies showed that after treatment of rat AR42J cells with 6.25-100mM ethanol for 24 or 48 hours, the pattern of CCN2 mRNA induction was correlated with that of IL-1 beta expression. IL-1 beta expression was not affected by an increase in CCN2 expression, however, expression of chemokine (C-C motif) ligand 20 (CCL20) and C-C chemokine receptor type 6 (CCR6) were significantly increased.

Conclusions: Unrelated to its pro-fibrotic role in PSC, CCN2 is robustly expressed in mouse acinar cells during chronic pancreatic injury, the timing corresponds to increased infiltration of activated macrophages as well as of acinar IL-1 beta expression. Cultured rat acinar cells demonstrate co-expresison of CCN2 and IL-1 beta in response to ethanol. Although IL-1â was not CCN2 dependent it is also known to increase levels of CCL20 and CCR6 suggesting a possible upstream role of IL-1 beta in CCN2 signaling during the classic inflammatory cascade. Taken together, these data support a role for acinar cell-derived CCN2 as a modulator of the immune response. We conclude that CCN2 regulates components of the immune response during pancreatic injury prior to exerting its fibrotic role.

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PGF2alpha Stimulates the Expression IL8 and other Chemokines: A Potential Role in Corpus Luteum Regression Through Recruitment of Neutrophils.

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Early pregnancy is maintained by progesterone produced by the ovarian corpus luteum (CL). In unfertile reproductive cycles and at the end of pregnancy the uterus produces prostaglandin F2 alpha (PGF), which causes regression of the CL in ruminants and other species, including humans. PGF receptors are GPCRs that couple to Gq and provoke calcium, PKC and ERK signaling in steroidogenic cells. Recent studies suggest chemokines and cytokines may contribute to the process of luteal regression. Our objective was to identify chemokines induced by PGF in vivo and to determine their effect on specific luteal cell types in vitro. Mid-luteal phase cows were injected with saline or a luteolytic dose of PGF and ovaries containing CL were removed after 0.5, 1, 2 or 4 hours. Tissue was processed for protein or RNA isolation. Gene expression was analyzed using Affymetrix bovine WT microarrays and validated by quantitative real time PCR. Purified steroidogenic cells, endothelial cells and fibroblasts were prepared for analysis of signaling molecules by western blot and progesterone production was determined by radioimmunoassay. Bovine neutrophils were purified from peripheral blood and were analyzed for activation and migration. Analysis of gene array data by Ingenuity Pathway Analysis indicated patterns of genes associated with the inflammatory response and cell-to-cell signaling. PGF treatment in vivo rapidly increased the expression of the chemokines IL8, CXCL2, CCL2, and CCL8. Four hours after treatment CXCL2 had increased 7-fold, CCL2 increased 12-fold, and CCL8 increased 30-fold. IL8 expression increased 10-fold within an hour and 35-fold after 4 hours. Expression of IL8 mRNA in steroidogenic luteal cells was also stimulated following in vitro PGF treatment. IL8 rapidly activated the ERK and NFkB signaling pathways in neutrophils, but did not activate either signaling pathway in steroidogenic cells, endothelial cells or fibroblasts. IL8 significantly induced neutrophil migration and degranulation. In conclusion, PGF rapidly induced chemokine expression in vivo and in vitro. IL8 stimulated migration and degranulation of neutrophils. The rapid induction of chemokines by PGF suggests that the process of luteolysis may be a controlled inflammatory response. Support: VA, NIFA 2011-67015-20076.

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Central obesity is associated with a lower β2-adrenoceptor expression in peripheral blood mononuclear cells.

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Background and aim: Sympathetic nervous activation plays a major role in the onset and development of obesity and cardiovascular comorbidities. A large part of the sympathetic nervous system-mediated energy expenditure occurs via coupling of adrenaline (AD) and noradrenaline (NA) with β2-adrenoceptors (β2-AR) [1]. Central fat–related activation of the innate immune system [2] and insulin resistance are implicated in the pathogenesis of cardiovascular diseases. The recent finding that immune cells and adipocytes are catecholamine-producing cells prompt us to investigate the association between central obesity (CO) and β2-AR expression in peripheral blood mononuclear cells (PBMCs) and innate inflammatory markers in a blood donor population.
Methods: We have studied 60 blood donors (33 men and 27 women) aged 20 to 63 years (mean 39) considering waist circumference (WC), inflammatory markers, namely plasma ultrasensitive PCR (usPCR) and monocyte subpopulations (CD14+ CD16− or CD14+ CD16+) and β2-AR expression in PBMCs. The latter was investigated by quantitative real-time PCR and monocyte subsets staining for CD14, CD16, CD36 and CD11b by flow cytometry analysis. CO was defined following IDF criteria by using WC measurement (≥80 cm for women and ≥ 94 cm for men) [3]. Student-t test was used to assess differences between groups ($p < 0.05$).
Results: Seventy-three percent of blood donors were centrally obese. CO was associated with a more proinflammatory monocyte pattern: less intensity of CD36 (0.4504 ± 0.001 vs. 0.549 ± 0.001) and CD14 (0.393 ± 0.024 vs. 0.536 ± 0.054) and lower side scatter signal (0.843 ± 0.010 vs. 0.881 ± 0.020), $p < 0.05$. Centrally obese subjects showed lower β2-AR expression in PBMCs (1.4 x 10-5 ± 6 x 10-6 vs. 6.5 x 10-5 ± 2.5 x 10-5; $p = 0.007$) and higher plasmatic levels of usPCR (2.5 ± 0.5 vs1.0 ± 0.3; $p = 0.049$), comparing with those without CO. Using regression analysis, we have found that in men β2-AR expression was negatively correlated with NA and usPCR plasmatic levels. However, none of these correlations were found for women.

Conclusion: In our population, waist circumference, a marker of central obesity, is associated not only with higher systemic levels of markers of innate immune activation, but also with β2-adrenoceptor expression in peripheral blood mononuclear cells. The pathophysiological relevance of this finding needs clarification, since lower β2-adrenoceptor expression might be associated with a higher cardiovascular risk, especially in men.

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Membrane Trafficking at the Synapse

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Synaptic activity stabilizes the post-synaptic density scaffold proteins SAP97 and AKAP79 in the neuronal dendritic spine.

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Scaffold and anchoring proteins play critical roles in regulating spatial and temporal regulation of signaling cascades. In excitatory neurons, regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors is central to the regulation of glutamatergic synaptic activity. Activity-dependent changes in AMPA receptors are initiated by regulation of lateral diffusion, endocytosis, recycling, and post-translational modifications. These events are controlled by a rich array of scaffold proteins expressed in the postsynaptic density (PSD), of which SAP97 and AKAP79 are the focus of this study. Using Fluorescence Recovery After Photobleaching (FRAP), we investigated how the mobility of these two scaffold proteins in postsynaptic dendritic spines is influenced by neuronal activity and cytoskeletal actin remodeling. We demonstrated that increasing neuronal activity by activating NMDA-type glutamate receptors or stabilizing the actin cytoskeleton with Jasplakinolide reduces the mobile fraction of SAP97 and AKAP79 in spines. The ratio of SAP97 and AKAP79 in spines versus dendritic shafts was greater after these treatments indicating that synaptic activity or a stabilized actin cytoskeleton reduced the net movement of SAP97 and AKAP79 out of spines. In particular, stabilization of AKAP79 in spines with increased synaptic activity required an internal domain that binds to SAP97 and the related scaffold protein PSD-95 as well as palmityolation of the AKAP N-terminal targeting domain that binds membranes and F-actin. Thus, activity-dependent stabilization AKAP79 may depend on coordinated regulation of AKAP interactions with the postsynaptic membrane cytoskeleton and other PSD scaffolds.

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MAP1A supports NMDA-receptor transport for synaptic plasticity.

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Microtubule-associated protein 1A (MAP1A) is one of the major components of the neuronal cytoskeleton. To examine the role of MAP1A, we generated mutant mice lacking MAP1A. Through analysis of their phenotypes, we found that MAP1A knockout mice exhibited severe memory disturbances. The MAP1A-knockout neurons revealed reduced surface expression of NMDA-receptors concomitant with a decrease in NMDA-dependent postsynaptic current and long-term potentiation (LTP). Reduced NMDA receptor transport underlay the altered receptor function. These results suggest that MAP1A supports the transport of NMDA-receptors and synaptic plasticity in neuronal dendrites(Y.T. and Y.K. contributed equally to this work).

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Soluble oligomeric amyloid-beta peptide causes defects on vesicle traffickings.

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Soluble oligomeric form of amyloid beta (oAbeta) has been linked to memory and cognitive deficits in Alzheimer disease (AD). The oAbeta is believed to be a key cytotoxic agent in AD, although correlation with disease progression and soluble Abeta levels is still controversial.

Recent growing evidences support the link between Abeta oligomers and synaptic function, while a direct role of oAbeta in presynaptic functions has not been established. Here we found that acute treatment of nanomolar concentration of oAbeta affects multiple steps of synaptic vesicle traffickings including exo-endocytosis and repriming. All of effects were recovered to the control levels with pretreatment of a specific antibody to Abeta. Our results suggest that oAbeta causes defects in the synaptic vesicle traffickings, which could affect to synaptic dysfunction associated with AD. More experiments to pinpoint the site of action are on the way.

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Chondroitin sulfate proteoglycans target TrkB to downregulate spine formation in the cortical neurons.

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Chondroitin sulfate proteoglycans (CSPGs) are components of the extracellular matrix inhibitory for axonal sprouting and experience-dependent plasticity. CSPGs are required for closure of the critical period in the visual cortex, whereas the molecular mechanism underlying the limitation of neuronal plasticity by CSPGs remained poorly understood. In addition, although Protein Tyrosine Phosphatase σ (PTPσ) was proven to be a receptor for CSPGs, its downstream signals remained elucidated. Here we show that CSPGs target and dephosphorylate tropomyosin-related kinase B (TrkB), the receptor of brain-derived neurotrophic factor (BDNF), via PTPσ in the embryonic cortical neurons in vitro. PTPσ interacted with TrkB in the neurons. Although BDNF promoted dendritic spine formation of the embryonic cortical neurons, CSPGs abolished the effects of BDNF and even eliminated dendritic spines. The latter effect was dependent on the p75 receptor, suggesting that BDNF binding to the p75 receptor elicits elimination of dendritic spines. These results highlight the counteracting effects of CSPGs against neurotrophins at the receptor level on the formation of dendritic spines.

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Regulation of AMPA receptor-mediated transmission by the Arf6-GEF BRAG1

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Activity-dependent modifications of excitatory synapses contribute to synaptic strength and maturation, and are critical for learning and memory. Consequently, impairments in synapse formation or synaptic transmission are thought to contribute to several types of mental disability. BRAG1 is a guanine nucleotide exchange factor (GEF) for the small GTP-binding protein Arf6 and localizes to the postsynaptic density of excitatory synapses. Mutations in BRAG1 have been identified in 4 separate families with X-linked intellectual disability (XLID). These mutations mapped to either the catalytic domain or a precicted calmodulin-binding IQ-like motif, however the pathophysiological basis of these mutations remains unknown. Here, we show that the BRAG1 IQ motif binds to apo-calmodulin, and that calcium-induced calmodulin release triggers a reversible conformational change in BRAG1. We demonstrate that BRAG1 activity, stimulated by activation of NMDA-sensitive glutamate receptors, acts to depress AMPA receptor-mediated transmission. Importantly, a BRAG1 mutant that fails to activate Arf6 also fails to depress AMPA-R signaling, indicating that Arf6 activity is necessary to this process. Conversely, mutation of the BRAG1 IQ-like motif impairs calmodulin binding and results in constitutive, NMDA-R-independent depression of AMPA-R activity. Together these findings suggest that BRAG1 GEF activity is critical for synaptic transmission, and is regulated by its interaction with calmodulin downstream of NMDA receptor activation. Our findings reveal a role for BRAG1 in

response to neuronal activity with possible clinical relevance to nonsyndromic X-linked intellectual disability.

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VAC14, a Protein that Regulates the Phosphoinositide PI(3,5)P2, Localizes to Synapses and Regulates Synaptic Function.

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The signaling lipid, phosphatidylinositol 3,5-bisphosphate (PI(3,5)P_2), is regulated by a protein complex composed of FAB1/PIKfyve, VAC14, and FIG4. Mice hypomorphic for VAC14 and FIG4 exhibit degeneration in the central and peripheral nervous systems. Moreover, mutations in human *FIG4* are associated with Charcot-Marie-Tooth syndrome and Amyotrophic Lateral Sclerosis. How the nervous system is susceptible to loss of PI(3,5)P_2 was not clear. Here we use cultured hippocampal neurons and show that endogenous VAC14 partially overlaps with early and late endocytic markers in both the soma and neurites. Unexpectedly, a significant fraction of VAC14 localizes to synapses, suggesting a role in regulating synaptic function. Indeed, the amplitude of miniature excitatory synaptic currents is enhanced in *Vac14*$^{-/-}$ neurons. This defect is rescued by overexpression of VAC14 in the postsynaptic neuron. Consistent with this finding, surface GluA2, a glutamate receptor subunit, is elevated in *Vac14*$^{-/-}$ neuron. Thus, VAC14 plays a role in controlling postsynaptic function, possibly through regulation of glutamate receptor internalization or recycling.

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Local palmitoyltransferase activity induces nucleation of postsynaptic protein assembly.

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Palmitoylated PSD-95, a representative postsynaptic scaffold protein, clusters at specialized postsynaptic membranes and plays a critical role in synaptogenesis and synaptic plasticity. However, how the position of PSD-95 clusters is determined in polarized neurons and what triggers their formation are not completely understood. To investigate this process, we selected a recombinant antibody that specifically recognizes the palmitoylated conformation of PSD-95. Its use as a GFP-tagged intrabody in live imaging allowed spatiotemporal visualization of palmitoylation state of endogenous PSD-95. We discovered previously unidentified postsynaptic structures: multiple submicron clusters of PSD-95 in a dendritic spine. These submicron clusters were colocalized with AMPA-type glutamate receptors and plasma membrane-inserted PSD-95 palmitoyltransferase, DHHC2. Knockdown of DHHC2 blocked multicluster formation while forced accumulation of DHHC2 on the dendritic membrane generated PSD-95 clusters. Thus, highly localized palmitoylating activity at the plasma membrane directly nucleates protein assembly to organize postsynaptic protein assembly.

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Palmitoylation of an AKAP Scaffold Protein Regulates Dendritic Endosomal Targeting and Synaptic Plasticity Mechanisms.

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LTP and LTD regulate AMPA receptor (AMPAR) phosphorylation and postsynaptic localization to control synaptic strength during learning and memory. The A-kinase anchoring protein (AKAP) 79/150 signaling scaffold regulates AMPAR phosphorylation and trafficking during LTP/LTD. AKAP79/150 is targeted to dendritic spine plasma membranes by an N-terminal domain that binds phospholipids, actin, and cadherins. However, we do not understand how regulation of AKAP targeting controls AMPAR endosomal trafficking. Here we report that palmitoylation of the AKAP N-terminal domain targets it to endosomes and is regulated by LTP/LTD and seizure activity. With chemical LTP induction we observe palmitoylation-dependent AKAP spine recruitment that requires Rab11-regulated endosome trafficking coincident with spine enlargement and AMPAR surface delivery. Importantly, expression of an AKAP palmitoylation-deficient mutant prevents spine enlargement, AMPAR surface delivery, and synaptic potentiation. These findings emphasize the emerging importance of palmitoylation in controlling synaptic function and reveal novel roles for the AKAP79/150 signaling complex in endosomes.

Host-Pathogen/Host-Commensal Interactions I

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"Journey to the Center of Translation: In Situ Visualization and Characterization of Compartmentalized Protein Synthesis in Cells and Tissues."

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Protein translation is a central cellular function considered for decades as the domain of hardcore biochemists. Recently, translation has attracted attention from cell biologists integrating gene product-specific information into a systems view of cellular function. Protein synthesis is a highly varied process with global adaptations for varied circumstances, including distinct modifications tailored for environmental and infectious stressors and differentiation states. Recent studies imply that translation is compartmentalized to allow for local expression of specific mRNAs in order to optimize processes such as cell migration, embryogenesis, neuronal synapse formation, and viral infection. To study translational compartmentalization, we developed the RiboPuromyclation Method (RPM), which visualizes translating ribosomes in cells by standard immunofluorescence. RPM is based on ribosome-catalyzed puromycylation of nascent chains immobilized on ribosomes by chain elongation inhibitors and detection of the puromycylated nascent chains with a puromycin-specific mAb. Ribosome catalyzed puromycylation occurs in live and digitonin permeabilized cells and cell extracts. Due to its high sensitivity, RPM enabled us to visualize translation sites and distinguish protein synthesis sub-compartments based on their localization and their composition. We corroborated the significance of local protein synthesis in several key cellular mechanisms including activation, differentiation, stress, antigen presentation and viral infection. Applying RPM *in vivo*, we demonstrated that protein synthesis is anatomically regulated in lymphoid organs -and even more strikingly in specific cell subsets- following viral infection.

Overall this study demonstrates the remarkable and tremendously intricate organization of the protein synthesis machinery. In addition, it clearly illustrates the strength of RPM as a unique and powerful tool for **visualizing and characterizing** translation sites that will facilitate understanding of translation compartmentalization across many disciplines in biological research, including eukaryotic and simple prokaryotic cell biology.

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Phagosome volumes similar to infection by L. amazonensis, altered the production of IL-12 via ER-Golgi in phagocytosis of latex particles, and also could be secreted by endocytic pathway.

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The intracellular pathway to secrete immune effectors from macrophages and its relationship with vesicular trafficking are poorly described. The objective of this work was to study the effect of phagosomes of different volumes in the intracellular route and trafficking of interleukin 12 (IL-12). J774A.1 macrophage-like cells were infected with L. amazonensis or exposed to latex beads of different diameter and at different proportions to generate phagosomes of different sizes. Then J774.A1 were exposed to interferon gamma (INF-g) and lipopolysaccharide (LPS) to induce classical activation. IL-12 expression was determined by real time PCR, its intracellular location by confocal microscopy and its secretion by flow cytometry. Co-labeling of components of the endocytic pathway or classical secretion was also done.
There was not constitutive secretion of IL-12 but it was induced after exposure to INF-g and LPS. Secretion was dose dependent and biphasic with a first peak at two hours and second pick at six hours post-activation. IL-12 was detected early after activation and co-localized with Calnexin indicating a endoplasmic reticulum (ER) localization. By four hours IL-12 co-localized with ICAM-1 to the plasma membrane. Neither infection nor phagocytosis induced per se IL-12 secretion. However there was detection at ER of IL-12 after L. amazonensis infection. At phagosome volumes above 160 mm3 the quantity of IL-12 detected was less compared to activated macrophages, despite the nature of the phagocytic load. Not only, the amount of IL-12 varied, this interleukin co-localized in these groups with GM-130 suggesting retention at Golgi.
These results suggest that large phagocitic volumes, alter intracellular IL-12 route by the classical secretion pathway via RE-Golgi, compromising its secretion. IL-12 is secreted in vesicles t-SNAREs containing SNAP-23 in the Leishmania infection and alternative RE-Golgi routes for IL-12 secretion may be used by the macrophage.

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GPI PbPga1p of P. brasiliensis is a surface antigen that binds to extracellular matrix components and activates murine macrophages releasing TNF-α and NO.

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Paracoccidioides brasiliensis, a dimorphic fungi, is the etiologic agent of paracoccidioidomycosis (PCM), one of the most prevalent mycosis in Latin America. *P. brasiliensis* cell wall components interact with host cells and can influence the pathogenesis of PCM. In *P. brasiliensis* and other fungi, most of the proteins that compose the cell wall are GPI anchored. These proteins are involved in cell-cell adhesion and cell-tissue adhesion, and have a key role in the interaction between fungal cells and host cells. The protein PbPga1 is a GPI

anchored protein from P*. brasiliensis* whose function is unknown. In the present study, we examined the gene expression of PbPga1 by RT-PCR and observed that PbPga1p is up-regulated in the yeast pathogenic form. A recombinant protein rPbPga1p was then produced in *Pichia pastoris,* purified on a NI-NTA column and an antibody anti-rPbPga1p was produced in chickens. The ability of rPbPga1p to bind to extracellular matrix components and to activate murine macrophages was also examined. rPbPga1p was able to bind to laminin, fibronectin, collagen type I and type IV in a dose-dependent manner. These results suggest that PbPga1 can facilitate the adhesion of *P. brasiliensis* to host tissues, which is one of the crucial steps in the pathogenic process and a pre-requisite for host colonization. Interestingly, rPgPga1p induced mouse peritoneal macrophages to release high levels of TNF-α and nitric oxide (NO) *in vitro*. Moreover, anti-rPbPga1p was able to recognize a protein located on the yeast cell surface at the neck between the mother cell and the bud, covering the surface of the new buds. Taken together these results indicate that PbPga1 is a surface antigen that may play an important role in PCM pathogenesis.

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Bacillus anthracis Stimulates the Formation of Neutrophil Extracellular Traps.

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Bacillus anthracis Stimulates the Formation of Neutrophil Extracellular Traps
Clinical findings from the 2001 bioterrorist attacks revealed normal or minimally elevated peripheral polymorphonuclear neutrophils (PMN) counts at time of hospital admission, despite high-levels of bacteremia. PMNs are the first cells recruited to sites of infections, yet surprisingly little is known about their role in anthrax pathogenesis. The poly-γ-D-glutamic acid capsule of *B. anthracis* serves as a virulence factor that inhibits phagocytosis by PMNs allowing anthrax to grow unimpeded. In addition to phagocytosis, activated PMNs release extracellular traps (NETs) that contribute to resistance by degrading virulence factors and killing bacteria. NETs provide high local concentrations of antimicrobial proteins to bind, disarm, and kill microbes extracellularly, independent of phagocytic uptake. OBJECTIVE: We sought to determine if *B. anthracis* stimulates NET production and if NETs are able to efficiently capture and kill both the spore and vegetative forms of *B. anthracis*. METHODS: In this study we infected PMNs with spores or vegetative bacilli of a fully virulent encapsulated or nonvirulent unencapsulated *B. anthracis* strain. We used methods that facilitate the visualization and measurement of NETs using different *in vitro* cell culture techniques and functional assays. RESULTS: We were able to show for the first time that both spores and vegetative bacilli are able to stimulate the induction of NETs. After blocking phagocytosis with cytochalasin D, an inhibitor of actin polymerization, we found an extracellular mechanism of killing. Unencapsulated bacilli but not encapsulated bacilli were killed through entrapment of bacilli in NETs and the antimicrobial activity of histones located on the NETs. Interestingly, both NET and phagocytosis-mediated killing occurred independent of reactive oxygen species. Virulent encapsulated bacilli, although trapped in NETs were resistant to killing. Spores from either strain were not readily captured by the NETs even though they stimulate NET production. CONCLUSIONS: We showed, for the first time, that PMNs are able to readily form NETs in response to *B. anthracis* infection. Unencapsulated bacilli are killed by entrapment in NETs and the antimicrobial activity of histones. The poly-γ-D-glutamic acid capsule appears to protect *B. anthracis* from PMN-mediated NET killing, and we are presently exploring the mechanism of action. These results suggest a significant role for NETs in neutrophil-mediated innate immunity.

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Yeast Entry into Host Cells: Studying Cryptococcus neoformans-macrophage Interaction.

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Cryptococcus neoformans is an encapsulated yeast that causes disease in immunocompromised and, less commonly, immunocompetent patients. Cryptococcosis is considered an AIDS-defining condition, and it is the third most frequent neurological complication in AIDS patients. During the last decade, studies have focused on how this yeast can survive and proliferate within macrophages. However, no studies have been made to better understand which mechanisms the yeast uses upon entering host cells. Therefore, we have started our studies observing C. neoformans interactions with peritoneal macrophages using scanning electron microscopy (SEM) and fluorescence microscopy.

C. neoformans strains ATCC28957 and ATCC52817 (CAP67 – acapsular mutant) were put to interact with peritoneal macrophages, previously adhered on glass cover slips, in a ratio of 25:1 (yeast:host cell) for 2, 4 and 6 hours. The cells were then processed for SEM or fluorescence microscopy, where actin was fluorescently labeled with AlexaFluor 488 phalloidin and a-tubulin was imunnolabeled with anti-a-tubulin AlexaFluor 546 conjugate.

In order to better understand the morphology of the phagocytic uptake structures involved in C. neoformans-macrophage interactions, we used SEM. Macrophages represent the first line of defense for the immune system, and the phagocytic uptake of invading microorganisms is a prerequisite for their efficient elimination. Different structures were observed during interaction with both strains, such as ruffle-like uptake structures and zipper-like internalization processes in infected macrophages. These findings suggest that macrophages can engage different mechanisms to engulf C. neoformans yeasts and the presence or absence of capsule does not seem to influence these uptake structures. Analyzing how the cytoskeleton is involved in this internalization process, a-tubulin was observed surrounding endossomes containing yeasts, whereas actin did not appear to be recruited after yeast internalization. Together, these results contribute to an early understanding on how C. neoformans-macrophage interaction occurs. Further studies are ongoing and will aid on the unraveling of this opportunistic yeast's survival in host.

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Host-specific Cell Surface Proteomes of African Trypanosomes.

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The African trypanosome, *Trypanosoma brucei*, is a protozoan parasite that causes sleeping sickness in humans and related debilitating diseases in animals. Sleeping sickness is fatal if untreated, yet current treatments are old, toxic and difficult to administer. Thus, there is a pressing need to better understand these parasites and facilitate development of new therapeutic interventions. *T. brucei* alternates between mammalian and tsetse fly hosts and must integrate signals from diverse external environments for survival, development and pathogenesis. Parasite surface proteins protect against host immune defenses, allow acquisition of nutrients and provide sensory and signaling functions important for host-parasite

interactions and pathogenesis. Cell surface proteins are thus critical for the parasitic lifestyle of *T. brucei* and make attractive targets for therapeutic intervention in sleeping sickness. The flagellar membrane in particular, is a sub-domain of the cell surface specialized for host-parasite interaction in *T. brucei* and important for signaling in a wide variety of other organisms. We recently published a flagellum surface proteome from bloodstream form parasites obtained by combining affinity purification of surface-exposed proteins with state-of-the-art proteomics, however no systematic study of the entire parasite surface has been conducted and only a few key surface-exposed proteins have been characterized. This represents a major gap in our understanding of trypanosome biology and the host-parasite interface. We have now capitalized on our proteomics approach to define the cell surface proteomes of *T. brucei* from insect and mammalian bloodstream form parasites. From these data, we have identified life cycle stage-specific cell surface proteins that may provide host-specific adaptations, including several life cycle stage-specific adenylate cyclases. Ongoing experiments will test the impact of host-specific proteins on infection of the mammalian host and address whether protein diversity on the cell surface is concentrated within the flagellum membrane. Together, the proposed experiments will increase understanding of the role of trypanosome surface proteins during infection and advance efforts to understand and exploit the flagellum as a key host-parasite interface. While our focus is on the host-parasite interface of *T. brucei*, we expect our results to be of wide interest for the community studying fundamental biology and pathogenesis of parasitic protozoa.

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A putative ion channel is required for flagellum attachment in Trypanosoma brucei.

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Trypanosoma brucei is the etiological agent of sleeping sickness, which causes significant human mortality and limits economic development in sub-Saharan Africa. Critical to trypanosome development and disease pathogenesis is the parasite’s single flagellum which drives cell motility and participates in host-pathogen interactions, immune evasion and cell morphogenesis. A unique and defining feature of T. brucei cell architecture is the flagellum attachment zone (FAZ), a region of membrane-cytoskeleton linkages that provide for lateral attachment of the flagellum to the cell. Disruption of the FAZ results in flagellum detachment and is lethal. Only a handful of proteins have been localized to the FAZ, some of which function in maintenance of attachment and others that participate in surface remodeling. However, the nature of the attachment architecture and the involved components remain enigmatic.

We discovered that Goldengate, a putative calcium channel, localizes to the FAZ region and is required for flagellum attachment. Goldengate is a massive 304 kDa multi-spanning membrane protein with orthologs in many eukaryotes. We in situ tagged Goldengate in both procyclic and bloodstream form parasites and showed FAZ localization and membrane partitioning by immuno-fluorescence and biochemical fractionation respectively. Flagellum-detachment experiments suggested that Goldengate resides on the flagellar side of the FAZ. Surprisingly, RNA-interference knockdown of Goldengate results in flagellar detachment and is lethal in both life stages. Precise localization of Goldengate by immuno-electron microscopy and structure-function experiments are underway to distinguish between ion channel functions and structural functions in flagellum attachment.

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The gen LbrM01_V2.0210 of Leishmania braziliensis codifies for a chloride channel.

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Leishmaniasis is a complex of diseases of high relevance, caused by protozoan parasites of the genus *Leishmania*. To complete its life cycle *Leishmania* is exposed to different temperature, osmolarity, ion concentration and pH conditions. As a promastigote, extracellular stage inside the gut of a sand fly vector, the parasite is at 28°C, ~300 mOsm and pH of 7,0-8,0, but, as an amastigote, the intracellular stage, the environment is at 35°C, ~150–250 mOsm and pH 4,5-5,5 inside the parasitophorous vacuole. Parasite adaptation to this pH gradient and osmolarity changes while, securing nutrient transport depends on P-type H^+-ATPases function, coupled to anionic transport. We recorded voltage-dependent chloride currents in *Xenopus laevis* oocytes after injection of *Leishmania* mRNA. A group of four putative sequences for chloride channels is reported in the *Leishmania braziliensis* genome. In this work we present evidence of expression of these sequences in *Leishmania* promastigotes and recorded chloride currents from HEK 293 cells transfected with *LbrM01_V2.0210.*
Specific primers were designed to amplify partial and total coding regions of the genes *LbrM01_V2.0210, LbrM04_V2.1010, LbrM32_V2.3670* (GeneBank and GeneDB). Total RNA was extracted from various *Leishmania* species and different parasite stages with and cDNA obtained, that was used as template for PCR. The *LbrM01_V2.0210* PCR product was purified, cloned into the mammalian expression vector pcDNA3.1/CT-GFP-TOPO, and verified by sequencing (Macrogen USA), before transfection into HEK 293 cells. Chloride channel activity was recorded by patch clamp.
Products of the expected molecular weight were detected for *LbrM33_V2.1260, LbrM01_V2.0210, LbrM04_V2.1010, LbrM32_V2.3670* in stationary promastigotes of *L. braziliensis, L. amazonensis, L. panamensis* and *L. major.* Partial sequence showed high similarity to those reported in GeneDB. Expression of *LbrM33_V2.1260, LbrM01_V2.0210* was also found in *L. braziliensis* amastigotes. Bioinformatic analysis of these genes reported homology to mammalian intracellular ClC channels, particularly ClC3 and ClC6. Promastigote labeling with anti-ClC3 antibodies shows fluorescence in the parasite plasma membrane and flagellar pocket. Whole cell recordings of HEK 293 cells transiently transfected with *LbrM01_V2.0210* showed outwardly rectifying voltage currents. The amplitude of these currents was modulated by hipotonicity and low pH. Inhibitors of anionic channels block the current by 50-70%. These electrophysiological properties are consistent with a chloride channel type ClC that may support pH and osmolarity regulation in *Leishmania*.

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Host cell egress by intracellular protozoan parasites.

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Leishmania sp. and Plasmodium falciparum are obligatory intracellular parasites, responsible of major tropical diseases. Their life cycle include a stage in a vertebrate host, within which they localized intracellularly, confined into a parasitophorus vacuole (PV). In the PV, these parasites divide and are released in order to infect new cells. The egress model has not been fully established, but two basic mechanisms are proposed: (1) egress by lysis of the parasitophorous vacuole membrane (PVM) and the host cell plasma membrane (HCPM) or (2) egress by fusion of the PVM and the HCPM. We explore both mechanisms in these two models.

Monitoring infection by light microscopy indicates that parasite exit occurs between 72-78 hours post-infection (hpi) for Leishmania and at 48 hpi for Plasmodium. The period measure for Leishmania coincides with an increase in macrophage's capacitance, suggesting incorporation of membrane to the HCPM, which could come from PVM fusion. Lactate dehydrogenase (LDH) activity, distribution of the fluorescent probe Calcein AM and FM464 and FM143 labeling were carried out to infer the egress mechanism. During parasite exit, LDH activity was detected in culture supernatants but it was of less magnitude to that of total HCPM lysis. Calcein was partially distributed to supernatants with an important amount left in host cells. The evidence indicates that these parasites might use both mechanisms as has been shown for Chlamydia.

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Stereoscopic transmission electron microscopy of Maurer′s Clefts in ″unroofed″ specimens of *Plasmodium falciparum*-infected erythrocytes.

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Intraerythrocytic stages of human malaria parasite, *Plasmodium falciparum* (*Pf*), modify and remodel their host cell environment in numerous ways. Among these modifications are Maurer′s Clefts (MCs), which develop in erythrocyte cytoplasm and promote the trafficking of parasite proteins including Pf erythrocyte membrane protein 1 (PfEMP1). The fine structure of MCs and their relationships with the erythrocyte membrane are still poorly understood. In this study, we investigated the structure of MCs by stereoscopic transmission electron microscopy (TEM) of snap frozen and ″unroofed″ specimens of parasitized erythrocytes. Our images show that MCs have spherical/oval bodies with sizes of approximately 200 nm to 500 nm in width and 300 to 700 nm in length. These shapes are consistent with a previously proposed 3D model reconstructed from TEM section images. In addition, we observe tethers/strings which interconnect adjacent MCs and connect to the erythrocyte membrane skeleton. The presence of these previously unobserved extensions and tethers in our images demonstrates the value of unroofing techniques and their ability to provide new information on the structural modifications of parasitized erythrocytes.

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PfEXP-250, a Novel P. falciparum-exported Protein, Associated with the Maurers clefts is Expressed in the Exoerythrocytic Stages.

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After invading a host erythrocyte, *Plasmodium falciparum* induces extensive modification of the host erythrocyte. Thus far, the expression and localization of *Plasmodium*-exported has only been studied in the erythrocytic stage. Very little is known about the *Plasmodium* proteins exported during the liver stage. We have previously characterized PfEXP-250, a unique *P. falciparum*-exported protein containing a PEXEL motif located at the carboxy-terminus of the protein. PfEXP-250 is highly conserved and has paralogues in *P. vivax*, *P. yoelii* and *P. berghei*. We have found PfEXP-250 to be exported to the erythrocyte cytosol and to be associated with Maurer's cleft resident proteins. Surprisingly, we have also found EXP-250 to be expressed in sporozoites and in the exo-erythrocytic forms of *P. berghei*. However, EXP-250 does not appear to be exported in the sporozoite and exo-erythrocytic forms. We have examined the expression of skeleton-binding protein 1 (SBP-1) and ring-exported protein 1 (REX-1) in *P. berghei* and

found both not to be expressed in the liver stage. These data suggest that PfEXP-250 may be playing a unique role in the parasite. Its mechanism of export is currently under investigation

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The Adaptor Protein MyD88 is Essential For Development of Murine Placental Malaria.

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Placental malaria (PM) is associated with a panel of complications affecting both mother and fetus. Many of the injuries induced by the infection during pregnancy are related with the accumulation of infected red blood (iRBC) cells in the placenta and the interaction between parasite and host cells. It has been described that members of the Toll Like Receptors (TLR) family, recognize components of Plasmodium sp. and affect the host immune response. In common, TLR signal through MyD88, which leads to induction of NF-kB-dependent inflammatory cytokine production. The objective of this study was to evaluate the effect TLR activation by iRBC in the placenta. Initially, to ascertain if TLR could recognize P. berghei NK65 iRBC, human embryonic kidney epithelial (HEK) cells were cotransfected with mouse CD14 and TLR2 or TLR4, or transfected with TLR9. For luciferase reporter assay, cells were also cotransfected with ELAM-1-firefly luciferase and beta-actin-Renilla luciferase plasmids. Our results shown that iRBC were able to activate TLR9 and with less intensity activate TLR4-CD14, but not TLR2-CD14 transfected cells. To verify the effects of TLR activation, we infected pregnant MyD88 knockout and C57BL/6 mice with P. berghei NK65 on the 13th gestational day. Histopathological analysis of the placentas shows a decrease of vascular spaces in C57BL/6 infected mice when compared with placentas from non-infected mice (38% vs. 48% of vascular space). On the other hand, placentas from infected and non-infected MyD88 did not presented differences. Moreover, we evaluate the placental expression of pro-inflammatory cytokines IL-1β and IL-6 mRNA. Our results show that only C57BL/6 infected mice present an increase in the expression of both cytokines. Placental inflammation is correlated with abortions and preterm delivery, in fact in our results, C57BL/6 infected mice presented an increased number of abortions when compared with non-infected animals (1.8 vs. 0.2 abortions/female) whereas in MyD88KO mice no abortions were observed. In summary, this study illustrates the importance of a severe inflammatory response in the development of PM pathogenesis and, additionally, indicates the adaptor protein MyD88 as potential targets for therapeutics intervention.

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A Live-Cell Approach to Investigating the Microsporidia-Host Interface.

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The Microsporidium, *Anncaliia algerae (Brachiola algerae),* is an obligate eukaryotic intracellular parasite and an opportunistic human pathogen that can cause morbidity and mortality among immune-compromised individuals including patients with AIDS and those undergoing chemotherapy. There is little known about the Microsporidia-host cell interface in living host cells, due to current approaches being limited by the lack of fluorescent reporters for detecting the parasite lifecycle. Here we have developed and applied novel fluorescent parasite labeling methodologies in conjunction with fluorescent protein-tagged reporters to be able to track simultaneously the dynamics of both parasite and host cell specific components, including the secretory and endocytic trafficking pathways, during the entire infection time period. We have found dramatic changes in the dynamics of host secretory trafficking organelles during the course of infection. The Golgi compartment is gradually disassembled and regenerated adjacent

to Endoplasmic Reticulum exit sites. Importantly we find that individual microsporidial progeny are each associated with these *de novo* formed mini-Golgi structures. These host structures appear to create a membrane bound niche environment for parasite development. Our studies presented here are an important first step in understanding the biology of Microsporidial parasites in the living host.

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Essential Roles for Soluble Virion-Associated Heparan Sulfonated Proteoglycans and Growth Factors in Human Papillomavirus Infections.

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A subset of human papillomavirus (HPV) infections is causally related to the development of human epithelial tumors and cancers. Like a number of viruses, HPV entry into target cells is initiated by first binding to heparan sulfonated proteoglycan (HSPG) cell surface attachment factors. The virus must then move to distinct secondary receptors, which are responsible for particle internalization. Despite intensive investigation, the mechanism of HPV movement to and the nature of the secondary receptors have been unclear. We report that HPV16 particles are not liberated from bound HSPG attachment factors by dissociation, but rather are released by a previously unobserved process for pathogens. Virus particles reside in infectious soluble high molecular weight complexes with HSPG, including syndecan-1 and bioactive compounds, including growth factors. MMP inhibitors that block HSPG and virus release from cells interfere with virus infection. Using a co-culture assay, we demonstrate HPV associated with soluble HSPG-growth factor complexes can infect cells lacking HSPG. Interaction of HPV-HSPG-growth factor complexes with growth factor receptors leads to rapid activation of signaling pathways important for infection, whereas a variety of growth factor receptor inhibitors impede virus-induced signaling and infection. Depletion of syndecan-1 and removal of serum factors reduce infection, while replenishment of growth factors restores infection. Our findings support an infection model whereby HPV usurps normal host mechanisms for presenting growth factors to cells via soluble HSPG complexes as a novel method for interacting with entry receptors independent of direct virus-cell receptor interactions.

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Purified the Human T-Cell Leukemia Virus Type 1 via Centrifugation.

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Human T-Cell Leukemia Virus Type 1 (HTLV-1) is a human retrovirus. It is the cause of two types of human diseases: adult T-cell leukemia (ATL) and tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM). Currently, there are no therapeutic agents for HTLV-1 infection. Our hypothesis is that there are cellular factors packaged within HTLV-1 virion, which are important for successful viral replication. In order to investigate this, purified virus must be used. Therefore, we have performed tripartite transfection to produce VSV-G[HTLV-1] virions. HEK 293T-cells were transfected with three plasmids, which contain the necessary viral genes to produce VSV-G[HTLV-1]. Next, western blots have been performed, which confirm that VSV-G[HTLV] virions were produced and processed correctly. We have developed a purification method involving dialysis and centrifugation to purify the viruses from producer cells. As a control MT-2 cells, which are chronically infected with HTLV-1, have been used. Currently, we are using microscopy and western blotting techniques to identify cellular proteins that are packaged inside of the VSV[HTLV-1].

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PI4P/Cholesterol enriched Membrane Platforms are required for viral RNA synthesis.

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Many RNA virus infections induce complex subcellular membrane structures, on which they assemble their replication enzymes and synthesize viral RNA. These replication membrane platforms are unique in protein and lipid composition from that of host membranes. Previously we had demonstrated that enteroviruses and flaviviruses hijack host phosphatidylinositol-4-kinase III beta (PI4KIIIb) enzymes to generate replication platforms enriched in phosphatidylinositol-4-phosphate (PI4P) lipids, which in turn facilitated viral RNA synthesis. In this study we demonstrate that in addition to PI4P lipids, the sterol cholesterol also plays an important role in generating the viral replication platforms. We show that in the early stages of infection viral RNA enzymes are assembled on pre-existing membrane domains of the TGN that are enriched in both PI4P and cholesterol. The replication organelles that are generated later during peak replication kinetics also continue to be enriched in both PI4P and cholesterol. We show that the cholesterol at these membrane sites is trafficked from the plasma membrane via Rab8 and Rab11 dependent endocytic pathways. Furthermore the presence of cholesterol binding proteins such as Caveolin, OSBP and CERT can compete and interfere with the assembly of viral replication complexes on these platforms. Taken together, these findings indicate that PI4P/cholesterol platforms can facilitate viral RNA synthesis and these domains may be critical for helping assemble the viral replication machinery components in the early stages of infection when they are in low abundance in the cell.

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Interaction of soluble S proteins of *transmissible gastroenteritis coronavirus* (TGEV) with its receptor pAPN and sialic acids.

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Transmissible gastroenteritis virus (TGEV) is a porcine coronavirus that infects the epithelium of the intestinal tract leading to gastroenteritis. It affects pigs of all ages and the cellular receptor - the porcine aminopeptidase N (pAPN) - is required to initiate the infection of target cells. Binding to pAPN is mediated through the spike glycoprotein (S) of TGEV. The S protein also possesses a sialic acid binding activity which is connected with the enterotropism of TGEV. It has been shown in previous studies that TGEV recognizes a mucin-type glycoprotein in a sialic acid-dependent fashion.

To analyze the binding activity - to both the cellular receptor pAPN and the high molecular weight sialoglycoprotein - we generated a soluble S proteins comprising the S ectodomain connected either to the human Fc backbone or to the trimerization domain derived from the GCN4 leucine zipper. The soluble proteins were expressed by baby hamster kidney cells (BHK-21) or human embryonic kidney cells (293T). These S proteins bound to BHK cells transiently expressing pAPN as shown by indirect immunofluorescence and FACS analysis. The binding to porcine cell lines originating from testis (ST) or gut epithelium (IPI-2I) was also demonstrated. Pretreatment of the cells with neuraminidase (from Clostridium perfringens) showed a significant increase in binding of the soluble S proteins. Furthermore, we used swine erythrocytes for an erythrocyte pull-down assay to demonstrate the pull-down of soluble S proteins after incubation with erythrocytes. As shown by Western blot analysis, the pull-down-assay did not provide evidence for interaction of sol S proteins with sialic acids of erythrocytes.
Thus, the soluble S protein provides an interesting tool to investigate the interaction of TGEV with porcine aminopeptidase N.

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Live imaging of membrane compartments interacting with herpes simplex virus during viral production: Evidence for HSV1- recruitment of fast vesicular transport machinery.

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A major controversy exists as to the molecular mechanism(s) of outbound transport of nascent herpes simplex virus after replication in the nucleus. Infectious herpes simplex virus type 1 (HSV1) particles have four concentric compartments: a double-stranded DNA core within a proteinaceous capsid, encased in tegument proteins and enveloped in a glycoprotein-rich lipid membrane. Whether virus assembles before, during or after transport is not understood. To address this question, we performed live confocal time-lapse imaging of HSV1 packaging and transport in cells synchronously infected over a one-hour window. By labeling cellular membrane systems with lipid dyes, DiI, DiO and DiD, transfecting cells with cellular membrane proteins labeled with fluorescent proteins, and infecting with HSV1-VP26GFP that carries GFP label on capsids, we directly witness the dynamic interplay between cellular membranes and nascent virus. These studies show that at early time points, most viral capsids assemble with membranes in the perinuclear region (71%) and then travel together with membranes to the periphery, with multiple reversals and zig-zags. A few capsids also travel at fast transport rates independent of detectible membranes or cellular membrane proteins. At later time points when the Golgi has fragmented and drifted towards the cell periphery, more capsids travel outwards for longer distances without membranes. We conclude that viral transport is most often but not necessarily coupled with secondary envelopment, that the stage of infection influences transport mechanisms, and that HSV1 thus may use multiple redundant mechanisms to attract cellular motors mediating its exit from cells. NS062184; RO1 NS046810 and P5OGM08273.

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Intranuclear Pathways Targeted by Influenza Virus.

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Influenza A viruses are negative-sense, segmented RNA viruses, which cause about 500,000 deaths worldwide per year. Genomic studies have shown that the non-structural protein (NS1) of influenza A virus is a major virulence factor that is essential for pathogenesis. NS1 is a multifunctional protein localized in the nucleus and in the cytoplasm. In the cytoplasm, NS1 inhibits host signaling pathways that result in down-regulation of interferon expression and innate immune response. In the nucleus, NS1 represses host gene expression by down-regulating mRNA processing and export. We showed that NS1 binds the mRNA complex constituted of NXF1/TAP, NXT/p15, Rae1, and E1B-AP5, which are key components of the mRNA export machinery. By targeting this complex, NS1 blocks host mRNA export, and cells become highly permissive to virus replication. Another intranuclear pool of NS1 was found to interact with a host protein termed NS1-BP (NS1-Binding Protein), which has been suggested to play a role in pre-mRNA splicing. However, the functions and mechanisms involving NS1-BP and their impact in the host and for the influenza virus life cycle remained to be elucidated. To investigate the function of NS1-BP, we first identified its binding partners by immunoprecipitation followed by mass spectrometry. We then functionally validated these interactions and showed that NS1-BP has roles in transcription and mRNA processing. These functions direct impact both virus replication and host intranuclear functions that regulate host mRNA levels. Thus, we identified here NS1-BP as a regulator of virus replication and as a host factor that is required for

proper expression of specific subsets of host mRNAs. These functions regulate both the virus life cycle and the host immune response.

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Enhanced invertase activities in the callus-like structures induced by BSCTV infection on Arabidopsis.

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Geminiviruses are single-stranded DNA viruses that infect a number of monocotyledonous and dicotyledonous plants. *Arabidopsis* is susceptible to infection with the *Curtovirus* group of geminiviruses including *Beet severe curly top geminivirus* (BSCTV). *Arabidopsis* infected with BSCTV exhibits systemic symptoms such as stunting of plant growth, callus induction on shoot tips, and curling of leaves and shoot tips. The regulation of sucrose metabolism is essential for obtaining the energy required for the process of viral replication and the development of symptoms in BSCTV-infected *Arabidopsis*. We evaluated the enhanced transcript level and enzyme activity of invertases in the inflorescence stems of BSCTV-infected *Arabidopsis*. These results were consistent with the increased pattern of ribulose-1,5-bisphosphate carboxylase/oxygenase activity and photosynthetic pigment concentration in virus-infected plants to supply more energy for BSCTV multiplication. The altered gene expression of invertases during symptom development was functionally correlated with the differential expression patterns of D-type cyclins, E2F isoforms, sucrose metabolism-related genes, and auxin-associated genes. Taken together, our results indicate that sucrose sensing by BSCTV infection may regulate the expression of sucrose metabolism and result in the subsequent development of viral symptoms in relation with the activation of cell cycle regulation.

Imaging Technologies

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Automated Correlation of Fluorescent and Electron Micrographs of Astrocyte Cells.

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The accurate correlation of fluorescent and electron data is becoming increasingly popular among cell biologists but is often impeded by the lack of automation and certainty in their correlative approach. Our work focused on the automatic correlation of images taken with a fluorescent light microscope (FLM) and scanning transmission electron microscope (STEM). Astrocyte cells expressing enhanced green fluorescent protein (EGPF) collected from the hippocampus of transgenic mice were used for this experiment. The cells were also immunolabeled for EGFP with nano gold particles. Although EGFP expression was apparent in the light micrographs, images of the ultrastructure were also needed to confirm EGFP expression was confined to astrocyte cells. With the aid of newly designed software, images acquired from the FLM and an overview image from the STEM were accurately aligned. This subsequently allowed for automatic acquisition of higher resolution STEM images in selected EGFP expressing areas. Using this method it was possible to accurately locate the electron dense tags in the cellular ultrastructure and determine that the expression of EGFP was confined to only astrocyte cells.

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Advances in serial block face DualBeam electron microscopy for the exploration of cardiovascular and muscular tissues.

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Traditionally serial section transmission electron microscopy has been the only method available in which images are acquired from sections that are cut and mounted on grids. These image representing a 3D volume can be used to reconstruct and analyze the structure of the elements within. This method is very labor intensive, requiring a greater deal of manual dexterity, and when small mistakes occur, a continuous dataset is lost.

Recent studies have explored an alternative method for serial image acquisition in which the block face of resin-embedded neural tissue was imaged within a FEG SEM [1]. Sections were removed from the imaged face in the microscope using an ultra microtome and then immediately imaged by the SEM. This provides a series of aligned images of the tissue in the block face and has the clear advantage that image acquisition can be fully automated. However the quality of the sectioning is critically dependent on homogenous resin hardness which is difficult to maintain when the electron beam is being used at higher resolution and part of the block suffers from the differential heating effects.

We have continued [2] to explore an alternative approach that is seemingly unaffected by variations in resin quality that uses a focused ion beam directed perpendicular to the block face. This is used to mill the surface of the resin from which serial images can be acquired in either an arbitrary location or in a selected area of specific interest.

We have used automated repetitive cutting and imaging on the DualBeam (Slice and View) with consistent sectioning intervals to as low as 5 nm. I two separate experiments we used en EPON embedded vascular sample with an endothelial cell layer with an atherosclerotic plaque and block of heart muscle tissue to demonstrate the specific site 3D analysis (plaque region) as well as a bulk 3D analysis for the muscular tissue.

Results show that this focused ion beam technique is capable of reliably sectioning tissue, and using the back scattered electrons we were able to visualize the detailed ultrastructure. Organelles like ER, mitochondria, myosin are clearly visible. In a fully automated mode we were able to collect serial images that allowed us to make detailed morphological analyses of the cellular architecture in 3D.

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Spatio-temporal control of intracellular ROS concentration in PDGF signaling revealed by single Eu^{3+}-doped nanoparticle imaging.

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For many cell functions, notably those requiring an asymmetric response, such as directed migration, spatio-temporal organization of signaling pathways is important for the cell response regulation. PDGF (Platelet Derived Growth Factor) induces migration in numerous contexts, such as reparation of vascular lesions or metastasis formation, inducing ROS (Reactive Oxygen Species) production as second messenger. The potential lethality of concentrated ROS and the importance of intracellular organization for migration require a tight control of their concentration. However, the dynamics of ROS production and organization is so far mostly unknown. By imaging single Eu^{3+}-doped nanoparticles[1], we probed the intracellular ROS response with high temporal and spatial resolution. We thus measured the absolute ROS concentration in normal or tumoral cells and revealed specific temporal patterns of ROS production under PDGF stimulation. We measured an integration time of several minutes required for the ROS response formation. This response formation is shorter for tumoral cells. We satisfyingly explained this fact by modeling the diffusion-limited dimerization of PDGFRs. This may constitute a temporal filtering mechanism for preventing cell responses to transient signals and its impairment in tumoral cells could be of great physiological relevance for the metastatic transition. We moreover quantitatively measured a transactivation of EGFR by PDGF stimulation and revealed its role in the dynamics of the cell response. By using a microfluidic system, we furthermore apply spatially controlled PDGF stimulation and displayed the maintenance of asymmetric ROS concentration in the cell under a PDGF gradient. This likely relies on a balance between ROS diffusion and degradation. This balance controls the local ROS concentration and thus the cell response. Altogether, our results reveal the tight regulation of the ROS spatio-temporal organization by the cell, which illustrates how the spatio-temporal control of transduction pathways is crucial for the buildup of the cell response.

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The Living Cell Perceives the Difference between Magnetic and Nonmagnetic Isotopes of Magnesium.

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Some chemical elements have two kinds of stable isotopes, magnetic and non-magnetic ones. The question arises of whether the living cell can perceive the difference between magnetic and non-magnetic nuclei of the same element? Among three stable magnesium isotopes, ^{24}Mg, ^{25}Mg and ^{26}Mg with natural abundance about 79, 10 and 11%, only ^{25}Mg has nuclear spin and, hence, nuclear magnetic field. We revealed the isotope difference in parameters of growth of *E. coli* on different isotopes of magnesium. The adaptation period of the cells to the liquid media supplied with magnetic ^{25}Mg was found to be shorter than the adaptation period to the media supplied with nonmagnetic ^{24}Mg or ^{26}Mg. The cells enriched with ^{25}Mg demonstrate less activity of superoxide dismutase (SOD) when compared to the cells enriched with the nonmagnetic

isotope. Since the SOD level is adjusted to the level of superoxide radicals ($O_2^{\bullet-}$), it can be considered as evidence for the lower production of $O_2^{\bullet-}$ as by-product of respiration. On the solid nutrient media, the cells that were previously grown on ^{25}Mg demonstrate essentially higher viability (determined by counting CFU) than the cells grown on the nonmagnetic isotopes (Koltover et al., 2011). Furthermore, in experiments with *S. cerevisiae* we revealed the isotope difference in parameters of recovery of yeast cells after UV irradiation. The rate of post-radiation recovery was found to be twice higher for the cells enriched with ^{25}Mg as compared to the cells enriched with the nonmagnetic isotope (Grodzinsky et al., 2011). Magnetic isotope effects are known in chemistry (Buchachenko et al., 2008). We have uncovered, for the first time, the magnetic isotope effects *in vivo*. These results demonstrate the feasibility of new antistress therapy including radio-protectors based on the stable magnetic isotopes. [Supported by RFBR, projects 10-03-01203a and 10-04-90408-Ukr_a].

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Fibroblasts in Unloaded Tendon Tissue In Vitro Have Warmer (Mid-Infrared) Cytoplasm Than Nucleus.

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Fibrillar extracellular type I collagen of dense connective tissue and osteoid is the main structural element bearing the load of tissue and organism weight in gravity. Fibroblasts and osteocytes in these tissues produce, degrade, organize and maintain type I collagen. Fibroblasts and osteocytes breakdown native type I collagen with a specific collagenase, matrix metalloproteinase I. I asked if fibroblasts and osteocytes also use thermal energy to denature adjacent collagen to gelatin and control very localized collagen breakdown through general proteolysis? In the present preliminary study tendon tissue was imaged in vitro to evaluate the distribution of 3-5 µm thermal energy in the extracellular matrix (ECM) and cells. Freshly isolated adult rat tail tendon was placed unloaded in an uncovered petri dish on top of a gauze pad containing complete culture medium. The tendon was barely covered on top by a thin layer of culture medium between it and the camera but was able to absorb nutrients from the culture medium below. This set up was placed on a heating plate set at 37° C. A FLIR mid-wavelength (3-5 um) infrared camera, SC7650E with 1X and 3X close-up lenses, was used to image the tendon. Images were captured with FLIR ExaminIR software and analyzed using Image J. The temperature range across the thickness of the tendon went from 32.25 °C to 34.5° C. Fibroblasts could be distinguished from the surrounding ECM of the tendon. In all regions of the tendon the cells were 0.2° C cooler than the adjacent ECM. In addition, the center of the fibroblasts were 0.1° C cooler than their periphery. Cell processes connecting fibroblasts in the tendon tissue could not be distinguished at 1X or 3X. These results suggest that the ECM of tendon is 0.2° C warmer than the fibroblasts in the tissue. However, since tendon is a bright white tissue, it is likely that ECM is reflecting IR which leaves unresolved evaluation of its temperature. The results also show that the periphery of the fibroblasts where the cytoplasm, cytoskeleton and organelles reside is 0.1°C warmer than the center of the cells where the nucleus is. This may reflect increased heat production in the periphery of the fibroblast due to metabolic processes and cytoskeletal force generation. And finally, these results show that imaging finer details of the fibroblasts will require greater than 3X magnification.

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Metabolic labeling of newly synthesized proteins and their post translational modification reveals differential involvement of cellular degradative pathways.

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After translation by the ribosome cellular proteins have a finite lifespan. This lifespan is governed by one of two major degradative pathways: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP). Deficits in either pathway alter protein homeostasis and can cause proteins to build up in the cytoplasm, which can be detrimental to the cell. In an extreme case these proteins form aggregates and these have been associated with cellular pathologies including neurodegenerative disorders. Here we report the metabolic labeling of proteins with a methionine analog in conjunction with tagging of post-translation modification with mannosamine analogs. Using metabolic labeling of proteins we show the lifetime of metabolically labeled proteins in HeLa and A549 cells. We also quantify the relative contribution of the UPS and ALP to protein clearance and show that under regular growth conditions the UPS is the predominant route for protein clearance. Finally we use this approach to demonstrate the accumulation of ubiquitin-positive protein aggregates in the cytoplasm of cells treated with inhibitors of the UPS and ALP. We also show the accumulation of post-translation products upon inhibition of either the UPS or ALP, thereby highlighting the role of these two pathways in recycling moieties attached to proteins following translation.

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Microfluidic devices for live imaging of gently immobilized C.elegans and for high-resolution imaging of early embryos during acute drug treatment.

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A major challenge for studying dynamic developmental processes in C. *elegans* has been difficulties associated with long-term high-resolution imaging of worms. We use a micro-machined elastomer chip that is pushed against a thin layer of a silicone gel on a glass coverslip by the application of vacuum to trap worms between the elastomer and gel. Loading the device is simple and minimizes the loss of worms. The device is compatible with short working distance microscope objectives and has an array of continuously perfused compartments with individual worms sequestered in them. The pushing force is widely adjustable by varying the level of vacuum, making the device suitable for a variety of worm sizes and enabling a user-defined degree of immobilization. Upon reduction of the vacuum, the worms recover their normal behavior, while staying confined in their compartments. Worms can be easily recovered from the device into a minimal volume of buffer for further propagation, by separating the elastomer chip from the gel substrate. In pilot experiments, we have successfully used this device to completely immobilize and image worms at different developmental stages without loss of viability. Specifically, we have imaged individual neurons and the developmental time course of the gonad region, using DIC and two-color fluorescence confocal microscopy.
Small molecule inhibitors are a valuable tool for the analysis of fundamental cellular functions and an entry point for the development of therapeutic agents. In C. *elegans* embryos the use of small molecule inhibitors has been limited by eggshell impermeability. We identified a gene whose inhibition renders the eggshell permeable without disrupting events during the early embryonic division and built a specialized microdevice for in situ worm dissection and high resolution imaging of embryos. The microdevice has a rectangular well with an array of

microwells at the bottom and an integrated dissection board nearby. Worms are placed on the board, cut with a scalpel and the fragile embryos are gently swept towards the microwell array. Embryos settle onto the bottom of the microwells, where they rest on a coverslip, being protected from flow by the microwell walls. Permeable embryos are acutely exposed to drugs after the medium in the microwells is exchanged by aspiration of the existing medium and dispensing of a medium with the drug into the well with a pipette. The technique was tested with 4 common inhibitors: the microtubule inhibitor nocodazole, actin inhibitor latrunculin A, myosin inhibitor blebbistatin, and proteasome inhibitor c-lactocystin-β-lactone. All 4 inhibitors had the expected effects on permeable embryos and no effect on control impermeable embryos.

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Label-free imaging of the trabecular meshwork of the eye using Coherent Anti-Stokes Raman Scattering (CARS) microscopy.

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Objective: Dysfunction of fluid outflow through the trabecular meshwork (TM) region of the eye is the primary cause of glaucoma. Our objective was to image the human TM using a non-invasive, non-destructive technique without the application of exogenous label.
Methods: Flat-mounted trabecular meshwork samples from a human cadaver eye were imaged using two non-linear optical techniques: coherent anti-Stokes Raman scattering (CARS) and two-photon autofluorescence (TPAF). In TPAF, two optical photons are simultaneously absorbed by autofluorescence molecules such as collagen and elastin. The CARS technique uses two laser frequencies to specifically excite carbon-hydrogen bonds, allowing the visualization of lipid-rich cell membranes. Multiple images were taken along an axis perpendicular to the surface of the TM for subsequent analysis.
Results: Analysis of multiple TPAF images of the TM reveals the characteristic overlapping bundles of collagen of various sizes. Simultaneous CARS imaging revealed elliptical structures of ~7x10 µm in diameter populating the meshwork which were consistent with TM cells. Irregularly shaped objects of ~4 µm diameter appeared in both the TPAF and CARS channels, and are consistent with melanin granules.
Conclusions: CARS techniques were successful in imaging live TM cells in freshly isolated human TM samples. Similar images have been obtained with standard histological techniques, however the method described here has the advantage of being performed on unprocessed, unfixed tissue free from the potential distortions of the fine tissue morphology that can occur due to infusion of fixatives and treatment with alcohols. CARS imaging of the TM represents a new avenue for exploring details of aqueous outflow and TM cell physiology.

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Real-time hyperspectral fluorescence imaging of β-cell insulin-secreting dynamics with Image Mapping Spectrometer (IMS).

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Ca2+ and cAMP are important second messengers that are involved in the regulation of insulin secretion [1]. Although previous studies have suggested that Ca2+ signal pathway directly

interacts with cAMP production, the underlying mechanisms remain unresolved, especially regarding the role of Ca2+ in regulating the cAMP oscillation following both glucose and hormone secretion. Recently, a range of fluorescent-Protein (FP)-based biosensors have been developed [2], which allows the dynamics of cAMP to be imaged in living cells. However, since most FP-based biosensors spectrally overlaps with Ca2+ indicator – Fluo4, it is difficult for traditional cameras to image FP-based biosensor and Ca2+ indicator Fluo4 simultaneously.
In our study, a novel hyperspectral imaging device – Image Mapping Spectrometer (IMS) has been utilized to monitor insulin-secreting cellular dynamics. The IMS is a widefield method acquiring full spectral information simultaneously from every pixel in the field-of-view [3]. It works by spatially distributing (i.e. mapping) neighboring image zones to create space between them. A spectrally dispersive element then spreads the wavelength content from each zone into this space which is recorded by a CCD detector. Since no scanning is employed in the IMS, The IMS system offers significant advantages in spectral imaging speed (currently up to 7.2 fps) and signal collection (~ 85% optical throughput) [4].
To demonstrate the capabilities of the IMS in cellular applications, we measured the drug response of cAMP production (indicated by a FP-based biosensor - Epac) and Ca2+ flow (indicated by Fluo4 dye) in living MIN6 ß-cells. Then, the cAMP oscillations in the islet and their relation to Ca2+ oscillations have been investigated as a function of glucose and other perturbative factors in the insulin secretion pathway. Our data indicate that, upon initialization of cAMP production, the signaling cascades between Ca2+ oscillations and cAMP oscillations are interconnected at the time scale of a sec. These findings prove that significant progress in elucidating the roles of Ca2+ and cAMP in ß-cell function is possible with the simultaneous monitoring provided by the IMS system.

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High-speed quantum dots tracking reveals small-scale short-term compartmentalized diffusion in plasma membranes.

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In this work, we show that tracking of single quantum dots (QDs) is possible at image acquisition rates up to ~2,000 Hz. We image with a spatial precision of ~40 nm for several seconds (9,000 frames) at this high-speed imaging rate using a standard wide-field fluorescence microscope and an EMCCD camera.
Using this system, we show that membrane proteins and lipids, which have been exogenously labeled with functionalized QDs display three types of motion in the plasma membrane of live cells; free diffusion, confined diffusion, and compartmentalized diffusion where the molecules moves between compartments of ~100 nm in diameter within a lifescale of ~100-200 ms (also termed hop-diffusion). We further investigate the cholesterol dependence of the compartmentalized diffusion.
Such observed dynamic features will always be dependent on three parameters; the diffusion rate of the investigated molecule, the compartment size, and the image acquisition rate. If not imaged at sufficient speed, compartmentalized diffusion will appear either as free or confined diffusion but at a slower diffusion rate. Therefore, we compare our experimental result with

computer simulations to find the relation between the diffusion rate, the compartment size and the image acquisition frequency.
The QDs used in this imaging system has a signal brightness and stability advantage as compared to conventional fluorophores allowing for long-term fast imaging, and a size advantage to gold particles used for high-speed single particle tracking.

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Polarized TIR-FM of sub-resolution membrane curvature during endocytosis and viral budding.

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Induction of membrane curvature is an essential step in the formation of endocytic vesicles and viral particles. Electron microscopy can be used to visualize membrane curvature in fixed cells, however, these techniques cannot be used to visualize the dynamics of the biochemical events influencing bilayer structure. Axelrod and co-workers have shown that certain fluorescent dye molecules can orient in the lipid bilayer and can be selectively excited by polarized light thereby enabling detection of membrane curvature in a fluorescence microscope. Therefore, we investigated whether polarized Total Internal Reflection Fluorescence Microscopy (TIR-FM) could be used to visualize endocytosis and viral budding in cells labeled with the lipophilic fluorophore DiI. s-pol and p-pol TIRF fields were created by positioning a beam laser at distinct azimuthal positions in the back focal plane using a commercial two-dimensional scan head. s-pol images preferentially excited DiI molecules in membrane parallel to the coverglass and p-pol preferentially excited DiI molecules orthogonal to the coverglass. Ratios of p/s were calculated to generate images of membrane curvature and images of p+2s allowed visualization of total membrane. Images of clathrin-YFP and HIV Gag-YFP were collected simultaneously to visualize sites of endocytosis and viral assembly.

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***In vivo* Imaging of Plasmid DNA Reporters in Skeletal Muscle.**

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To date, skeletal muscle differentiation and development has been heavily researched and intense focus has been placed on understanding these processes as they relate to disease. While recent advances have yielded many insights into the transcriptional and post-transcriptional mechanisms that control these processes, the difficulty of mammalian *in vivo* experiments has limited many of these findings to *in vitro* models. A system which would allow the real-time measurement of transcriptional and post-transcriptional regulation events both spatially and temporally, in living animals, would greatly augment our current cellular and molecular understanding of skeletal muscle. In an attempt to achieve this we have developed a novel system to visualize reporter activity in skeletal muscle myofibers. Using hydrodynamic limb vein injection (HLV) of plasmid DNA, combined with *in vivo* fluorescent and bioluminescent imaging, we can monitor the activity of reporters and quantify the signal using Region of Interest (ROI) analysis. This allows measurement of promoter regions temporally during regeneration in C57BL/6 mice and also in the mdx4cv muscular dystrophy model. There is also an interest in understanding the pathophysiological role that microRNAs play in skeletal muscle disorders, and in muscle regeneration following injury. Here we show that this technique can also be used to monitor in real time the activity level of endogenous microRNAs by using complementary microRNA sequences inserted into the firefly luciferase 3’-UTR.

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Quantification of homo and hetero oligomerization between paralogous proteins in Saccharomyces cerevisiae.

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Introduction

A trascendental aspect related to the quaternary organization of paralogous proteins is the study of the transition from homomeric to heteromeric structures and the importance of this transition in the appearance of new functions. Such functional versatility may imply a mayor stability of the complexes, the acquisition of different kinetic properties or changes in the interaction networks with other proteins. The simultaneous existence of different isoforms between products of duplicated genes, their impact in the physiology and biochemistry of *Saccharomyces cerevisiae* were assessed in this study.

Methodology

Saccharomyces cerevisiae strains Y8205 MATα y BY4741 MATa were used in this study. The analysis of the different interactions between paralogous proteins was assessed by BiFC. This technique consists in the phusion of complementary half's of GFP to the paralogous proteins. If there is an interaction between these proteins, the GFP is reconstituted producing a fluorescence signal which can be quantified by confocal microscopy and flow cytometry.

Results

The pertinent constructions to determine the formation of oligomers of the following paralogous proteins pairs: *ENO1, ENO2, GDH1, GDH3, HXK1, HXK2, LEU4, LEU9, LYS20, LYS21, PYC1, PYC2, TDH1, TDH2, TDH3, URA7, URA8* were made. Using flow cytometry the dynamic fluctuations of the formation of different isoforms (homo- and hetero-oligomers) was evaluated for the first time.

Conclusions

A very important aspect in the study of protein-protein interactions is the quantitative analysis to determine the frequency of homomeric and heteromeric interactions. Recently the dynamic of the protein associations in *S. cerevisiae* was assessed by BiFC quantifying the product of this interaction with flow cytometry which allows to:

Identify that the hetero-oligomerization between products of duplicated genes in *S. cerevisiae* occurs frequently.
Describe the simultaneous existance and the relative prevalence of the different oligomeric states between paralogous proteins.
For certain paralogous proteins the hetero-oligomeric form predominates in at least one metabolic condition.

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PhenoRipper: A Novel Platform For Rapid Image Analysis.

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Fluorescence microscopy provides a high-content molecular profiling platform to investigate the relationship between complex cellular phenotypic states of biomarkers with quantities of biological and clinical interest, such as tumor type or disease progression. Automated analysis of microscopy images has the potential to act as a standardized aid for tumor classification and

biomarker discovery, particularly for subtle or uncharacterized phenotypes. While many computational tools exist to characterize cellular phenotypes, they require significant sample dependent fine tuning. In particular, cellular segmentation, a prerequisite for most analysis schemes, remains a challenging problem in general, and is often intractable on tumor samples. Thus without dedicated image-analysis experts, there is a significant barrier to automated image analysis by life scientists.
Here we present PhenoRipper, an easy to use, image analysis software package that enables rapid exploration and interpretation of microscopy data. PhenoRipper requires minimal user input or training, effectively making it a turnkey solution for the comparison and classification of microscopy images. Users can also correlate PhenoRipper results with image phenotypes and any metadata provided by the user.
In tissue samples, we show that PhenoRipper can distinguish tumors from non-tumor regions. In HeLa culture, we show that different groups of perturbations can be grouped together: drugs with similar mechanism of action cluster and knockdowns of related genes are found to have similar phenotypic effects in a siRNA screen. Despite requiring virtually no tuning of algorithmic parameters, the classification performance of PhenoRipper rivals that of established methods, while taking an order of magnitude less computational time. Additionally, PhenoRipper provided insights into the presence of subpopulations and their fractional changes across conditions.
PhenoRipper is an unsupervised, exploratory tool for analysis of high-content images which will enable bench-scientists to perform rapid exploratory analysis of their data soon after acquisition. We believe that the rapid turn-around between analyses and imaging made possible by PhenoRipper will make it a useful tool during assay development and for bio-marker selection while PhenoRipper's simplicity will encourage automated quantification of samples whose complexity has so far limited such analysis.

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Quantitation of collagen alignment: tools for characterizing cancer progression and invasion.

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Collagens are the most abundant proteins in mammalian bodies and a major protein constituent of the extracellular matrix (ECM), which principally maintains shape and structural integrity for cells and tissues, and plays an important role in wound healing, tissue repair and morphogenesis. Of particular interest is the involvement of stromal ECM in the formation, growth and progression of tumors. It has been shown that the physical characteristics of stromal collagen influence and are influenced by tumor initiation and local invasion into the stroma. An important facet of this relationship is the local reorganization of collagen fibers at the tumor-stroma boundary that facilitates invasion of transformed epithelial cells into the surrounding ECM, with tumor cells migrating directly along collagen fibers. Using the nonlinear optical imaging techniques of multiphoton laser-scanning microscopy (MPLSM) and second harmonic generation (SHG) imaging, specific and consistent changes in stromal collagen organization at various stages of breast cancer progression have been detected. Of particular relevance, two of these signatures differentiate non-invasive from invasive characteristics of the primary tumor and, as such, correlate with metastatic potential. Specifically, non-invasive regions are contained by collagen fibers oriented parallel to the tumor boundary while regions of local invasion possess areas where collagen has been realigned perpendicular to the tumor boundary to facilitated local invasion. Therefore, it has been hypothesized that the angle of

collagen fibers relative to the tumor boundary may be used as a predictor of imminent invasion and metastasis. Hence, the ability to quickly and accurately quantify both fiber angle and any related structural changes of the collagen matrix, in conjunction with cellular morphology and concurrent molecular events, could result in an effective experimental and diagnostic tool. To this end, we have developed two semiautomated tools based on the Fast Discrete Curvelet Transform (FDCT) for detecting the orientation of collagen fibers in biological Issues.

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Minimizing Detection Errors in Single Molecule Localization Microscopy.

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Fluorescence microscopy using single molecule imaging and localization (PALM, STORM, and similar approaches) has quickly been adopted as a convenient method for obtaining multicolor, 3D superresolution images of biological samples. Using an approach based on extensive Monte Carlo simulations, we examined the performance of various noise reducing filters required for the detection of candidate molecules. We determined the best noise reduction method among those tested, and derived an optimal, nonlinear threshold which minimizes detection errors introduced by conventional algorithms. We also present a new technique for visualization of single molecule localization microscopy data based on adaptively jittered 2D histograms. We used our new methods to image both Atto565-phalloidin labeled actin in fibroblast cells, and mCitrine-erbB3 expressed in A431 cells. The enhanced methods developed were crucial in processing the data we obtained from these samples, as the overall signal to noise ratio was quite low. Low SNRs in SMLM data can result in significant numbers of missed molecules, and worse, an unacceptably high rate of false positive detections. Systematic analysis of the algorithms used for the detection of candidate molecules can significantly reduce the problem. Our results also suggest the use of a nonlinear threshold boundary on the measured data, combining both the signal to noise ratio and the imaged sizes of the detected objects. This approach minimizes detection errors in an automated and unbiased way.

P. Křížek, I. Raška, and G. M. Hagen, 2011, "Minimizing detection errors in single molecule localization microscopy," *Optics Express*, 19, p. 3226.

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Functional and molecular diversification of the endocytic pathways and their cell-to-cell variability.

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Endocytosis has evolved from a simple pathway in single-cell eukaryotes into a highly complex network of uptake mechanisms and intracellular trafficking routes in mammalian cells. We currently do not have a systematic functional genomics view of this complexity and how it has emerged. We conducted parallel RNAi screens targeting 1,183 genes on the endocytosis of Transferrin, Cholera Toxin B, Low Density Lipoprotein, Epidermal Growth Factor, GPI-anchored GFP, Dextran via fluid phase endocytosis, and Dextran via EGF-induced macropinocytosis in human tissue culture cells. This was complemented with screens on subcellular patterns of molecular markers of early endosomes, late endosomes, the Golgi complex, and caveolae. Using automated image analysis of app. 10^6 single cells per screen, combined with novel multivariate statistics incorporating population context-determined cell-to-cell variability, we uncover that endocytosis routes have different, mutually exclusive patterns of cell-to-cell variability. By accounting for this cell-to-cell variability, we reveal a first unbiased quantitative molecular classification of the endocytic pathways. From the classification tree, we identified 200 genes determining the core diversification machinery. Evolutionary analysis of these genes

in 18 genomes covering most branches of the tree of life, revealed hierarchical organization of conserved endocytic modules. Besides the core machinery of diversification that acts in all cells, we also reveal a regulatory machinery, enriched in protein kinases and regulators of GTPases, which controls endocytic pathways in subsets of cells as a function of population context, coordinating the establishment of mutually exclusive patterns of cell-to-cell variability of endocytosis routes.

MONDAY, DECEMBER 5

Actin and Actin-Associated Proteins II

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The small GTPase Rif triggers formation of actin stress fibre in epithelial cells.

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The Rho family of small GTPases is the critical regulators of the actin cytoskeleton. We found that small Rho GTPase Rif triggers formation of one kind of key element of the actin cytoskeleton; actin stress fibres. Actin stress fibres are composed of bundles of actin filaments, which allow the cell to apply contractile force to its body. It is widely accepted that stress fibre formation is controlled by the small GTPase RhoA and two highly related proteins, RhoB and RhoC, representing the canonical stress fibre formation pathway. The work we have done showed that Rif GTPase triggers the stress-fibre formation in epithelial cells in an alternative pathway. Rif is distantly related to RhoA; however, we show that the two proteins share a common downstream partner in stress-fiber formation - the Diaphanous-related formin mDia1. Rif-induced stress fibers also depend on the activity of the ROCK protein kinase. This study establishes Rif as a general regulator of Diaphanous-related formins and shows how non-classical Rho family members can access classical Rho pathways to create new signaling interfaces in cytoskeletal regulation.

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Differential regulation of actin microfilaments by human MICAL proteins.

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The Drosophila melanogaster MICAL (D-MICAL) protein is essential for the neuronal growth cone machinery that functions through plexin- and semaphorin-mediated axonal signaling. D-MICAL is also involved in regulating myofilament organization and synaptic structures, and serves as an actin disassembly factor downstream of plexin-mediated axonal repulsion. In mammalian cells there are three known isoforms: MICAL1, MICAL2 and MICAL3, as well as the MICAL-like proteins MICAL-L1 and MICAL-L2, but little is known of their function, and information comes almost exclusively from neural cells. In this study we show that in non-neural cells human MICALs are required for normal actin organization, and all three MICALs regulate actin stress fibers. Moreover, we provide evidence that the generation of reactive oxygen species by MICAL proteins is critical for their actin-regulatory function. However, while MICAL1 is auto-inhibited by its C-terminal coiled-coil region, MICAL2 remains constitutively active and affects stress fibers. These data suggest differential but complementary roles for MICAL1 and MICAL2 in actin microfilament regulation.

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Filamin CH domains bind to F-actin in an open conformation.

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Filamin is a large non-muscle protein participating in numerous cellular processes which lead to reorganization of the actin cytoskeleton. This protein crosslinks actin filaments into bundles or a

three-dimensional gel. The protein contains an N-terminal F-actin-binding domain (ABD) and an elongated flexible segment with numerous immunoglobulin-like repeats. The ABD contains two calponin homology (CH) domains similar to those in α-actinin. Like α-actinin, filamin forms a homodimer.
We used electron microscopy and three-dimensional reconstruction to study the complex of F-actin with the filamin ABD. The reconstruction shows that the filamin tandem CH domains bind to F-actin in open conformation, where only one CH domain is bound while the other is disordered. This is very similar to what we observed with the tandem CH domains of α-actinin when bound to F-actin (Galkin *et al.*, Nature Struct. Biol. **17**, 614-616, 2010). This result confirms our earlier suggestion that the opening of these domains when binding to F-actin may be one of the main regulatory mechanisms for actin-binding proteins containing tandem CH domains.

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Profilin I and II are both influencing SRF-dependent signalling in B16 melanoma cells and loss of Profilin I interferes with cell migration.

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Cellular functions such as migration, adhesion, endo/exocytosis, intracellular trafficking and the maintenance of cell shape and polarity are all dependent on the actin microfilament system - its dynamic remodelling and force-generation.

Profilin is a key regulator of actin polymerization. It binds to actin monomers and forms the profilin:actin (P:A) complex, which delivers actin monomers to growing filament (+)-ends in a process that is enhanced by nucleation and elongation promoting factors like Ena/Vasp and the formins. Profilin binds a number of other proteins and also lipids of the phosphatidylinositol family. It has been reported that both actin and profilin are present in the nucleus, however it is less clear to what extent the P:A complex is formed and functions in the nucleus.

Actin is coupled to gene expression control through the serum response factor (SRF)-pathway. SRF is a transcription factor which controls a large number of genes, including many encoding microfilament associated proteins like actin itself and profilin. It 'senses' cytoplasmic changes in actin dynamics through its co-activator megakaryocytic acute leukemia (MAL), which is an actin monomer binding protein that rapidly shuttles to the nucleus when dissociated from the actin. It competes with profilin for actin binding and upon serum stimulation and dissociation from actin, it accumulates in the nucleus where it cooperates with SRF to induce gene expression.

In this study we report on the connection between SRF/MAL and actin dynamics with respect to the role of profilin (isoforms I and II); we have observed that down-regulation of profilin I expression using siRNA interferes with the nuclear accumulation of MAL fused to GFP. Furthermore, siRNA-depletion of profilin I and II represses SRF/MAL-controlled transcription as seen using a SRF reporter system. Migration of profilin-depleted cells was slightly reduced.

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The Forked Proteins of Drosophila and Their Relationship to the Espin Actin-Bundling Proteins of Vertebrates.

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The forked proteins are required to assemble large parallel actin bundles in the developing neurosensory bristles of *Drosophila* pupae. Although the forked proteins are putative orthologs of the espin actin-bundling proteins of vertebrates, relatively little is known about their activities. Espins, which are present in a variety of parallel actin bundle-containing structures (hair cell stereocilia, microvilli, and Sertoli cell junctions), cross-link F-actin into parallel actin bundles and are the target of the jerker deafness mutation, which causes espin deficiency and results in abnormally thin, unstable stereociliary actin bundles in mice. Here, we uncover some activities of the forked proteins and clarify their relationship to the espins. When expressed in transfected LLC-PK1-CL4 epithelial cells the major forked isoform, forked A, was targeted to the nucleus and assembled large nuclear actin bundles. This unexpected result was also observed in Cos-7 cells and *Drosophila* S2 cells. Untagged forked A also formed nuclear actin bundles. An examination of forked A fragments revealed that the protein is composed of two major functional domains: an N-terminal domain that targets the nucleus, owing to a previously unrecognized nuclear localization signal, and a C-terminal domain that targets microvillar actin bundles (LLC-PK1-CL4) or stress fiber-like actin bundles (Cos-7), by analogy to espins. Accordingly, the purified recombinant C-terminal domain of forked A bound and bundled F-actin in vitro similarly to espin constructs. The identification of the forked peptides and amino acids required revealed a degree of homology to espins greater than that appreciated previously. Since certain espin isoforms contain functional nuclear localization signals, we suggest that the forked and espin proteins constitute a family of evolutionarily related actin-bundling proteins that can transit between cytoplasmic actin bundles and the nucleus (NIH DC004314 to JRB).

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Cortactin knockdown reduces tubulobulbar complex length.

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Tubulobulbar complexes are podosome-like double membrane structures that internalize intercellular junctions in the seminiferous epithelium of mammalian testis. These actin-based projections possess a similar protein network to podosomes that includes N-WASp, Arp2/3, cortactin and dynamin. Cortactin depletion by RNA interference results in a loss of podosomes in osteoclasts. Here, we test the prediction that knockdown of cortactin in the seminiferous epithelium will cause a similar loss of tubulobulbar complex formation. To target cortactin, we used an *in vivo* knockdown strategy using SD strain rats. In each animal, one testis was surgically exposed and injected with RNAi reagents. The other testis served as a control by receiving the same treatment with a non-targeting reagent. After three days and in each experiment, control and experimental testis were removed and prepared for analysis by electron microscopy, immunofluorescence and immunoblotting. Two animals were used for each analysis. Immunofluoresence microscopy revealed a remarkable phenotype in RNAi-treated testis in comparison to control testes. Rather than a complete loss of tubulobulbar complexes, the complexes appeared shorter. Immunoblots confirmed that cortactin expression was less in experimental testes in comparison to control testes. To be certain that the phenotype was caused by siRNA, we repeated the experiment with a dye modification on one of the four targeting siRNA sequences. On cryosections of seminiferous epithelium, dye clusters could be detected in Sertoli cells confirming that reagents entered the cells. Our results indicate that normal levels of cortactin are essential for tubulobulbar complexes to acquire or maintain their normal length. Supported by an NSERC Discovery grant to AWV.

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The prognostic role of fascin-1 in cancer progression-Insights from epidemiology and cell biology.

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Fascin-1 is a 55-kDa actin-bundling protein that functions in forming parallel actin bundles in cell protrusions. Fascin-1 is low or absent in most normal adult epithelia, yet its expression in carcinomas from many tissues correlates clinically with aggressive disease and reduced patient survival. This has raised interest in fascin-1 as a novel biomarker and potential therapeutic target for aggressive, metastatic carcinomas. However, many clinical studies have been based on relatively small numbers of samples. The carcinoma types in which fascin-1 might be of most relevance remain unclear. We are therefore taking combined epidemiological and cell biology approaches to analyse the role and regulation of fascin-1 in carcinomas. A systematic review and meta-analysis of the association of fascin-1 expression with cancer-specific survival in breast, colorectal and oesophageal cancer has identified that up-regulation of fascin-1 protein is associated with increased risk of cancer-specific mortality in breast, colorectal and oesophageal cancer patients. In parallel, we are studying molecular mechanisms of *FSCN1* transcription. CREB and AhR are major, specific regulators of FSCN-1 promoter activity in human breast and colon carcinoma cells [Hashimoto et al. PLoS One 2009. **4**: e5130]. We have extended analysis to androgen responsive and non-responsive prostate carcinoma cells as new early biomarkers for aggressive disease are urgently needed in prostate cancer. With luciferase promoter reporter assays, we demonstrate that the *FSCN1* promoter region -219/+1 is active in androgen responsive PC3 and DU-145 cells and non-responsive LNCaP cells. The conserved CREB and AhR binding motifs within this promoter region are required for promoter activity in prostate carcinoma cells. These results suggest that regulation of FSCN1 promoter activity in different carcinomas occurs by common transcriptional regulatory pathway mechanisms which depends on CREB and AhR. The meta-analysis results highlight the potential value of fascin-1 as a novel biomarker and therapeutic target and the need for more clinical studies to elucidate role of fascin-1 in progression of breast and prostate carcinomas. *Supported by the Wellcome Trust.*

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CARMIL Removes Capping Protein from the Barbed End by an Allosteric Mechanism.

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Regulation of capping protein (CP) is important for controlling polymerization at free barbed ends of actin filaments. In lamellipodia of migrating cells, CP bound to F-actin dissociates quite rapidly, suggesting the possibility of uncapping. A CP-interacting motif (CPI) capable of inhibiting capping and promoting uncapping is found in a set of otherwise unrelated proteins. The protein CARMIL contains a CPI motif plus another CP-binding motif specific to CARMIL, termed CSI (CARMIL-specific interacting). In vitro, a 115-aa CP-binding region (CBR) of CARMIL, which contains the CPI and CSI motifs, binds to CP with high affinity, inhibits capping, and promotes uncapping. We asked how CARMIL binding to CP inhibits capping and promotes uncapping. Structural and biochemical studies have shown that the actin-binding surface of CP is located on the top of the mushroom-shaped molecule, and that the CPI and CSI motifs both

bind to the under surface of the mushroom cap. Here, we used a collection of mutants affecting the actin-binding surface of CP to test the possibility of a steric-blocking model, which had remained open because a region of CBR between the CPI and CSI is unresolved in the CBR / CP co-crystal structure. The CP actin-binding surface mutants bound CBR normally, arguing against a steric-blocking model. In addition, a mutant form of CBR in which all the amino acids of the unresolved region had been changed showed nearly normal binding to CP, also arguing against steric blocking. To test the possibility of an allosteric model, we used molecular dynamics and principle component analysis on free CP as well as CP bound to actin and bound to CBR. We conclude that the CBR of CARMIL promotes uncapping by binding to a freely accessible site on CP bound to a filament barbed end and inducing a change in the conformation of the actin-binding surface of CP. This biochemical process has physiological relevance for actin assembly in migrating cells.

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N-WASP independent role of CR16 in the formation of filopodia through IRSp53.

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The Neural Wiskott Aldrich syndrome protein (N-WASP) belongs to the WASP/WAVE family of proteins and is a key regulator of actin dynamics, which is critical for many cellular processes such as cell migration and cell adhesion. The mammalian verprolins are known regulate the actin cytoskeleton through WASP dependent and WASP independent pathways. The primary objective was to identify the role of corticoid regulated (CR16) protein in remodeling the actin cytoskeleton, in an N-WASP independent manner. CR16 is a proline rich protein which interacts with N-WASP through the C-terminal WBD (WASP binding domain). In order to identify other binding partners of CR16 we performed a yeast two-hybrid screen using CR16, as bait. Using this screen, we identified IRSp53 as one of the interactors of CR16. The activity of CR16 was characterized by expressing CR16 together with IRSp53 in N-WASP-/- mouse embryonic fibroblasts (MEFs), which showed that CR16-IRSp53 complex, caused a significant increase in filopodia induction. IRSp53 is an adaptor protein with an IMD (IRSp53 and MIM (missing in metastases) homology Domain) located at the N-terminus, a GTPase Binding Domain (GBD) and a SH3 domain. IRSp53 mutants with mutations in the IMD domain (IRSp534A), the CRIB domain (IRSp53I267N) and the SH3 domain (IRSp532A) were further transfected along with CR16 and a reduction in the filopodia induction was observed. The interaction between CR16 and IRSp53 is regulated by Cdc42 (member of Rho family of GTPase) as expression of CR16 and IRSp53 with constitutively active form of Cdc42 (cdc42G12V) resulted in membrane localization however when expressed in conjunction with dominant negative mutant of Cdc42 (Cdc42T17N) a loss of localization was observed. In conclusion, CR16 interacts with IRSp53 in concert with Cdc42 and induces filopodia formation independent of N-WASP.

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Identification and characterization of a unique region of WASP in Jurkat T-cells chemotaxis.

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Wiskott-Aldrich syndrome protein (WASP) and its homologue Neural-WASP (N-WASP) are the members of WASP family of proteins which act as nucleation promoting factors and integrate receptor signalling with actin cytoskeleton reorganization. WASP is expressed predominantly in hematopoietic cells compared to N-WASP which is expressed ubiquitously and absence of WASP expression in hematopoietic system leads to Wiskott-Aldrich syndrome (WAS). WASP and N-WASP share more than 50% sequence homology, having similar binding partners and

basic functions. Although WASP and N-WASP both are expressed in hematopoietic system, N-WASP can only partially compensate for the loss of WASP in WASP knockout hematopoietic cells suggesting that WASP may have some unique functions compared to N-WASP or their activity may be regulated by different mechanism. Here we have identified a unique region of WASP which is rich in proline residues. Using yeast two hybrid system, we have found that this region of WASP interacts with 8 SH3 domain containing proteins out of 17 known WASP Proline Rich Region interacting proteins tested. In resting state, WASP has been shown to adopt a closed conformation in which an auto-inhibitory loop is formed through the binding of the VCA domain to the upstream GTPase binding domain (GBD). Using Bi-molecular Fluorescence Complementation (BiFC) assay; we found that deletion of this region of WASP relieved WASP from its closed conformation even in the presence of WIP. We further identified the role of this region of WASP in WASP knockdown human Jurkat T-cells. Knockdown of WASP expression in Jurkat T-cells does not affect the expression of N-WASP. We tested the chemotactic response of WT and WASP knockdown Jurkat T-cells in response to chemokine SDF-1á using Dunn chamber and found to be impaired in WASP knockdown Jurkat T-cells which were rescued by expression of WT WASP. We will use the WASP knockdown Jurkat T-cells to determine the the role of the unique region in chemotaxis and T-cell activation.

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Cooperative binding of drebrin to actin filaments.

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Drebrin A is a major brain-specific actin-binding protein localized in dendritic spines – the dynamic, actin-rich dendrite-specific structures that participate in signal transmission to the cell body of neurons. Reduced drebrin levels have been reported in patients with Alzheimer’s disease and Down syndrome. This study tested the possible cooperativity of drebrin interaction with F-actin on a single filament level. Atomic force microscopy (AFM) experiments revealed that N-terminal drebrin construct (sequence 1-300, Drb1-300) causes the same morphological changes in actin filaments as the full length drebrin A. This result is in a good agreement with our previously reported solution binding data and makes Drb1-300 a suitable tool to investigate the effects of drebrin on F-actin morphology and dynamics. High resolution AFM imaging showed that under sub-saturating conditions Drb1-300 exhibits a cluster-like decoration of actin filaments, revealing positive binding cooperativity. Moreover, we observed the propagation of the drebrin-induced conformational changes on the neighboring ‘bare’ actin regions. Binding cooperativity was farther confirmed in a two-color TIRF experiments with Drb1-300KCK construct labeled by Cy3 fluorescent dye and then visualized on Alexa 488 palloidin-stabilized actin filaments. Cluster-like F-actin decoration was observed upon rapid complex dilution into F-buffer followed by its immobilization on polylysine coated coverslips. Our results provide evidence for cooperative binding of drebrin to actin filaments. Possible mechanisms and biological implications will be discussed.

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Drebrin Induces Stabilization of Actin Filaments.

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Drebrin is a mammalian neuronal protein that binds to and organizes filamentous actin (F-actin) in dendritic spines, the receptive regions of most excitatory synapses whose morphology was shown to play a crucial role in higher brain functions. Here, structural effects of drebrin on F-actin were examined in solution. Depolymerization and differential scanning calorimetry (DSC)

assays show that F-actin is stabilized upon decoration by drebrin. Along with full length drebrin, C-terminal truncated constructs of the protein were employed to clarify the domain requirements for the observed stabilization effects. Drebrin inhibits depolymerization at both barbed ends and pointed ends of a filament and increases thermal stability of F-actin by 0.5^{o}C. Because previous Atomic Force Microscopy (AFM) analysis showed that drebrin-A induced an increase in the helical pitch and stiffness of F-actin, we explored the molecular details of the observed changes by probing the effects of drebrin on lateral and longitudinal contacts in actin filaments. We have found that drebrin binding rescues the polymerization of V266G/L267G, a hydrophobic loop (H-loop) yeast actin mutant with an impaired lateral interface. In good agreement with these findings, Drebrin actin binding domain ($DrbABD_{233-300}$) increases the inter-strand disulfide cross-linking between Cys265 (H-loop) and Cys374 (C-terminal) in yeast S265C mutant F-actin. To probe the longitudinal interface, we use assembly incompetent tetramethyl rhodamine (TMR) labeled-actin, T203C/C374S yeast actin mutant and ECP cleaved actin (specifically cleaves the DNaseI-binding loop between Gly42 and Val43). Our results show drebrin rescues the polymerization of the ECP cleaved actin. Our data suggest that drebrin stabilizes actin filament through its effect on the inter-strand and intra-strand contacts in F-actin.

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Role of the formin FMNL3 in filopodia assembly and cell-cell adhesion.

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Formins are actin assembly proteins that can nucleate actin filaments as well as control filament elongation. Mammals possess 15 formin proteins, and the cellular functions of many formin isoforms are poorly understood. FMNL3 (also called FRL2) belongs to a sub-family of formins that can bundle actin filaments in vitro, in addition to its effects on nucleation and elongation. We have previously shown that over-expression of constitutively active FMNL3 constructs causes a dramatic increase in filopodia assembly in a variety of cell types (Harris et al (2010) Cytoskeleton). In this poster, we show that endogenous FMNL3 enriches strongly at the leading edge plasma membrane in NIH 3T3 cells, with particular enrichment at filopodia tips. The most dramatic enrichment in FMNL3 occurs in filopodia that are making contact with a neighboring cell. Live-cell microscopy shows that FMNL3-GFP-containing filopodia at the leading edge are are highly dynamic, growing and retracting on a timescale of minutes. In contrast, filopodia in contact with another cell are not dynamic, maintaining constant length for minutes. Following up on these observations, we are currently studying the role of FMNL3 in cell migration and cell-cell adhesion.

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Investigating the Mechanism of INF2-mediated Actin Filament Severing and Depolymerization.

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Formins are a class of proteins that accelerate actin nucleation, then influence actin filament elongation rate by remaining at the barbed end. INF2 is a biochemically unique mammalian formin in that it accelerates both actin polymerization and depolymerization. Importantly, mutations in INF2 lead to the kidney disease focal and segmental glomerulosclerosis (FSGS). I am elucidating the molecular mechanism of INF2’s unique depolymerization activity, using TIRF microscopy and other biochemical assays of actin dynamics. Prior work in our lab has shown that depolymerization requires both the FH2 and WH2/DAD sequences, and occurs in two steps: a) a severing step, which requires phosphate release from the actin subunits; and b) a depolymerization step, which requires the WH2/DAD. I used two INF2 constructs to test this

mechanism further: the FH1FH2C construct (containing the WH2/DAD); and the FH1FH2 construct (without the WH2/DAD). Using TIRF microscopy, I find that severing occurs throughout the length of the filament, and that the individually severed filaments depolymerize rapidly thereafter. This result suggests that INF2 binds filament sides prior to severing, and is supported by direct binding assays. INF2 can bind filament sides that are both phosphate-bound and phosphate-free, suggesting that phosphate release is not required for side binding but is required for subsequent severing. The FH1FH2 construct alone has depolymerization activity, albeit at much reduced levels compared to the FH1FH2C construct. I postulate that filament side binding by the FH2 domain changes the twist of the filament, which promotes severing and changes monomer dynamics at filament ends. I postulate further that the WH2/DAD enhances severing by insertional binding between actin subunits in the filament.

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Different modes of Spir-actin interaction.

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Spir is a WH2-containing actin nucleator, which is essential for establishing an actin mesh during Drosophila oogenesis. Spir was reported to not only nucleate filaments but also sever them and sequester actin monomers in a stable complex. Here we provide new evidence for Spir's ability to sever, albeit weakly, and depolymerize filaments. We measured Spir's severing activity in real-time with TIRF microscopy. Significant severing was only observed at concentrations of Spir stoichiometric to actin, and its severing activity was very slow compared to that of cofilin. We also observed significant depolymerization at the barbed end of actin filaments in these assays. When Spir was added to actin filaments in bulk assays, it induced their rapid depolymerization. Based on our single filament assays, we attribute this to Spir's sequestration activity rather than its weak severing activity. To probe the positioning of monomers by Spir into a nucleating complex, we performed velocity sedimentation and crosslinking experiments, and monitored excimer emission from pyrene-labeled actin mutants, Q41C (which reports longitudinal contacts) and S265C (to report lateral contacts). Under polymerizing conditions, both S265C and Q41C produce high excimer signals in the presence of Spir suggesting both lateral and longitudinal proximities of labeled sites in these complexes. Interestingly, we only observed crosslinking when Spir was mixed with S265C actin. Taken together, these data indicate that although the orientation of adjacent monomers is not precisely filament-like, they do form lateral contacts in the presence of Spir. Velocity sedimentation data show different binding modes of Spir-ABCD, which contains all four WH2 domains, and Spir-CD, which contains only two WH2 domains. A broad distribution of species can be seen with up to three monomers bound to ABCD. A narrower distribution of CD bound predominantly to two monomers was observed. Combining the crosslinking, spectroscopic, and velocity sedimentation data provides insight into the complex and dynamic Spir-bound lateral and longitudinal actin structures.

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Regulation of actin dynamics by the formin FMNL3 requires the C-terminus: Relationship to the formins INF2 and mDia1.

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Actin filaments are integral components in at least 15 cellular structures. Due to actin's widespread use, cells must rigorously control when and where new actin filaments assemble. Formin proteins are attractive candidates for site-specific actin polymerization regulation, being able to accelerate actin nucleation and to regulate filament elongation. Mammals possess

genes for 15 formin proteins. FMNL3 is a mammalian formin that can bundle actin filaments, in addition to influencing actin polymerization rates. In this work, we aim to delineate the molecular mechanism of FMNL3's effects on actin polymerization. Here, we find that the C-terminal 74 amino acids of FMNL3 dramatically accelerates actin polymerization and filament production by the FH2 domain, similar to recent work on INF2 and mDia1. We characterize an actin-binding motif within the C-terminus of FMNL3 that directly interacts with both actin monomers and actin filament barbed ends, slowing monomer polymerization and inhibiting filament elongation. This motif bears some similarity to WH2 motifs, although one conserved aliphatic residue has diverged in FMNL3. Both profilin and WH2 domains from other proteins (VopL and ciboulot) compete with FMNL3-Cterm for actin monomer binding, suggesting a common binding interface. Specific point mutations within this motif strongly inhibit the actin binding ability of the C-terminus. Curiously, however, these mutations do not influence the ability of the FH1-FH2-C construct to accelerate actin polymerization. Thus, the high affinity actin binding site in the C-terminus is not necessary for actin polymerization acceleration by FMNL3. In the context of the formin family, this work illustrates that, while the C-termini of three different formins (FMNL3, INF2, mDia1) can interact with actin monomers, these interactions differ greatly in both affinity of the interaction and consequence for actin polymerization.

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Effects of the actin cytoskeleton on Golgi architecture through the formins INF2 and mDia1.

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Formins represent a widely expressed family of actin assembly factors, accelerating nucleation and regulating barbed end elongation. Mammals have 15 formin genes. One mammalian formin, INF2, has the unique ability to accelerate both actin polymerization and depolymerization. INF2 can be expressed in two C-terminal splice variants, either the prenylated CAAX isoform, which is ER-localized, or the non-prenylated nonCAAX isoform, which localizes in an actin-dependent meshwork pattern. We have shown that INF2 expression is cell-specific, with the CAAX variant highly expressed in mouse fibroblasts, and the nonCAAX isoform expressed in U2OS human carcinoma and Hela cells. In this study we focused on elucidating cellular functions of the INF2-nonCAAX variant, particularly on its recently identified role in Golgi morphology. INF2 knockdown in U2OS cells causes dispersion of Golgi stacks, both the cis and trans compartments. In contrast, treatment with LatrunculinB for 30 min causes Golgi compaction, and reverses the INF2 knockdown effect. These results suggest an actin-dependent action of INF2 on maintenance of Golgi architecture, and we wished to examine this possibility in more detail. Analysis of confocal sections of U2OS cells reveals actin patches closely associated with the Golgi stacks. The number of these patches decreases about 30 % in cells with INF2 knockdown. Another formin, mDia1, which is known to interact with INF2, has an opposite effects on Golgi morphology and actin patch number. Knockdown of mDia1 results in Golgi compaction (similar to recently published work by Zilberman et al (2011) MBoC), and increases the number of Golgi-associated actin patches to 153% from control. INF2 and mDia1 also have different effects on some aspects of actin stress fiber morphology. INF2 knockdown causes a decrease in dorsal stress fibers, an increase in ventral stress fibers, and an increase in focal adhesion number and size. Additionally, ventral stress fibers in INF2 knockdown cells show greater resistance to Latrunculin B treatment than in control cells. In contrast, mDia1 knockdown reduces the number of ventral stress fibers and focal adhesions, but does not influence dorsal stress fiber number. Taken together, these data show that INF2 and mDia1 have opposing effects on both Golgi and stress fiber architecture. Currently, we are examining the relationship between Golgi, Golgi-associated actin patches, and stress fibers.

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Cooperation between Drosophila Spire and Cappuccino.

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The actin nucleators Spire (Spir) and Cappuccino (Capu) interact directly and are both essential for polarity establishment in the Drosophila oocyte. We are interested in why there are two nucleators in a single actin regulatory pathway. One possible model is that Spir acts as the filament-nucleating site, while Capu is the elongation factor, protecting the growing barbed end from capping protein. To test this model we are studying the interaction using a combination of binding assays, pyrene-actin polymerization assays and single filament fluorescence imaging to localize Spir and Capu on growing filaments. We found that the kinase non-catalytic C-lobe domain (KIND) of Spir binds with nanomolar affinity to a short (~30 aa) sequence at the very C-terminus of Capu, adjacent to the actin filament-nucleating FH2 domain. Although this sequence is distinct from the Diaphanous auto-regulatory domains (DADs) of Diaphanous-related formins, we found that the C-terminal tail of Capu plays a critical role in actin assembly similar to that recently described for DADs (Gould et al, 2011). Additionally, both DADs and the Capu tail bind to G-actin. We also found that in the presence of Spir-KIND, a construct containing the FH1, FH2, and tail domains of Capu was unable to protect the barbed end from capping protein. These observations explain how Spir binding to the Capu tail inhibits nucleation but not why processive elongation is blocked. Therefore, we are now investigating the role of the tail in Capu's barbed end elongation and F-actin binding activities. Our findings indicate that in order for the nucleation/elongation cooperation model to be true, there must be another component in the pathway that breaks the Spir-Capu interaction.

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Involvement of Formin in the Suppression of a Saccharomyces cerevisiae Mutant Actin-Associated Phenotype by a Mutant Profilin.

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In the S. cerevisiae actin-profilin interface, actin A167 of the barbed end W-loop and H372 near the C-terminus form a clamp around a profilin segment from residue R81 to Y79. Modeling suggests that altering steric packing in this interface may regulate actin activity. An actin A167E alteration, which occurs in muscle actin, should increase interface crowding and alter actin regulation. A167E actin causes a severe growth defect resulting in large part from mitochondrial dysfunction accompanied by loss of mt DNA staining and aggregation of mitochondrial fragments near the bud neck. To test this packing hypothesis, we altered Y79 to S hoping its decreased mass would relieve interface strain and rescue the mitochondrial phenotype. Y79S profilin alone caused no growth defect in WT actin cells but did rescue the mitochondrial defects caused by A167E actin. We then investigated the molecular basis of this rescue. WT profilin accelerates actin ATP exchange rate about 3X. Y79S profilin did not accelerate the ATP exchange rate of WT actin but slightly increased that of A167E actin. Thus, acceleration of actin nucleotide exchange by profilin does not appear to be important for WT yeast cells. Both mutant and WT profilins similarly inhibited either WT or A167E actin filament nucleation. Thus, Y79S profilin rescue does not appear to result from direct effects on actin polymerization. The yeast formin Bni1 elongates actin filaments using the actin-profilin complex. We thus tested the impact of A167E actin and Y79S profilin on formin-regulated actin polymerization. The Bni FH1-FH2

fragment accelerated WT actin polymerization more efficiently than A167E actin. WT profilin inhibited bulk Bni-mediated WT actin polymerization in a concentration-dependent fashion over the entire range tested. Lower WT profilin concentrations inhibited Bni-mediated A167E actin polymerization. However, with A167E actin, profilin/actin ratios higher than 1:1 produced a sigmoidal rather than the hyperbolic polymerization curve observed with WT actin. Y79S profilin exhibited approximately the same inhibition of formin-dependent WT actin polymerization as did WT profilin. However, Y79S profilin converted the sigmoidal behavior seen with WT profilin and A167E actin to the hyperbolic behavior associated with the WT protein mixture. This result indicates a rescue of the system by the mutant profilin to a WT-like state. In vivo, as visualized by binding to GFP-ABP140, WT actin cables moved from the neck to the base of the mother cell with an average rate of 1.8 μm/sec, whereas A167E actins moved with a rate of 2.2 μm/sec. However, cables in both WT and A167E cells expressing Y79S profilin moved significantly slower (1.5 and 1.4 μm/sec respectively). In summary, the phenotype caused by the A167E actin mutation appears to result from an inappropriate actin-profilin complex affecting its utilization by the formin Bni1 in regulating actin filament dynamics. The rescue observed with Y79S profilin seems to occur from restoring the appropriate use of this complex by formin.

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Autoregulation of the Drosophila Actin Nucleator Cappuccino.

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Formins are a conserved class of proteins known to enhance actin polymerization. Five of the seven groups of metazoan formins are regulated by autoinhibitory interactions between the C-terminal diaphanous autoregulatory domain (DAD) and the N-terminal diaphanous inhibitory domain (DID). Based on the lack of DID/DAD sequence homology, the formin group of formins is not predicted to be autoregulated. Previously published data for the *Drosophila* formin Cappuccino (Capu) is consistent with this prediction (Rosales-Nieves et al., NCB 2006). However, the mammalian ortholog of Capu, Formin-1, is autoinhibited (Kobielak et al., NCB 2004). We, therefore, re-examined Capu and found that it is, in fact, autoinhibited. In pyrene-actin polymerization assays, Capu's N-terminal half (Capu-NT) inhibits the C-terminal half's (Capu-CT) ability to stimulate actin polymerization. Furthermore, truncating the recently described C-terminal tail of Capu (Vizcarra et al., PNAS 2011) completely abolished Capu-NT's inhibition activity, suggesting Capu has an autoregulatory domain situated in a position similar to the DAD of other formins. Consistent with this observation, adding purified Capu tail to the polymerization assay in the presence of both Capu-CT and Capu-NT restored Capu-CT's activity. This result indicates that Capu tail binds to Capu-NT, freeing Capu-CT to stimulate polymerization. We measured a tight interaction between the tail and Capu-NT by fluorescence anisotropy (K_D~ 300 nM). Using truncations and limited proteolysis approaches, we mapped the Capu tail binding site or Cappuccino inhibitory domain (CID) to the first 321 residues of Capu. Interestingly, the Capu tail also binds to the Spire KIND domain (Pechlivanis et al., JBC 2009; Vizcarra et al., PNAS 2011). None of the single Capu tail point mutations sufficient to abolish the tail/KIND interaction affect the tail/CID interaction. We have identified two residues that contribute to tail/CID binding and the double mutant greatly diminishes the tail/CID interaction. Future experiments will be aimed at understanding the role of Capu autoinhibition in the presence of Spire and *in vivo*.

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Regulation of Actin Dynamics I

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Actin-dependent regulation of Golgi integrity by the formin, INF2.

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INF2 is a unique formin which can both polymerize and depolymerize actin filaments, with depolymerization requiring a severing step that depends upon ATP hydrolysis and phosphate release from the actin filament. Mutations in INF2 cause the kidney disease focal and segmental glomerulosclerosis. INF2 can be expressed as two C-terminal splice variants: CAAX and nonCAAX. The CAAX isoform contains a C-terminal prenyl group, and is tightly bound to the endoplasmic reticulum (ER). The localization pattern and cellular function of the nonCAAX isoform is not clear. Recently, INF2 was shown to interact with and inhibit another formin, mDia1. Our objective in this study was to understand the cellular consequences of INF2-mediated actin dynamics, and how INF2 activity is regulated. We find that INF2-CAAX and nonCAAX isoforms have dramatically different functions. While INF2-CAAX is ER-localized in an actin-independent manner, INF2-nonCAAX localizes in an actin-dependent meshwork pattern distinct from ER. INF2-nonCAAX is loosely attached to this meshwork, being extracted by brief digitonin treatment. Suppression of INF2-nonCAAX causes fragmentation of the Golgi apparatus, and this effect is counter-acted by treatment with the actin monomer sequestering drug, Latrunculin B. We find a reduction in actin patches around the Golgi apparatus upon INF2-nonCAAX depletion. Coupled with recent results showing that mDia1 causes Golgi dispersal, our hypothesis is that INF2 and mDia1 are counter-acting factors in maintenance of a cytoplasmic actin meshwork that influences Golgi integrity. We are currently testing this theory biochemically and in cells. Using full-length mouse INF2 expressed in insect cells, we show that purified INF2 is not subject to autoinhibition, despite possessing autoinhibitory sequences. However, INF2 expressed in a variety of culture cells does not cause aberrant actin polymerization unless the autoinhibitory regions are mutated or deleted, suggesting that additional factors are necessary for inhibition. The inhibitory DID region of mDia1 binds tightly to INF2’s C-terminal DAD sequence, but mDia1-DID alone does not inhibit INF2. We are currently testing reciprocal regulation between INF2 and mDia1 using full-length purified proteins.

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ITB3 encodes a novel regulator of ADF and is required for normal actin organization and Golgi body development in *Arabidopsis.*

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Introduction Actin filament turnover is required for many actin-dependent cellular processes including cell motility and membrane trafficking. Members of the actin depolymerizing factor/cofilin (ADF) family of actin-binding proteins are essential for severing and depolymerizing actin filaments. The predominant mode of regulation of ADF activity is by phosphorylation of a

conserved Ser residue near the N-terminus, which inhibits ADF binding to actin. We identified a protein of unknown function by map-based cloning of the *IRREGULAR TRICHOME BRANCH 3* (*ITB3*) gene in *Arabidopsis*. The *ITB3* gene is responsible for normal epidermal hair (trichome) shape; *itb3* mutants show reduced trichome expansion and branching. To uncover the function of ITB3, we carried out a yeast 2-hybrid screen for interacting proteins. We found that ADF interacts with ITB3.

Objectives The objectives of this study were to determine if ITB3 regulates ADF activity, and to determine if *itb3* mutants show defects in actin organization. Finally, we aimed to conceptually connect the trichome cell expansion defects observed in *itb3* mutants with putative changes in ADF activity.

Methods Pull-down assays showed that ITB3 and ADF interact in vitro. Actin organization in developing wildtype and *itb3* trichomes was observed using fluorescent phalloidin and confocal microscopy. *In vitro* actin polymerization assays were used to examine ADF activity in the presence of ITB3. Golgi bodies were observed in plants stably transformed with a YFP-sialyltransferase fusion protein.

Results We verified that ITB3 interacts with ADF *in vitro* and inhibits the interaction of ADF with actin. We found that developing trichomes of *itb3* mutants showed severe actin defects including an increase in the number of thick actin filament bundles culminating in an “actin knot” surrounding the cell nucleus. The *itb3* mutants also showed an increase in Golgi body number, but no discernible effect on Golgi body motility.

Conclusions Our results demonstrate that ITB3 functions as an inhibitor of ADF activity, and loss of ITB3 leads to severe actin organization defects *in vivo*. The major players in plant cell expansion –cellulose synthase, non-cellulosic polysaccharides, and cell wall proteins– are trafficked through the Golgi on their way to the plasma membrane and cell wall. Our finding that *itb3* mutants show Golgi body abnormalities suggests a connection between actin dynamics and cell expansion that goes beyond myosin-based transport of Golgi stacks.

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Roles for ADF/cofilin in membrane blebbing and new actomyosin cortex assembly.

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The contractile actomyosin cortex is important for cell morphogenesis and crucial for a form of cell motility based on dynamic plasma membrane bleb protrusions. The mechanism for new actomyosin cortex assembly is poorly understood. We show here that ADF/cofilin actin dynamizing proteins are essential for cortical actomyosin remodeling and new cortex assembly during blebbing. In human cells depletion of ADF/cofilin results in dynamic or persistent blebs dependent on residual levels of ADF/cofilin. At blebs, ADF/cofilin proteins are essential for modulating actomyosin turnover and for preservation of a pool of actin filaments that are utilized for new cortex assembly and consequently for bleb retraction. Efficient bleb retraction requires dynamic turnover of the newly assembled cortex, indicating further roles for ADF/cofilin proteins in this process. We present a model for the involvement of ADF/cofilin in the regulation of various stages of the bleb life cycle. Our findings implicate key roles for ADF/cofilin in the motility of cells, during normal development and cancer pathogenesis, which rely on dynamic membrane bleb protrusions.

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Phospho-Regulation of Cofilin is Essential for Shear Stress-Mediated Actin Microfilament Realignment.

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In vivo and *in vitro* evidence indicates that fluid shear stress (FSS) causes endothelial cell and actin microfilament alignment in the direction of FSS. Regulation of actin microfilament dynamics depends in part upon the Actin Depolymerizing Factor (ADF) family of proteins, of which cofilin is a prominent player. It is well-documented that phospho-cofilin is dephosphorylated by cofilin phosphatases, including chronophin and the slingshot (SSH) family of protein phosphatases which are known to be regulated by various external stimuli. Once dephosphorylated, cofilin induces the disassembly of actin filaments, promoting actin reorganization. Utilizing immunofluorescent staining and laser scanning confocal microscopy, it was previously determined that exposing bovine aortic endothelial cells (BAECs) to 15dynes/cm^2 FSS results in a decrease in cytoplasmic phospho-cofilin levels. This decrease in cytoplasmic phospho-cofilin levels was accompanied by an increase in nuclear phospho-cofilin and a slight increase in cytoplasmic total cofilin. These results suggest that under FSS, phospho-cofilin translocates to the nucleus, a finding seen in other cell types and under various cellular stresses. Working with the knowledge that cofilin location and possibly activity is altered during FSS, two cofilin mutants were employed to further understand its role in FSS-mediated actin microfilament realignment. A constitutively active, phosphorylation defective cofilin (Serine-3-Alanine – S3A) mutant construct and a constitutively inactive, phosphomimic cofilin (Serine-3-Aspartic Acid – S3D) mutant construct both disrupt multiple stages of the actin realignment process. The S3A cofilin mutant cells exhibit largely normal actin structures at 15 min and still form a cortical actin band at 30 min of 15dynes/cm^2 FSS, albeit the actin is less organized. The actin in the S3D mutant cells is disorganized at 15 min and fails to form cortical actin bands at 30 min of 15dynes/cm^2 FSS. Both S3A and S3D mutant cells fail to elongate and form stress fibers in the direction of FSS at 60 min. The S3D mutant cells also possess non-traditional actin structures which appear to be short actin clumps near cell nuclei. These results suggest that cofilin activity is involved in FSS-induced actin realignment and that the regulated phosphorylation and dephosphorylation of cofilin is necessary for proper realignment of the whole cell and actin microfilaments in the direction of FSS. Research in the Lowe-Krentz lab is supported in part by NIH grant HL54269.

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Effects of Caldesmon and Cofilin on the Depolymerization of Actin Filaments.

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We have previously reported that actin undergoes an obligatory conformational transition (maturation) during polymerization; caldesmon, or its C-terminal fragment, H32K, if present before the transition, inhibits this process and arrests the actin filament at the pre-transitional young state (Huang et al., JBC 285:71, 2010). Since phosphorylated caldesmon is localized to the cell periphery where the actin cytoskeleton undergoes active remodeling, it is thought that caldesmon therein could stabilize young actin filaments and promote actin dynamics. To test this hypothesis we have investigated actin filaments disassembly by monitoring the fluorescence change of pyrene-labeled actin. Upon dilution into either G- or F-buffer, actin filaments were protected against depolymerization by unphosphorylated H32K, but not by the mutant D3D4 that mimics ERK-phosphorylated protein, or in the presence of Ca^{2+}/calmodulin. The actin depolymerizing factor cofilin, when added to fully polymerized mature F-actin, resulted in a dose-dependent decrease of pyrene emission, with a stoichiometry of 1 per actin monomer.

The rate of the pyrene-actin fluorescence decrease was fast (~0.2 min^{-1}) and independent of the cofilin concentration. D3D4 showed no protection against cofilin-induced depolymerization. For H32K-bound mature filaments, addition of cofilin also resulted in a large drop of the pyrene fluorescence, but the rate was cofilin concentration-dependent and slower than that of actin alone, indicating the caldesmon fragment exerts some protection, especially when the amount of cofilin is low. For H32K-stabilized young filaments, cofilin apparently resulted in both displacement of H32K, thus allowing filament to mature, and subsequent severing of the filament. Since the mature filament exhibited unattentuated pyrene emission, the net fluorescence change depended on the concentration of cofilin relative to that of actin. Our results are consistent with that the ERK-phosphorylated caldesmon facilitates cofilin-mediated disassembly of actin filaments by maintaining the filament at the young state, which binds cofilin with a higher affinity, yet permitting severing to proceed. Supported by an NIH grant.

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Novel phosphorylation site(s) of cofilin by PKCα and its negative role in the degranulation from RBL-2H3 mast cells.

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Mast cells are known as the major effector cells in allergic diseases. Protein kinase C (PKC) activity is essential to the IgE-induced the release of histamine from mast cells. However, the role of individual PKC isoform is controversial and downstream molecules of PKCs are unknown in the degranulation. Here we show that PKCβI is a positive but PKCα is a negative regulator of degranulation from RBL mast cells using specific inhibitors and kinase-negative mutants of PKCα and βI. In addition, mass spectrometric analysis and mutagenesis technique revealed that PKCα phosphorylates cofilin at Ser23 and/or Ser24 during the degranulation. Furthermore, a mutant of cofilin mimicking the phosphorylation did not able to bind F-actin and lost F-actin depolymerizing and/or severing activity, resulting in the F-actin polymerization. These results indicate that the PKC-mediated novel phosphorylation site(s) of cofilin contribute to F-actin polymerization, which is necessary for proper termination of the degranulation. The novel phosphorylation site(s) are conserved within mammals and birds but not in fishes, suggesting its importance for specified function of cofilin in degranulation in higher organisms.

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Unexpected roles for Srv2/CAP in driving cofilin-mediated actin filament severing and turnover.

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Srv2/CAP has been one of the most enigmatic components of the eukaryotic actin machinery. While highly conserved, ubiquitously expressed, and critical for maintaining normal actin cytoskeleton organization and turnover in a wide range of model organisms, its specific functional roles in regulating actin dynamics have remained elusive. Further, the functional importance of Srv2/CAP hexamerization has been unclear. Here, we show that Srv2/CAP performs two distinct and biochemically/genetically separable roles in driving actin turnover. The C-terminal half of the protein forms dimers and potently stimulates nucleotide exchange on ADP-actin monomers, overcoming cofilin's inhibition of this process. The N-terminal half forms hexamers and performs a highly unanticipated role in directly enhancing cofilin-mediated severing and disassembly of actin filaments, observed in bulk fluorescence and TIRF

microscopy assays. Consistent with these activities, we found that mutations in the N-terminus but not the C-terminus of Srv2/CAP are synthetic lethal with mutations in Aip1, another co-factor in cofilin-mediated actin disassembly. These genetic observations emphasize that in vivo cofilin is not sufficient to drive actin filament disassembly, and that Srv2/CAP and Aip1 share an equally essential role in this process. Consistent with their distinct activities, both halves of Srv2/CAP were required for rapid cofilin-mediated F-actin turnover in phosphate release assays and bead motility assays. Remarkably, the two halves of the protein functioned in trans with equal efficiency to intact Srv2/CAP, further supporting their distinct roles. These results were corroborated in vivo by co-expression of each half of Srv2 in cells. Finally, we used electron microscopy and single particle analysis to show that the hexameric N-terminal half of Srv2 forms a wheel with six symmetrical protrusions that are sites of cofilin/actin-interaction. Disruption of hexamerization by a deletion of the N-terminal coiled coil domain in Srv2/CAP compromised its biochemical activities and genetic functions, suggesting that hexamerization is important for enhanced actin disassembly and raising intriguing possibilities for the severing mechanism.

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Mutant Huntingtin Causes Defective Actin Remodeling During Stress: Nuclear Shuttling of Cofilin is Essential to the Cofilin-Actin Rod Stress Response.

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Huntington's disease (HD) is caused by a polyglutamine expansion in the 350 KDa huntingtin protein. Cellular stresses can trigger the release of huntingtin from the endoplasmic reticulum (ER), allowing huntingtin nuclear entry. We have discovered that full-length huntingtin localizes to nuclear cofilin-actin rods during stress and is required for the proper stress response involving actin re-modeling. Mutant huntingtin induces a dominant, persistent nuclear rod phenotype. Using live cell temporal studies, we show that this stress response is similarly impaired when mutant huntingtin is present, or when normal huntingtin levels are reduced. In HD patients, we have quantitatively detected cross-linked complexes of actin and cofilin with complex formation varying in correlation with disease progression. By live cell studies FLIM-FRET and western blot assays, we quantitatively observed that stress-activated tissue transglutaminase 2 (TG2) is responsible for the actin-cofilin covalent cross-linking observed in HD. These data support a direct role for huntingtin in nuclear actin re-organization, and describes a new pathogenic mechanism for aberrant TG2 enzymatic hyperactivity on cofilin-actin rods in neurodegenerative diseases.
We have characterized the active nuclear export of cofilin through a leptomycin-B sensitive, CRM1-dependent, nuclear export signal. Using FLIM-FRET between cofilin moieties and actin, as well as automated image analysis in live cells, we have defined subtle mutations in the amino-terminal half of the cofilin nuclear localization signal that allow cofilin to maintain its actin binding ability but affect cofilin dynamics during stress. We propose that active nuclear shuttling is critical for the role of cofilin in the cofilin-actin rod stress response.

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Nuclear import and export mechanisms of actin.

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Actin is a major cytoskeletal protein that is also present in the nucleus. Nuclear actin is a vital component of important nuclear complexes and influences all stages of the gene expression, but the mechanistic connection is not known. For example, the form of actin responsible for individual stages of the gene expression process is debatable. In the cytoplasm, actin exists in

dynamic equilibrium between monomeric (G) and filamentous (F) forms; controlled by numerous actin-binding proteins. However, the relation between nuclear G-actin and F-actin is unclear although many major actin-regulating proteins are localized into the nucleus. Moreover, it is unclear how the nuclear and cytoplasmic actin pools are connected. Two export receptors, exportin 6 and Crm1, have been linked to nuclear export of actin, but the nuclear import mechanism has not been characterized.
Here we show by Fluorescence Recovery after Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP) that actin actively shuttles between the nucleus and the cytoplasm and the rate of movement is dependent on the availability of actin monomers. This suggests that nuclear and cytoplasmic actin pools are dynamically linked, and that although actin (43 kDa) could potentially diffuse through the nuclear pores, it uses an active mechanism for both nuclear entry and exit. We have further utilized RNAi in both Drosophila and mammalian cells to identify proteins responsible for nucleo-cytoplasmic shuttling of actin, and show that nuclear export of actin is dependent on exportin-6, but not Crm1. We also identify importin 9, a member of the importin beta superfamily, as the nuclear import receptor for actin. Also cofilin, an actin-binding protein, is required for the active nuclear localization of actin. Importin 9 interacts with both cofilin and actin and the interaction is sensitive to RanGTP. We further show that the active maintenance of nuclear actin levels by importin 9 is required for maximal transcription. Taken together, these results establish a dynamic connection between nuclear and cytoplasmic actin networks and identify an active nuclear import mechanism for actin, which is required to drive essential nuclear processes.

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Sequential action of formin family proteins during fibroblast adhesion and spreading

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Closely examining the initial attachment and spreading of cells have gained many important insights into cell migration. This process can be broken down into three consecutive phases: the initial cell attachment (P0), fast increase in spread area (P1) and lateral waves of protrusion and retraction events (P2). All three phases depend on actin nucleation, polymerization, depolymerization, bundling and crosslinking and multiple proteins regulate each of these processes. Actin nucleation and polymerization factors are grouped into several classes, such as the ARP2/3 complex or the formin family proteins. Although formins are known to participate in the regulation of actin dynamics in fibroblasts, their role during adhesion and spreading is still not clear. Here we investigate a potential role of formins during fibroblast adhesion and spreading by using smiFH2, a specific formin inhibitor. Furthermore, we investigate the expression and activation pattern of formin family members by RT-PCR, confocal microscopy, live cell imaging, western blotting and co-immunoprecipitation and find sequential action of different formins, amongst them FHOD1, mDia1 and mDia2.

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The Effect of Formin Actin Assembly Properties on Fission Yeast Cytokinesis.

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Formins stimulate actin assembly through a novel mechanism by remaining processively associated with the elongating barbed end and driving the rapid addition of profilin-actin. Eukaryotic cells typically express multiple formin isoforms that are differentially required for diverse cellular processes. We hypothesize that in addition to activation at the right time and place, the specific actin assembly properties tailor formin isoforms for a particular cellular role.

We are investigating the physiological importance of actin assembly properties in fission yeast, which expresses three formins with unique cellular roles: Cdc12 (cytokinesis), For3 (polarization) and Fus1 (mating). We found that all three formins share fundamental biochemical properties, however particular reaction rates vary significantly such as nucleation efficiency and barbed end elongation. We then constructed fission yeast strains that exclusively express cytokinesis formin Cdc12 chimeras, in which its actin assembly domains were replaced with the actin assembly domains from biochemically diverse formins (SpFus1, SpFor3, ScBni1, MmDia1, MmDia2, and CeCYK-1). With the exception of SpFus1, all strains are viable, however they exhibit a large range of cytokinesis defects from mild to severe. Quantitative time-lapse imaging reveals significant delays in contractile ring assembly and maturation. Furthermore, the mechanism of contractile ring assembly varies significantly between formin chimera strains from the search, capture, pull, release (SCPR) pathway to the leading cable (spot) pathway.

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Concerted Effects of Multiple Formin Regulators on Yeast Actin Cable Architecture.

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While the role of formins as actin assembly factors is well established, a key unanswered question is how their potent activities are controlled in vivo with the spatial and temporal precision required to assemble actin networks that have particular architectures and dynamics. After their initial release from an autoinhibited state, formins must still be tightly controlled in a temporal manner to produce actin networks with proper filament length and organization. We recently identified two different inhibitors of the yeast formin Bnr1, which exhibited distinct biochemical activities and mutant phenotypes. Bud14 displaced the FH2 domain of Bnr1 from filament ends, and loss of *BUD14* in vivo led to abnormally long, buckled cables that were resistant to latrunculin A (Chesarone et al., 2009). In contrast, Smy1 slowed the rate of filament elongation by Bnr1 without displacing the formin from filament ends, and loss of *SMY1* in vivo led to wavy/kinked actin cables that grew abnormally fast and fluctuated in thickness (Chesarone-Cataldo et al., 2011). Consistent with these inhibitors having distinct regulatory effects on Bnr1, *bud14Δ smy1Δ* double mutants also showed compounded defects in cell growth and actin cable organization. More recently, we have introduced point mutations into Smy1 that weaken its inhibitory effects on Bnr1 in vitro, and we are in the process of analyzing their effects in vivo. Further, we have identified an important functional motif in Smy1 that is shared with Bud14, despite the differences in their biochemical and cellular activities. We hypothesize that this may be a commonly used FH2-binding motif that is combined with other sequences in the contexts of different proteins to produce distinct formin regulatory effects. Finally, we are performing a surface scanning mutagenesis of the Bnr1 FH2 domain to identify its regulatory surfaces, and dissecting the mechanistic relationship of Bud14 and Smy1 and their coordinated effects on Bnr1.

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Regulation of the Diaphanous-related formin, DAAM1, in mammalian cells.

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Diaphanous-related formins (DRFs) are a highly conserved family of proteins critical to the regulation of the cytoskeleton. The regulation of DRFs involves an autoinhibitory process in which the binding of the C-terminal Diaphanous- autoregulatory domain (DAD) to the N-terminal

Diaphanous-inhibitory domain (DID) keeps the protein in an inactivated state. Upon binding of an activated Rho GTPase to the DRF GTPase binding domain (GBD), the DID-DAD interaction is released, thereby activating the DRF protein. One DRF family member that has been shown to be localized to both axons and dendrites of neuronal cells is the Dishevelled-associated activator of morphogenesis-1 (DAAM1). DAAM1 has also been demonstrated to interact with the Rho GTPases, RhoA and Cdc42, and play an important role in a variety of biological processes and pathways, including gastrulation and the development of the tracheal cuticle in the respiratory system. Our laboratory has created constitutively active full-length DAAM1 and mDia3 to elucidate the localization and cellular effects of the protein in cells. Here, analogous to the M1041A and M1182A mutations in the DAD regions of mDia2 and mDia1 respectively, we show that the F1032 residue (DAAM1) and M1053 (mDia3) are critical to the DID-DAD autoregulatory interaction of the proteins. Fluorescence anisotropy demonstrates that these mutations in DAD result in the complete inability to bind to the DID region. The loss of DID-DAD binding is consistent with the localization and impact of full-length constitutively active DAAM1 and mDia3 in mammalian cells. Expression of constitutively active versions of DAAM1 and mDia3 in three different cell lines (NIH3T3/mouse fibroblast, PC12/rat pheochromocytomas, N1E-115/mouse neuroblastomas) resulted in cells with an increased number of abnormally-shaped filopodia and cellular protrusions. The F1032A DAAM1 was found to be evenly distributed throughout the entire filopodia, which was in contrast to the constitutively active mDia3 being mostly localized to the tips of the filopodia. Interestingly, the inhibition of Rho GTPases by C3 transferase did not decrease the ability of constitutively active DAAM1 and mDia3 to cause cellular protrusions, nor did it significantly inhibit the formins' ability to activate serum response factor in cells. Lastly, the localization of the activated mDia3 at the filopodia tips was dependent on its ability to nucleate actin. Together, these results demonstrate the critical contribution of F1032 (DAAM1) and M1053 (mDia3) to the autoregulation process, as well as shed some light on the cellular effects and localization of full-length constitutively active DAAM1 and mDia3.

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Probing the role of phosphorylation in the mechanism of formin mDia2.

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Diaphanous-related formins are a highly conserved family of proteins that influence numerous cellular processes by regulating cytoskeletal dynamics. One such way that formins affect cytoskeletal dynamics is through nucleating actin filaments, by recruiting actin monomers as the filament elongates and decreasing actin depolymerization at the barbed end. Since the formins are an important focal point which affect so many cellular processes, it is vital that they are tightly regulated and only activated in response to cellular signals, as uncontrolled formins can result in dire consequences for a cell. The regulation of one specific mammalian formin, mDia2, is regulated by intramolecular interactions between the N- and C-terminus, in order to inhibit the domain responsible for actin polymerization (FH2). The autoinhibitory binding occurs between the diaphanous inhibitory domain (DID) at the N-terminus and the diaphanous auto regulatory domain (DAD) at the C-terminus. Thus far, it is understood that formins are activated when a Rho GTPase binds to the GTPase binding domain, but binding of a GTPase may not fully activate the protein. Other signals seem to be necessary in order to fully activate the formin. Previously, we identified a specific cellular protein, p21 activated kinase (PAK1), that phosphorylates mDia2 and potentially serves to regulate the formin in cells. We have identified the specific amino acid sites on mDia2, in both the DID and DAD domains, that are modified by PAK1. In addition, we have verified that PAK1 phosphorylates mDia2 in mammalian cells. Using isothermal titration calorimetry and fluorescence anisotropy, we have shown that PAK1 binds to both DID and DAD regions of mDia2, and this binding does not seem to have a significant effect

on the DID-DAD affinity. Lastly, we have attempted to trace the interplay of the different formin domains with PAK1 by measuring actin polymerization in vitro. Preliminary data suggest that the presence of PAK1 may inhibit the promotion of actin nucleation by mDia2 FH1FH2DAD by potentially enhancing DID-DAD interactions. With the observation of DRF phosphorylation by kinases that are known to interact closely with Rho GTPases, we hope to provide valuable insight into the mechanism of mDia2 regulation.

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Single Molecule Visualization of Cooperation Between the Actin Nucleators mDia1 and APC.

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Evidence for cooperation between actin nucleators and elongation factors in vivo is growing. Such mechanisms have been postulated for formins interacting with Spire, the tumor-suppressor adenomateous polyposis coli (APC), Bud6, and Arp2/3 complex. However, the mechanisms underlying this proposed cooperation have remained obscure. Here we employed triple-color TIRF microscopy to reconstitute and visualize actin filament formation by APC and the formin mDia1 in real-time and at the single molecule level. TIRF microscopy on SNAP-tagged fluorescently labeled mDia1 and APC-C showed that each protein is a dimer, and enabled us to track their dynamics during actin filament formation. Capping protein and profilin were included to suppress spontaneous nucleation and elongation of filaments and mimic the cellular environment. Using this experimental setup, we visualized for the first time the processive movement of single formin molecules (dimers) on the rapidly growing ends of actin filaments. SNAP-mDia1 and SNAP-APC directly interacted to form transient prenucleation complexes with actin monomers. Upon initiation of filament polymerization, the APC-mDia1 complex was immediately released, resulting in rapid processive translocation of SNAP-mDia1 on the barbed end, leaving SNAP-APC at the pointed end. Notably, Latrunculin B treatment resulted in long-lived prenucleation complexes, suggesting that processive translocation of mDia1 at the barbed end provides the driving force for mDia1-APC separation. Higher concentrations of SNAP-APC led to filament bundling and the formation of APC-clusters from which multiple mDia1-elongated filaments sprouted into filament ‘bouquets’. Finally, APC-mDia1 interactions required sequences in mDia1 C-terminal to the FH2 domain, suggesting parallels between APC-formin and Spire-formin. Thus, the mechanisms visualized here may recapitulate general principles of cooperation between formins and other actin nucleators.

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Structure, Mechanism, and Regulation of the Formin Nucleation Promoting Factor Bud6.

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Recent studies indicate that cells often bring together two different actin assembly-promoting factors with complementary strengths to overcome physiological barriers to polymer formation such as profilin and capping protein. While formins efficiently protect growing actin filament ends from capping protein, their nucleation activities in the presence of profilin are weak. Thus, the ability of formins to overcome the profilin barrier to nucleation in vivo may require nucleation-promoting factors (NPFs), and indeed, this has recently been suggested for formins that interact with Spire and adenomatous polyposis coli (APC). Here, we show that the yeast formin-binding

protein Bud6 represents a functional counterpart to these metazoan NPFs. Using TIRF microscopy, we demonstrate that Bud6 elevates formin nucleation activity without affecting rate of filament elongation. Through the generation of separation-of-function alleles, we show that Bud6 NPF activity in vitro and in vivo requires two distinct domains in Bud6 that bind to the formin DAD domain and actin monomers. We demonstrate that Bud6 is a dimer, which can bind two actin monomers. Thus, each formin dimer, with its two DAD domains, can recruit two Bud6 dimers, and in turn four actin monomers, which we propose are organized into a nucleation seed from which the formin FH2 domain elongates a filament. We present the crystal structure of a core segment of Bud6 (residues 550–688) that binds to the formin. The structure reveals a novel triple helical coiled-coil dimer, and we identify conserved surface residues that mediate DAD binding and are critical for NPF activity. Finally, we show that Bud6 interacts with the two yeast formins (Bni1 and Bnr1) through distinct mechanisms, and that these associations are differentially regulated in vivo by a novel cellular binding partner of Bud6 that we identified. These unexpected results demonstrate that a single NPF can be employed in vivo to differentially control the activities of two distinct formins depending on the availability of specific binding partners.

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Formin proteins have distinct mechanisms of microtubule/actin interaction.

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A number of cellular processes utilize both microtubules and actin filaments, but the molecular machinery linking these two cytoskeletal elements remains to be elucidated in detail. Formins are actin-binding proteins that have multiple effects on actin dynamics, as well as influencing microtubule stability and positioning in interphase and mitotic cells. Although several formins have been shown to bind microtubules directly, the binding mechanisms are poorly understood. Here, we show that three formins, INF2 (active in the secretory pathway), mDia1 (focal adhesion/stress fiber assembly), and mDia2 (cytokinesis), display important fundamental differences in their interactions with and effects on microtubules. All three formins bind microtubules with high affinity (K_d < 100 nM), and require the formin homology 2 (FH2) domain for this interaction. However, only mDia2 binds microtubules with 1:1 stoichiometry, while INF2 and mDia1 display saturating binding at 1:3 (formin dimer:tubulin dimer). INF2 is a potent microtubule bundling protein, an effect that results in a large reduction in catastrophe rate. In contrast, neither mDia1 nor mDia2 bundle microtubules appreciably. The C-termini of mDia2 and INF2 have different functions in microtubule interaction, with mDia2's C-terminus required for high affinity binding and INF2's C-terminus required for bundling. These formins also differ in their abilities to bind actin and microtubules simultaneously. Microtubules strongly inhibit actin polymerization by mDia2, while they moderately inhibit mDia1 and have no effect on INF2. Conversely, actin monomers inhibit microtubule binding/bundling by INF2, but do not affect mDia1 or mDia2. Our conclusion is that, while all three formins require the FH2 domain for high affinity microtubule interaction, they differ markedly in their effects on microtubules and the ability to interact with actin and microtubules simultaneously, with INF2 assembling extensive actin/microtubule co-bundles whereas mDia1 and mDia2 do not. These biochemical differences result in substantial differences in the abilities of these three formins to interact with actin and microtubules simultaneously in cells.

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Evidence that rapid release of VCA from filament-bound Arp2/3 complex is required for actin branch formation.

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During cell locomotion and endocytosis, actin filament nucleation by Arp2/3 complex is stimulated by the VCA domain of WASP family proteins, which likely exist in multimeric clusters on membrane surfaces. The mechanism of actin filament nucleation by Arp2/3 complex has been well studied using bulk measurements. While these studies have revealed many details regarding the nucleation mechanism, the kinetic mechanism of how VCA association with Arp2/3 complex, Arp2/3 complex binding to an existing filament and new filament nucleation are coordinated remains unclear. Here, we address this challenge by using a colocalization single molecule spectroscopy (CoSMoS) approach to directly visualize the assembly of actin filament branches with fluorescently labeled actin, Arp2/3 complex and VCA in vitro. Preceding nucleation, VCA is often bound to Arp2/3 complex at the time of docking on a pre-existing filament, but then is released very quickly (lifetime <0.05s for monomeric VCA). Release of dimeric WASP VCA from the nascent branch site is somewhat prolonged, but its lifetime is still short (0.2-0.4 s). The majority of Arp2/3-diVCA-filament interactions are unproductive in nucleation, and result in rapid release of Arp2/3-diVCA complexes from the filament. Further, using a set of N-WASP VCA dimers with engineered point mutations we find that weaker affinity for Arp2/3 complex corresponds with higher nucleation activity. From these results we hypothesize that rapid release of VCA from the nascent branch is required for daughter filament growth. Such a mechanism could explain how mother filament binding and daughter filament nucleation are coordinated.

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α-Catenin: An Inhibitor of Arp 2/3 Complex-Mediated Actin Polymerization.

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Cell-cell adhesion occurs through cadherins on cell membranes, which link to the internal actin cytoskeleton. β-Catenin (β-cat) binds to the cytoplasmic domain of E-cadherin (E-cad) and α-catenin (α-cat) binds to β-cat, and they collectively form the ternary cadherin-catenin cell-cell adhesion complex. α-Cat binds and bundles F-actin and is considered the primary link between cadherin-mediated cell-cell adhesion complexes and the actin cytoskeleton. However, the E-cad/β-cat/α-cat ternary complex binds weakly to F-actin *in vitro*, whereas α-cat alone can bind F-actin and regulate actin dynamics. We postulated that α-cat regulates actin dynamics at cell-cell junctions by dissociating from the ternary complex and binding F-actin. To test this hypothesis, we coated beads with an equimolar mixture of ActA – an activator of the Arp2/3 complex – and the E-cad cytoplasmic tail in the absence or presence of β-cat and α-cat (i.e., the E-cad/β-cat/α-cat ternary complex); these beads were then incubated with a mixture of fluorescent G-actin, capping protein, and Arp2/3 complex to promote actin network assembly. We found that reconstitution of the E-cad/β-cat/α-cat complex on beads significantly reduced the rate of Arp2/3 complex-dependent actin network assembly. In addition, the presence of the

ternary complex altered gross F-actin morphology, consistent with inhibition of Arp2/3 complex activity. Removal of α-cat from the complex (E-cad/β-cat alone) restored actin assembly rates to control (no complex) levels, indicating that α-cat is required for inhibition of actin network formation by the E-cad ternary complex. To visualize α-cat localization during actin network growth, we reconstituted the ternary complex with EGFP-labeled α-cat (E-cad/β-cat/EGFP-α-cat). We observed EGFP-α-cat throughout the F-actin network and distal to the bead surface, suggesting that α-cat can dissociate from the ternary complex and subsequently bind F-actin. We propose that unbinding of α-cat from E-cad ternary complex and binding to actin filaments inhibits Arp2/3 complex-dependent actin assembly. These results have implications for mechanisms involving the E-cad ternary complex and α-cat in the regulation of actin dynamics at cell-cell adhesions.

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Activation of the Arp2/3 complex by nucleation promoting factors.

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Branched actin networks are critical for cellular processes such as endocytosis, vesicle trafficking and membrane protrusion. The Arp2/3 complex, which is an evolutionarily conserved seven protein complex consisting of Arp2, Arp3 and ARPC1-5 subunits, is a central factor in the construction of branched actin networks. Upon activation by a nucleation promoting factor (NPF), the Arp2/3 complex binds to the sides of existing actin filaments and nucleates new daughter filaments to form Y-branches. Distinct NPFs harness Arp2/3 activity for different processes within the cell. A key step in Arp2/3 activation is NPF binding, and recent evidence indicates that two NPF-binding sites exist on the complex. However, a variety of experimental approaches have suggested more than two possible regions to which NPFs may bind. To localize NPF binding on the complex, we have used site-directed mutagenesis to engineer recombinant human Arp2/3 complexes that are mutated in candidate NPF-binding sites, including a basic patch on Arp3 and hydrophobic residues of Arp2. Pyrene actin polymerization assays demonstrate that both of these sites are important for Arp2/3-mediated actin nucleation and suggest that they represent two distinct NPF binding sites. In the future, affinity measurements of mutant complexes for different NPFs will directly address the importance of each site in Arp2/3-NPF binding and also determine if distinct NPFs exhibit binding site preferences. Additionally, experiments examining the role of each NPF-binding site in Arp2/3 activation in the context of its cellular functions, such as membrane protrusion and lamellipodia dynamics, will dissect the contributions of two NPF binding events and the associated recruitment of two actin monomers to Y-branched actin network formation in cells.

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Arp2/3 complex and myosin II both contribute to the dynamic behavior of actin filament veils in neuronal growth cones.

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Arp2/3 complex nucleates actin filaments and generates actin filament networks at the leading edge of motile cells, but the role of Arp2/3 complex in neuronal growth cones has been questioned. The growth cone's peripheral domain (P-domain) contains a treadmilling network of actin filaments that assembles at the leading edge and moves centripetally by a process referred to as "retrograde actin flow". Normal rates of retrograde flow depend both on actin assembly and the actions of more centrally localized non-muscle myosin II. When myosin II is

inhibited, retrograde flow persists at reduced rates that now fully depend on "pushing" forces generated by leading edge actin assembly. A specific role for Arp2/3 complex in retrograde flow and/or possible interdependencies of Arp2/3 complex and myosin II have not been reported. Here, we investigated the effects of a small molecule Arp2/3 complex inhibitor, CK666, on actin filament structure and dynamics in growth cones. CK666 treatment delocalized Arp2/3 complex from the leading edge, markedly decreased the density of actin filament barbed ends in the P-domain, reduced actin density in veil networks and resulted in actin veil retraction. CK666 treatment left structure and dynamics of filopodia remarkably intact and significantly increased residual actin retrograde flow rates in the P-domain. In contrast, inhibition of both myosin II activity and Arp2/3 complex decreased retrograde flow and veil retraction was no longer observed. Together, these results suggest Arp2/3 complex and myosin II both contribute to actin veil structure and dynamics.

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Coupling Arp2/3 Complex-mediated Actin Branching and Membrane Deformation by the Exocyst Component Exo70.

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Actin dynamics and membrane remodeling are tightly coordinated spatially and temporally to generate cell surface protrusions for morphogenesis and migration. However, the molecular mechanism for this coordination is unclear. Here we report that Exo70, a component of the exocyst complex, couples actin assembly and membrane curvature induction. Using pyrene actin assay and dual-color total internal reflection fluorescence (TIRF) microscopy, we found that Exo70 synergizes with WAVE2 to kinetically stimulate the Arp2/3 complex-mediated actin polymerization and branching. We also found that Exo70 is able to generate tubular invaginations toward the lumen of in vitro synthesized large unilamellar liposomes (LUVs) and giant unilamellar liposomes (GUVs). In cells, Exo70 induces actin-free filopodial protrusions, which are subsequently stabilized by actin filaments as observed by correlative light and electron microscopy. Cells expressing Exo70 mutants deficient in interacting with the Arp2/3 complex or bending membranes are defective in lamellipodia formation and directional cell migration. Together, our findings provide a novel mechanism for the coordination of actin organization and plasma membrane remodeling during cell morphogenesis and migration.

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Interactions Among IQGAP1, Cdc42/Rac1, N-WASP and Actin Quantified by In vitro Reconstitution and Live Cell, Three-Color FRET Microscopy.

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IQGAP1 stimulates branched actin filament nucleation by activating N-WASP, which in turn activates the Arp2/3 complex. N-WASP can be activated by other factors, including GTP-bound Cdc42 or Rac1, which also bind IQGAP1. In pyrene-actin assembly assays in the presence of N-WASP and the Arp2/3 complex, Cdc42 and IQGAP1 cooperatively stimulated actin filament nucleation. Cdc42 also stimulated binding of IQGAP1 to N-WASP in a dose dependent manner. In contrast, Rac1 showed an inhibitory effect of IQGAP1 binding to N-WASP. The two G

proteins and their association with IQGAP1 therefore have a distinct impact on N-WASP and the downstream nucleation of actin filaments. To assess the physiological significance of these *in vitro* findings we developed a novel three-color FRET microscopy assay, in which live MDCK cells simultaneously expressed various combinations of fluorescent fusion proteins of Cdc42, Rac1, N-WASP, IQGAP1 and actin by co-transfection. Cdc42 fluorescence was not correlated with the distance between IQGAP1 and actin, whereas increasing Rac1 fluorescence correlated with increasing IQGAP1-actin distance. In the presence of IQGAP1, N-WASP fluorescence levels decreased with rising Rac1 fluorescence, while the opposite was true for Cdc42. Moreover, the distance between IQGAP1 and N-WASP decreased with increasing Cdc42 fluorescence, but rose as Rac1 fluorescence rose. The live cell triple FRET data therefore confirm the conclusion from *in vitro* reconstitution experiments that Cdc42 and Rac1 respectively strengthen and weaken the binding of IQGAP1 to N-WASP, and suggest corresponding positive and negative effects on actin polymerization. More broadly, these data establish three-color FRET microscopy as a powerful tool for studying dynamic multi-molecular interactions in live cells.

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PIP2 dynamics couples Calcium/InsP3 and N-WASP/F-actin oscillations to coordinate secretory vesicle recruitment and fusion at the plasma membrane.

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Secretory granules have to pass through the actin cortex with a mesh size typically much smaller than average vesicle diameter, suggesting that the actin cortex acts as barrier for secretory granules. Yet Myosin Va a transport motor that is important for vesicle recruitment and docking, requires the actin cortex for its function, suggesting that the cortex acts as a carrier of secretory granules. How does the cell consolidate these seemingly opposing roles of the actin cortex? Here we show that during the FceRI response in RBL-2H3 cells, the levels of F-actin at the cell cortex oscillate. The oscillations of cortical actin are a result of oscillations in the levels of plasma membrane recruitment of N-WASP. We demonstrate that these F-actin/N-WASP oscillations are coupled to the cell's Ca2+/InsP3 oscillations via oscillatory changes in the levels of PIP2 in the plasma membrane. The oscillatory dynamic of the actin cortex allows temporal partitioning of the barrier and carrier roles of the actin cortex, thereby consolidating the two opposing roles of the actin cytoskeleton during secretory exocytosis.

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The WAVE Regulatory Complex Links Neuronal Receptors to the Actin Cytoskeleton.

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Wiskott-Aldrich syndrome protein WAVE activates Arp2/3 complex to stimulate actin polymerization adjacent to membranes. This activity is important for many membrane-associated cellular activities, including cell migration and adhesion. In vivo, WAVE exists as inhibited in a 400kD heteropentameric complex, known as WAVE regulatory complex (WRC) consisting of Sra, Nap, Abi, HSPC300 and WAVE. Although a few known regulators can activate WRC, what ligands link its activity to specific cellular processes is largely unknown. Recently a neuron-specific adhesion receptor protocadherin10 (PCDH10) was reported to interact with WRC via its cytoplasmic tail (CT), which then recruit WRC to cell-cell contact site. Here we report that the unstructured PCDH10 CT contains a highly conserved WRC interacting protocadherin sequence (WIPS) that binds WRC. We present crystal structures of WRC complexed with synthetic WIPS peptides, in which the peptides bind to a highly conserved hydrophobic pocket formed by Sra and Abi. Point mutations revealed a consensus WIPS that

exists in many other proteins of various functions. We further confirmed that among these proteins many neuron-specific receptors, including protocadherins, neuroligins and glutamate receptors, bound WRC using WIPS in their cytoplasmic tails. This broad, diverse and highly conserved WIPS-specific interactome for WRC may provide a general mechanism that links actin cytoskeleton to different cellular activities.

Microtubule Dynamics and Its Regulation II

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CENP-E interacts with SKAP to orchestrate accurate chromosome segregation in mitosis.

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Mitotic chromosome segregation is orchestrated by the dynamic interaction of spindle microtubules with the kinetochore. During chromosome alignment, bi-oriented kinetochores move chromosomes by regulating the plus-end dynamics of the attached microtubules. Although previous studies show that mitotic kinesin CENP-E forms a link between attachment of spindle microtubule to the kinetochore and the mitotic checkpoint signaling cascade, the molecular mechanism underlying dynamic kinetochore-microtubule interactions in mammalian cells remains elusive. Here we identify a novel interaction between CENP-E and SKAP which functions synergistically in governing dynamic kinetochore-microtubule interactions. SKAP binds to the C-terminal tail of CENP-E in vitro and is essential for an accurate kinetochore-microtubule attachment in vivo. Immuno-electron microscopic analysis indicates that SKAP is a constituent of the kinetochore corona fibers of mammalian centromeres. Depletion of SKAP or CENP-E by RNA interference results in a dramatic reduction of inter-kinetochore tension, which causes chromosome mis-segregation with a prolonged delay in achieving metaphase alignment. Importantly, SKAP binds to microtubules in vitro, and this interaction is synergized by CENP-E. Based on these findings, we propose that SKAP cooperates with CENP-E to orchestrate dynamic kinetochore-microtubule interaction for faithful chromosome segregation.

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Molecular delineation of TIP150 function underlying kinetochore microtubule dynamics during mitotic chromosome segregation.

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Microtubules are cytoskeletal elements that are essential for an array of intracellular processes such as mitosis and cellular division. Mammalian plus-end-tracking proteins (+TIPs) localize to the growing ends of microtubules, and regulate their dynamic behavior and interactions with kinetochores during mitosis. Tip150 is a novel microtubule plus end binding protein essential for dynamic kinetochore-microtubule attachments and proper cellular division. Previous biochemical and immunofluorescence analyses show that TIP150 interacts with plus end bind proteins EB1 (end bind protein 1) and MCAK (mitotic centromere associated kinesin). However, the molecular behavior of TIP150 on microtubules and how it regulates microtubule dynamics remains elusive. To understand the molecular biophysics underlying TIP150-elicited end-tracking, we reconstituted an in vitro particle tracking experimentation system using purified

+TIP proteins, which allowed the delineation of EB1-TIP150 interactions on microtubule ends at the nanometer scale. This total internal reflection fluorescence microscopy assay enables us to observe dynamic plus end tracking in vitro, and the quantitative analysis of single +TIP molecule turnover at the growing microtubule ends in the presence or absence of mitotic kinases. The consolidation of those in vitro molecular dynamics of +TIPs coupled with specific protein-protein interaction manipulation in vivo will allow us to build a model accounting for the function of +TIPs in mitotic chromosome segregation.

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PLK1-MCAK interaction orchestrates kinetochore microtubule dynamics essential for faithful chromosome segregation.

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During cell division, interaction between kinetochores and dynamic spindle microtubules governs chromosome movements. The microtubule depolymerase mitotic centromere-associated kinesin (MCAK) is a key regulator of mitotic spindle assembly and dynamics. However, the regulatory mechanisms underlying its depolymerase activity during the cell cycle remain elusive. We have recently showed that PLK1 is a novel regulator of MCAK essential for accurate chromosome dynamics in mitosis (Zhang et al., 2011. J. Biol. Chem. 286, 3033-3046). MCAK interacts with PLK1 in vitro and in vivo. The neck and motor domain of MCAK associates with the kinase domain of PLK1. MCAK is a novel substrate of PLK1, and the phosphorylation stimulates its microtubule depolymerization activity of MCAK in vivo. Overexpression of a polo-like kinase 1 phosphomimetic mutant MCAK causes a dramatic increase in misaligned chromosomes and in multipolar spindles in mitotic cells, whereas overexpression of a nonphosphorylatable MCAK mutant results in aberrant anaphase with sister chromatid bridges, suggesting that precise regulation of the MCAK activity by PLK1 phosphorylation is critical for proper microtubule dynamics and essential for the faithful chromosome segregation. We reasoned that dynamic regulation of MCAK phosphorylation by PLK1 is required to orchestrate faithful cell division, whereas the high levels of PLK1 and MCAK activities seen in cancer cells may account for a mechanism underlying the pathogenesis of genomic instability. Using a novel MCAK activity reporter coupled with chemical inhibitors, we are illustrating how spatiotemporal dynamics of MCAK activity is coordinated with kinetochore mechanics during mitotic chromosome movements.

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The Kinesin-8 Kip3 Controls Anaphase Spindle Length.

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Kinesin-8 is a conserved family of microtubule motors and regulator of microtubule dynamics with important roles in chromosome alignment and spindle function in a wide range of organisms. Inhibition of Kinesin-8 was reported to produce increased anaphase spindle lengths often having a buckled morphology. These defects have long been attributed to a proposed function for Kinesin-8 in spindle disassembly following mitosis. Combining molecular genetics with fluorescence imaging revealed a novel role for the budding yeast Kinesin-8, Kip3, prior to spindle disassembly in which it actively restrains inappropriate spindle elongation during anaphase. When challenged with an anaphase arrest, spindles stopped elongating when they obtained a length approximately equal to the cell diameter. However, in the absence of Kip3, anaphase spindles failed to stop elongating, buckled, and continued to hyper-elongate throughout the arrest. To determine whether the rigid cell wall limits spindle length, we tracked

spindle elongation in cells lacking Ipl1 (Aurora B), which are known to have buckled spindles. Spindles in cells lacking Ipl1, unlike those lacking Kip3, buckled but did not hyper-elongate over extended periods of anaphase arrest. These results indicate Kip3 does not function to prevent spindles from overcoming cortical resistance and buckling but instead, can limit buckled spindles to the appropriate length. To define the mechanism by which Kip3 halts spindle elongation we measured the rates of spindle hyper-elongation. Interestingly, these rates were similar to typical anaphase rates suggesting that the same activities that drive anaphase elongation continue to drive hyper-elongation. Additionally, midzone lengths in Kip3 deletion strains with straight, buckled, and hyper-elongated spindles were unaltered compared to the midzone in control cells, demonstrating that excess microtubule polymerization at the midzone is coupled to spindle hyper-elongation. Taken together, our results reveal that, independent of a role in spindle disassembly, Kip3 plays a novel role to actively restrain inappropriate spindle elongation and maintain spindle integrity, likely through limiting microtubule polymerization at the midzone. RR was funded by T32 HL094282; KD was funded by The Biological Sciences Collegiate Division Research Endowments at the University of Chicago.

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Genetic suppressors provide insight into PP2A subunit interactions in *Caenorhabditis elegans.*

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Phosphorylation plays an important role in regulating many mitotic events. We are studying a specific protein phosphatase 2A (PP2A) complex that regulates centrosome-based microtubule outgrowth during mitotic spindle assembly. In *C. elegans*, RSA-1 (regulator of spindle assembly) is a PP2A B'' regulatory subunit that is required for robust microtubule outgrowth from the centrosomes. RSA-1 protein locates to the centrosomes of *C. elegans* embryos, suggesting that this regulatory subunit plays an important role in centrosome-specific phosphatase activity. Embryos depleted of RSA-1 exhibit a severe reduction in microtubule outgrowth from centrosomes and a spindle-collapse phenotype whereby the centrosomes move toward the chromosome mass during spindle assembly[1]. However, the centrosomes in *rsa-1* mutants appear to have all major components and they are otherwise competent to support microtubule outgrowth[1]. In order to further characterize this complex, we performed a genetic screen for suppressors of a temperature sensitive allele of *rsa-1(or598ts)* [2]. Progeny from *or598ts/or598ts* adults exhibit 100% lethality at 26°C but only 20% lethality at 15°C, making this allele ideal for our suppressor screens. Using EMS, we have screened an estimated 150,000 mutagenized haploid genomes in three independent rounds of mutagenesis. Twenty-four candidate suppressors were isolated and 21 have since been identified. Currently all identified suppressors have been either intragenic or in *paa-1*, the structural subunit of the PP2A complex. These suppressors suggest that the *rsa-1(or598ts)* lesion disrupts the protein-protein interaction between RSA-1 and PAA-1. Yeast two-hybrid analysis has been used to confirm this hypothesis. Preliminary results suggest that the suppressor mutations in *paa-1* are capable of restoring the protein interaction with *rsa-1(or598ts)*. Immunofluorescence imaging of RSA complex components in the mutant and suppressed C. elegans embryos will be used to test this hypothesis. The remaining suppressors are currently being mapped and further characterized.

1. Schlaitz AL, et al. (2007) The *C. elegans* RSA Complex Localizes Protein Phosphatase 2A to Centrosomes and Regulates Mitotic Spindle Assembly . Cell (128): 115-127.
2. O'Rourke SM, et al. (2011) A Survey of New Temperature-Sensitive, Embryonic-Lethal Mutations in *C. elegans*: 24 Alleles of Thirteen Genes. PLoS ONE 6(3): e16644.

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MCAK activity controls microtubule dynamics and directed cell migration.

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Cell polarization during directed cell migration is coordinated by (MT) growth toward the leading edge. Cells can locally control MT growth or shortening through the regulation of depolymerizing kinesins (MCAK), which catalyze the destabilization of MT plus ends, causing MTs to transition from growth to shortening (catastrophe). However, it is not known if MCAK regulation affects polarized MT growth dynamics that mediate cell migration. We hypothesized that MCAK is locally inhibited to establish preferential MT growth toward the leading edge to promote directed migration. To test this hypothesis, we performed high resolution imaging of fluorescently tagged EB3 that dynamically associates with growing MT plus-ends, coupled with automated image-based tracking of MT growth speeds and growth lifetimes (1/catastrophe frequency). We found that knockdown of MCAK (MCAK-KD) promoted increased MT growth lifetimes globally, abolishing the polarized differences in MT growth lifetime in the cell center and edge seen in control cells. As a result, directional cell migration was significantly reduced, suggesting that local regulation of MCAK activity is critical for establishing polarized MT growth and directing cell migration. To determine how local regulation of MCAK might be achieved, we investigated the role of Aurora-A kinase, which when activated by phosphorylation localizes to mitotic centrosomes and behaves as a phospho-inhibitor of MCAK depolymerase activity. Phospho-Aurora-A immunoprecipitation from cell extracts revealed interaction between MCAK and the phospho-active form of Aurora-A. Live-cell measurements of MT growth dynamics showed that overexpression of Aurora-A promotes long-lived MT growth similar to MCAK-KD, suggesting that Aurora-A regulates the depolymerase activity of cytoplasmic MCAK. To identify upstream activators of Aurora-A, we expressed either constitutively active- (CA-Rac1) or dominant negative-Rac1 (DN-Rac1) and measured changes in active Aurora-A via immunolabeling with a phospho-specific Aurora-A antibody. Compared to control, cells expressing CA-Rac1 displayed a 4-fold increase of phospho-active Aurora-A, while in cells expressing DN-Rac1, phospho-active Aurora-A levels were decreased by 3-fold. In addition, live-cell imaging revealed that like GFP-MCAK, GFP-Aurora-A tracks with a subset of growing MT plus-ends in CA-Rac1 cells, but not in DN-Rac1 cells. Finally, while CA-Rac1 promoted fast and long-lived MT growth, this effect was lost by pharmacologic inhibition of Aurora-A. Together, these results suggest that Aurora-A-mediated regulation of MCAK depolymerase activity is achieved downstream of Rac1 signaling to elicit regional regulation of MT dynamics and thereby promote polarized MT growth and directional cell migration.

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Microtubule Length Regulation by Depolymerizing Kinesins.

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Microtubules are dynamic filaments whose plus ends alternate between periods of slow growth and rapid shortening as they explore intracellular space and move intracellular organelles. A key question is how regulatory proteins such as the depolymerizing kinesins modulate catastrophe, the conversion from growth to shortening. To study this process, we reconstituted microtubule dynamics in the absence and presence of the kinesin-8 Kip3 and the kinesin-13 MCAK. Surprisingly, we found that even in the absence of the kinesins, the microtubule catastrophe frequency depends on the length and age of the microtubule, indicating that catastrophe is a multistep process. Kip3 slowed microtubule growth in a length-dependent manner and

increased the rate of catastrophe-inducing feature formation. In contrast, MCAK did not change the feature formation rate, but instead transformed catastrophe into a single step process. Thus, both kinesins are catastrophe factors, but influence microtubule length distribution in different ways: Kip3 mediates fine control of microtubule length by narrowing the distribution of maximum lengths prior to catastrophe, whereas MCAK promotes rapid restructuring of the microtubule cytoskeleton by making catastrophe a first-order random process.

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Kinesin-13 targeting and functional specificity is governed by intrinsic factors and associated proteins.

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Microtubule dynamics are essential throughout mitosis to ensure correct chromosome segregation. Microtubule depolymerization is controlled by microtubule depolymerases, including the kinesin-13 family of proteins. In humans, there are three closely related kinesin-13 isoforms that are highly conserved in their primary sequences. However, these enzymes display different localization and non-overlapping function. To understand the molecular basis for the observed differences, we conducted a comparative analysis of each isoform and we defined molecular components that determine the specificity of the kinesin-13 family members. Chimeric proteins containing the divergent N-termini of each member together with the catalytic core of another kinesin-13 indicate that the N-terminus is the primary determinant of cellular localization, and that the individual catalytic domains display different functional properties. We also identify three novel interacting partners for Kif2b that form subpopulations of Kif2b complexes in vivo. We show that these Kif2b-associated proteins display robust microtubule binding activity that is regulated by CDK activity. Thus, these associated proteins provide Kif2b complexes with a second microtubule binding site and thereby modify Kif2b functional properties. This represents a molecular paradigm for kinesin function, where it was previously thought that motors were the sole contributors to microtubule binding. Overall, we show that the kinesin-13 functional specificity and diversity in the cell originates from divergent protein domains and unique protein-protein interactions.

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Control of neuronal differentiation by USP47-mediated stabilization of katanin p60.

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Katanin p60 as a member of the AAA family of ATPases has a microtubule-stimulated ATPase and microtubule-severing activities in the absence of p80 subunit. Katanin p60 is essential for robust axonal growth of neurons and regulation of spindle pole during mitosis and meiosis. USP47 is a deubiquitinating enzyme that is conserved in Bilateria and predominantly expressed in neuronal tissues. Here we show that katanin p60 subunit is degraded by the ubiquitin-proteasome pathway. Conversely, USP47 specifically deubiquitinates katanin p60 and thereby stabilizes it. USP47 expression was detected in both axon and dendrite of hippocampal neurons in the early stage. In the late stage, USP47 was found to localize in the mature spines of neurons and partially colocalize with PSD-95. Moreover, overexpressioin of USP47 led to an increase in the number of dendrites and length of axon. Overexpression of katanin p60 showed a similar phenotype to that of USP47. These results suggest that the morphological changes in the neurites are regulated by USP47-mediated stabilization of katanin p60.

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Testing a Novel Drug against the Beta-III Isotype of Tubulin.

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Tubulin is the target of some of the most successful anti-tumor drugs, such as the taxanes and Vinca alkaloids. Tubulin is a heterodimer of α and β subunits, each of which consists of numerous isotypes differing in amino acid sequence and encoded by different genes. The taxanes and Vinca alkaloids appear to target the βII isotype. The logic of this is that βII occurs in many tumors and is relatively scarce in normal tissues, except in nerves and muscles. The latter aspect may play a role in the neurotoxicity associated with these drugs. We hypothesize that βIII would be a better target since it is associated with many aggressive and metastatic tumors and is less widely distributed in normal tissues than is βII. In nerves, βIII occurs only in neurons and we have shown that silencing βIII is much less deleterious to neurons than is silencing βII. Our results and those of others suggest that βIII may protect microtubules from free radicals. Using modeling, we designed and synthesized a novel colchicine derivative, CH-35, to bind better to βIII. Testing against cancer cell lines showed IC50's in the 3-4 nM range. We are now testing it in a mouse model of breast cancer and also in cultured breast cancer cell lines with different levels of βIII. In the former system we find that CH-35 is about equally effective as taxol in inhibiting growth of mouse mammary tumors. Our objective with cell lines is to measure the effect of βIII level on the sensitivity of the cell lines to CH-35. We also intend to test the effect of irofulven, an inhibitor of thioredoxin and thioredoxin reductase to see if it can synergize with CH-35. [Supported by Grants from the US Army (DOD BCRP) to Dr. Richard F. Luduena and Dr. John C. Lee].

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Inhibition of HDAC6, a Microtubule Deacetylase, Increases Microtubule Stability.

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In this study, we investigated the role of acetylation in regulating microtubule stability and dynamics by inhibiting the enzyme HDAC6, a known microtubule deacetylase. HDAC6 was inhibited using different strategies namely pharmacological inhibition by two small molecule inhibitors, tubastatin A and trichostatin A (TSA), and inhibition of HDAC6 gene expression using siRNA. We found that the inhibition of HDAC6 increased the acetylation level of microtubules in MCF-7 cells. Microtubules were found to resist nocodazole and cold-induced depolymerization in the presence of tubastatin A and TSA. Microtubules that resisted the destabilizing effects of nocodazole or cold-treatment were found to be predominantly acetylated. In TSA and tubastatin A treated cells, the proportion of detyrosinated tubulin increased indicating that the microtubules were stabilized in presence of these agents. Further, the effect of inhibition of HDAC6 on microtubule dynamics was determined using live cell imaging of microtubules in EGFP-tubulin transfected MCF-7 cells. Tubastatin A was found to suppress the dynamics of individual microtubules of MCF-7 cells. For example, the growth and shortening rates of microtubules were decreased by 25 and 32%, respectively in the presence of 15 μM tubastatin A. Microtubules spent 37 and 68% time in the pause state neither growing nor shortening detectably in the absence or the presence of tubastatin A, respectively. In addition, tubastatin A reduced the dynamicity (dimer exchange per unit time) of microtubules by 64%. Like tubastatin A, TSA also suppressed the dynamic instability of microtubules in MCF-7 cells. While the depletion of HDCA6 using siRNA did suppress the nocodazole induced depolymerization of microtubules, the extent of microtubule stabilization was not similar to that exhibited in the presence of TSA or tubastatin A suggesting that the presence of HDAC6 might partly stabilize

microtubules. The results together suggested a role of HDAC6 in microtubule dynamics and stability. (The work is supported by a grant from Department of Atomic Energy, Government of India).

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Tubulin posttranslational modifications - how to approach them?

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Posttranslational modifications of tubulin, such as polyglutamylation, polyglycylation, detyrosination and acetylation are known for many years now. These intriguing modifications, which take place on alpha- and/or beta-tubulin, are expected to regulate multiple functions of the microtubule cytoskeleton. Despite their potentially high impact on microtubule functions, only little insight into the roles of these modifications has been gained so far.
One of the reasons only few researchers have undertaken to study the role of tubulin posttranslational modifications is that only a limited number of tools and techniques are available, which are furthermore often cumbersome and difficult to access for outsiders of the field. Here we present an overview of tools and techniques to analyze tubulin modifications, and discuss recent advances frontiers in this research field.

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Microtubule acetylation induced by chronic alcohol consumption impairs nuclear translocation of STAT5B, but not SMAD 2/3, in polarized hepatocytes.

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Although alcoholic liver disease is clinically well-described, the molecular basis for alcohol-induced hepatotoxicity is not well understood. Previously we found that alcohol exposure led to increased microtubule acetylation and stability in polarized, hepatic WIF-B cells and in livers from ethanol-fed rats. Because increased microtubule acetylation is known to impair STAT5B nuclear translocation, we chose to explore a paradox regarding growth hormone signaling in alcohol-exposed hepatocytes. Although the circulating levels of growth hormone are increased in alcoholics and ethanol-fed animals, its hepatoprotective and hepatomitogenic responses are greatly reduced. Does alcohol-induced tubulin acetylation impair STAT5B nuclear translocation leading to impaired signaling? STAT5B and Jak2 (STAT5Bâ€™s activating kinase) protein levels did not change in ethanol-treated WIF-B cells or in livers from ethanol-fed rats indicating that decreased signaling cannot be simply explained by decreased protein amounts. Although STAT5B is partially cytosolic in control cells both biochemically and morphologically, increased cytosolic levels were observed in ethanol-treated cells with a reciprocal decrease in nuclear amounts. STAT5B nuclear levels also decreased to similar extents by addition of taxol (a microtubule stabilizing drug) or trichostatin A (TSA; a deacetylase inhibitor), agents that promote microtubule acetylation in the absence of alcohol. These results suggest that the alcohol-induced impairment of STAT5B nuclear translocation can be explained by increased microtubule acetylation. We also assayed nuclear translocation directly in control and treated cells after growth hormone addition. All three treatments impaired STAT5B nuclear translocation to similar extents both biochemically and morphologically indicating that microtubule hyperacetylation was responsible for the defect. Interestingly, only ethanol-treatment impaired STAT5B phosphorylation indicating that microtubule acetylation is not important for its activation by Jak2. Furthermore, nuclear exit and dephosphorylation were not changed by ethanol, taxol or TSA exposure indicating these processes are also independent of microtubule acetylation. Interestingly, the nuclear translocation of Smad2/3 (a transcription factor whose translocation is

prevented by intact microtubules), was not changed in ethanol-treated cells indicating different mechanisms regulate the microtubule-dependent distribution of these two classes of transcription factors. These results also raise the exciting possibility that agents that promote lysine deacetylation (e.g., resveratrol) may be effective therapeutics for the treatment of alcoholic liver disease.

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Effects of Disruption of Tubulin Post-Translational Modification on Cell Polarity.

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Microtubule post translational modifications are thought to provide directional cues to molecular motors for the delivery of cargoes to specific areas in cells. The precise delivery of cargoes is particularly important for the establishment and maintenance of epithelial cell polarity as different sets of proteins are needed in the apical and basal domains. Previously, we have shown that two post-translational modifications of tubulin, acetylation and detyrosination, define functionally different populations of microtubules in epithelial cells. We showed that there is a switch in the predominant form of tubulin modification from detyrosination to acetylation as cells become polarized, and that the two populations of modified microtubules are localized to distinct regions of MDCK cells at each stage of polarization. If these modified microtubules do provide the directional cues for polarized cargo traffic, then the establishment and maintenance of cell polarity may depend upon the integrity of the modified tubulin network. Here we have disrupted the balance between tubulin acetylation and detyrosination by altering the cellular levels of a tubulin acetyltransferase and a tubulin deacetylase and assessed the effects on cell polarity though immunofluorescence of the tight junction protein, ZO-1, as well as other apical markers.

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Microtubules Modified by Different Tubulin Post-Translational Modifications Show Differential Stability.

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Microtubules with tubulin post-translational modifications have long been correlated with the population of stable microtubules that does not undergo dynamic instability, but the precise relationship between tubulin modifications and microtubule stability has remained a mystery. Previously we have shown that in epithelial cells, there are multiple subpopulations of microtubules, some of which are acetylated only, some of which are detyrosinated only, some of which appear to only have polyglutamylation, and some with combinations of each of these modifications. We went on to show that these subpopulations were differentially localized in cells. Here we show that these microtubule subpopulations show differential sensitivities to both nocodozole-induced depolymerization and cold-induced depolymerization, and further that the sensitivities to nocodozole and cold differ from one another. We also separate the soluble tubulin pool from the polymer tubulin pool after both nocodozole and cold depolymerization and show that the reversal rates of different tubulin post-translational modifications is different. Tubulin re-tyrosination is relative rapid in these cells, but de-acetylation and de-glutamylation are slow. In addition, these results suggest that there may be significant soluble pools of tubulin dimers with these modifications. Together, these data suggest that there are multiple subpopulations of microtubules that differ in their subunit composition, their localization within the cell and their dynamic characteristics. This complexity may provide specificity for subcellular localization of microtubule-based activities, such as trafficking by molecular motors.

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A TOG:αβ-tubulin complex structure reveals conformation-based mechanisms for a microtubule polymerase.

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Microtubules are highly regulated, dynamic polymers of αβ-tubulin that have essential roles in intracellular organization and chromosome segregation. Proteins in the evolutionarily conserved Stu2p/XMAP215/Dis1 family are the major regulatory factors that promote fast microtubule growth in vivo. These proteins use multiple tubulin-interacting TOG domains to bind unpolymerized αβ-tubulin and to selectively recognize one end of the microtubule, where they catalyze microtubule elongation. The molecular details of TOG domain function remain poorly understood, in part because the structural basis of TOG:tubulin interations has not yet been defined. Here we report the 2.9 Å resolution structure of a complex between the TOG1 domain from Stu2p and a polymerization-blocked mutant of yeast αβ-tubulin that we developed to facilitate crystallization. The structure reveals that the TOG1 domain makes significant contacts with both α- and β-tubulin, and that the conformation of GTP-bound αβ-tubulin resembles a 'curved', microtubule-incompatible conformation observed previously in the structure of αβ-tubulin bound to a stathmin-like protein. The TOG1-interacting epitopes on α- and β-tubulin revealed by the structure do not overlap αβ-tubulin polymerization interfaces, and biochemical experiments demonstrate that TOG1 discriminates between αβ-tubulin conformations, binding preferentially to a curved one. We propose a conformation-based hand-off mechanism that explains how Stu2p family proteins can bind tightly to unpolymerized ab-tubulin and still promote their incorporation into the microtubule.

Cilia and Flagella II

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Regulation of intraflagellar transport by the MAP kinases MAK, MRK, and MOK.

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Cilia are microtubule-based organelles that can be found on the surface of almost all vertebrate cells. They were first noted for their function in motility, however were soon found to have a sensory function as well. Cilia are assembled and maintained by intraflagellar transport (IFT), which uses IFT particles to move cargo bi-directionally along the axoneme of the cilium. Regulation of IFT does not only allow modulation of cilia length, but also influences the different signaling pathways that initiate in the cilium.

We recently identified and characterized the *C. elegans* gene *dyf-5*. The *dyf-5* gene encodes a conserved MAP kinase. Loss of function and overexpression studies showed that DYF-5 affects IFT, and negatively regulates ciliary length. DYF-5 has three mammalian homologues: MAK (male germ cell-associated kinase), MRK (MAK-related kinase) and MOK (MAPK/MAK/MRK overlapping kinase). In this study, we aim to determine if MAK, MRK and MOK have similar functions as *C. elegans'* DYF-5.

We have generated GFP fusion constructs for MAK, MRK and MOK to determine their subcellular localization. All three *dyf-5* homologues localize along the microtubule axis of the cilium of IMCD3 (inner medullary collecting duct) and RPE (retinal pigment epithelium) cells, and show accumulation at the distal tip. Knockdown of MRK in IMCD3 cells, which express MRK and MOK, results in longer cilia. In RPE cells, which express all three *dyf-5* homologues, the knockdown of MOK also results in longer cilia. Overexpression of MAK and MRK in IMCD3

cells results in short and stumpy cilia. Interestingly, overexpression of MOK does not affect cilia length in IMCD3 cells. These effects on cilia length are in line with our data from *dyf-5* loss of function and overexpression worms.
We have generated IMCD3 cell lines that stably express GFP-fusion constructs of different components of the IFT complex: Kinesin-II (mCit-Kif3b), Kif17 (Kif17-mCit), complex A (IFT43-YFP), complex B (IFT20-GFP), and the BBSome (GFP-BBS8). This allows us to visualize the IFT particles, and measure their velocity. Preliminary experiments show an average anterograde speed of ~0,3 μm/s and a retrograde speed of ~0,4 μm/s for these GFP fusion proteins. We are currently performing knockdown experiments of the dyf-5 homologues in these stable cell lines to see whether they also affect IFT rates in mammalian cells.

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Temporary dikaryons in Chlamydomonas reveal that transport of I1 dynein in the cilium requires the intermediate chain subunit IC140 and Ida3p.

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To determine mechanisms of assembly and transport for ciliary dynein motors, we focused on the Chlamydomonas inner dynein arm, I1, as a model. Fractionation of cytoplasmic extracts from wild type (WT) cells revealed that I1 dynein forms a 20S complex in the cytoplasm prior to entry to the ciliary compartment. Analysis of cytoplasmic extracts from ida7 (IC140-null) reveals that the intermediate chain subunit IC140 is required for assembly of the 20S I1 complex. In another mutant that fails to assemble I1 dynein in the axoneme, ida3, the 20S I1 dynein complex assembles in the cytoplasm as in WT but fails to enter the cilium. We used cytoplasmic complementation in dikaryons to test the hypothesis that I1 dynein is transported to the tip of the cilium for assembly in the axonemes (Johnson and Rosenbaum, JCB 1992). In dikaryons formed between WT and I1 dynein mutants (or between ida1 x ida7 or ida1 x ida3), rescue of I1 assembly began at the distal tip of the mutant cilium and proceeded from tip to base. Thus, I1 dynein complexes are transported, possibly by IFT, to the distal end of the cilium prior to assembly in the axoneme. However, in contrast to other dikaryon combinations, I1 dynein assembly was significantly delayed in dikaryons between the ida7 (IC140-null) and ida3 (predicted to be defective in Ida3p). We postulate that IC140 / Ida3p form an obligate complex and new protein synthesis is required for rescue of I1 dynein assembly in axonemes from ida3 x ida7 dikaryons. Consistent with this hypothesis, cyclohexamide completely blocked rescue of I1 dynein assembly in ida7 x ida3 dikaryons. Cyclohexamide did not block rescue of I1 dynein assembly in any other combination of WT or I1 dynein mutants. We conclude that: [1] assembly of the 20S I1 dynein complex in the cytoplasm requires IC140; [2] I1 dynein is transported to the tip of the cilium prior to assembly in the axoneme; and [3] transport is dependent on a complex between IC140 and Ida3p. We postulate that IDA3 encodes a novel adapter protein, Ida3p, which links I1 dynein to the IFT machinery for transport in the cilium.

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Genetic analysis of individual IFT proteins in the transport of Hedgehog signaling components.

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Primary cilia extend from the apical surface of most vertebrate cells and function in a sensory manner to translate extracellular cues into intracellular responses. The IFT particle is a highly conserved multiprotein complex required for assembling primary cilia. Mutations in IFT proteins impair Hedgehog signaling due to either defective assembly of cilia or defective transport of

signaling components. However, the underlying contributions of individual IFT proteins to these processes remain obscure. We are using a cell culture system of immortalized cell lines derived from IFT mutant mice to decipher the molecular mechanisms that make individual IFT proteins indispensable for Hedgehog signaling. Ift20 mutant cells completely lack cilia, Ift140 mutant cells assemble short structurally abnormal cilia and Ift25 mutant cells assemble normal cilia that are defective for Hedgehog signaling. We are using these cells to test the ability of deletion and point mutants to rescue important phenotypes such as IFT particle integrity, cilia assembly, and ciliary trafficking of Hedgehog components.

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IFT46, IFT52, and IFT88 play different roles in IFT Complex B assembly, stability, and apical basal body localization in Chlamydomonas reinhardtii.

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Cells form cilia, hair-like appendages projecting from the cell membrane, in a process known as ciliogenesis. The key mechanism for ciliogenesis and maintenance of cilia is called intraflagellar transport (IFT). The main components necessary for effective IFT are Complexes A and B, anterograde motor kinesin-II, and retrograde motor IFT-dynein, although other proteins are involved. Direct protein-protein interaction analysis has shown that the core of Complex B is divided into three main subcomplexes: IFTs 52, 88, 46, and 70; IFTs 74, 81, and 72; and IFTs 27 and 25. By analyzing complex B mutants, in this study, we investigate the different roles of IFT46, IFT52, and IFT88 in IFT Complex B assembly and stability in Chlamydomonas reinhardtii. Our results show that a cell lacking IFT52 is not able to form Complex B, and this is most likely the reason why flagella do not form in bld1, an IFT52 null mutant. Cells without IFT88 also do not form flagella, even though this mutant allows the formation of a smaller Complex B. The reason for the lack of flagella in ift88 null mutants may be due to the fact that IFT88 is important for allowing proper localization of the IFT particles to the base of the flagella. Unlike bld1 and ift88, ift46 null mutants form short flagella perhaps because they still form an operable Complex B. The reason the cilia are so short and there are less IFT particles in ift46 mutants, is probably due to the importance of IFT46 in the stability of Complex B. These results clearly show that IFT46, IFT52 and IFT88, three subunits within the same subcomplex B, have distinct roles in complex B assembly, stability and apical basal body localization. This research is supported by the National Science Foundation.

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The RABL5 homolog IFT22 regulates the cellular pool size and the amount of IFT particles partitioned to the flagellar compartment in Chlamydomonas reinhardtii.

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Cilia and flagella, sensory and motile structures protruding from the cell body, rely on the continuous bidirectional traffic of intraflagellar transport (IFT) particles to ferry flagellar precursors into flagella for assembly. Cells synthesize a large pool of IFT particle proteins in the cell body, but only a small portion engages in active transport within the flagella at any given time. The atypical small G protein Rab-like 5 (RABL5) has been shown to move in an IFT-like manner in the flagella, but its function in ciliogenesis is controversial. In this report, we demonstrate that IFT22, the Chlamydomonas reinhardtii homolog of RABL5, is a bona fide IFT particle complex B subunit. Although the amount of IFT22 remains unaffected by depletion of either complex A or B, depletion of IFT22 leads to a smaller pool of both complex A and B. Strikingly, the smaller cellular pool of IFT particles does not lead to a reduced distribution of IFT particles to flagella. Instead, the amount of IFT particle proteins, including IFT22 itself, increase

in the flagella. Moreover, severely shortened flagella filled with IFT particles are observed in cells over-expressing IFT22 several-fold. Taken together, these data indicate that, in C. reinhardtii, IFT22 controls the cellular levels of both complex A and B, thus plays a critical role in determining the cellular availability of IFT particles. In addition, although IFT22 may not directly carry any precursors for flagellar assembly, it controls how many IFT particles out of the cellular pool participate in ferrying precursors into flagella.

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Control of IntraFlagellar Transport dynamics by the Planar Cell Polarity effector Fuzzy.

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Multi-ciliated cells are critical for development and homeostasis in many vertebrate organs, and Planar Cell Polarity (PCP) proteins have recently emerged as key regulators of multi-ciliated cell function, governing both ciliogenesis and the generation of polarized ciliary beating. However, it remains unclear how PCP is connected to the core cellular machinery underlying the formation and maintenance of cilia, the IntraFlagellar Transport pathway (IFT). We have developed an in vivo method for reliably imaging the dynamics of IFT within the multi-ciliated cells of the *Xenopus* embryonic muco-cilliary epidermis using high-speed confocal microscopy. Utilizing this method, we have characterized the dynamics of IFT within the axonemes of these cells and have found that IFT proceeds at rates comparable to those reported in studies of other ciliated cell types. Specifically, we find that IFT proceeds at a rate of 0.84 μm/sec in the anterograde direction and 0.87 μm/sec in the retrograde configuration. We also note that these rates have a wide distribution, with rates varying from 0.3 μm/sec to 1.4 μm/sec, likely reflecting differences in train composition or the molar ratio of attached motor proteins. In addition to our rate quantitation, we have qualitatively observed behaviors at the level of single IFT trains, including release and return of trains from the peri-basal body pool, as well as pausing of trains before they reach the distal tip of the axoneme. Excitingly, we found that the PCP effector Fuzzy (Fuz) is important for proper axonemal IFT function. When Fuz function is impaired, IFT trains accumulate along the length of the axoneme in large, extremely static foci, indicating that Fuz is a critical regulator of vertebrate IFT dynamics. Additionally, we have shown that loss of Fuz function leads to strong defects in patterning the distal--but not proximal--domain of multi-ciliated cell axonemes, supporting a role for Fuz in the regulation of axonemal IFT. This work presents the first detailed analysis of IFT behaviors in a multi-ciliated cell population, and provide the basis for a more thorough analysis of in vivo IFT behavior at the resolution of single trains. Our findings also argue that Fuz is an important regulator of vertebrate IFT, providing a key link between PCP signaling and the conserved machinery of ciliogenesis.

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A Source and Sink Model for IFT Cargo Transport and Unloading.

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The assembly of eukaryotic cilia and flagella requires intraflagellar transport (IFT) to move ciliary precursors (cargo) from their place of synthesis in the cell body (source) to the sites of assembly in the cilium (sink). To investigate how defects in the sink affect IFT, we analyzed three *Chlamydomonas* mutants having large-scale defects in the axoneme: *pf15*, defective in katanin p80 and lacking the central pair apparatus; *pf14*, defective in radial spoke protein RSP3 and lacking radial spokes; and *oda1*, defective in the dynein docking complex component DC2 and lacking outer dynein arms. We observed that IFT complex A- and B-particle proteins and anterograde and retrograde IFT-motor components are highly enriched in the flagella of all three

mutants compared to wild type. Further, a much larger proportion of the IFT proteins in the mutant flagella is less mobile and less soluble by detergent extraction. In *pf14* and *pf15*, the insoluble IFT material has accumulated in the space normally occupied by the radial spokes or central pair, respectively. Concomitant with accumulation of IFT particles, there is an accumulation of unassembled IFT cargos, including those that are not components of the defective structure. Based on these observations, we propose the following model for IFT cargo transport: In the cell body, free IFT particles and cargos are in an equilibrium with IFT particle-cargo complexes. The latter, however, can enter the flagellum, removing IFT particle-cargo complexes from the equilibrium and ensuring efficient loading. Within the flagellum, IFT particle-cargo complexes dissociate and reassociate, reestablishing the equilibrium. At the tip of wild-type flagella, free cargos are removed by binding to appropriate high-affinity sink structures, and free IFT particles are removed by activation of the retrograde motor and export from the flagellum. However, in the mutant flagella, the absence of an appropriate cargo sink causes the equilibrium to shift toward IFT particle-cargo complexes, which are not efficiently removed from the flagellum. The resulting increase in IFT particles also affects the equilibrium between IFT particles and cargos that are not part of the missing structure, so that these cargos also accumulate. While other factors may control details of this process, the described principle could be the driving force for IFT cargo delivery and flagellar assembly. The model could explain previously enigmatic features of the mutants, including their slower flagellar regeneration kinetics and shorter flagella.

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Phospholipase D Requires the BBSome and Retrograde IFT for Export from *Chlamydomonas* Flagella.

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Bardet-Biedl syndrome is a rare ciliopathy caused by defects in the BBSome. This conserved seven-subunit protein complex is required to maintain proper composition of the ciliary membrane; how the BBSome regulates ciliary membrane composition is unknown. The flagella of *Chlamydomonas bbs* mutants accumulate certain membrane-associated proteins. For example, phospholipase D (PLD) is accumulated ~150x compared to wild-type flagella, which contain only traces of PLD. PLD is abundant in wild-type cell bodies and reduced by about 50% in *bbs4* mutant cell bodies. These observations suggest that the BBSome efficiently prevents PLD accumulation in flagella. Lipidomic analysis of *bbs* mutant flagella revealed large changes in flagellar membrane lipid composition; these changes included a 30% decrease in the PLD substrate phosphatidylethanolamine and 50% and 4000% increases in the PLD products phosphatidic acid and ethanolamine respectively, and a 500% increase in the downstream product diacylglycerol. Therefore, PLD is active in mutant flagella and its presence there is likely to contribute to the non-phototactic phenotype characteristic of *Chlamydomonas bbs* mutants. To learn more about the role of the BBSome, we determined the kinetics of PLD accumulation in *bbs4* mutant flagella. Only small amounts of PLD are present in newly assembled mutant flagella; PLD accumulates over the course of several hours as the flagella age. We also observed that some flagellar proteins, e.g. carbonic anhydrase, were initially present in *bbs4* flagella at near wild-type levels but were lost over time, suggesting that their loss is a secondary effect. The continuous accumulation of PLD implicates the BBSome in either PLD flagellar entry control or PLD flagellar export. To distinguish between these possibilities, wild-type BBSomes were introduced into *bbs* mutant flagella by mating *bbs4-1* gametes with wild-type gametes to generate cells possessing 4 flagella, 2 of which originated from wild type and two from the mutant, in a common cytoplasm. PLD was removed from the mutant flagella within minutes after cell fusion. This rapid removal suggests an efficient BBSome-dependent flagellar export

mechanism for PLD. PLD also accumulates in flagella of *Chlamydomonas dhc1b*-ts, a hypomorphic mutant for the retrograde IFT motor dynein1b. This strain has elevated levels of flagellar BBS4/BBSomes, indicating that BBSome transport by retrograde IFT is required for PLD flagellar export. Our data show that BBSome-dependent ciliary protein export is important for maintaining a proper ciliary membrane.

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IFT and precursor pool regeneration in long and short flagella mutants of Chlamydomonas reinhardtii.

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The flagella of Chlamydomonas provide an excellent model system in which to investigate the basic question of organelle size control. The powerful genetics of this organism have allowed a number of mutations to be identified in which flagellar length is increased or decreased, but while many of these genes have been cloned, the mechanisms by which they alter length remain to be determined. Here, we investigate the long and short flagella mutants of Chlamydomonas using quantitative assays. We find that the four existing long flagella mutants (lf1 - lf4), all show increased levels of intraflagellar transport as judged by live cell TIRF microscopy. We hypothesize that these mutants have long flagella because of increased intraflagellar transport, which would support a key role for IFT in length control. We also have analyzed a set of short flagella (shf) mutants, and found that mutations in the PF15 gene, which encodes the p80 subunit of katanin, appear to have short flagella due to a defect in induction of the flagellar precursor pool. Although we do not know precisely why pool regeneration is defective, we suggest it could reflect a competition between cytoplasmic and flagellar microtubules for a common pool of tubulin, such that decreased microtubule severing in the cytoplasm leads to increased sequestration of newly synthesized tubulin, forcing shorter flagella to grow.

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A role for the Rab-interacting lysosomal protein family in cilia function.

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Many cell types in the mammalian body have a single non-motile primary cilium, a microtubule-based sensory organelle. Disruption of cilium form or function is the cause of a diverse set of human diseases collectively known as ciliopathies. Other specialized mammalian cell types have multiple motile cilia. Though these types of cilia are distinct, they share several structural and functional features. Most importantly, each cilium is nucleated by a centriole, the microtubule structure at the heart of the centrosome. To identify genes that are important for cilium structure and function we determined the transcriptional profile of ciliogenesis in cultured mouse tracheal epithelial cells (MTECs), which form hundreds of motile cilia per cell. One gene that is significantly upregulated during ciliogenesis is Rab-interacting lysosomal protein like 2 (Rilpl2). Rilpl2 is a member of a family of proteins that share two unique domains of sequence similarity. The founding family member, Rilp, is a Rab7/Rab34/Rab36 effector involved in vesicle trafficking to the lysosome. The only known role for Rilpl2 is an interaction with MyoVa that affects dendritic spine formation in neurons. There is no known function for the remaining family member Rilpl1. We show that Rilpl1 localizes to the mother centriole and both Rilpl1 and Rilpl2 localize to the primary cilium in fibroblast and epithelial cell lines. The C-terminal domains

of Rilpl1 and Rilpl2 are sufficient for centriolar and ciliary localization. In addition, the C-terminal domain of Rilpl2 can block ciliogenesis. Depletion of Rilpl1 or Rilpl2 alone or in combination does not affect ciliogenesis, but does prevent proper epithelial cell organization in three-dimensional culture, suggesting a disruption of cilium-dependent signaling. Our biochemical studies show that Rilpl2 self-interacts and we are currently assessing potential interactors. In summary, Rilpl2 was initially identified in our motile cilium formation model and has now been localized to the primary cilium along with its related protein Rilpl1. These data along with Rilpl2's role in neurons and the function of the related protein Rilp, suggest that Rilp-like proteins may be involved in trafficking or cytoskeletal rearrangements required for cilia function.

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IFT20 is a common component in macroautophagy and cilia formation.

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Mouse embryo fibroblasts (MEFs) in culture grow primary cilia upon serum starvation for 6-48 h. Macroautophagy (MA), a process that mediates degradation of cellular components in lysosomes through the formation of autophagic vacuoles (AV), is also activated in most cells by starvation at the time of ciliogenesis. We hypothesized that ciliary growth and MA are interrelated, sharing certain common components. One such component might be IFT20, a protein that shuttles between the Golgi apparatus and ciliary IFT particles (Follit et al., MBC, 2006). When IFT20 is knocked down MEF clones do not grow cilia. Similarly formation and clearance of AVs is reduced in IFT20 KD MEFs. These results suggest that interfering with MA will increase IFT20 available for ciliogenesis. To test this, MEFS defective in Atg5, an essential component of MA, are used. In the presence of serum, ciliary growth is minimal in wt MEFS. However, Atg5 KO MEFS have increased cilia formation and changes in the intracellular distribution of IFT20. Upon starvation, the reduction in IFT20 levels observed in wt cells is markedly attenuated in Atg5 KO cells and the % of ciliated cells is greatly increased. We conclude that IFT20 is a common component in ciliogenesis and MA.

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Ift88 Orchestrates Cell Polarization During Development And Repair Of The Corneal Endothelium.

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Directed cell migration generally requires polarization of cellular organelles such as the basal body (bb) and the Golgi apparatus. Very little is known, however, about how extracellular cues instruct intracellular polarity. Corneal endothelial cells (CEC) migrate or stretch with a precise directionality during development and repair of the corneal endothelium (CE), the cellular monolayer of the cornea facing the anterior chamber of the vertebrate eye. Our recent studies have shown that primary cilia are required for CE patterning and assemble during development and repair of CE but disassemble during normal adult CE steady state (1). Here we utilize the in vivo paradigm offered by the mouse CE to test the hypothesis that cilia are required for cell polarization during directed cell migration.

To assess cell polarization during postnatal development of the CE we have determined the position of the bb and IFT20 within CEC located at the center and periphery of the cornea. IFT20 is a component of the intraflagellar transport machinery required for assembly and

maintenance of cilia that not only localizes at the bb and cilia like all the IFTs but also at the Golgi. In central CEC of wild-type mice the majority of the bb and the IFT20 were localized near the cell center. In contrast, the bb and IFT20 were shifted toward the periphery in CEC located at the CE periphery. Strikingly, the position of the bb and distribution of IFT20 in peripheral CEC was random in orpk mice that carry a mutation in the IFT88 gene and show short cilia and defective CE patterning. A similar cell polarization was also found in CEC involved in in vivo wound healing of a mechanical injury. After 30h healing cytoplasmic IFT20 also polarized toward the leading edge of cells close to the wound whereas in intact CE, IFT20 localized perinuclearly and at the bb. We have found that the cilium instructs cell polarization of CEC involved in directed cell migration during development. We are currently using a conditional mutant for IFT88 to determine whether cellular polarization during repair also requires an intact cilium.

1) A. L. Blitzer et al., Proc Natl Acad Sci U S A 108, 2819 (Feb 15, 2011).

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An IFT74 truncation alters the balance of complexes A and B in flagella and leads to increased numbers of central microtubules.

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Intraflagellar transport (IFT), originally discovered as a bidirectional movement of large protein particles within *Chlamydomonas reinhardtii* flagella, is required for ciliary assembly in numerous organisms. IFT particles contain ~20 different proteins and are separable into two complexes known as complex A and complex B. Despite recent progress on involvement of IFT in specific signaling pathways, identification of some IFT cargoes, and elucidation of protein-protein interactions within complexes A and B, detailed information on the functions of individual IFT proteins is still largely lacking. In our ongoing screen for *Chlamydomonas* mutants defective in ciliary assembly and function, we have isolated an insertional mutant with a disruption in exon two of the gene encoding the complex B protein IFT74. Western blotting and sequencing of *ift74* cDNA indicated that this mutant expresses a truncated IFT74 protein missing the N-terminal 196 amino acids. Importantly, this mutant assembles flagella of ~40% wildtype (WT) length, allowing a more detailed analysis of the resulting flagella than is possible with complex B mutants lacking flagella. IFT particles visible by high-resolution DIC microscopy are dramatically decreased in frequency in the mutant flagella compared with WT. Sucrose density gradient centrifugation of flagellar membrane-plus-matrix showed that the truncated IFT74 protein enters flagella as part of a complex B with altered mobility in the gradient. Moreover, the molar ratio of complex A to complex B is reduced in mutant flagella, suggesting a requirement for the N-terminus of IFT74 for normal association of complexes A and B and transport of complex A into flagella. Transmission electron microscopy indicated that *ift74* cells are missing the wedge-shaped microtubule-membrane connectors visible in longitudinal sections of WT transition zones. Since a number of other *Chlamydomonas* flagellar assembly mutants also lack these structures, this may be a secondary effect of having partially assembled flagella. In addition, ~25% of *ift74* axonemes have increased numbers of central pair microtubules, with most of these having a 9+3 or 9+4 structure. Extra central pair microtubules are occasionally observed in some radial spoke mutants; however, radial spoke head proteins RSP1 and RSP10 are present at normal levels in the *ift74* flagella. Several reproducible protein differences between mutant and WT flagella visible on SDS-PAGE gels may hold clues to the mechanisms behind the imbalance between complexes A and B and the increase in central pair microtubules. We are currently analyzing these differences.

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Chlamydomonas IFT20 is essential for flagellar assembly but dispensable for IFT subcomplex assembly.

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Intraflagellar transport (IFT), the bidirectional movement of particles along the length of flagella and cilia, is required for the assembly and maintenance of these organelles. IFT particles are sub-divided into complex A and complex B. The protein IFT20 is part of complex B, and in mammalian cells IFT20 localizes to basal bodies, cilia, and the Golgi apparatus. To learn more about the function of IFT20, we are studying a *Chlamydomonas* insertional mutant that contains a complete deletion of the *IFT20* gene. The *ift20* null cells mostly lack flagella; constructs encoding wild-type, HA-, and GFP-tagged IFT20 rescue the mutant phenotype. The tagged proteins co-localize and co-immunoprecipitate with other IFT complex B proteins, but do not display Golgi localization, a pattern confirmed in wild-type cells using antibodies to endogenous IFT20. Consistent with this, *Chlamydomonas* lacks a homologue of GMAP210, which is required to localize IFT20 to the Golgi in mammalian cells. In contrast to deletions in all other IFT genes we have analyzed, loss of IFT20 does not affect the whole cell levels of other IFT proteins; we also found that IFT subcomplexes assemble normally in the cytoplasm in the absence of IFT20. This is consistent with IFT20 being peripherally associated with complex B (Lucker et al, 2005, J. Biol.Chem. 280:27688). Another study demonstrated that IFT20 binds to kinesin II (Baker et al., 2003, J. Biol.Chem. 278:34211), which functions in the anterograde movement of IFT particles. Together with our observation that IFT20 is dispensable for IFT particle subcomplex assembly, we hypothesized that IFT20 is an essential linker that bridges the IFT particle with the kinesin motor. To test this, we treated living cells with DSP, a membrane-permeable protein cross-linker that is cleaved in the presence of reducing agents, and then immunoprecipitated the cross-linked IFT complexes and analyzed their content by SDS-PAGE. Treatment of wild-type or rescued cell lines resulted in cross-linking of kinesin to IFT particles; this association was lost in *ift20* mutant cells. In addition, complex B was cross-linked to complex A in wild-type cells; this association was also not observed in *ift20* mutant cells. We are now testing two models that could explain these results: a) IFT20 interacts directly with both kinesin II and complex A, or b) complex B must first bind to either complex A or kinesin before binding the other, and one of these interactions is dependent on IFT20.

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RIIa and Dpy-30 domains dock discrete effectors to two amphipathic helices in a flagellar AKAP.

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A-kinase anchoring proteins (AKAPs) contain an amphipathic helix (AH) that anchors the dimerization and docking (D/D) domain, RIIa, in cAMP-dependent protein kinase (PKA). This localization mechanism for PKA has been exploited to discover many proteins with a RIIa-binding AH. Yet the D/D domain has broader applications, since RIIa and a similar Dpy-30 domain are adjacent to disparate molecular modules in proteins implicated in unrelated processes, like flagellar beating, membrane trafficking and epigenetic regulation. These molecules with a putative D/D domain and their anchoring proteins remain poorly characterized. We found that a dimeric AKAP, RSP3, in the flagellar radial spoke form a structural scaffold extending throughout the RS. It harbors two discrete AHs for anchoring RIIa and Dpy-30

domains in four spoke proteins that mediate the assembly and modulation of the complex. Interestingly, one AH can bind both types of D/D domains in vitro. In the same vein, Dpy-30 domain in Set1-like histone methyltransferase complex also interacts with a sequence containing a similar AH. Thus AHs and D/D domains constitute a versatile and promiscuous system for localizing various effector mechanisms. These results greatly expand the current concept about anchoring mechanisms and AKAPs.

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The vertebrate DYF-13 homologue undergoes IFT and is required for cilia formation and maintenance.

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Primary cilia are hair-like organelles that protrude from the surface of most cells in our body and are composed of nine parallel microtubule doublets surrounded by an extension of the plasma membrane. Recent studies have shown that the primary cilium acts as a sensory organelle to detect a wide variety of extracellular signals. Ciliary defects have been implicated in a diverse spectrum of diseases including retinal degeneration, polycystic kidney disease, left-right asymmetry defects and hydrocephalus. Primary cilia are assembled and maintained by the process of intraflagellar transport (IFT), a highly conserved mechanism in almost all eukaryotes. We analyzed the ciliary protein TTC26, which is the vertebrate homologue of *C. elegans* DYF-13. Mutants of *dyf-13* cause a dye-filling defect in *C. elegans* sensory neurons. GFP-labeled TTC26 localizes to the basal bodies and primary cilia of cultured mammalian cells. In addition, TTC26-GFP undergoes bidirectional movement in primary cilia. Moreover, tandem affinity purification analysis indicates that TTC26 makes a complex with other IFT proteins. Knockdown of TTC26 in zebrafish embryos produces short cilia within the Kupffer's vesicle and pronephric ducts and produces typical cilia-related defects including pronephric cysts, hydrocephalus, and the randomization of left-right asymmetry. We hypothesize that TTC26 is an IFT protein that is required for cilia formation and maintenance.

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Dual-colour live imaging in amphid channel cilia of *Caenorhabditis elegans* to study intraflagellar transport.

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Primary cilia are conserved extensions that protrude from most post-mitotic vertebrate cells and they play important roles in sensory processes. Intraflagellar transport (IFT) is a specialized transport mechanism inside cilia, required to transport proteins from the base of the cilium to the tip and back. Two kinesin motor complexes mediate anterograde transport in cilia of *C. elegans*; kinesin-II and OSM-3. Together these motors transport particles in the middle segment of cilia, while only OSM-3 enters the distal segment where it moves at a higher speed. The motors are attached to a complex of IFT proteins, which can be separated biochemically into complex A and B. The BBS proteins stabilize the two complexes and modulate the coordination of the two motor proteins. Several mutants have been identified, including the *bbs*, *dyf-5* and *gpa-3*, in which the two anterograde motors move at different speeds, and thus seem uncoordinated. The composition of the IFT particles in these mutants and in wild type animals has been mostly derived from speed measurements of different fluorescently-tagged IFT proteins independently. It has not been shown directly that IFT particles are transported by the two kinesins together, nor has it been shown what happens to the IFT particle composition in e.g. *bbs* mutants. Our objective is to determine the composition of IFT particles in the amphid cilia of *C. elegans* in

both wild type and mutant worms using dual-colour live imaging of fluorescently-tagged IFT proteins.
Constructs have been generated to express fluorescently-tagged OSM-3, KAP-1 (kinesin-II subunit), XBX-1 (dynein subunit), DAF-10 (complex A), CHE-13 (complex B) and BBS-8 in the cilia of one pair of amphid channel neurons. Interestingly, the dynamics of the fluorescently-tagged IFT protein is affected by its expression level. When the *osm-3::gfp* concentration is too high, its speed in the middle segment is higher than reported, while too much *kap-1::gfp* results in a reduced speed. This is likely caused by an uncoupling of the motor proteins. These results are in accordance with the current model of kinesin-II and OSM-3 moving together and support that the stoichiometry of these proteins is important for their coordination. We are currently testing whether overexpressing complex A, B, XBX-1 or BBS proteins affects IFT dynamics. In addition, we are generating transgenic animals co-expressing two different fluorescently-tagged IFT proteins.

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Endocytosis genes facilitate protein and membrane transport in C. elegans sensory cilia.

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Cilium formation and function relies on efficient and regulated targeting of proteins and membranes to the organelle. Transport almost certainly involves multiple modalities such as intraflagellar transport and there is increasing evidence for involvement by the exocytic and endocytic pathways. Although endocytic events near the cilium/flagellum base are suggested to facilitate membrane and ciliary protein trafficking, this model is not well tested. Thus, we examined the role of endocytosis in ciliogenesis and ciliary protein transport in the genetically tractable nematode, *C. elegans*. In worms, cilia extend from the distal dendrite tips of 60 highly polarized sensory neurons, house sensory signaling molecules, and enable the worm to sense environmental stimuli. Here we describe a ciliary endocytic zone (CEZ) at the base of sensory cilia; this zone appears to be a morphologically distinct compartment of the distal dendrite and contains pools of endocytosis-associated proteins such as the clathrin light chain, AP-2 clathrin adaptor, dynamin and RAB-5. Disruption of endocytic gene function causes expansion of membrane area both at the CEZ and in specialized olfactory cilia, as well as differential effects on the localization and transport dynamics of ciliary transmembrane, membrane-associated and IFT proteins. Expansion of ciliary membranes in dynamin and AP-2 mutants requires the functions of BBS-8 and RAB-8. Together, these data implicate an endocytic sorting station at the base of *C. elegans* sensory cilia, and suggest that this station facilitates the retrieval of ciliary membrane in a manner that may be counter-balanced by BBS-8 and RAB-8-mediated membrane delivery.

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Ciliary proteins are found on isolated cytoplasmic membrane vesicles and bioactive membrane vesicles are released from flagella.

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We are using the flagellated algal model system, Chlamydomonas reinhardtii, to compare the protein composition of 1. cytoplasmic vesicles targeted to the cilium, 2. plasma membrane, 3. flagellar membrane, and 4. membrane vesicles released from flagella. Western blot analysis of

these membrane compartments show several membrane and axonemal proteins including PKD2, IFT polypeptides, axonemal precursors and tubulin to be enriched in a specific cytoplasmic membrane compartment, and also in the flagellar membrane. IFT polypeptides and tubulin are not enriched in the plasma membrane. The results lend support to our hypothesis that almost all ciliary polypeptides, both membrane and axonemal, are first associated with cytoplasmic vesicles, some passing through the RER and Golgi, and others derived from free polysomes which piggyback on the Golgi vesicles, and that these vesicles then go through exocytosis in the pericentriolar region, and their polypeptides moved through the ciliary transition zone by IFT onto the flagellar membrane. The enrichment of ciliary membrane and axonemal proteins in the purified flagellar membrane fraction indicates that these ciliary precursors, IFT and its motors, form a complex with the flagellar membrane during their movement to the flagellar tip assembly site.

Real-time observation by DIC or fluorescence microscopy of fluorescently-tagged flagellar membrane shows flagellar membrane is released into the medium. This pinching off of flagellar vesicles was also documented by electron microscopy. Vesicles isolated from the culture medium are enriched for a subset of flagellar polypeptides such as α-tubulin (but not β), 14-3-3, PKD2 and high molecular weight ubiquitinated proteins, suggesting that flagellar membrane vesicle release is an active, selective process. Indeed, the rate of flagellar vesicles released increases when Chlamydomonas gametes attach to each other by their flagella, the first step in the mating process. Flagella are also required for the release of daughter cells from the mother cell wall, and this is dependent on the release of a vesicle-associated protease from the flagella. Cells without flagella are not released from the mother cell wall. We hypothesize that a similar release of biologically active vesicles probably occurs from many types of cilia, thereby signaling adjacent cells during development. Supported by NIH grant GM14642.

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The plasma membrane is a pool for some, but not all flagellar membrane proteins.

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Flagellar membrane proteins are associated with a variety of functions, including signaling and cell-cell interactions. Protein transport is dynamic, with selected surface proteins moving into the cilium upon stimulation by extracellular signals (Rohatgi & Snell, 2010) or when surface proteins are removed (Nakamura et al, 1996). Pulse-labeling revealed rapid incorporation of newly synthesized proteins (Song & Dentler, 2001; Stephens, 2001), interpreted as turnover. To determine if flagellar membrane lipids may turnover, Chlamydomonas cells were treated with up to 36μM Brefeldin A (BFA). BFA disrupted Golgi and induced flagellar shortening; BFA removal restored Golgi and flagella regrew, suggesting that Golgi-derived membrane lipids may be required to maintain flagella. We could not detect lipid recycling but discovered that flagellar membranes and surface proteins are released to the medium during an 8-hour period. If membrane and membrane proteins are released, then we predict that they must be replaced to maintain flagellar length. Possibly “turnover” is one-way: membrane and proteins are delivered to the flagellar base and released at flagellar tips. BFA may deplete the pool of available membrane lipids but what about membrane proteins? Despite rapid incorporation of newly synthesized membrane protein (above), new protein is not required to maintain flagella: they maintain normal length for more than 8 hrs. if protein synthesis is inhibited by cycloheximide. This suggests that there is be a pool of flagellar membrane proteins that does not depend on Golgi-delivery. To determine if the plasma membrane is the source of this pool, flagellated cells were biotinylated with NHS-LC Biotin, flagella were amputated, and new flagella were allowed to regenerate. Biotinylated proteins were detected on western blots of labeled and regenerated flagella and cell bodies. Regenerated flagella contained the same labeled bands that were present in initially biotinylated flagella, although the levels of some proteins were reduced. A

second deflagellation and regeneration revealed that the plasma membrane still served as a reservoir for selected flagellar proteins. These results reveal that the plasma membrane is a reservoir for most (surface accessible) membrane proteins and that flagella may draw on two pools of membrane protein, one of which is stationed on the plasma membrane and one that either is not accessible to biotinylation or must be delivered via membrane trafficking.

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Assembly and persistence of primary cilia in dividing *Drosophila* spermatocytes.

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Observations in many cell types suggest that the basal body must be freed from the cilium and transition to a centriole to organize functional centrosomes in dividing cells. Hence, dividing cells appear unable to form primary cilia. This has lead to wide acceptance of the principle of a mutually exclusive centriole/basal body life during cell cycle progression. Contrary to this general view, we show here that cilia assemble at G_2 phase of the cell cycle in *Drosophila* primary spermatocytes, and persist through two meiotic divisions. Using markers for centrioles and a marker specific for basal bodies (uncoordinated (UNC), the *Drosophila* ortholog of vertebrate OFD1), we show that basal bodies form in G_2 phase in spermatocytes. Acetylated tubulin marks cilia axonemes distal to spermatocyte basal bodies concomitantly with UNC recruitment to centrioles. Remarkably, all four centrioles assemble primary cilia, in contrast to most cell types that typically assemble only one primary cilium from the mother centriole. At prophase, all four centriole-cilium complexes disengage from the plasma membrane, and transit into the cytoplasm encased in a packet of membrane. Centrioles then recruit centrosomal material into microtubule-organizing centers, and persist at the spindle poles through division. Thus, at cell division, spermatocyte centrioles organize centrosomes and cilia simultaneously. These cilia persist through two meiotic divisions, partially disassemble in new spermatids, and then reassemble into the long spermatid flagellum. Moreover, spermatocyte cilia have a unique “9+1” ultrastructure, a rare departure from the usual cilium architecture. Cilium resorption may therefore not be necessary to enter the cell cycle. These findings challenge the prevailing view that cilia antagonize cell cycle progression, and raise the possibility that cilium retention in dividing cells may occur in other organisms and in certain cell types.

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A Size-Exclusion Permeability Barrier and Nucleoporins Form a Ciliary Pore Complex that Regulates Transport into Cilia.

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The primary cilium is a microtubule-based organelle with a unique complement of proteins for motility and signaling functions. Entry into the ciliary compartment is proposed to be regulated at the base of the cilium. Recent work demonstrated that components of the nuclear import machinery, including the RanGTPase and importins, regulate ciliary entry. We hypothesized that the ciliary base contains a ciliary pore complex (CPC) whose molecular nature and selective mechanism are similar to the nuclear pore complex (NPC). By microinjecting fluorescently-labeled dextrans and recombinant proteins of various sizes, we characterize a size-dependent diffusion barrier similar to that of the NPC, for the entry of cytoplasmic molecules into primary cilia in mammalian cells. We demonstrate that nucleoporins localize to the base of primary and motile cilia in mammalian cells and that microinjection of anti-nucleoporin antibodies restricts the ciliary entry of KIF17 motors. Together, this work demonstrates that the physical and molecular

nature of the CPC is similar to the NPC, and further extends functional parallels between nuclear and ciliary import.

Centrosomes

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Myosin II-Dependent Traction Forces Regulate Centrosome Positioning.

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Fibroblasts maintain centrally localized centrosomes on 2D surfaces but show off-centered centrosomes when embedded in a 3D collagen gel. This study aims to investigate the mechanism of centrosome positioning and the reason why it differs between cells in 2D and 3D. By combining substrate micropatterning and traction force microscopy, we show that cells with a highly elongated shape, similar to those seen in collagen gels, have significantly decreased traction forces and off-centered centrosomes. Blebbistatin, an inhibitor of myosin II-dependent traction forces, also causes centrosomes to scatter, indicating that traction forces are required for maintaining centrosome location. Furthermore, when plated on micropatterned substrates to create well-spread cells with small focal adhesions, cells also show reduced traction forces and scattered centrosomes, suggesting that shape per se is not a determining factor. Our results indicate that actomyosin contractility, in addition to forces generated by microtubules and dynein, plays an important role in centrosome positioning.

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Determining the role of Nek2 in regulating β-catenin function at centrosomes.

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Beta-catenin is a multifunctional protein with critical roles in cell-cell adhesion, Wnt-signaling and bipolar spindle formation. Whereas the roles of β-catenin in adhesion and Wnt-signaling have been extensively studied, the mechanism(s) by which β-catenin regulates bipolar spindle formation is poorly understood. The centrosomal NIMA-related protein kinase 2 (Nek2) binds and phosphorylates β-catenin and stimulates centrosome separation. Nek2, like β-catenin, is required for bipolar spindle formation. Using *in vivo* and *in vitro* approaches, we analyzed the effects of Nek2 on β-catenin localization, function and stability at centrosomes. Nek2 phosphorylates regulatory sites in the N-terminus of β-catenin. Nek2 inhibits β-catenin ubiquitylation and promotes β-catenin stability independent of its kinase activity. Nek2 depletion dramatically reduces β-catenin levels at centrosomes, inhibits centrosome separation, and delays mitotic progression. Over-expression of kinase-dead Nek2 also inhibits centrosome separation but does not appear to strongly affect centrosomal β-catenin levels. These results indicate that although Nek2 protein is required for β-catenin recruitment and stability at centrosomes, its kinase activity is not. In order to assay the function of Nek2-phosphorylated β-catenin, we have developed an *in vitro* assay for centrosome separation (loss of centrosome cohesion). This assay confirms previous findings that Nek2 activity is required for centrosome separation and allows us to further elucidate the key role of Nek2 and β-catenin in regulating centrosome cohesion during the cell cycle.

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Voltage Dependant Anion Channel 3 (VDAC3), a novel centrosomal protein regulating ciliogenesis and centriole assembly.

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In cycling cells centrosomes undergo duplication to serve as the poles of the mitotic spindle, whereas in quiescent cells the maternal centriole is converted to a basal body in order to organize a primary cilium. Since the cilium must be resorbed upon cell cycle re-entry, a mechanism must exist to control the inter-conversion of basal body and centriole in order to coordinate the centriole duplication cycle and ciliary assembly-disassembly. We have demonstrated earlier that the centrosomal pool of Mps1 is crucial for the proper control of centriole duplication, but how Mps1 is targeted to centrosomes is not known. In an attempt to characterize the mechanisms of Mps1 targeting to centrosomes, we used yeast two-hybrid to identify protein(s) that can bind to the Mps1 centrosome localization domain. One of the candidates identified from this screen was VDAC3, a member of mitochondrial porin family of proteins, which was also found in the sperm flagella outer dense fiber. The molecular interaction between VDAC3 and Mps1 was validated by in vitro pull down assays and co-immunoprecipitation between Mps1 and GFP-VDAC3 expressed in HeLa cells. Moreover, GFP-VDAC3 localizes to centrosomes and a VDAC3-specific antibody predominantly stains the mother centriole, suggesting that VDAC3 is a bonafide centrosomal protein. VDAC3 depletion led to a significant reduction in the centrosomal Mps1 levels and an inhibition of centriole duplication as judged by an increase in the number of S-phase cells with unreplicated centrioles. Depletion of VDAC3 also leads to aberrant formation of primary cilia in growing human RPE-1 cells while the ectopic expression of GFP-VDAC3 in quiescent RPE-1 cells suppressed their ability to form cilia. Depletion of Mps1 also induced aberrant formation of cilia but in a smaller number of cells compared to VDAC3 depletion. Thus, our observations suggest that VDAC3 regulates a decision between ciliogenesis and centriole assembly, at least in part by regulating the recruitment of Mps1 to centrosomes.

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CEP135 carries two functional domains and is required for centriole assembly.

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The centrosome is the primary microtubule-organizing center (MTOC), which is composed of two centrioles surrounded by an electron-dense matrix known as pericentriolar material. Centrioles are usually composed of nine triplets microtubules (MTs) organized around a cartwheel structure, which is located at the proximal end of the centriole and is required for the initiation of daughter centriole assembly. Recently, several key conserved proteins including PLK4, hSAS-6, CPAP, and CEP135 have been reported to be involved in the early event of centriole duplication in human cells. In *Chlamydomonas reinhardtii,* both Bld10p and SAS6 function in the formation of the nine-fold symmetrical cartwheel and are essential for an early step of centriole/basal body assembly. CEP135, the human ortholog of Bld10p, was reported to be required for PCM integrity, centrosome cohesion, and PLK4-induced centriole amplification. However, the molecular basis of how CEP135 functions in the cartwheel and participates in centriole assembly is not clear. Here, we show that CEP135 directly interacts with hSAS-6 and is associated with microtubules (MTs). Using co-immunoprecipitation, GST pulldown, yeast two-hybrid, and MT cosedimentation assays, we found that the C-terminal domain of CEP135 (residues 896-1140) interacts directly with hSAS-6 (residues 146-586) and the N-terminal

domain of CEP135 (residues 1-460) is cosedimented with microtubules. Interestingly, overexpression of either N-terminus or C-terminus of CEP135 perturbs centriole duplication, suggesting that these truncated polypeptides exert a dominant negative effect on centriole duplication. Furthermore, depletion of CEP135 results in inhibition of normal centriole duplication and reduction of centriole amplification induced by overexpression of PLK4, hSAS-6, or STIL. CEP135 depletion also inhibits CPAP-induced centriolar microtubule assembly, implying that CEP135 may have addition roles in stabilizing the microtubules cylinder. From electron microscopic analysis, we further observed defective centrioles with abnormal numbers of triplets and reduced centriole length in CEP135-depleted cells. In light of these findings, we propose that CEP135 plays an essential role to connect the inner cartwheel to outer microtubules triplets during the initiation of daughter centriole assembly, followed by CPAP-mediated centriole elongation at later stage.

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The Role of Mps1 Kinase in Centrosome Duplication in Human Breast Cells.

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Each centrosome contains two symmetric centrioles that strictly duplicate once every cell cycle and mediate the proper segregation of chromosomes during mitosis. Errors in this duplication produce extra centrioles that lead to chromosome mis-segregation and aneuploidy in cultured cells. Centrosome amplification has been found in pre-malignant lesions, in-situ tumors of the breast and in over 70% of invasive breast tumors. However, the factors that contribute to aberrations in centriole duplication and the correlation between aneuploidy and tumorigenesis remain elusive. Studying centrosomal proteins and their functions will shed light on potential mechanisms of centrosome amplification. Our long-term goal is to improve breast cancer prognosis and widen our knowledge on potential targets for therapeutic intervention. Mps1 is a protein kinase that localizes to the centrosomes and is involved in centriole duplication. Microarray data shows strong positive correlations between high levels of Mps1 mRNA and breast tumor grade. Preventing the degradation of Mps1 at centrosomes is sufficient to cause centriole re-duplication during prolonged S-phase arrest in various cell lines. We have found that stable expression of the non-degradable form of Mps1 (Mps1Δ12/13) in 293 cells leads to the production of excess centrosomes in cycling cells, and leads to colony formation in limiting dilution plating assay. These data suggest that Mps1 is an important regulator of centrosome numbers and studying its functions may contribute to a better understanding of the possible link between centriole duplication and tumorigenesis. Owing to the frequent presence of centrosome defects early in the progression of breast cancer, we sought to study the precise role of Mps1 in centriole duplication in non-transformed human mammary cells. Preliminary data suggests that transient over-expression of both Mps1 and Mps1Δ12/13 in MCF10A cells leads to centrosome re-duplication with the number of extra centrosomes being higher in the latter. We hypothesize that deregulation of the degradation of Mps1 will cause centriole re-duplication and chromosomal instability in cycling breast cells, contributing to tumorigenesis. To test the hypothesis, we are generating breast cell lines that inducibly express the non-degradable Mps1 mutant (Mps1Δ12/13), and we will examine centrosome numbers and ploidy upon induction of Mps1Δ12/13. Further, the effect of Mps1Δ12/13 on in-vitro transformation and tumor formation in mice will be studied.

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Mps1 in the Plk4 Centriole Biogenesis Pathway.

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Even upon culmination of all the recent advances in the structural and functional understanding of the centriole, and despite remarkable conservation, the exact molecular mechanisms of the Plk4 induced centriole biogenesis pathway in human cells remains unclear. Plk4, the presumptive orthologue of *C. elegans* ZYG-1, is thought to be the major driver of centriole assembly. However, the physiological substrates of Plk4 remain to be identified, and Plk4 is not functionally interchangeable with ZYG-1. Moreover, unlike the case in *C. Elegans,* Plk4 has not been shown to phosphorylate hSas6, nor is it required for recruitment of hSas6 to the procentriole, though Plk4 does have a direct or indirect role in the maintenance of hSas6 at the procentriole. Overexpression of Mps1, Plk4, or HsSas6 is capable of producing extra centrioles, indicating the importance of strict regulation of these proteins throughout the cell cycle. Our preliminary data suggests that centriole amplification in human cells caused by overexpression of hSas6 does not require Plk4. This suggests that other centrosomal kinases can compensate for the absence of Plk4, and one study has suggested that ZYG-1 is more closely related to Mps1 than it is to Plk4. While the requirement of Mps1 in centrosome duplication remains controversial, it is clear that Mps1 plays several roles in centriole assembly, and defects in its regulation promote centrosome re-duplication. We have found that Mps1-dependent re-duplication requires Plk4, suggesting that the two kinases cooperate to promote centriole overproduction. However, the regulation between these two centrosomal kinases remains unclear. In this study, we explore the potential interplay between Mps1 and Plk4 using various combinations of overexpression and depletion of these two kinases.

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The molecular interplay between CEP63 and CEP152 in the vertebrate centrosome.

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We have recently established CEP63 as a protein required for maintaining normal centrosome numbers in vertebrate cells. In this study, we carried out a proteomics screen to find that CEP63 interacts with CEP152, a highly conserved centrosomal protein essential for centrosome duplication in flies and mammals. Furthermore, we identified C-terminal 321 amino acids of CEP63 as CEP152 interaction domain. In line with CEP63-CEP152 interaction, we found that CEP63-deficient centrosomes were unable to accumulate CEP152, a defect most pronounced in mitosis. Tethering CEP152 to CEP63-deficient centrosomes restored normal centrosome numbers to CEP63-knockout cells, indicating that CEP63 controls centrosome duplication by targeting CEP152 to the centrosome. In addition, centrosomal localisation of CEP152 seems to be regulated by Polo like kinase 1 (PLK1), a kinase implicated in centriole biogenesis. We found that inhibition of PLK1 with a small-molecule inhibitor, BI-2536, dramatically increased centrosomal CEP152 levels in mitosis. Overall, our data suggest that centrosomal levels and distribution of CEP152 are positively regulated by CEP63 and negatively by PLK1 activity. To gain further insight into the molecular roles of CEP63 and CEP152, we have also disrupted the CEP152 gene in vertebrate cells and will also present characterisation of centrosome function in this cell line.

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PLK1 phosphorylation of pericentrin initiates centrosome maturation at the onset of mitosis.

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The microtubule-organizing activity of centrosome oscillates during cell cycle, reaching the highest at M phase. At the onset of mitosis, the centrosome undergoes maturation, which is characterized by a drastic expansion of the pericentriolar matrix (PCM) and a robust increase in microtubule organizing activity. It is known that PLK1 is critical for the initiation of centrosome maturation. Here, we report that pericentrin (PCNT), a PCM protein, is specifically phosphorylated by PLK1 during mitosis. The phospho-resistant point mutants of PCNT did not recruit centrosomal proteins, such as CEP192, GCP-WD, gamma-tubulin and Aurora A into the centrosome during mitosis. The centrosomal recruitment of PLK1 is also dependent on phospho-PCNT most likely in a positive feedback mechanism. However, centrosomal recruitment of CEP215 depends on PCNT irrespective of the phosphorylation status. Furthermore, ectopic expression of the PLK1-PCNT fusion proteins induces the centrosomal accumulation of CEP192, GCP-WD and gamma-tubulin even in interphase cells, mimicking centrosome maturation. Based on these results, we propose that PLK1-mediated phosphorylation of PCNT initiate centrosome maturation by organizing the spindle pole-specific PCM lattice.

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SAS-6 dimers adopt a folded conformation required for centriole assembly.

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SAS-6 is a component of the universal module required for centriole assembly. SAS-6 has an N-terminal globular domain, a central coiled-coil, and a C-terminal domain that is predicted to be unstructured. Recent structural work revealed that SAS-6 dimers associate with each other through their N-terminal globular domains to form a 9-fold symmetric circular plate that resembles the cartwheel seen in EM cross-sections of centrioles. In the current model, the N-terminal domains form an ~23 nm diameter circular hub, and the SAS-6 coiled-coils extend out from the hub as rigid rods to form the cartwheel spokes. No function has yet been ascribed to the predicted unstructured C-terminal domain of SAS-6. We have analyzed SAS-6 full-length and the SAS-6 coiled-coil alone using a combination of analytical ultracentrifugation, small angle X-ray scattering (SAXS), and electron microscopy. Surprisingly, single particle EM revealed that the SAS-6 dimer is not a rigid rod, but folds back upon itself to adopt a bent conformation. This observation was consistent with SAXS analysis, which indicated that full-length SAS-6 is more compact (smaller radius of gyration and maximum distance between scatterers, Dmax) than the SAS-6 coiled-coil alone. The folded SAS-6 conformation requires the unstructured C-terminal tail, and a weak interaction between the C-terminal tail and the rest of SAS-6 can be detected *in vitro*. Using a single copy transgene insertion system in *C. elegans*, we found that the C-terminal tail is essential for centriole assembly. Mutational analysis identified a small region in the tail that is essential. Perturbation of the tail or the more defined region does not affect the interaction of SAS-6 with SAS-5, excluding disruption of this interaction as a potential reason for the centriole assembly failure. We propose that this region in the unstructured SAS-6 C-terminus interacts with a region in the coiled-coil to generate a folded conformation that is important for centriole assembly. We are currently investigating the precise step at which centriole assembly fails when this intramolecular interaction is disrupted.

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A Dual Role For Bld10 During Basal Body Assembly.

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Centriole dysfunction causes ciliopathies and has been implicated in cancer. These multifunctional organelles form the core of centrosomes and are required for nucleation of cilia. Centrioles that function to assemble and maintain cilia are referred to as basal bodies. Due to the torque generated by the beating of motile cilia, basal bodies require proteins to stabilize the structure. Thus both basal body assembly and stability are integral to ciliary function. While much has been done to illuminate the proteome of basal bodies there remains a substantial gap of knowledge concerning the mechanism of basal body assembly. Basal body structure is well conserved across eukaryotes with a key domain being the cartwheel. The cartwheel is composed of nine symmetrically spaced spokes radiating out from a central hub. Attached to the end of each spoke are triplet microtubules which form the length of the basal body. Formation of the cartwheel is the first identified step in basal body assembly. The central hub and the spokes of the cartwheel are made up, at least in part, of the conserved basal body protein Sas6. Bld10/Cep135 is an additional conserved protein that localizes to and is required for cartwheel assembly. This large coiled-coil domain containing protein is required for basal body formation in most systems tested. However the functional role that Bld10/Cep135 plays in basal body assembly is uncharacterized. We identified a single conserved Bld10/Cep135 gene, TtBld10, that encodes a 171 kDa protein in the ciliate Tetrahymena thermophila. We find that Bld10 localizes to the periphery of the cartwheel which raises the possibility that Bld10 helps attach microtubules triplets to the cartwheel. Additionally, we find that Bld10 has two distinct incorporation profiles. One population of Bld10 incorporates in a stable manner only during new basal body assembly. Another population of Bld10 accumulates at the basal body slowly with time. Once assembled, both of these populations remain stable at the centriole. We propose that the early localizing population of Bld10 is required for assembly of basal bodies and the late accumulating population of Bld10 is required for basal body stability. A complete genomic knockout of Bld10 disrupts cell swimming and causes pronounced cortical shape irregularities. Cell lethality occurs by 48 hours in bld10Δ cells. Furthermore, we find that assembly of new basal bodies is inhibited in bld10Δ cells. In addition to inhibition of new basal bodies assembly, we find that existing basal bodies in bld10Δ cells disassemble with time suggesting that Bld10 is required for basal body stability. Thus we find that Tetrahymena Bld10 functions in both the assembly and stability of basal bodies.

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Centrosome Loss in the Evolution of Planarians.

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The centrosome, a cytoplasmic organelle formed by cylinder-shaped centrioles surrounded by a microtubule-organizing matrix, is a hallmark of animal cells. The centrosome is conserved and essential for the development of all animal species described so far. We show that, unlike the rest of animals, planarians and possibly other flatworms as well completely lack centrosomes. We found that planarians assemble centrioles de novo in ciliated cells, but lack the pathway for centriole duplication underlying centrosome reproduction in other animal species. This unique characteristic allowed us to identify a large set of conserved proteins required for centriole assembly in animals, as well as the centrosome signature proteins missing from the planarian

genome. Our study uncovers the molecular architecture and evolution of the animal centrosome and emphasizes the plasticity of animal cell biology and development.

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Genes and post-translational modifications required for acentrosomal mitosis.

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Centrosomes serve as the major microtubule-organizing centers (MTOC) for animal cells at mitosis. In *Drosophila*, centrosomin (Cnn) is required for recruitment of the pericentriolar material (PCM) and for mitotic MTOC activity. Cells that are deficient in Cnn, either *in vivo* or in cell culture, assemble mitotic spindles by an alternative "acentrosomal" pathway. Cells divide efficiently using this alternative mode of mitosis, and are capable of supporting development into an adult fly. Synthetic lethality between *cnn* mutations and mutations in other non-lethal genes have been reported, and suggests that an alternative pathway is involved in acentrosomal mitosis. Regulation of this pathway remains unclear. We have employed a genetic screen to dissect the genes involved in this pathway, and a proteomic approach to identify changes in protein levels or modifications that are different between centrosomal and acentrosomal mitosis. By using this combination of genetic and proteomic approaches, we will define the differences between these pathways. Using 2D-Difference Gel Electrophoresis (2D-DIGE), we found 15 proteins that are differentially expressed or post-translationally modified between wild-type and *cnn* mutant tissues. For the genetic approach, we identified 26 mutations that are synthetic lethal with *cnn*. Two of these mutants exhibit non-lethal phenotypes (infertility) when present in a *cnn* heterozygous background. The preliminary results suggest that at least one of these genes is required for proper spindle assembly in acentrosomal mitosis, but not in centrosomal mitosis. By combining these genetic and proteomic approaches we can define the major differences between centrosomal and acentrosomal mitosis, and gain a mechanistic understanding of mitotic spindle assembly *in vivo*. These results could lead to new targets for halting cell division in a therapeutic setting.

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Analysis of fungal homologs of Mozart1, a newly identified spindle pole protein.

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Mozart1 is a small (82 aa) centrosomal protein that was identified by high-throughput genome-wide screening of animal cells. It plays an important role in mitosis including establishment of bipolar spindles (Hutchins et al., Science 2010, 328, 593). Orthologs of Mozart1 were found in the genomes of a wide range of eukaryotes including most fungi suggesting that the function of this protein has been conserved throughout evolution. We analyzed the function of the Mozart1 orthologs in the filamentous fungus *Aspergillus nidulans* (MZTA) and the fission yeast *Schizosaccharomyces pombe* (Mzt1). In *A. nidulans,* a homolog of Mozart1 that exhibits about 50% amino acid identity is encoded by a single gene (*mzt*A). Tagging MZTA with fluorescent proteins revealed that MZTA localizes to spindle pole bodies (SPBs) indicating that this polypeptide is likely to be an functional homolog of Mozart1. Strains carrying a deletion of *mzt*A were viable but exhibited a weak temperature sensitivity. In *mzt*A deletants, fewer cytoplasmic microtubules were observed suggesting that nucleation of the microtubules is suppressed in the absence of MZTA. We investigated the functional relationship between MZTA and the γ-tubulin complex proteins (GCPs). Double deletants of *mzt*A and nonessential GCP genes (*gcp*D~F)

exhibited a variety of synthetically sick phenotypes. While the absence of these nonessential GCPs alone does not affect growth significantly, depleting both MZTA and any one of these GCPs at the same time caused a significant growth reduction. *S. pombe* Mzt1 is essential for growth, and colocalizes with the γ-tubulin complex to the SPBs throughout the cell cycle and to the eMTOC, a site for cytoplasmic microtubule formation upon mitotic exit. We created a number of temperature-sensitive *mzt1* mutants and found that *mzt1* mutant cells are defective in bipolar spindle assembly and elongation, and formation of interphase arrays of cytoplasmic microtubules. Our data indicate that the homologs of Mozart1 play an important role in organizing microtubules in fungi by interacting with γ-tubulin and/or γ-tubulin complex proteins.
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Plant Homologues of GCP2, 3, 4, 5, and 6 Proteins are Components of Gamma-tubulin Complexes in Acentrosomal Plant Cells.

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Gamma-tubulin complexes that present major microtubule nucleators from centrosomes are believed to be conserved in all eukaryotes. Microtubules are nucleated in acentrosomal plant cells from sites dispersed in cytoplasm, with membranes, and with pre-existing microtubules. There are orthologues of genes encoding all mammalian gamma-tubulin complex proteins (GCPs) present in Arabidopsis genome, except recently reported GCP8 protein. However, data on composition and function of plant gamma-tubulin complexes are still largely missing. We identified Arabidopsis GCPs homologues AtGCPs 2, 3, 4, 5, and 6 among proteins that co-purified either with endogenous or with expressed gamma-tubulin-GFP. Here we provide data on homologue of GCP3 protein, a member of the small g-TUSC complexes, and on AtGCP4 protein that is a representative of the large g-TURC in animal cells. We found that AtGCP3 and 4 were together with gamma-tubulin spun down with taxol polymerized plant microtubules. Association of AtGCP3 and AtGCP4 with microtubules was proven on cellular level, immunofluorescent staining further revealed localization of both GCPs in the nuclei. Complexes of gamma-tubulin with AtGCP3 and AtGCP4 proteins were purified from the cytosolic and microsomal fraction. Presence of AtGCP3 and AtGCP4 proteins in size heterogeneous gamma-tubulin complexes with maximum >1 MDa was further indicated by size exclusion chromatography. Our data suggest that AtGCP2-6 proteins are components of large gamma-tubulin complexes. Our proteomic analysis identified further proteins that belong to the cell cycle signaling and Ran GTPase pathway co-purified with gamma-tubulin. Regulatory functions of gamma-tubulin that recently emerged in cells equipped with centrosomes or spindle pole bodies might be therefore preserved also in acentrosomal plant cells.
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Functional characterization of CP148, a novel key component for centrosome integrity in Dictyostelium.

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The Dictyostelium centrosome consists of a layered core structure surrounded by a microtubule-nucleating corona. A tight linkage through the nuclear envelope connects the cytosolic centrosome with the centromeres that are clustered in the pericentrosomal region of the nuclear matrix. At the G2/M transition the corona dissociates and the core structure duplicates yielding

two spindle pole bodies. CP148 is a novel centrosomal component identified in a proteomic screen. It is a coiled coil protein with no significant homology to other proteins. Confocal deconvolution microscopy revealed that CP148 is a component of the centrosomal corona. Moderate expression of GFP-CP148 demonstrated a cell cycle-dependent centrosomal localization. The fusion protein is present at the centrosome during interphase but it is absent from mitotic centrosomes. Its cell cycle-dependent presence and absence at the centrosome correlates with dissociation of the corona in prophase and its reformation in late telophase. Live cell imaging revealed the appearance of many cytosolic GFP foci in early telophase, which coalesced later in telophase and finally joined the centrosome. This explains the hypertrophic appearance of the corona in interphase cells upon strong overexpression of GFP-CP148, which was confirmed by electron microscopy. Since CP148 is a corona protein and the time frame of GFP foci formation coincides with corona formation, we concluded that CP148 is required for corona organization and functions as glue for corona proteins. To confirm this idea, we depleted endogenous CP148 by RNAi. This resulted in a virtual loss of the corona and consequently disorganization of interphase microtubules. Surprisingly, formation of the mitotic spindle and astral microtubules was unaffected by CP148-RNAi, which indicates that microtubule nucleation complexes employ different means of association with centrosomal core components during interphase and mitosis, respectively. Furthermore, CP148 RNAi caused dispersal of the usually clustered centromeres and altered distribution of Sun1 at the nuclear envelope suggesting a role of CP148 in the nuclear envelope-spanning linkage of centrosomes and centromeres. Taken together we have shown that the novel Dictyostelium protein CP148 is an essential factor for the formation and organiziation of the microtubule-nucleating centrosomal corona and suggest that centrosome/nucleus attachment occurs through corona components.

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Aurora Kinase and the formation of the blepharoplast, a centrosome-like particle that nucleates *de novo* formation of basal bodies in *Marsilea vestita*.

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The development of the *Marsilea vestita* microspore into motile sperm occurs in 11 hours at 20°C, with all of the cellular divisions occurring during the first 5 hours. The blepharoplast is a structure that arises *de novo* ~3.5 hours after the dry spores are placed in water. The particle disappears then reappears at 4 hours and functions as a MTOC in a centrosome-like fashion for the spindle during the final mitotic division. After this division, it matures and eventually serves as the *de novo* assembly site for ~140 basal bodies. We are interested in the role and function of Aurora Kinases in the *de novo* synthesis of the blepharoplast. Aurora Kinases are a conserved family of serine/threonine kinases that are essential in regulating the replication machinery of most organisms. Aurora is essential in the orientation and function of the mitotic spindle and in the attachment and segregation of chromosomes. We have identified three isoforms of Aurora Kinase in *M. vestita*. These isoforms all contain the large central protein kinase domain that is conserved in all members of the Aurora family and have unique 3' and 5' ends. Each of the 3 isoforms have different mRNA expression patterns during the first 8 hours of development in the gametophyte, with all 3 being present at 3 to 4 hours after the onset of development. This time point coincides with the formation of the blepharoplast. When Aurora knocked down using RNAi, both the development and formation of the blepharoplast were blocked. In severe knockdowns, the gametophytes mitosis was totally inhibited, and the nuclei were enlarged and irregularly shaped. Less severe knockdowns allowed a few of the early divisions to occur, but the formation of the blepharoplast was not observed. The blepharoplast either failed to form, or it assembled but failed to develop and segregate basal bodies at the appropriate time. This suggests that Aurora Kinase is necessary for the proper progression of

cell cycles in the microspore, and for the assembly of the blepharoplast and *de novo* basal body formation. This research was supported by NSF grant 0842525 to S.M.W.

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Polo-like kinase 4 stability is controlled by autophosphorylation of multiple residues within its downstream regulatory element.

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Polo-like Kinase 4 (Plk4) is a conserved regulator of centriole duplication and performs the particularly crucial function of initiating the sequence of events culminating in the assembly of a new daughter centriole. Previous work by our lab has demonstrated that Plk4 protein levels oscillate during the cell cycle, peaking in mitosis when we believe Plk4 is active but practically vanishing during the rest of the cycle. Except for the brief mitotic spike, the absence of Plk4 prevents centriole reduplication and is brought about by the E3 ubiquitin-ligase complex, SCF^{Slimb}. Plk4 autophosphorylation promotes its recognition and ultimate destruction by SCF^{Slimb}, and therefore we performed a detailed study of the Plk4 autophosphorylated residues and their roles in Plk4 stabilization. First, we used mass spectrometry to identify autophosphorylated residues. Plk4 autophosphorylates its consensus Slimb-binding domain (SBD) and multiple residues flanking this region. To understand what effect autophosphorylation has on Plk4 regulation, we chose 11 phosphorylatable residues flanking the SBD and systematically mutated them to non-phosphorylatable alanines. These mutants were expressed in cultured *Drosophila* S2 cells, with or without Slimb depletion by RNAi. Our data show that residues besides those found in the canonical SBD are involved in SCF^{Slimb} recognition of Plk4 and additionally, that these residues may be used by an alternative degradative pathway to regulate Plk4 levels.

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SV40 small tumor antigen exploits PP2A's role in stabilizing Plk4 to induce centriole amplification.

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Centriole duplication is a tightly-regulated process that must occur only once per cell cycle, otherwise supernumerary centrioles can lead to chromosomal instability and tumorigenesis. Initiation of this process is a key point of regulation and is initiated by the conserved enzyme, Polo-like kinase 4 (Plk4). Consequently, regulation of centriole duplication is achieved by control of cellular Plk4 protein levels. Throughout interphase, autophosphorylation of Plk4 triggers its ubiquitin-mediated proteolysis, thus preventing centriole amplification. However, Plk4 activity is required during mitosis for proper centriole duplication, but the mechanism stabilizing Plk4 is unknown. Here we identify a mechanism which increases Plk4 activity: Drosophila Protein Phosphatase 2A (PP2A) (with the regulatory subunit Twins) counteracts Plk4 autophosphorylation, thus stabilizing Plk4 and promoting centriole duplication. Twins is a rate-limiting component in this regulatory mechanism. Like Plk4, Twins protein levels peak during mitosis and are required for centriole duplication. However, untimely Twins expression stabilizes Plk4 inappropriately, inducing centriole amplification. Paradoxically, expression of tumor-promoting SV40 small t-antigen (ST), a well-established PP2A inhibitor, was shown to promote centrosome amplification by an unidentified mechanism. We solve this mystery by

demonstrating that ST does not inhibit all PP2A activities but instead functionally mimics Twins in stabilizing Plk4 and inducing centriole amplification.

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Autoregulated instability of Polo-like kinase 4 limits centrosome duplication to once per cell cycle.

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Accurate control of the number of centrosomes, the major microtubule-organizing centers of animal cells, is critical for the maintenance of genomic integrity. Abnormalities in centrosome number can promote errors in spindle formation that lead to subsequent chromosome missegregation and extra centrosomes are found in many cancer cells. Centrosomes are comprised of a pair of centrioles surrounded by an amorphous pericentriolar material and centrosome duplication is controlled by centriole replication. In vertebrates and invertebrates, the conserved protein kinase Polo-like kinase 4 (Plk4) plays a key role in initiating centriole duplication and overexpression of Plk4 promotes centriole overduplication and the formation of extra centrosomes. Using isogenic stable cell lines and an analogue sensitive Plk4 allele, we show kinase active Plk4 is inherently unstable and targeted for degradation by the proteasome. Plk4 multiply self-phosphorylates within a 24 amino-acid “phospho-degron” that is required to integrate Plk4 kinase activity with protein stability. Using gene targeting in human cells we demonstrate the autoregulated instability of Plk4 self-limits Plk4 activity and forms an important mechanism to limit normal centriole duplication to once per cell cycle. Preventing Plk4 auto-regulation leads to centrosome amplification that inhibits proliferation of non-transformed cultured cells. However, cells carrying amplified centrosomes can be induced to proliferate through expression of the viral oncogene SV40 large-T antigen, suggesting compensatory mutations may be required to allow the propagation of cells with extra centrosomes.

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Inhibition of Clathrin Dependent Endocytosis Results in Senescence-like phenotype and Plk4-mediated Centrosome Overduplication.

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Coordination of DNA synthesis, centrosome duplication and cell division is essential for unperturbed mitosis and accurate chromosome segregation between daughter cells. Supernumerary centrosomes and aneuploidic genome are common features of many malignant tumors. Recent reports suggest that several proteins involved in endocytosis also play a role in centrosome and mitotic spindle function. One of them is cyclin G-associated kinase (GAK) that interacts with clathrin heavy chain (CHC) and plays crucial role at several steps essential for vesicular trafficking and clathrin-mediated endocytosis (CME). Knockout of GAK interrupts cell cycle progression and prohibits both primary and transformed mouse embryonic fibroblasts from entering S phase, leading to senescence and overduplication of centrosomes. This arrest is associated with p53 phosphorylation on ser15 and induction of CDK inhibitor p21. Still, CDK2 is activated by thr160 phosphorylation and despite p21 binding, residual activity of CDK2/cyclin E complex is retained. Cells arrested in late G1 phase undergo multiple rounds of centrosome duplication, which shows that centrosome duplication cycle is uncoupled from cell cycle progression. Early stages of centrosome duplication cycle require Plk4 activity at the centrosome; this activity is temporally restricted, which prevents overduplication. In GAK-

depleted cells Plk4 is found on the majority of centrosomes showing that these cells are arrested at the cell cycle stage where Plk4 is active at centrosomes. Structural integrity of the resulting supernumerary centrosomes is not perturbed since they contain both centrioles and pericentriolar material. This indicates that the control of centrosome number is impaired rather than centrosomal maturation. In GAK-depleted cells centrosome overduplication is dependent on CDK2 activity and serum stimulation, but may be induced by EGF alone. GAK knockout phenotype is largely reproduced by CHC knockdown and dynamin inhibition. We conclude that perturbation of CME results in uncoupling of centrosome duplication cycle from DNA synthesis and interferes with transduction of mitogenic signaling, demonstrating that CME-independent mitogenic signaling is sufficient for triggering centrosome duplication but not for DNA synthesis

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Kinesin-1 prevents capture of the oocyte meiotic spindle by the sperm aster.

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Centrioles are lost during oogenesis and inherited from the sperm at fertilization. In the zygote, the centrioles recruit pericentriolar proteins from the egg to form a mature centrosome that nucleates a sperm aster. The sperm aster then captures the female pronucleus to join the maternal and paternal genomes. Because fertilization occurs before completion of female meiosis, some mechanism must prevent capture of the meiotic spindle by the sperm aster. Here we show that in wild-type C. elegans zygotes, maternal pericentiolar proteins are not recruited to the sperm centrioles until after completion of meiosis. Depletion of kinesin-1 heavy chain or its binding partner resulted in premature centrosome maturation during meiosis and growth of a sperm aster that could capture the oocyte meiotic spindle. Kinesin-1 heavy chain, light chain and a light chain binding protein were all concentrated in a shell surrounding the sperm DNA and centrioles during female meiosis. Microtubules were required for kinesin-dependent movement of the sperm DNA/centriole complex within the zygote but microtubules were not required for kinesin-dependent suppression of centrosome maturation. These results support a model in which kinesin prevents recruitment of pericentriolar proteins by coating the sperm DNA and centrioles and thus prevents triploidy by a non-motor mechanism.

Dynein

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The 2.8- Å crystal structure of the dynein motor domain.

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Dyneins are microtubule-based motor complexes that power a wide variety of biological processes within eukaryotic cells, including the beating of cilia and flagella, cell division, cell migration, and the intracellular trafficking. Most recently, structures of the dynein motor domain have been reported at 5-6 Å resolution, which have provided invaluable information at the secondary structure level. However, a higher resolution analysis is required to reveal complete atomic structure of the motor domain.

Here we report the crystal structure of the 380-kDa motor domain of *Dictyostelium* cytoplasmic dynein at 2.8 Å resolution. This atomic structure shows details of functional units constituting the motor domain, such as the ATP-hydrolyzing ring composed of six AAA+ modules, the long

coiled-coil microtubule-binding stalk, and the force-generating rod-like linker. Our analysis visualizes four ADP molecules bound to the first four AAA+ modules of the ring, among which three are active ATP hydrolysis site and one is a unique ADP/ATP binding site. The structure also uncovers how the linker and stalk interact with the ring unit, which should be critical for dynein's motor activity. This long sought atomic structure will open up new avenues for investigating and understanding how dynein produces force and movement.

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In vivo 3D structural analysis of axonemal dynein in flagella/cilia by electron cryo-tomography.

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Eukaryotic flagella/cilia are bending organelles to cause cellular motility or extracellular flow. They have a common “9+2” structure, in which nine microtubule doublets surround two single microtubules and adjacent doublets are connected by dynein motor proteins. There are a number of isoforms of dyneins and they form inner and outer arm complexes. Questions still to be answered are (1) mechanism of dynein power stroke, (2) cause of bending and (3) determination of waveforms. To address these questions, we employed the technique of electron cryo-tomography for structural analysis of dynein in flagella/cilia in vivo without purification. We revealed overall dynein arrangements in flagella/cilia (Bui et al. (2008) J. Cell Biol. 183, 923) and their conformational changes induced by ATP (Movassagh et al. (2010) Nat. Struct. Mol. Biol. 17, 761). Our analysis proved that the dynein ATPase head shift toward the distal end (plus end of the microtubule) at 8nm during power stroke, while there is no rotation of the ring detected (Winch mechanism). In the presence of ADP.Vi (non-hydrolysable ADP.Pi analogue), dynein molecules do not change structure in the synchronized manner: about half of the dyneins stay as non-nucleotide conformation, while the other half switch to the nucleotide-bound form. Interestingly these two structures coexist, forming clusters. This result suggests that torsion is generated at the boundary of the two clusters to cause bending. In this presentation, we will discuss the flagellar/ciliary bending mechanism based on the structure and mapping of dynein isoforms to discuss the determination of waveforms.

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Submolecular structure of cytoplasmic dynein tail.

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Cytoplasmic dynein is a microtubule-based motor protein and plays important roles in vesicle transport, organelle positioning, neuronal migration and mitosis. Cytoplasmic dynein molecule is a homo dimer of heavy chains and has two headed structure. As for cytoplasmic dynein 1, the motor domain (the head) consists of C-terminal two third of heavy chains, while the tail region is composed of a N-terminal third of heavy chains as well as intermediate, light intermediate and light chains. Although dynein tail domain is important for the specific binding and the regulation of various cargos, the submolecular structure of the tail remains to be unveiled.

To investigate the molecular architecture of dynein tail, we observed the recombinant dynein molecules by negative staining electron microscopy using nanogold-labeling which is specific to the introduced tag. The position and the orientation of intermediate and light intermediate chains in the tail were determined. Notably, the relative position of these two chains and the orientation of intermediate chain were opposed to the previously advocated model. Furthermore, single

particle analysis of dynein tail region revealed that the tail consists of three globular domains. These findings provide important information for the cargo binding and targeting to the specific location in the cell, as well as the binding of regulatory proteins including dynactin complex, LIS1 and NDEL1.

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Recombinant human cytoplasmic dynein heavy chain 1 and 2.

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Cytoplasmic dynein is a microtubule (MT) motor protein and moves by the energy derived from ATP hydrolysis. Cytoplasmic dynein comprises two classes: dynein-1 and dynein-2. Dynein-1 is involved in various activities in cells, including cell division, cargo transport, and neural development. In contrast, cytoplasmic dynein-2 is reported to work exclusively in intraflagellar transport (IFT), which is a bidirectional transport of particles along axonemes in cilia and flagella. Dynein-2 is a putative retrograde IFT motor, which transports IFT particles from the ciliary tip toward the cell body.
To elucidate in vitro properties of dynein-1 and dynein-2, we have purified recombinant human dynein-1 and dynein-2 complex by expressing streptavidin-binding peptide (SBP)-tagged human dynein-1 HC and dynein-2 HC in HEK-293 cells. Both purified dynein-1 and dynein-2 showed minus-end-directed motor activities as shown in an in vitro MT gliding assay, and revealed two-headed structure by electron microscopy. These results are the first to describe the structure and motor activity of dynein-2 in vitro. Our results have also shown the structural and motile differences between dynein-1 and dynein-2. Firstly, human dynein-1 was able to drive robust gliding of MTs in an MT gliding assay, however, the MT gliding of dynein-2 was not robust, and MTs easily dissociated from the dynein-2 coated glass surface. The average velocity of dynein-1-driven MTs was 905 ± 152 nm/sec (mean ± SD, n = 120) and the average velocity of dynein-2 was 69.6 ± 41.0 nm/sec (mean ± SD, n = 53). Secondly, unlike dynein-1, which had the dense tail domain, the tail domain of dynein-2 was obscure and some of the dynein-2 molecules had thin tail structures. These results might reflect motile and structural diversity between the two classes of cytoplasmic dynein.

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Studying Multiple Motor Ensembles using DNA Nanotechnology.

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*Brian Goodman and Nathan Derr contributed equally to this work.

In eukaryotic cells, many cargos are transported by groups of microtubule-based molecular motors. Some of these cargos move bidirectionally and can be observed switching directions during movement, but ultimately, cellular contents are sorted with spatial and temporal precision. The biophysical mechanisms that allow ensembles of same- or opposite-polarity motors to coordinate their behaviors are not known. The major technical challenge for dissecting this problem in vitro has been the inability to control the spacing, number, and type of motor present on individual cargo molecules. Using three-dimensional DNA origami, we have designed a synthetic cargo, to which precise numbers and geometries of DNA-linked dynein and kinesin motors can be attached in varying combinations of 1 to 7 motors. We find that synthetic cargo bearing multiple dynein motors can move farther, but not faster than single dynein molecules. Under physiological conditions, multiple dynein motors can move cargo

processively, while single dynein motors cannot. Our results suggest that dynein motors may coordinate to move cargo efficiently. Studies of "tug-of-war" scenarios are underway to determine whether cargos driven by both dynein and kinesin exhibit stochastic bidirectional movements.

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Structural Mechanism for Dynein Control by Lis1.

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Cytoplasmic dynein, the large microtubule-based motor protein, is carefully controlled in cells, enabling it to be deployed to specific sites, collect cargoes, and transport them towards the microtubule minus end at set times. Dynein's "engine" is evolved from ring-shaped AAA+ ATPases, and its microtubule-binding domain lies at the tip of a coiled-coil stalk, but how these elements might be acted upon to achieve control remains unknown. Here, using purified proteins from *Saccharomyces cerevisiae*, we dissect how cytoplasmic dynein is controlled by two of its ubiquitous regulators: Lis1/Pac1 and Nudel/Ndl1. By single-molecule microscopy, we find that Lis1 slows dynein velocity, prolongs its encounters with microtubules, and is tethered to dynein by Nudel. High-precision analysis shows that dynein-Lis1 undergoes frequent "anchored" cycles, during which ATP is consumed without the usual microtubule release and forward motion. Unexpectedly, the structural basis for these changes involves Lis1 binding at the interface between dynein's ATPase domain and its microtubule-binding stalk. Lis1 is thus ideally situated to alter allosteric communication in cytoplasmic dynein, which could facilitate its control and function in cells. *J. Huang and A. Roberts contributed equally to this work.*

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Inhibition of cytoplasmic dynein motility by a microtubule-binding protein, She1.

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She1 (sensitive to high expression-1) has been implicated in modulating cytoplasmic dynein function in *S. cerevisae* by regulating the association between dynein and dynactin at microtubule plus ends and the cell cortex. We find that overexpression of She1 in yeast severely compromises dynein activity and results in the mislocalization of dynein along the length of astral microtubules, suggesting that She1 affects the microtubule-binding behavior of dynein. To test the effects of She1 on dynein motility, we have produced a recombinant HALO-tagged variant of She1 from bacteria. She1-HALO binds directly to microtubules with high affinity and exhibits diffusive behavior along reconstituted microtubules. Using a GFP-tagged full-length dynein heavy chain (Dyn1-GFP) purified from yeast, we examined the ability of She1 to affect dynein motility in vitro at single-molecule resolution using time-lapse total internal reflection fluorescence microscopy. Remarkably, She1 reduced the velocity and increased the dwell time of Dyn1-GFP in a concentration-dependent manner. Using a motility-competent tail-less dynein construct (GST-Dyn1$_{MOTOR}$-GFP), we found that this effect is mediated via the motor domain, not the N-terminal tail domain where dynactin associates. We demonstrated that the effects of She1 are specific to dynein, since She1 had no effect on the motile behavior of either Kip2, a yeast kinesin-7 that walks along astral microtubules, or a human conventional kinesin (kinesin-1). The effects of She1 on dynein motility are unique from those previously described for Tau, which causes reversals of direction for dynein and dissociation of kinesin-1 from the microtubule track. Thus, She1 affects dynein activity in budding yeast by at least two distinct mechanisms:

1) regulating the association between dynein and dynactin, and 2) regulating the motility of the dynein motor along astral microtubules.

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Rotation of microtubules driven by *Tetrahymena* 22S outer arm dynein and its sub-particles.

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Axonemal dynein is the molecular motor that provides motive force in cilia and flagella. *Tetrahymena* 22S outer arm dynein, which is localized between the outer-doublet microtubules, consists of three different heavy chains, termed α, β and γ, and is decomposed into two-headed βγ sub-particle and one-headed α sub-particle by chymotryptic digestion. While motile activity of βγ sub-particle has been already reported, motile activity of α sub-particle has not been shown yet. In this study, we examine whether the α sub-particle has the motile activity, using three-dimensional tracking of a quantum dot attached to microtubules in an *in vitro* motility assay [1] to directly visualize the motion of the microtubule driven by α sub-particles. We found that surface-attached α sub-particles drove not only on-axis microtubule sliding, but also clockwise rotation of each microtubule around its axis. Rotational pitch determined by fitting the *xy*-position of a quantum dot with a sine function varied from 0.8 μm to 0.4 μm with increasing ATP concentration from 1 μM to 10 μM. Applying this method to intact 22S dynein, we also found that the 22S dynein induced rotation of sliding microtubules. The pitch varied from 1.7 μm to 0.7 μm with increasing ATP concentration from 1 μM to 10 μM. These corkscrewing motions of microtubules have not been seen previously for both 22S dynein and its α sub-particle, and it demonstrates that both 22S dynein and its α sub-particle produce torsional forces as well as axial sliding forces. Our results suggest that torque generation by the three-headed 22S dynein is not due to cooperation between each of the three heads, but due to the intrinsic property of individual heads. The relatively longer pitch caused by 22S dynein than by its α sub-particle may be attributed to the competition among three different heads. To comprehend regulation of cilia beating, it may be necessary to consider influence of the torsional forces generated by axonemal dyneins.

[1] Yajima, J. et al. Nat. Struct. Mol. Biol. 15 (2008) 1119-21.

905

Neurodegenerative Mutations in Dynactin Disrupt the Initiation of Transport from the Distal Axon.

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Dynactin is a required cofactor for the minus-end directed microtubule motor cytoplasmic dynein. Mutations in the CAP-Gly domain of the $p150^{Glued}$ subunit of dynactin cause two distinct neurodegenerative diseases, an inherited form of Parkinson's disease, Perry syndrome, and distal hereditary motor neuropathy 7B (HMN7B). The mechanism by which the CAP-Gly domain promotes dynein motor function remains unclear and how these distinct point mutations within the same protein domain cause two disparate diseases is unknown. Here, we report a novel function of the CAP-Gly domain of dynactin. We show that wild-type $p150^{Glued}$accumulates in distal neurites, while the N-terminal truncation construct, ΔCAP-Gly $p150^{Glued}$, does not. These data show that the CAP-Gly domain is necessary to enrich dynactin in distal neurites. Using live-cell imaging in primary neurons we show that the CAP-Gly domain is not necessary for sustained vesicle transport. Knockdown of $p150^{Glued}$ inhibits the motility of lysosomes while rescue with either wild-type or ΔCAP-Gly $p150^{Glued}$ completely prevents this disruption. However, using fluorescence recovery after photobleaching, we show that expression of wild-

type p150^{Glued} is necessary for efficient flux of lysosomes from the distal axon, as expression of ΔCAP-Gly p150^{Glued} decreases flux from the distal neurite by 65%. These data suggest that the CAP-Gly domain is critical to facilitate the initiation of transport from the distal axon. We studied the disease-associated mutations using biochemical and live-cell imaging assays to further investigate the function of dynactin in neurons. We show that the different mutations induce distinct disruptions in intracellular motility. We show that the HMN7B mutation destabilizes the CAP-Gly domain, preventing efficient incorporation of the mutant protein into the dynein-dynactin complex and effectively disrupts axonal transport by acting as a dominant-negative inhibitor of dynein motor function. In contrast, we show that mutations in dynactin linked to Perry syndrome cause a loss of CAP-Gly function that does not affect retrograde transport but results in a failure to localize p150^{Glued} in distal processes, leading to an inhibition of transport initiation. Together, our observations indicate that dynactin enhances dynein-mediated transport and the efficient initiation of axonal transport through distinct mechanisms. These distinct mechanisms provide the basis to explain the cell-type specific degeneration observed in these two diseases.

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PKA phosphorylation of cytoplasmic dynein activates recruitment to adenovirus.

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Adenovirus 5 (Ad5) enters the host cell after initial binding to cell surface receptors (e.g. CAR and α5 integrins), which increases intracellular PKA and p38/MAPK activity and leads to receptor-mediated endosomal uptake of virions. After endosomal escape, Ad5 relies on cytoplasmic dynein for transport from the cell periphery to the nucleus. We previously reported that Ad5 recruits dynein through direct interactions of the dynein IC and LIC subunits with the major adenovirus capsid protein hexon (Bremner et al., *Cell Host Microbe*, 2009). These interactions depended on short-term low pH exposure of hexon, suggesting that passage through the acidic lumen of the endosome primes the adenovirus capsid for dynein recruitment. We now find that exposure of the dynein complex to phosphatases substantially reduces the interaction of the motor protein with hexon and that phosphorylation of dynein with PKA rescues this effect. These data suggest that an important result of adenovirus-induced PKA activation (Suomalainen et al., *EMBO J*, 2001) is to promote dynein recruitment. The underlying molecular events accompanying this activity are under investigation.

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Regulation of Cytoplasmic Dynein in T cell Activation-Induced Microtubule-Organizing Center (MTOC) Reorientation.

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Upon activation, T cells rapidly reorient their MTOCs towards the immunological synapse (IS), the contact site with the antigen-presenting cell. This creates a target for directional transportation of cytokines and lytic factors towards the IS, ensuring the specificity of secretion and preventing bystander effects. Cytoplasmic dynein is recruited to the IS downstream of T cell receptor (TCR) stimulation and is involved in driving MTOC reorientation. However, how dynein is recruited and regulated at the IS remains unclear. In this study, we combined photoactivation of the TCR with total internal reflection fluorescence (TIRF) microscopy to analyze dynein recruitment dynamics and function during MTOC polarization to the IS in primary mouse T cells. Using fluorescently labeled dynein intermediate chain (IC), we found that dynein was recruited to the IS prior to MTOC reorientation. This recruitment was independent of the microtubule

network, as it could not be inhibited by the microtubule depolymerizing agent nocodazole. Silencing of dynein IC by shRNA greatly reduced MTOC reorientation in T cells, confirming a requirement for dynein in the process. In a subsequent screen to identify potential dynein regulators at the IS, we found that two dynein co-factors, dynactin and Lis1, were recruited to the IS at the same time as dynein. To determine the role of dynactin in MTOC reorientation, we used shRNA to silence the dynein binding subunit $p150^{Glued}$ and observed only a subtle defect in MTOC reorientation, suggesting that dynactin is not absolutely required for the process. Consistent with this result, we observed a substantial reduction in dynactin recruitment to the IS in dynein IC silenced cells, indicating that dynactin recruitment is dependent on dynein. To determine the role of Lis1, we transduced T cells homozygous for a floxed Lis1 allele with cre recombinase. We found that suppression of Lis1 dramatically impaired MTOC reorientation, indicating that it is functionally important for the process. Currently we are investigating the role of Lis1 in recruiting dynein to the IS. Taken together, our results suggest that dynein is involved in positioning the MTOC at the IS, and its function at the IS is differentially regulated by dynactin and Lis1.

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Human T-cell Leukemia Virus type-1 Binds Microtubule-dependent Motor Protein, Cytoplasmic Dynein.

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The Human T-cell leukemia virus type-1 (HTLV-1) affects an estimated 10-20 million people worldwide. It is believed to be the causative agent of adult t-cell leukemia (ATL) and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). To initiate a successful infection HTLV-1 must bind to the cell, penetrate into the cytosol, deposit its genome into the host cell cytoplasm and progress to the nucleus in order for the virus to replicate. Previous studies have shown HTLV-1 infection is dependent upon interactions between the virus and host cell cytoskeleton. Specifically, researchers have illustrated that HTLV-1 intracellular movement is dependent upon microtubules. Our laboratory proposes that HTLV-1 utilizes microtubule-based motor protein, cytoplasmic dynein, to translocate from the cell periphery to the nucleus. We have conducted immunocytochemistry and immunopreciptation experiments. Results from these experiments suggest that HTLV-1 interacts with cytoplasmic dynein two hours post infection. Our lab is embarking to understand how the recruitment of cytoplasmic dynein to human T-cell leukemia virus occurs. We have purified the HTLV-1cytoplasmic dynein from infected HEK 293 cells. Currently, we are investigating if dynactin, a cytoplasmic dynein-binding partner, is a component of the HTLV-1 cytoplasmic dynein protein complex.

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Differential roles for NudE and NudEL in nuclear migration in the developing brain.

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Radial glial progenitor cells in the developing neocortex undergo cell cycle dependent interkinetic nuclear migration (INM). We have found apical nuclear migration to be carried out by dynein, and basal nuclear migration by kinesin-3 (Tsai et al. *Nat. Neurosci.* 2010, 13:1463-71). A number of dynein regulatory factors are also involved in brain development, including LIS1 and its interactors NudE and NudEL. NudE has been found to recruit LIS1 to dynein to adapt it for high load transport (McKenney et al. *Cell.* 2010, 141(2):304-14). NudE and NudEL are closely related (55% identity; 70% similarity), but the brain developmental phenotypes of NudE and NudEL knockout mice are very different, either due to differences in expression or function. Using *in utero* electroporation followed by fixed and live cell imaging, we have tested the effects

of NudE and NudEL RNAi on INM. NudE RNAi resulted in strong early inhibition of INM with severe defects in apical nuclear migration and a complete failure to enter mitosis. NudEL RNAi, in contrast, had no effect on INM and cells divided as normal at the ventricular surface. Both NudE and NudEL RNAi, however, led to an accumulation of multipolar cells in the SVZ, whereas further migration through the IZ to the cortical plate appeared to be blocked, an effect rescued by RNAi-insensitive NudE expression. These results identify NudE as the isoform specifically required for INM, and identify both common NudE and NudEL functions as regulators of the multipolar to bipolar transition and migration of postmitotic neurons. Supp. by NIH HD40182.

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Single-Particle Tracking of Dynein-mediated EGF transport through the endosomal system.

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Endocytosed cargo destined for degradation must be sorted through multiple compartments in the endosomal system before its arrival in perinuclear lysosomes. Cargo endocytosed at the edge of the cell may travel tens of microns on a complicated cytoskeletal network of actin and microtubules in order to reach lysosomes. Isolated endosomal compartments contain multiple types of actin and microtubule motors, suggesting the cargo coordinates motors for transport during its journey. To better understand how an endogenous cargo uses motors and the cytoskeleton for transport we used live-cell imaging of endocytosed epidermal growth factor attached to quantum dots (EGF-Qdots), a cargo that is destined for the lysosome. Low-angle oblique, simultaneous multicolor acquisition microscopy has allowed us to track EGF-Qdot movements in epithelial cells with high temporal (50 ms) and spatial (20 nm) resolution relative to endosomal and cytoskeletal markers. Tracking the path of EGF-Qdots from time of binding to cells to perinuclear accumulation shows that they are transported in short bursts of fast centripetal movement with very few anterograde runs. The fast movement is along microtubules and is punctuated by long pauses or bouts of diffusive motion. 79% of the EGF-Qdot track lifetimes were spent paused and 21% spent moving. The average speed during the runs was 2.5um/sec and the average run time was 4 seconds. Disruption of the dynein/dynactin complex, the major retrograde motor in the cell, leads to loss of these fast runs consistent with dynein driving centripetal movement of early endosomes to the perinuclear region for maturation and sorting. Dynein/dynactin disruption also results in dispersion of these static early endosomes throughout the cell. In contrast, lysosomes respond to dynein/dynactin loss by accumulating at the cell periphery and anterograde runs can still be observed, suggesting the highly biased centripetal early endosome movement might be due to the lack of competing anterograde motors seen on lysosomes. These observations suggest that high resolution tracking of single cargos moving through the endosomal system will provide new insights into the cytoskeletal and motor interactions that drive this process.

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Microtubule Motor Proteins Drive Nuclear Rotation and Translocation in Developing Skeletal Muscle Cells.

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During development, dynamic interactions between the cytoskeleton and nuclear envelope proteins drive the movement and positioning of nuclei. In skeletal muscle, nuclei become

distributed evenly along the length of the mature syncytial myofiber. Here, we aim to understand the mechanisms driving nuclear movement in developing skeletal muscle cells. Using the well-characterized C2C12 mouse cell line as a model of myotubes, we show that nuclei actively translocate along the long axis of the cell. During translocation, we find that nuclei often rotate in three-dimensions; both the nuclear envelope and internal structures such as nucleoli rotate together. Within a single myotube, nuclei rotate independently with variable angular velocities and directions. To investigate the contribution of the microtubule (MT) cytoskeleton to nuclear dynamics, we performed live cell imaging of myotubes expressing either GFP-tubulin or GFP-EB3. While most MTs project along the long axis of the myotube in an anti-parallel arrangement, MTs can also polymerize between closely apposed nuclei, as well as along channels or invaginations on the nuclear surface. Depolymerization of the MT network abolishes nuclear rotation entirely, although some translocation persists. Immunofluorescence analysis reveals the presence of both kinesin-1 and dynein on the nuclear envelope. Knock-down of dynein using siRNA caused a ~50% reduction in sustained rotation of the nuclei (>30°/15min). Knock-down of KIF5B or overexpression of a dominant negative kinesin-1 tail domain construct also markedly inhibited rotation, indicating that both plus- and minus-end directed MT motors drive nuclear rotation in myotubes. Moreover, in cells with reduced dynein or kinesin-1 function, nuclei were abnormally clustered, suggesting that proper nuclear dynamics are required for normal distribution of nuclei. We hypothesize that the motors bind to the nuclear envelope through associations with LINC complexes, and that the competition between oppositely-directed microtubule motors is essential for effective nuclear positioning in myotubes.

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Dynein affects muscle function through mechanistically distinct regulation of muscle size and myonuclear position.

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Each cell within an organism bears a distinct morphology that is defined by its size, shape and intracellular organization, all features that are regulated by the cytoskeleton. Muscle is an ideal system for studying morphogenesis because it displays a highly ordered and repetitive structure and the physiological output of its morphogenesis can be readily assessed. The impact of muscle size on function is clear, but defects in myonuclear positioning have also been correlated with muscle disorders, such as centronuclear myopathies and muscular dystrophies. Using Drosophila we have examined the mechanistic contributions of Dynein to embryonic morphogenesis and the impact of altered morphogenesis on muscle function. We have found that Dynein is essential for both muscle extension/growth and myonuclear positioning. Mechanistically, Dynein regulates muscle growth and myonuclear positioning through similar but distinct sets of factors. The Dynein light chain, Dlc90F (Tctex-1), and the Inscuteable/Pins polarity complex are necessary for both Dynein dependent muscle growth and Dynein dependent myonuclear positioning. However, Lis1 is necessary only for Dynein dependent muscle growth, whereas CLIP-190 contributes only to Dynein dependent myonuclear positioning. Moreover, CLIP-190 and Lis1 do not functionally interact illustrating that Dynein dependent muscle growth and Dynein dependent myonuclear positioning are indeed distinct pathways. These morphogenetic defects are maintained through larval development: Lis1 mutations affect muscle growth, CLIP-190 mutations affect nuclear spacing, and Dynein mutations affect both muscle growth and nuclear spacing. Finally, defects in muscle size, myonuclear positioning or both, result in larvae that crawl more slowly than controls, indicating that muscle function is also impaired. These data illustrate for the first time that Dynein regulates two distinct aspects of muscle morphogenesis, muscle size and myonuclear positioning, and that both processes are necessary for proper muscle function. Moreover, we

have shown that the contribution of Dynein to these two processes is mechanistically distinct and have identified CLIP-190 and Lis1 as the factors that specify Dynein activity in these contexts. The separation of function provides a system to further investigate the molecular mechanisms controlling growth and myonuclear positioning and evaluate their relative importance to muscle function.

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Muscle differentiation and function require the interplay between microtubules and MAPs to correctly position myonuclei.

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Proper cellular organization and morphogenesis are critical for cell function. Nuclear positioning is a poorly understood process that is particularly important in multinucleated cell types such as muscle. Within each syncytial myofiber, the nuclei are positioned to maximize internuclear distance. Disruptions of this organization, whereby the nuclei become clustered together, are associated with disease states. Nevertheless, little is known about the mechanisms underlying proper myonuclear positioning and whether mispositioned nuclei are a cause or consequence of muscle disease. To study this process, we used the genetically and optically tractable model organism, Drosophila melanogaster. In wild-type embryos, the nuclei in the lateral transverse (LT) muscles are evenly distributed along the length of the myofiber. Through mutational analysis, we have found that the spacing of nuclei is microtubule (MT) dependent, and we have identified Kinesin, cytoplasmic Dynein, and Ensconsin (Ens/MAP7) as necessary proteins for proper nuclear positioning. Because Ens is a MAP that can regulate interactions between the motor protein Kinesin and MTs in the ovary, we hypothesized that the interplay between Ens and motor proteins also regulates myonuclear positioning. We found that both cytoplasmic Dynein and Kinesin functionally interact with Ens during myonuclear positioning. These mutants had correct nuclear numbers and tendon attachments, suggesting that the effect on nuclear position is specific. Most importantly, we found that defects in myonuclear position tightly correlate with muscle weakness as mutant Drosophila larvae crawl more slowly than unaffected larvae toward a stimulus. Collectively, these data suggest that the process of myonuclear positioning is microtubule dependent, requiring the motor proteins Kinesin and cytoplasmic Dynein, and that proper myonuclear positioning is essential for muscle morphogenesis and function.

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Identification of novel pathways that cooperate with kinesin-5 to drive bipolar spindle assembly.

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Bipolar spindle assembly is an essential process during cell division. Spindle bipolarity is setup in two steps. During prophase, the two centrosomes migrate around the nuclear envelope (NE) to opposite sides. Second, during prometaphase the NE disassembles and the characteristic bipolar spindle assembles. It is known that the highly conserved homotetrameric microtubule motor kinesin-5 (also known as Eg5), which can slide microtubules apart, is absolutely essential both for centrosome separation in prophase and for bipolar spindle assembly. However, there is evidence suggesting that additional redundant pathways might exist as well. To identify additional pathways for bipolar spindle assembly, we performed a series of â€œdirected evolutionâ€ experiments in vitro in which cells were treated with increasing concentrations of

the kinesin-5 inhibitor STLC, to generate cells can form bipolar spindles independently of kinesin-5 activity. Strikingly, these cells not only form bipolar spindles in the absence of kinesin-5 activity, but also are able to separate their centrosomes during prophase. By characterizing these cells in more detail, we identified several redundant mechanisms that cooperate with kinesin-5 to drive centrosome separation and bipolar spindle assembly.

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The MAP Kinase Pathway Regulates Cytoplasmic Dynein Binding to Endosomes.

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Dynein is the motor for vesicle trafficking toward microtubule minus-ends but little is known about how dynein binding to specific vesicles is regulated. PC12 cells and neurons recruit dynein to signaling endosomes formed in response to neurotrophin binding to receptor kinases (Trks). Using mass spectrometry, we identified a novel phospho-serine on the IC2 subunit of dynein isolated from neurotrophin-treated PC12 cells. In addition, the comparable phospho-IC1 site was found in dynein from cultured neurons. In both cell types, a phospho-IC specific antibody showed a time dependent increase in IC phosphorylation in response to neurotrophin. The MAP kinase, Erk1/2, a major downstream effector of Trk, was proposed to phosphorylate the sites by kinase prediction algorithms. The MAP kinase kinase (MEK) inhibitor, U0126, and the Trk kinase inhibitor, K252a, but not inhibitors of other downstream effectors of Trk, blocked the neurotrophin induced IC phosphorylation in both cell types. To investigate the role of the phospho-IC in living cells, we made mRFP-tagged IC-WT, S/A dephospho- and S/D phospho-mimic mutants. The S/A dephospho-mimic, but not the S/D, mutant had significantly less co-localization with endosomes relative to WT. In PC12 cells, IC2 S/A mutant co-localization with TrkA was reduced 50%. In cultured neurons, IC1 S/A mutant co-localization with TrkB and rab7 (late endosomes) was reduced by 70% and 40% respectively relative to WT. When neurons were treated with U0126 under conditions that significantly reduced the amount of IC1 phosphorylation (~50%), the motilities of TrkB, rab7, and IC1 in axons were comparably reduced. In addition, there was a 2.4 fold enrichment of the phosphorylation on ICs that co-purify with vesicles relative to cell lysates, and treatment of PC12 cells with U0126, before neurotrophin addition, reduced by 70% the amount of dynein that co-purified with signaling endosomes. Collectively these studies show that MAP kinase phosphorylation of the ICs is necessary for dynein binding to and transport of endosomes.

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The Drosophila homolog of ARFAPTIN2 (DARFIP2) reveals a novel role for dynactin function at the golgi during presynaptic growth.

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Analysis of motor neurons in DCTN1/P150/Glued mutants in mice and flies have revealed a number of neuronal phenotypes including reduced synapse growth, reduced synaptic stability, impaired axonal transport, and disrupted Golgi structure. It is unclear whether these disparate phenotypes are the result of a singular mechanism or represent the combined effects of multiple dynactin-dependent mechanisms within the motor neuron. Previous studies suggest that individual dynactin-dependent processes including synapse growth and synaptic stabilization can be genetically separated but evidence for this is lacking. For example, we predict that there exists a unique set of genes that function to specify dynactin complex function during synapse growth independent of other dynactin-dependent processes such as synaptic stability and

axonal transport. To address this hypothesis, we have performed a large genetic screen to identify critical molecules responsible for specifying dynactin complex function during synapse growth at the NMJ. A motor neuron specific enhancer/suppressor screen of 3663 independent P-element insertion lines was performed to identify insertions that modified the viability of dynactin complex mutants. Secondary analyses isolated 40 individual enhancers and suppressors that were subsequently processed for synapse growth, synaptic stabilization, and axonal transport. This approach has identified mutations in the *Drosophila* homolog of *Arfaptin2* (*Darfip2*) that specifically impair dynactin-dependent synapse growth but not synaptic stabilization or axonal transport. The Darfip2 protein belongs to an interesting class of proteins that contain a BAR domain. BAR domains are protein sequences that mediate membrane binding and have been implicated to function during vesicle formation and membrane trafficking. Analysis of *Darfip2* mutants finds that Darfip2 is required presynaptically for normal synapse growth, but not axonal transport or synapse stability. Biochemical analysis demonstrates that Darfip2 is present in dynactin complexes isolated from fly brains. In addition, Darfip2 is able to mediate the association of the dynactin complex with isolated membranes. Localization studies in motor neurons find that Darfip2 is concentrated at the TGN and that this localization requires a functional BAR domain. Importantly, Darfip2 localization to the TGN is required for normal synapse growth. These studies of Darfip2 suggest a novel regulatory mechanism linking the coordination of dynactin complex function and vesicle formation at the TGN during presynaptic growth.

Mechanotransduction I

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Understanding the Cooperative Interaction between Myosin II and Actin Crosslinkers Mediated by Actin Filaments during Mechanosensation.

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Myosin II is a central mechanoenzyme in a wide range of cellular morphogenic processes. Its spatial distribution is dependent not only on signal transduction pathways, but also on mechanical stress. In experiments, we found that myosin II and cortexillin (an actin-crosslinking protein) displayed cooperative accumulation at highly deformed sites in response to external forces and the extent of accumulation increased with the applied force. Additionally, the accumulation rate increased with time under constant mechanical stimuli. We suggest that this stress-dependent accumulation is the result of both the force-dependent binding to actin filament and cooperative interactions between bound myosin heads. The majority of the accumulated myosin comes in monomer form from other regions by diffusion and this kind of transport involves the turnover dynamics of basic functional unit of myosin II, the bipolar thick filament (BTF). For a quantitative interpretation of the in vivo cell behaviors, we developed a multi-scale model that integrates the cooperative interactions and the BTF turnover dynamics. By assuming that the binding of myosin heads causes local conformational changes in the actin filaments which enhances myosin II binding locally, we successfully simulated the cooperative binding of myosin to actin observed experimentally. In addition, we can interpret the cooperative interactions between myosin and actin-crosslinking proteins observed in cellular mechanosensation, provided that the same mechanism operates among different proteins. Finally, we present a scheme that couples cooperative interactions to the assembly dynamics of myosin bipolar thick filaments and accounts for the transient behaviors of the myosin II

accumulation during mechanosensation. This mechanism is likely to be general for a range of myosin II-dependent cellular mechanosensory processes.

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Interplay between cytoskeletal forces, membrane tension, and hydrostatic pressure in rapidly migrating cells.

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In motile cells, membrane pressure at the membrane/cytoplasm interface is balanced by the cytoskeletal forces and hydrostatic pressure. In particular, it is believed that protrusion of the lamellipodia is controlled by the balance of membrane pressure and the pressure generated by actin polymerization, while bleb formation results from the balance of membrane pressure and hydrostatic pressure. However, exact contributions of these forces to the cell shape and motion are not known. Membrane pressure is a product of tension and local curvature of the membrane; thus, to know the membrane pressure one has to know the exact three-dimensional shape of the cell. We have developed a simple approach to measure cell vertical profile and volume, which is based on the displacement of the fluorescent cell-impermeable dye dissolved in the medium. To get insight into the balance of forces at the membrane interface, we measure membrane tension (with tether assay), protrusion velocity, vertical profile, and volume dynamics in migrating fish epidermal keratocytes subjected to cytoskeletal drugs and volume perturbations. Inhibiting myosin-dependent contraction with blebbistatin resulted in irregular cell shape, and reduced protrusion velocity and membrane tension (variable and low tether forces as compared to a force distribution centered around 35-40 pN in control cells). Remarkably, hypoosmotic treatment normalized blebbistatin-treated cells by restoring their tension, protrusion velocity, and shape, and also increased tension and protrusion velocity in control cells. In general, treatments inducing increase in membrane tension and hydrostatic pressure also increased cell velocity, while treatments decreasing membrane tension decreased cell velocity. Vertical profile measurements showed that hypoosmotic treatment induced persistent cell swelling and substantial increase in the height of the lamellipodia. We propose that hydrostatic pressure that could be generated due to either myosin-dependent contraction or osmotic gradient helps to maintains optimal vertical profile of the lamellipodium and thus cooperates with actin-dependent protrusion. Supported by Swiss National Science Foundation, Swiss SystemsX and NCCBI.

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Mechanotransduction response in corneal cells is mediated by YAP pathway.

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Corneal wound healing is influenced by the extracellular environment both through soluble signals and from biophysical cues such as substratum topography and matrix stiffness. These cues profoundly influence phenotypic characteristics of cells such as adhesion, migration, shape, size and proliferation. These changes are mediated by activation/inhibition of specific signaling pathways. Human corneal epithelial and stromal cells have been demonstrated to remodel their cytoskeleton and orient differentially on anisotropically patterned substrates possessing biological length-scale, accompanied by changes in nuclear height, volume, area and orientation. However, there still remains a considerable knowledge gap in understanding the role of cell signaling pathways in response to biophysical cuing and mechanotransduction.

YAP (Yes-associated protein) and TAZ (WWTR1; transcriptional coactivator with PDZ-binding motif), two important signaling molecules of the Hippo pathway, have recently been implicated as nuclear relays to mechanical cues sensed by cells. We investigated the effects of nano through micron scale topographic features and substrates of various elastic moduli in altering the YAP/TAZ pathway in human corneal epithelial and stromal cells.

Immortalized corneal epithelial cells (hTERT) and primary corneal stromal fibroblasts were cultured on planar or topographically patterned substrates with biologically relevant dimensions (pitch sizes of 400, 1400 and 4000 nm; pitch = ridge width + groove width) for 12 h, or on planar substrates of differing elastic moduli [Tissue Culture Plastic (TCP), acrylamide hydrogels of biomimetic elastic moduli (5 kPa, 25 kPa, and 75 kPa)] for 48 h. RNA was isolated and the expression of YAP, 14-3-3σ and Transforming Growth Factor beta-2 (TGFβ2) was determined by qPCR.

A cell dependent and differential response to topography was observed. Corneal epithelial cells were most responsive to 400 nm pitch topography with upregulation in YAP, 14-3-3σ, and TGFβ2. Alterations in YAP or 14-3-3σ were not detected in fibroblasts on any topographically patterned surface. TGFβ2 was upregulated in fibroblasts on 400, 1400 and 4000 nm pitches. While, YAP, WWTR1, and 14-3-3σ expression were significantly upregulated on 25 and 75 kPa hydrogels in epithelial cells, YAP remained unaltered in fibroblasts on all compliances. Interestingly, 14-3-3σ expression in fibroblasts was significantly down-regulated while TGFβ2 was up-regulated on 25 kPa hydrogels.

The biophysical attributes of the microenvironment of corneal cells cause changes in cell morphology and are capable of altering cell signaling. Our results strongly suggest that YAP, WWTR1, 14-3-3σ and TGFβ2 play fundamental roles in regulating how corneal cells respond to their biophysical microenvironment.

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Netrin-1 Regulates Pak1-mediated Phosphorylation of Shootin1: Modulation of Clutch Activity of Shootin1 for Axon Outgrowth.

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Axon outgrowth is a critical process for neuronal circuit formation in developmental brains. The clutch system is thought to account for how neurons generate traction force at the growth cone for neurite outgrowth. The clutch system consists of three components: retrograde flow of treadmilling actin filaments, cell adhesion molecules, and clutch molecules which transmit the driving force of actin filament retrograde flow to cell adhesion molecules. Previously, we reported that shootin1 functions as a clutch molecule which links actin filaments and L1-CAM to promote axon outgrowth. It is assumed that spatial-temporal regulation of clutch activity by extracellular signals is one of mechanisms involved in directional axon outgrowth and cell migration, however, how the clutch activity is regulated is poorly understood.
In this study, we show that p21-activated kinase 1 (Pak1) phosphorylates shootin1. Netrin-1, an attractive axon guidance molecule, stimulated Pak1-mediated phosphorylation of shootin1 in cultured neurons. Single-molecule fluorescent speckle analysis revealed that the phosphorylation of shootin1 is required for its association with actin filament retrograde flow at axonal growth cones. Overexpression of wild type and phosphomimic form of shootin1 facilitated axon outgrowth, whereas overexpression of the nonphosphorylated form did not. Inhibition of Pak1 activity suppressed the localization of shootin1 at growth cones and axon outgrowth. These results suggest that an extracellular signal regulates shootin1 association with

actin filament retrograde flow through its Pak1-mediated phosphorylation, thereby modulating its clutch activity for axon outgrowth.

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Modulation of mechanosensitive TRPC1 channels on filopodia activates calpain to guide spinal axons.

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Intracellular Ca^{2+} signals control the development and regeneration of spinal axons downstream of chemical guidance cues, but little is known about the roles of mechanical cues in axon guidance. Here we show that transient receptor potential canonical 1 (TRPC1) subunits assemble mechanosensitive channels on neuronal growth cones that regulate the extension and direction of axon outgrowth on rigid, but not compliant substrata. Reducing expression of TRPC1 by anti-sense morpholinos inhibits the effects of mechanosensitive channel blockers on axon outgrowth and local Ca^{2+} transients. Ca^{2+} influx through mechanosensitive TRPC1 activates the protease calpain, which likely cleaves Src family kinases and talin to inhibit adhesion turnover and outgrowth. This pathway may also be important in axon guidance decisions since asymmetric inhibition of mechanosensitive TRPC1 is sufficient to induce growth cone turning. Together our results suggest that Ca^{2+} influx through mechanosensitive TRPC1 on filopodia activates calpain to control growth cone turning during development.

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Myosin-independent and Rac1-mediated ECM rigidity sensing in fibroblasts.

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Cells modulate their function and behavior based upon chemical and physical signals that they receive from their extracellular matrix (ECM). For instance, the mechanical rigidity of the ECM impacts cell adhesion, spreading, migration, and proliferation in culture, and has been strongly correlated to tumor progression in situ. ECM rigidity sensing is thought to be mediated by feedback between force generation in the lamellar actomyosin network, maturation of focal adhesions, and resistance by the ECM. Here, we identify a myosin-independent and Rac1-dependent rigidity sensing pathway in NIH 3T3 fibroblasts that plays a crucial role in mediating ECM stiffness-dependent changes in cell adhesion, spreading, and protrusion. We identify a threshold in ECM stiffness required for cell spreading and sustained lamellipodial protrusion in NIH 3T3 fibroblasts. When the shear elastic modulus of the ECM is less than 2 kPa, cells are poorly spread, exhibit small lamellipodial protrusions, and show less frequent nascent adhesion formation. Above this critical ECM stiffness, cell spread area, edge protrusive activity, and adhesion assembly are similar for all ECM stiffnesses ranging from 2.8 kPa – 51 kPa. This ECM rigidity sensing is independent of myosin II activity, as a similar threshold in cell spread area is observed in the presence of saturating concentrations of blebbistatin, a myosin II ATPase inhibitor, or Y27693, a Rho-kinase inhibitor. By expressing a constitutively active Rac1 construct, we can drive spreading of cells adhered to soft matrices to areas comparable to those observed on stiff ECM. Our data is consistent with a mechanosensation pathway occurring in the lamellipodia. This pathway would likely involve feedback between nascent adhesion signaling and Rac1-mediated actin polymerization to drive leading edge protrusion and nascent adhesion formation.

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ICAP-1 and beta1 integrin modulate adhesion strength and traction force to sense matrix rigidity and control cell migration.

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It is well known that cell mechanical properties modulated by extracellular matrix rigidity are crucial in regulating cell migration, cell differentiation and tumour progression. However, it remains unclear how forces are transduced through focal adhesions into biochemical activities and biological responses at the molecular and cellular level. To elucidate the mechanisms which drive cell migration, we examine the organization of adhesions, myosin II, and the actin network and determine adhesive strength and traction force in osteoblasts migrating on deformable substrates. We investigate the contribution of b1 integrin and its cytoplasmic regulator ICAP-1 in the adaptation of the cell to migrate on controlled environment thanks to cells deficient in beta1 integrin and ICAP-1. We show that the deficiency of beta1 integrin is associated with a round and small shape, a reduced number of focal adhesions, a slower migration, low traction forces and low adhesive strength. Surprisingly the additional loss of ICAP-1 rescues the migratory phenotype by increasing cell migration and traction forces without any change in adhesive strength suggesting a new signalling pathway controlled by ICAP-1 and independent on its interaction with beta1 integrin. This beta1 integrin independent signalling pathway seems to be important for extracellular rigidity sensing and ROCK activity control. On the whole our results are consistent with a role of beta1 integrin in adhesive strength and a role of beta1 and ICAP-1 in the control of traction forces. We show that quantitative changes in either adhesive strength or traction forces through a control by beta1 integrin and ICAP-1 can switch osteoblasts among qualitatively distinct migration regimes.

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ICAP-1 monoubiquitylation regulates cell migration.

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Cell migration is a complex biological process that requires spatiotemporally regulation of integrin activation, assembly/disassembly of focal adhesion and dynamic organization of actin cytoskeleton. Extracellular matrix rigidity is an external factor influencing cell migration. ICAP-1, a beta1-integrin tail-binding protein, is essential for ensuring integrin activation cycle and focal adhesion formation by interfering with talin binding. In this study, we provide evidence that ICAP-1 function can be regulated by monoubiquitylation. We identified the monoubiquitylation site on ICAP-1. This modification is not involved in protein degradation via proteasome but rather might regulate the assembly and organisation of adhesion sites likely through modulation of protein interactions. Indeed, the monoubiquitylation of ICAP-1 prevents its beta1 integrin binding whereas the non-ubiquitinable form of ICAP-1 interacts with beta1 integrin. Expression of the non ubiquitinable form of ICAP-1 in ICAP-1 deficient cells impacts on beta1 integrin localisation and focal adhesion organization. By testing different matrix rigidities we show that cells expressing the non ubiquitinable form of ICAP-1 exhibit a modification in the matrix density and rigidity sensing which affect cell migration. The monoubiquitylation of ICAP-1 might play a key role in integrin-mediated cell migration through matrix rigidity perception. The using of ROCK inhibitor suggests that this specific role of ICAP-1 is linked to ROCK signalling pathway. Our results show that the ubiquitylation system is integrally involved in the process of cell migration acting as a nodal regulator between partners of beta1 integrin.

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The Regulation of Traction Forces through Calpain 4 Mediated Secretion of Galectin-3.

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As a cell migrates it generates contractile forces. These forces, referred to as traction forces, are generated by the acto-myosin cytoskeleton and transmitted onto the external environment via integrins. However, the signaling mechanism for the production and regulation of these forces are still unclear and is an area of intense study. We have previously found that calpain 4, the small non-catalytic subunit of the proteases Calpain 1 and 2, is involved in the production of traction force. In this function, calpain 4 acts independently of the proteolytic activity of the larger subunits. We are currently working to define the mechanism used by calpain4 in the production of traction force. Using 2-D gel electrophoresis we have identified reduced levels of tyrosine phosphorylation of the protein galectin-3 in calpain 4 deficient cells, when compared to lysates from control and cells deficient in calpain 1 or 2. Galectin-3 is a lectin binding protein and a known regulator of migration and focal adhesion dynamics. Galectin-3 is localized within the cytosol and transiently within the nucleus, and it is also secreted by a currently unknown mechanism. Secreted galectin-3 has been shown to enhance cell adhesion through integrin clustering and focal contact formation. We have discovered that galectin-3 secretion is impaired in calpain 4 deficient cells, possibly due to its defective tyrosine phosphorylation. It is our hypothesis that calpain4 is regulating traction forces by mediating the secretion of galectin-3. Our current studies indicate that recombinant galectin-3 added externally to the media rescues focal adhesion turnover and maturation defects seen in calpain 4 deficient cells. Similarly, addition of recombinant galectin-3 to the media also enhances the adhesion strength of weakly adhered calpain 4 deficient cells. Migration studies performed by time-lapse microscopy with cells cultured on fibronectin coated cover glass also suggest that secreted galectin-3 alters linear speed and directional persistence during migration. Finally, using traction force microscopy, preliminary results further indicate that addition of extracellular galectin-3 can rescue the traction force defect observed in calpain 4 deficient cells. Our results suggest that calpain 4 and galectin-3 are key players in the production of traction force. Together these proteins regulate traction force by a novel mechanism involving internal signaling to strengthen the adhesion structures on the extracellular face of the cell.

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A Direct Interaction between the Calpain Regulatory Subunit and an Inducer of Matrix-Metalloproteinases; A Potential Role in Cellular Traction Forces.

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The Calpain family of cysteine proteases has long been implicated in the process of cell migration. Calpain 2 localizes to focal adhesions and functions in the turnover of focal adhesion proteins. To form a catalytically active holoenzyme, the calpain 2 and calpain 1 heavy chains heterodimerize with a common non-catalytic 28-kDa small subunit (calpain 4/*capn4*). Given the function of calpains in the turnover of focal adhesions, we previously investigated the role of calpain in the generation of traction forces and in the sensing of mechanical stimulation from the external environment. Traction forces are generated by the contractile machinery of a migrating cell as it crawls across a substrate. In previous studies we found that perturbation of either calpain 1 or 2 had no effect on the generation of traction force. However, we were surprised to find that traction forces generated by the cell were reduced when the small subunit calpain 4 was silenced. Furthermore, we found that silencing of either calpain 1, 2 or 4 resulted in the inability of the cells to sense changes in mechanical stimulation. These results thus suggest that calpain 4 plays a role independent of the large subunits in traction force production and together

with calpain 1 and 2 in mechanosensing. To gain further insight into how calpain 4 regulates traction force generation and mechanosensing, we performed a yeast two hybrid screen using *capn4* as the bait. A total of eight previously unknown binding partners of calpain 4 were identified. Of particular interest was the membrane protein basigin, also known as CD147 or EMMPRIN (extracellular matrix metalloproteinase (MMP) inducer). Basigin is a single pass transmembrane glycoprotein that contains two Ig domains one of which binds to caveolin-1. It has been found to effect the migration of both normal and cancer cells and the release of MMP's. We have confirmed the direct interaction of calpain4 with basigin through pull-down experiments. We are currently performing migration assay, traction force microscopy and mechanosensing assays on cells silenced for basigin. Preliminary data indicates that basigin deficiency results in a loss of the ability to sense mechanical stimulation just as seen in calpain 4 deficient cells. In addition, early results suggest an increase in traction force in embryonic fibroblasts when basigin is knocked down. In this study we have made the novel discovery of a previously unknown protein-protein interaction between the calpain small subunit and basigin. Further studies are currently underway to define how this interaction contributes to the regulation of traction force.

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Endothelial Mechanotaxis in Vascular Remodeling.

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Migration and plasticity of venous endothelial cells (ECs) during development depend on hemodynamic forces and play an important role in vascular remodeling processes. Here, we examined the venous circulation of the zebrafish embryo and found that, in contrast to a widely-held view, venous ECs migrate against the direction of blood flow. Migrating venous ECs invaded intersegmental vessels of arterial origin, indicating that this novel form of mechanotactic migration is relevant to vascular remodeling processes. In a microfluidic in vitro model, we observed that confluent human venous ECs both migrate and polarize against the flow at shear stresses encompassing those in the venous circulation, from an unexpectedly modest 0.45 dyn/cm^2 to 14.5 dyn/cm^2. The polarization of confluent ECs against the flow can be explained by hydrodynamic drag pushing their nuclei downstream. This behavior is in sharp contrast with polarization and migration of non-confluent ECs in the direction of flow that can be due to confluence-dependent differences in the organization of the actin cytoskeleton. Specifically, non-confluent ECs have dense actin bundles in the lamellum preventing the downstream displacement of the nucleus by hydrodynamic forces. We found that in shear flow, disruption of lamellum actin structure in non-confluent ECs by inhibition of myosin II-dependent contractility or of actin polymerization resulted in rapid shift of the nucleus towards the downstream side of the cells and in their polarization against the direction of flow. This new mechanism of mechanotaxis was further confirmed in an experiment where rapid displacement of nuclei in confluent ECs caused their immediate polarization and subsequent directed migration under nearly zero shear stress.

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3D Morphogenesis of Epithelial Cells on a Laminin-rich Soft Substrate Induced by Cellular Contractile Force.

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Collective cell migrations are essential for many physiological events, such as embryonic development, wound healing and tumor invasion. It is known that epithelial cells form 3D

morphology like a cyst and a tube in 3D environment. Our previous studies revealed that MDCK cells formed 3D morphology on top of a laminin-rich soft substrate (Matrigel, BD Biosciences). The appearance of morphologies was like a tulip hat or a tube. Time-lapse observation showed that the cells tugged at the peripheral matrix and remodeled the gel surface morphology. However, the mechanism of the 3D morphogenesis on the soft substrate is unclear.

In the present study, we performed time-lapse imaging by interference reflection microscopy and confocal laser scanning microscopy to observe how MDCK cells form 3D morphology on top of the Matrigel. Fluorescence beads were embedded in the Matrigel to visualize cellular contractile force. Time-lapse imaging showed the 3D morphogenesis of MDCK cells seeded on the Matrigel accompanied with deformation of the gel surface. Furthermore, we focused on this multicelluar behavior with respect to the contractile force of stress fibers consisting of actin and myosin II filaments, dominant driving force of cell migration. Our recent study has revealed that diphosphorylation of myosin II regulatory light chain (MRLC) enhances the degree of traction force. To elucidate the spatial regulation of the traction forces, localization of diphosphorylated MRLC (2P-MRLC) and two isoforms of myosin II heavy chain (MHC-IIA and MHC-IIB) was visualized by immunofluorescence. 2P-MRLC localized along the periphery of the epithelial sheet, and MHC-IIA colocalized with 2P-MRLC, but MHC-IIB did not. These results showed that the contractile force was mainly induced by phosphorylation of MRLC binding with MHC-IIA. Treatment with Y-27632, to inhibit phosphorylation of MRLC and abrogate cellular contractility, stopped the 3D morphogenesis. These results indicated that the cellular contractile force and the deformation of the substrate are critical for 3D morphogenesis of epithelial cells.

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Viscoelastic matrix elicits coordinated epithelial migration through actin reorganization.

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Collective cell migration, fundamental in embryonic development and adult tissue homeostasis, is governed by coordination of cell-cell and cell-matrix adhesions, but the mechanism that regulates reciprocal dynamics of adhesions is unknown. We have recently found that on a viscoelastic matrix of physiological relevance, highly correlated cell movement in an epithelial monolayer is induced, an E- cadherin-dependent phenotype as opposed to autonomous migration observed on stiff substrates. How cells convey mechano-signal from the matrix to adherens junctions and accordingly migrate in a concerted fashion is the main focus of current study. Twelve inhibitors have been used to screen cellular components that are essential for viscoelastic substrate-elicited phenotypes. Our results strongly argue that actin dynamics and actomyosin activity are indispensable for coordinated migration, while PI3K, Src family kinases, β-catenin stability, and microtubule dynamics are possibly involved. Actin organization has subsequently been studied by live-cell microscopy in epithelial sheets migrating on viscoelastic substrates. By imaging Lifeact-GFP, we have observed abundant stress fibers (SFs) and 2D morphology that are indistinguishable from cells on stiff substrates. Nevertheless, deconvolved 3D confocal images reveal distinct distribution of SFs with respect to surface-coated Rhodamine-fibronectin. Although fibronectin coating is essential for cell attachment, on viscoelastic substrates SFs make little connection to fibronectin but lie along the ventral surface, while the tips of SFs often colocalize with fibronectin on stiff substrates. Our study suggests that viscoelastic matrix-induced actin architecture may provide mechanical supports mainly to cell-cell contacts, and hence constitute the mechanistic basis for adherens junction-dependent coordinated migration.

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Distinct regimes of mechanical propagation at the onset of collective cell migration.

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A variety of fundamental processes in morphogenesis, health, and disease, involve the transition of cell sheets from a static to a migratory phenotype. While the biochemical events that regulate this transition are increasingly well understood under the rubric of the epithelial to mesenchymal transition, the physical forces that initiate and maintain collective cell migration remain unknown. By using polydimethylsiloxane (PDMS) cell micropatterning, traction microscopy, and monolayer stress microscopy, we provide dynamic maps of such physical forces and of the resulting cell velocities. Systematic analysis of these maps reveals an unexpectedly rich physical picture in which a velocity wave propagates away from and back to the leading edge. The direction of propagation defines two distinct mechanical regimes. During inward propagation, cells become motile under low levels of cell-cell stress. By contrast, outward propagation occurs under high and long-ranged levels of tensional stress. These distinct mechanical regimes are paralleled by a dramatic reorganization of stress fibers and cell-cell junctions. Our results demonstrate coupling between cell velocities, traction forces, and stress transmission between cells at the onset of collective cell migration.

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Overcrowding of epithelia cells induces extrusion to maintain homeostatic cell numbers.

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For an epithelium to provide a protective barrier, the tissue must properly balance the number of dividing and dying cells to maintain homeostatic cell numbers. While cell division can be triggered by dying cells, how cell death may be utilized to relieve overcrowding due to proliferation is not known. After initiating apoptosis within epithelia, we have shown that dying cells are extruded to preserve a functional barrier. In order to extrude, apoptotic cells produce sphingosine-1-phosphate (S1P), which signals the cooperative assembly of an actin/myosin ring in surrounding epithelial cells that contracts to squeeze out the dying cell. Yet, what promotes cell death during homeostatic turnover in epithelial tissues is not clear. We show that overcrowding induces extrusion of live cells to control epithelial cell numbers. Extrusion of live cells occurs at sites where highest crowding occurs in vivo and can be induced by experimentally overcrowding monolayers in vitro. Like apoptotic cell extrusion, we find that live cell extrusion resulting from overcrowding also requires S1P signaling and ROCK-dependent myosin contraction but is distinguished by upstream signaling through stretch-activated channels. Moreover, we find that blocking stretch-activated channels in developing zebrafish prevents extrusion and leads to the formation of epithelial cell masses, indicating that stretch-activated signaling is critical for maintaining epithelial homeostasis via extrusion. Our findings reveal that during homeostatic cell turnover, division and growth of epithelial cells on a confined substratum causes overcrowding which leads to cell extrusion and subsequent death due to loss of survival factors. Taken together, these results suggest that live cell extrusion could be a tumor suppressive mechanism that prevents the accumulation of excess epithelial cells.

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Automated Surface Tracking and Morphodynamics Quantification of Endothelial Cells in 3D Gels.

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Many non-amoeboid cell types migrate in 3D matrices with complex, branched morphologies. To better understand how these complex changes in 3D cell shape occur over time at both local and global levels, we sought to develop quantitative cell-surface tracking tools that would enable us to quantify cell shape changes in 4D in an unbiased manner. Spinning disk confocal 4D imaging of primary mouse aortic endothelial cells expressing tdTomato-CAAX, a membrane-targeted fluorescent tag, allowed visualization of the cell surface in 3D collagen gels over time. We have developed novel computational image segmentation and analysis tools that allow automated tracking and measurement of pseudopodial branch protrusion dynamics, local cell surface curvature, and branch topological complexity (branch order). To understand the role of myosin II contractility in regulating cell morphodynamics in 3D, we analyzed the effects of pharmacological inhibition of contractility. This revealed that inhibiting contractility increased cell branch number, as well as increased branch topological complexity and protrusion initiation rates from higher order branches. Local cell surface curvature measurements of the plasma membrane demonstrated that increased average surface curvature (both positive and negative) was correlated with decreased myosin II function, even in non-branched regions. These data suggest that not only does myosin II function to inhibit branch complexity and curvature of the cell surface, but also that the local surface curvature variability may be a useful metric in predicting cortical contractility of the actomyosin cytoskeleton. Automated surface and branch tracking algorithm enabled these quantitative observations that would have been difficult or impossible to achieve manually.

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Mapping Protein Structure Changes in Live Cells by Cysteine Labeling and Mass Spec.

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Protein unfolding, disassembly, and aggregation underlie many diseases, but detailed study of these processes in intact cells has been limited. Cysteine Shotgun labeling utilizes cell-permeable fluorescent dyes to label exposed cysteine residues and has been applied to study specific protein structure changes in response to mechanical stress on cells. We have expanded the technique to identify multiple protein changes in live red blood cells and mesenchymal stem cells as a function of mechanical stress and in whole-cell lysates in native versus urea-denaturing conditions as a function of time and temperature (20-45 deg-C). Labeling rate constants are calculated for any given Cys site by normalizing the protein labeling kinetics to the rapid labeling under denaturing conditions in lysates, or static conditions versus shear in live cells. Proteins can be identified and further analyzed by mass spectrometry to pinpoint specific, susceptible domains involved. This analysis has been done on a proteomic scale in cell lysates, on alpha and beta spectrin in mouse red blood cells, and Filamin A and B, Talin 1, Myosin 9 and lamin A/C in MSCs. Protein-specific results from these studies verify our results against previous literature.

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Combining Laser Ablation and FRET to Measure the Tension Distribution of Individual Stress Fibers on Focal Adhesions.

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Generation of traction against the extracellular matrix by stress fibers (SFs) via focal adhesions (FAs) is critical to mechanosensing, motility, and tensional homeostasis. However, the spatiotemporal relationship between SF force generation and tension on individual FAs remains poorly understood. Here we directly measure the tension distribution of individual stress fibers in living cells by combining subcellular laser nanosurgery with a vinculin fluorescence resonance energy transfer (FRET) tension sensor. Our results demonstrate that the tension change in FAs following SF ablation is not restricted to the FAs that are in direct contact with the severed SFs but is exhibited by almost all the FAs in the cell: most FAs show a decrease in tension while a minority of FAs has slight increase in tension. This tension distribution evolves with time following SF disruption and varies with the spatial location of the SF, consistent with our previous finding that central and peripheral SFs exhibit different viscoelastic properties and contributions to cell shape stability (Tanner et al., Biophys J 2010). In addition, morphometric analysis reveals that there is a greater change in tension for the FAs aligned in a direction that correlates with the long axis of cell polarity or that of the severed SF. This study enhances our understanding of how cells couple SF contractility to tension on individual FAs, and to our knowledge represents the first combination of laser nanosurgery with a molecular FRET sensor.

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Actin cap associated focal adhesions and their distinct role in cellular mechanosensing.

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The ability for cells to sense and adapt to different physical microenvironments plays a critical role in development, immune responses, and cancer metastasis. However, how cells realize changes in mechanical compliance remains unclear. Here we identify a small subset of focal adhesions that terminate fibers in the actin cap, a highly ordered filamentous actin structure that is anchored to the top of the nucleus by the LINC complexes; these differ from conventional focal adhesions in morphology, subcellular organization, movements, turnover dynamics, and response to biochemical stimuli. Actin cap associated focal adhesions (ACAFAs) dominate early mechanosensing in human and mouse adherent cells, a ACAFA-specific function regulated by phospho-myosin and α-actinin content in the actin cap and by FAK, but not zyxin, talin, and paxillin in ACAFAs. LINC-mediated attachments of the actin cap to the nucleus are also critical to mechanosensing by ACAFAs. A computational model explains the more sensitive response of ACAFAs compared to conventional focal adhesions by the enhanced tension in actin cap fibers. These results establish the perinuclear actin cap and associated ACAFAs as mediators of early cellular sensing of substrate compliance and a critical element of the long-speculated physical pathway that can transduce extracellular mechanical cues all the way to the nucleus.

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Isoform-specific Contributions of Non-muscle Myosin II to Viscoelastic Properties of Stress Fibers.

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Stress fibers (SFs) are bundles of F-actin, actin-binding proteins, and non-muscle myosin II (NMMII) that enable cells to generate traction forces against the extracellular matrix. It has recently been shown that two isoforms of NMMII (NMMIIA and NMMIIB) play distinct roles in orchestrating cellular contractile forces, yet the specific contribution of each isoform to stress fiber viscoelastic properties remains incompletely understood. To address this open question, we used laser nanosurgery to disrupt single SFs in living cells in the setting of isoform-specific NMMII suppression. We used lentiviral shRNAs to knock down the heavy chains of NMMIIA and NMMIIB in U373 MG human glioma cells, severed individual mCherry LifeAct-labeled SFs, and tracked viscoelastic SF retraction kinetics. The retraction of the two severed ends of the SF was recorded and the retraction behavior was fit to a Kelvin-Voigt model described by a viscoelastic time constant (τ) and a plateau retraction distance (L_0). Our results demonstrate that NMMIIA and NMMIIB have different contributions to cell morphology and SF viscoelastic properties. NMMIIA suppression produced a dose-dependent morphology change characterized by the elucidation of spike-shaped processes and it also produced slightly faster SF retraction kinetics. By contrast, NMMIIB suppression did not dramatically alter cell shape but to a greater extent reduced the time constant associated with viscoelastic retraction, consistent with our previous findings with myosin light chain kinase inhibition (Tanner et al., Biophys J 2010). This may suggest a model in which NMMII isoforms provide different levels of resistance in SF retraction. To our knowledge, this represents the first attempt to resolve isoform-specific contributions of NMMII to stress fiber mechanics and provides new insight into the roles specific myosin II isoforms play in the mechanobiology of human glioma cells. Given recent observations that NMMII can promote glioma cell invasion through the brain parenchyma by generating high contractile forces, this study may also lend insight into the molecular biophysical basis of tumor invasion.

Signaling Networks Governing Cell Motility I

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The scaffold Protein Grasp Interacts with Dock180 Bridging the Activation of ARF6 and Rac1 in Epithelial Cell Migration.

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Epithelial cells only exhibit migratory behavior under few conditions: developmental processes, wound healing and cancer metastasis. They normally form defined and polarized structures characterized by cell-cell and cell-substratum junctions. Small GTPases play a role in altering the cortical actin cytoskeleton and promoting epithelial movement. ARF6 and Rac1 are two of these GTPases that appear to cross-talk in migrating epithelial cells. We found that efficient ARF6 induced Rac1 activation happens via cytohesin2 and Dock180, the two respective GEFs of these GTPases. We proposed that the exchange factors interact within a larger multiprotein complex. Additionally data revealed that the scaffold proteins IPCEF and Grasp play a central role in the GTPases crosstalk (White D et. al. 2010). We have previously shown that Dock180 and Grasp interact with each other. We report here that Dock180 and Grasp bind to each other

via the SH3 domain on Dock180 and the proline-rich domain of Grasp. We found that the binding is disrupted when the SH3 domain is truncated and when we produced mutations in the proline-rich domain of Grasp. We confirm these results by coimmunoprecipitation and bi-molecular fluorescence complementation (BiFC) using the Venus split system. This study demonstrates that Grasp is the scaffold protein that bridges ARNO and Dock180 in migrating epithelial cells.

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Multimodal visualization of motile glioma C6 cells and their response to P2Y2 nucleotide receptor induction.

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While microscopy is crucial for development of live sciences from decades, the molecular biology revolution put this technique into the shade. From twenty years, however the real microscopy revolution take place. The revolution aims to put biochemical and molecular biology techniques from the glassware on the laboratory bench back into the living cell.
Our scientific interest focuses on the process especially suited for use of the live microscopic methods: cell motility. To address requirements of the real time subcellular studies of motile cell, we have decided to construct multimodal system allowing us to make parallel studies using both fluorescence and transmitted light methods. The system is organized around three main microscopy methods: long term DIC Nomarski cell observation, IRM (Internal Reflection Microscopy) and fast multiwavelength fluoresce microscopy. The fluorescence microscopy mode is optimized for fast Fura2 measurements yielding up to 30 frame-pairs per second in full camera resolution. This configuration allows overlay of calcium concentration maps on Nomarski contrast motility tracks and IRM adhesion measurements resulting in almost complete motility analysis.
Presented results show the influence of P2Y2 receptor induction by UTP and resulting calcium signal on motility of Glioma C6 cells and it substratum adhesion visualization. The signal parameterization showed previously on this conference (Poster 171, ASCB 50th Annual Meeting 2010, Philadelphia) as well as adhesion pattern is overlaid onto DIC images. While in the control, motile cells calcium signal is strongly polarized, our results show clearly how signal polarization disappears after cell actomyoisin contractility inhibition by Y-27632, a selective ROCK kinase inhibitor.
The main aim of the system is to put our previous results of nucleotide dependent signal polarization into cell motility context, however being financed from EU POIG founds, we are open for collaboration with all groups willing to put their biochemical results into imaging context but lacking microscopy expertise or equipment.

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Silencing of DLC1 promotes PAI-1 expression and reduces normal cell migration.

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Deleted in liver cancer (DLC1) is a GAP (GTPase Activation Protein) domain containing tumor suppressor that localizes to focal adhesion. One of DLC1¡¦s known functions is to regulate cancer migration. However, its role in normal cell migration has not been well studies. Here, we show that silencing of DLC1 (shDLC1) in normal prostates epithelial cells reduce cell migration. This migration defect is mainly due to up-expression of plasminnogen activator inhibitor 1 (PAI-1), since further silencing of PAI-1 rescues the shDLC migration phenotype. Re-expression of

DLC1 in shDLC1 cells suppresses PAI-1 protein level and rescues the migration defect. In contrast, DLC1-K714E (GAP inactive) mutant neither decreases the PAI-1 level nor rescues shDLC migration defect. Interestingly, re-expression of DLC1-Y442F (tesin-binding and focal adhesion-localizing defective) mutant is able to suppress PAI-1 expression but does not rescue the migration defect. Furthermore, PAI-1 upregulation in shDLC cells is EGFR-MEK pathway dependent and is able to promote in vitro angiogenesis. Together, our results demonstrate that at least two new mechanisms are involved in DLC1-mediated normal cell migration. (A) DLC1 modulates the expression of PAI-1, which is a negative regulator for cell migration, in a GAP domain and EGFR-MEK dependent manner. (B) Independent to PAI-1, the interaction of DLC1 with tensin members positively regulates cell migration.

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Arpin, a Novel Protein that Inhibits the Arp2/3 Complex, Controls Lamellipodium Protrusion and Cell Migration.

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The Arp2/3 complex is a major actin nucleator of eukaryotic cells. It generates a branched actin network that generates a pushing force. This force powers the protrusion of the plasma membrane in a structure called the lamellipodium. The Wave complex is the major Arp2/3 activator in the lamellipodium, and the small GTPase Rac activates the Wave complex, when GTP-bound. Here we present a novel inhibitor of the Arp2/3 complex that regulates dynamics of lamellipodia and cell migration.

We interrogated public databases for proteins containing an acidic C-terminal domain and harboring a W at position -2. This characteristic motif involved in Arp2/3 binding allowed us to retrieve an uncharacterized human protein among known Arp2/3 activators. This protein indeed interacted with the Arp2/3 complex in a GST-pulldown, and inhibited its activation in a dose-dependent manner, as revealed by pyrene-actin assays and by actin filament branching monitored with TIRF microscopy. We call this protein 'Arpin' as a tribute to Monique Arpin, A.G. PhD supervisor, who retired earlier this year, and also because it is a good mnemonic for *Arp*2/3 complex *in*hibitor.

We found that Rac activity induces the interaction of Arpin with the Arp2/3 complex. Consistently, endogenous Arpin localized to the lamellipodium of migrating cells. Upon RNAi-mediated depletion of Arpin in human cell lines, lamellipodia protruded faster, consistent with its inhibitory role on Arp2/3 complex activity. Arpin depleted cells explored a larger territory than the controls, because of increased speed and directionality. A knock-out of the orthologous Arpin gene from *Dictyostelium discoideum* also resulted in faster and more directional amoeba, indicating that Arpin function is conserved from amoeba to man. Fish keratocytes are the best model for persistent migration. Microinjection of purified zebrafish Arpin into keratocytes resulted in cycles of suppression of the existing lamellipodium immediately followed by growth of a new ectopic one.

Together these results argue for an essential inhibitory role of Arpin in modulating Rac signalling toward the Arp2/3 complex. If the WAVE complex is the 'engine' of cell migration, Arpin would be a 'brake' and the 'steering wheel'.

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RhoC is crucial to ensure proper protrusion formation through spatial location of cofilin activity.

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The steps of invasion and intravasation during metastasis require tumor cells to migrate and degrade through dense basement membrane barriers. To accomplish this, tumor cells display actin-rich subcellular compartments, named lamellipodia and invadopodia, involved in leading edge protrusion and matrix degradation, respectively. Regulation of actin dynamics in lamellipodia as well as invadopodial protrusions is crucial to drive invasion. The actin severing protein cofilin is essential for directed cell migration and chemotaxis. Through its severing activity, cofilin increases the number of free barbed ends to initiate actin polymerization for actin-based protrusion in these distinct subcellular compartments. Cofilin severing activity is tightly regulated and multiple mechanisms are utilized to regulate cofilin activity. We have recently shown that RhoC regulates cofilin phosphorylation through a ROCK/LIMK pathway to control the ability of cofilin to generate actin barbed ends in invadopodia in invasive tumor cells. RhoC activity is spatially confined to a ring-like pattern surrounding the invadopodium that are formed during EGF stimulation. This has been shown to focus cofilin activity to the core of invadopodia. This spatiotemporal restriction of RhoC activity is controlled by “spatially distinct regulatory elements”, p190RhoGEF and p190RhoGAP.
Since lamellipodium formation also requires fine regulation of actin dynamics, we studied how RhoC contributed this process. In MTLn3 cells, RhoC is required for formation and elongation of EGF-stimulated protrusions. We have studied the spatiotemporal dynamics of RhoC during lamellipodium protrusion using a new RhoC biosensor. We found that RhoC is activated one micron behind the leading edge to focus the activity of cofilin toward the front of the cell. This spatial localization of RhoC activity appears necessary to place active cofilin at the very tip of lamellipodium to generate barbed ends, similar to what happens in the invadopodium core.
We propose a new model wherein areas of elevated RhoC activity form a barrier against the diffusion of active cofilin by enhancing the cofilin phosphorylation within this zone. This results in the restriction of elevated cofilin activity to only within the invadopodium core or within the very tip of the lamellipodium compartment to focus the protrusion and to increase cell migration and invasion.

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Synergistic Interaction Between Cofilin and Arp2/3 Enhances Actin Barbed Ends Generations in a Spatiotemporal Model of a Lamellipodium.

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Rapid polymerization from the barbed ends of actin filaments generates protrusive forces at the cell edge, leading to cell migration. Two important regulators of free barbed ends, cofilin and Arp2/3, have been shown to work in synergy based on experiments on mammary carcinoma cells. Specifically, cofilin is found to sever older F-actin (with ADP bound monomers) generating new free barbed ends from which new ATP-F-actin polymerize. Arp2/3 complexes, which bind preferentially to these new filaments, can then nucleate new branch points, allowing for further growth of the actin network in the lamellipodium.

To further quantify the level of synergy between cofilin and Arp2/3, we build and analyze a mathematical model for the spatiotemporal distribution of actin filaments at the leading edge of a motile cell. The model incorporates spatial diffusion of cofilin and Arp2/3, membrane protrusion, F-actin capping and ageing, severing by cofilin and branch nucleation by Arp2/3. The level of synergy is quantified by calculating the ratio between the total barbed ends produced in the presence of both, to the direct sum of those produced by cofilin and Arp2/3 acting on their own.

By varying the level of cofilin and Arp2/3 activated at the cell edge, we found that each can generate a large pulse of barbed ends on its own. However, a high synergy is observed only under low cofilin and low Arp2/3 stimulation. We found that the synergy is larger, and results in greater protrusion in the spatially distributed system (compared to the well-mixed case). Further, the relative timing of the cofilin and Arp2/3 activation is important: a slight delay in the Arp2/3 peak can significantly increase the peak of barbed ends following cofilin activation. The synergy is maximized if Arp2/3 is introduced when the level of new F-actin is high following a burst of barbed end generation from an initial cofilin activation.

Note: See abstract submission 542 by Bravo-Cordero, et al., RhoC is crucial to ensure proper protrusion formation through spatial location of cofilin activity, for experimental work on a similar system. We ask to be included as a back-to back presentation/same poster session with the Bravo-Cordero et al presentation.

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Cortactin Controls Cell Motility and Lamellipodial Dynamics by Regulating ECM Secretion.

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Branched actin assembly is critical for both cell motility and membrane trafficking. The branched actin regulator, cortactin, is generally considered to promote cell migration by controlling leading edge lamellipodial dynamics. However, recent reports indicate that lamellipodia are not required for cell movement, suggesting an alternate mechanism. Since cortactin also regulates membrane trafficking and adhesion dynamics, we hypothesized that altered secretion of extracellular matrix (ECM) and/or integrin trafficking might underlie motility defects of cortactin-knockdown (KD) cells. Consistent with a primary defect in ECM secretion, both motility and lamellipodial defects of cortactin-KD cells were fully rescued by plating on increasing concentrations of exogenous ECM. Furthermore, cortactin-KD cell speed defects were rescued on cell-free autocrine ECM produced by control cells but not on ECM produced by cortactin-KD cells. Investigation of the mechanism revealed that whereas endocytosed FN is redeposited at the basal cell surface by control cells, cortactin-KD cells exhibit defective FN secretion and abnormal FN retention in a late endocytic/lysosomal compartment. Cortactin-KD motility and FN deposition defects were phenocopied by KD in control cells of the lysosomal fusion regulator Synaptotagmin-7. Rescue of cortactin-KD cells by expression of cortactin binding domain mutants revealed that interaction with Arp2/3 complex and actin filaments is essential for rescue of both cell motility and autocrine ECM secretion phenotypes whereas binding of SH3 domain partners is not required. In conclusion, efficient cell motility, promoted by cortactin regulation of branched actin networks, involves processing and resecretion of internalized ECM from a late endosomal/lysosomal compartment.

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CD11b regulates insulin-induced THP-1 cell migration and adhesion through PI3K/Akt1 pathway.

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Diabetes is risk factor for cardiovascular diseases and strongly associated with the incidence of atherosclerosis. During the pathogenesis of atherosclerosis, monocyte adhesion to vascular endothelium and subsequent migration across the endothelium has been recognized as key process. However, regulatory mechanism by which diabetic status promotes atherosclerosis is not clear. Here, we showed that high concentration of insulin (10uM)-induced human acute monocytic leukemia (THP-1) cell migration and phosphorylation of Akt in time and dose-dependent manner. However, insulin-like growth factor-1 (IGF-1), which is structurally similar growth factor, was not effective. Insulin-induced THP-1 cell migration was attenuated by pharmacological inhibition of phosphatidylinositol3-Kinase and Akt. In addition, silencing of Akt1 abolished insulin-induced cell migration and adhesion of THP-1 monocyte cells. We also found that both migration and adhesion of bone marrow-derived cells (BMDCs) isolated from mice lacking Akt1 was significantly attenuated. Finally, surface expression of CD11b/CD18 was strongly promoted by the stimulation with insulin. Enhanced surface expression of CD11b/CD18 was abrogated by silencing of Akt1 or in BMDCs isolated from mice lacking Akt1. Given these results, we suggest that insulin-induced THP-1 migration and adhesion is specifically regulated by PI3K/Akt1 pathway during the pathogenesis of atherosclerosis.

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PAK regulates adhesion dynamics and membrane protrusion of the neuronal growth cone through its interaction with PIX.

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Guidance of axons during development is critical to the establishment of a properly inter-connected nervous system. Targeting of axons requires growth cones at the tips of extending axons to navigate through complex environments by integrating signals generated though interactions with soluble and bound molecular cues. P21-activated kinase (PAK) is one intracellular signaling factor that may influence axon outgrowth and guidance downstream of extracellular matrix (ECM) proteins and growth factors. Here we examined the interaction of PAK with PAK-interacting exchange factor (PIX), in the regulation of growth cone motility. PIX functions as a guanine nucleotide exchange factor (GEF) for Rac1 and Cdc42, as well as an adaptor for other proteins that target PAK to adhesion sites known as point contacts. We find that PAK is expressed in the developing spinal cord and targets to both paxillin containing growth cone point contacts and the extending tips of filopodia. Additionally, we observe that acute disruption of the PAK-PIX interaction with a cell-permeable peptide called PAK18 leads to immediate acceleration of Xenopus spinal neurite outgrowth on laminin. Accelerated axon outgrowth in response to PAK18 is also associated with dramatic lamellipodial expansion of the growth cone. Immunocytochemical staining of the PAK targets, phospho-cofilin and phospho-myosin light chain II, paired with the corresponding changes in the cytoskeleton (increased actin depolymerization and decreased retrograde flow) suggest that disrupting PAK binding to PIX with PAK18 leads to less PAK kinase activity in growth cones. Furthermore, live imaging of paxillin-GFP adhesions shows that disruption of PAK-PIX interactions strongly regulates growth cone adhesion dynamics. While the number of adhesions increases markedly, point contacts formed in the presence of PAK18 are more rapidly disassembled. Interestingly we also find that Rac1 activity has a partial role in mediating the effect of PAK18 in neurite outgrowth suggesting

that PAK and Rac1 may be competing for PIX binding. Lastly, growth cones expressing a PAK-PIX binding mutant exhibit increased basal outgrowth and fail to respond to PAK18 stimulation. Together our data suggests that regulation of PAK-PIX interactions in growth cones controls neurite outgrowth by influencing the activity of several important mediators of actin filament polymerization and retrograde flow, as well as integrin-dependent adhesion to laminin.

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Migrating fibroblasts reorient directionality by a metastable, PI3K-dependent mechanism.

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Mesenchymal cell migration as exhibited by fibroblasts is distinct from amoeboid cell migration and is characterized by dynamic competition among multiple protrusions, which determines directional persistence and responses to spatial cues. Localization of phosphoinositide 3-kinase (PI3K) signaling is thought to play a broadly important role in cell motility, yet the context-dependent functions of this pathway have not been adequately elucidated. By mapping the spatiotemporal dynamics of cell protrusion/retraction and PI3K signaling, monitored by TIRF microscopy, we show that fibroblasts reorient predominantly by *branching* of existing protrusions followed by *pivoting* of the two branches. Consequently, if the two branches propagate to their fullest extent, a 90 degree turn is achieved. PI3K inhibition does not affect the initiation of newly branched protrusions in fibroblasts, nor does it prevent protrusion induced by photo-activation of Rac. Rather, PI3K signaling is a dynamic response to, not a precursor to, local protrusion and is required for the lateral spreading and stabilization of nascent branches. Actin polymerization is required for the redistribution of the PI3K signaling pattern, but not its maintenance, suggesting a “passive” form of positive feedback. We further show that, during chemotaxis, the branch experiencing the higher chemoattractant concentration is favored, and thus the cell reorients so as to align with the external gradient.

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A non-canonical role for Rgnef (p190RhoGEF) in integrin-stimulated FAK activation.

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Rgnef is a Rho guanine exchange factor (GEF) for RhoA and RhoC that uniquely binds to focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase activated by integrins. This Rgnef-FAK signaling complex promotes adhesion formation downstream of integrins and enhances colon carcinoma tumor progression. To investigate Rgnef function, we generated a knockout mouse model. Rgnef-null mice are born at a lower frequency than the predicted Mendelian ratio and Rgnef-null mouse embryo fibroblasts (MEFs) exhibit defects in integrin-stimulated adhesion formation on fibronectin (FN), RhoA GTPase activation, and cell migration. These phenotypes are rescued by Rgnef re-expression and are consistent with canonical models of Rgnef function with GEF activation occurring approximately 60 min after MEF binding to FN. However, Rgnef-null MEFs form fewer adhesions at 15 to 30 min on FN and early FAK activation is significantly decreased in Rgnef-null MEFs compared to wild type. Moreover, FN-stimulated FAK activation is inhibited by over-expression of the Rgnef-C-terminal domain but not Rgnef-C-Δ1292-1301 (FAK-binding domain deletion) in wildtype MEFs. Rgnef localizes to leading-edge cell projections and Rgnef (but not RgnefΔ1292-1301) re-expression facilitates FAK localization to and activation at nascent adhesions formed at 15 to 30 min on FN. These data support a new and non-canonical role for Rgnef binding to FAK in promoting early FN-stimulated FAK activation. Taken together, our studies indicate that Rgnef has two temporally distinct but

cooperative functions which contribute to the precise regulation of FAK activation and focal adhesion formation to control cellular contractility and migration.

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The SH2-domain-containing inositol 5-phosphatase (SHIP) limits neutrophil motility and chemotaxis.

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The recruitment and activation of neutrophils into sites of injury or infection is essential for host defense against invading microorganisms. However, excessive neutrophil recruitment or activation can be damaging to the surrounding tissues and cause unwanted inflammation leading to a delay in, or even prevention of healing. Hence, the responsiveness of neutrophils needs to be tightly regulated. It has been shown that SHIP hydrolyzes $PI(3,4,5)P_3$ to $PI(3,4)P_2$ and that $PI(3,4,5)P_3$ is an important regulator of cell migration. Here we investigated the role of Ship 5'-phosphatase activity on leukocyte motility and chemotaxis *in vivo,* using the zebrafish as an animal model. Using time-lapse confocal microscopy and ratiometric imaging of bioprobes specific for $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ in neutrophils, we show that $PI(3,4,5)P_3$-$PI(3,4)P_2$ accumulates at the front of neutrophils while $PI(3,4)P_2$ also localizes to the rear of neutrophils migrating *in vivo*, correlating with the observed localization of SHIP in neutrophils. Depletion of SHIP phosphatases using morpholino (MO) results in an increase in leukocyte recruitment to wounds. Time-lapse imaging of neutrophils randomly migrating in the head also reveals increased 3D velocity in SHIP-deficient larvae. The increase in the leukocyte recruitment in SHIP-deficient zebrafish is rescued by treatment with a low dose of a PI3 kinase inhibitor. Moreover, overexpression of the SHIP phosphatase domain specifically in neutrophils impairs neutrophil migration, suggesting that SHIP inhibits leukocyte motility through its phosphatase activity. Taken together, our results suggest that in an *in vivo* context, SHIP limits neutrophil motility and chemotaxis by modulating local sub-cellular pools of phospholipids.

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Size and shape dependence of epithelial gap closure.

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Fundamental biological processes such as morphogenesis and wound healing involve the sealing of epithelial gaps. Epithelial sealing is commonly thought to be driven by two independent mechanisms: assembly and contraction of a supracellular actomyosin cable (purse-string model) and crawling of marginal and submarginal cells into the gap. Ample evidence supports the existence of each of these two mechanisms but their relative contribution to gap sealing remains unknown, mostly because of the complexity of the process and the high variability in the experimental approaches. Here, we present a new method to study systematically the sealing of gaps in an epithelial culture. Using masking strategies to block the surface available to the cells, we developed a pillar removal assay that allows us to finely control and standardize the size and shape of gaps within an epithelial monolayer. Upon pillar removal, cells actively respond to the presence of free space by extending lamellipodia and crawling into the gap. Cells at the gap edge polarize and elongate in the direction of migration. The decrease of gap area over time is strikingly linear and shows two different regimes depending on the size of the gap. For gaps larger than 15 µm in diameter, epithelial closure is dominated by lamellipodium-mediated cell crawling rather than actin purse-string assembly. Indeed, Rac inhibition (the main regulator for lamellipodial protrusion) induced a dramatic delay

in the collective migration of cells while inhibition of either MLCK or ROCK had no significant effect in gap closure. By contrast, for gaps smaller than 15 μm epithelial closure progressed independently of Rac, MLCK, and ROCK inhibition, suggesting a passive physical mechanism. By changing the shape of the gap, we observed that low curvature areas favored the appearance of lamellipodia, thus promoting a faster closure. Altogether, this study establishes that large epithelial gap closure is driven by lamellipodial-based migration whereas small gaps appear to close by adhesion forces, independent of active motility.

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Effects of p120 catenin and Rho GTPase interactions during zebrafish gastrulation.

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Gastrulation is the process during which cells migrate and tissue layers differentiate during early embryo development. Such a process requires cell-cell adhesion mediated by cadherin-catenin interactions at adherens junctions. We study p120 catenin (p120ctn) in zebrafish. p120ctn stabilizes cadherins at the plasma membrane and interacts with various GTPases both in its membrane-bound and cytoplasmic states. These GTPases include Cdc42 and Rac, which stimulate filopodia and lamellipodia formation respectively, and RhoA, which stimulates stress fiber formation. Using an antisense splice morpholino we have shown that loss of p120ctn results in developmental deficiencies. In an amphibian model it was previously shown that Cdc42 and Rac in their constitutively active GTP-bound states could partially rescue similar developmental deficiencies due to loss of p120ctn. Our lab has shown that injection of mRNAs from Cdc42 and Rac in their wild-type states, which are able to cycle between GDP and GTP bound states, can more fully rescue the embryonic development caused by knockdown of p120ctn. We hypothesize that Cdc42 and Rac in their constitutively GTP-bound states activate filopodia and lamellipodia throughout the entire cell periphery resulting in undirected motion and thus a decreased rescue of developmental deficiencies as cells cannot differentiate into tissue layers efficiently. However, wild-type Cdc42 and Rac form localized filopodia and lamellipodia in response to chemokine signals thereby allowing directed mesodermal cell migrations. We are currently studying the potential of RhoA to rescue deficiencies by studying its role in mesoderm cell migration. It is known that RhoA becomes GTP bound and thus activated when near the plasma membrane and that GTP-bound RhoA acts by activating RhoA kinase increasing the activity of myosin. Since myosin cross-links and stabilizes actin filaments, RhoA indirectly strengthens the adhesion of migrating cells. Over-expression of constitutively active RhoA mRNAs disrupts normal gastrulation. By knocking down p120ctn we will test the potential of RhoA in different GTP and GDP bound states to rescue developmental deficiencies. We also hypothesize that p120ctn is additionally regulated via phosphorylation, with phosphorylation on serine and threonine sites increasing adhesion and that on tyrosine sites activating cell migration. We are currently studying the roles of phosphorylation at specific sites of p120ctn and their interactions with Rho GTPases.

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Directional Cell Migration Regulates Tubule Morphogenesis.

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Previous studies have shown that planar cell polarity (PCP), including orientated cell division (OCD) and convergent extension (CE), plays an important role in establishing and maintaining kidney tubule diameter in higher vertebrates. In the embryonic kidney, epithelial cells elongate perpendicularly to the axis of the tubule, which suggests that mediolateral cell intercalation

during convergent extension elongates the embryonic kidney tubules. Here we show that cell elongation is randomized in normal postnatal tubules. Genetic removal of PC1 in mice leads to the loss of this randomized cell elongation such that cells are elongated mostly parallel to the tubular axis, indicating abnormal directed cell migration (convergent expansion). We report the identification of a novel PC1-Pacsin 2-N-Wasp protein complex in kidney epithelial cells, and the finding that PC1 modulates directional cell migration through Arp2/3-dependent actin cytoskeleton organization. Analyses of mice lacking either Pacsin 2 or Pkhd1 suggest that neither convergent expansion nor OCD defect alone is sufficient to cause cystogenesis. We propose that directional cell migration and OCD cooperate to maintain kidney tubule diameter and that this process requires the integrity of PC1-Pacsin 2-N-Wasp complex.

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Chromophore Assisted Laser Inactivation of ADF/Cofilin Demonstrates Its Function of Filament Severing and Depolymerization in Regulating F-actin Networks.

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There are numerous ways in which ADF/Cofilin effects actin dynamics: it can sever actin filaments, accelerate filament disassembly, act as a nucleation factor, recruit or antagonize other actin regulators, and control the pool of polymerization competent actin monomers. An issue with studying Cofilin has been that these rather straightforward biochemical activities have complex functional outputs in living cells. This has led to a number of reports showing conflicting roles for Cofilin in the same biological process. It has become evident that the timing and localization of Cofilin activity is carefully regulated within the cell, which means that global perturbations of Cofilin may not be sufficient to probe its true function. In an effort to better understand the spatiotemporal regulation of Cofilin, we have implemented fluorescent protein based Chromophore Assisted Laser Inactivation (FP-CALI) to knockdown Cofilin activity instantly with subcellular precision. We have combined FP-CALI with high resolution live-cell imaging so that changes to the actin network can be monitored in real time before, during, and after Cofilin inactivation. CALI of either wild-type Cofilin or a constitutively active mutant ($Cofilin_{S3A}$) decreases actin monomer mobility, indicative of a loss of the ability to sever actin filaments. Furthermore, CALI of $Cofilin_{S3A}$ has a number of effects on the lamellipodial actin network, these include: an increase in total F-actin, a broadening of the lamellipodia towards the cell body, lengthening of actin bundles, and a reduction of retrograde flow. We provide evidence that these phenotypic changes to actin are specifically due to inactivation of Cofilin and are not caused by collateral or non-specific photodamage. These findings not only continue to validate FP-CALI as an effective technique for instantaneous and specific knockdown, but also provide additional understanding into ADF/Cofilin's function in regulating actin dynamics.

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Regulation of Nonmuscle Myosin II Assembly and Function through Heavy Chain PKC and Casein Kinase II Phosphorylation Sites.

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Nonmuscle myosin-II is a hexameric complex comprised of two heavy chains (MHC), two essential light chains (ELC), and two regulatory light chains (RLC).There are three NMHC-II isoforms (NMHC-IIA, NMHC-IIB, and NMHC-IIC). Myosin IIA is enriched in leading-edge lamella during persistent polarized migration and RLC phosphorylation is known to play critical roles in regulating nonmuscle myosin II filament assembly during the migration, but the regulation and physiological relevance of myosin II heavy chain (MHC) phosphorylation is not well understood.

Myosin IIA can be phosphorylated on several C-terminal heavy chain residues, including a Protein Kinase C (PKC) consensus target site at position S1916 and a calcium-calmodulin-dependent casein kinase II (CK-II) consensus target site at position S1943.

S1943 phosphorylation has been established in earlier work as participating in regulation of myosin IIA filament assembly in leading edge protrusions. Mutation of S1943 impairs myosin IIA localization to lamellipodia during spreading of cells on fibronectin coated surfaces and during polarized migration/extension in response to the chemoattractant EGF. Although the involvement of myosin IIA phosphorylation on S1943 in assembly control is recognized, there have been few studies of phosphorylation on site PKC site, and virtually none in the context of live cells. The present study is focus on studying regulation of myosin IIA assembly by phosphorylation of PKC and CKII sites on the myosin IIA heavy chain (MHC). Using site-directed mutagenesis approaches coupled with expression GFP-tagged MHC IIA genes, we report here that the PKC target sites on MHC IIA are clearly involved in control of myosin IIA filament assembly in live cells. Initial studies suggest that phosphorylation at S1916 functions in coordination with phosphorylation at S1943 to control filament assembly during spreading and migration.

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The distinct role of the Arp2/3 complex in 3D motility.

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Arp2/3 is a seven-subunit protein complex that nucleates actin filament assembly in adherent cells crawling on planar substrates. However, in physio-pathological situations, cell motility typically occurs within a three-dimensional (3D) environment and little is known about the role of Arp2/3 in 3D motility. Here we find that, for a wide variety of cells in 3D matrix, the Arp2/3 and associated proteins N-WASp, Cortactin, and Cdc42, mediate the formation of pseudopodial protrusions that branch off into multi-generation dendritic protrusions into the matrix. 3D cell speed in matrix correlates with the degree of side branching, not mother protrusion activity. This study shows that the Arp2/3 complex plays a critical role in 3D cell motility by mediating the dendritic topology of protrusions.

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Myosin-IIA contractility is required for activated T lymphocyte trans-endothelial migration in vivo.

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To carry out their surveillance and effector functions, T lymphocytes must migrate from the vasculature into lymph nodes and tissues. We have previously shown that Myosin-IIA plays a role in T cell migration by modulating adhesion to the surrounding substrate and regulating the switch between fast amoeboid-like motility and slower mesenchymal-like motility. We have also shown that interstitial motility is affected in naïve T cells lacking Myosin-IIA leading to inefficient recirculation through the lymph nodes. However, the specific cytoskeletal requirements that underlie T cell trans-endothelial migration (TEM) and extravasation are not yet fully understood. Therefore, we sought to analyze the contribution of Myosin-IIA in TEM and in extravasation of T cells. Lack of Myosin-IIA in naïve T cells did not significantly impair their extravasation potential. However, Myosin-IIA depletion caused a significant defect in the ability of activated T cells to

complete TEM both in vitro and in vivo. Our data suggest that, in addition to the effects of Myosin-IIA on adhesion that we previously described, Myosin-IIA contractility provides the force necessary to squeeze the nucleus-containing body of the T cell through the endothelial cell layer. Furthermore, our results showed that Myosin-IIA plays a significant role in the ability of effector T cells to reach inflamed peripheral tissues in vivo. Overall, these results suggest a critical role for Myosin-IIA in promoting TEM and in migration through constrictive environments, as is necessary for the extravasation of activated T cells.

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Dual Functions of p21-activated Kinase in Regulating Myosin Light Chain Phosphorylation.

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Intestinal edema and subsequent decreased intestinal contractile activity often occur under various pathologic circumstances. In an in vivo intestinal edema rodent model, our laboratory showed a significant decrease in intestinal contractile activity and corresponding decreases in both myosin light chain (MLC) and myosin light chain phosphatase targeting subunit (MYPT1) phosphorylation in edematous tissue. Our data also indicated in edematous tissue, P21-activated kinase (PAK) activity is increased while Rho kinase (ROCK) is decreased. Moreover, intestinal tissue contractility was rescued by inhibition of PAK activity in vivo.
To investigate the role of PAK in edema-induced intestinal contractile dysfunction, a human primary intestinal smooth muscle cell (hISMC) model was developed. HISMCs were subjected to either basal cyclical stretch (CCS) or an increasing cyclical stretch (ECS), mimicking pathologic changes in edematous tissues. ECS induced significant decreases in phosphorylation of both MYPT1 and smooth muscle myosin light chain (MLC) mimicking the in vivo intestinal edema findings. The role of PAK in regulating MLC phosphorylation has been investigated by transfecting hISMCs with two constitutively active PAKs (caPAK) or dominant negative PAK (dnPAK). In ECS group, transfection with caPAK induced further decrease in MLC phosphorylation while dnPAK produced the opposite effect. In contrast, in CCS group, caPAK transfection increased MLC phosphorylation and dnPAK decreases MLC phosphorylation. The PAK activator (BPIPP) and the PAK inhibitor (IPA-3) induced similar results as transfection with caPAK and dnPAK, respectively. While IPA-3 does not affect MYPT1 phosphorylation, BPIPP decreases MYPT1 phosphorylation in hISMCs. We conclude from this data that under physiologic environment, PAK is responsible for maintaining MLC phosphorylation; however, increased PAK activity under pathologic conditions switches PAK signaling to a pathway inhibiting MLC phosphorylation through decreased MYPT1 phosphorylation.

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Transfection with fluorescent constructs impairs the mechanical behavior of cells.

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To visualize cell movement and to analyze cytoskeletal and focal adhesion dynamics, the transfection of cells with fluorescently tagged protein expression vectors is a common method. Generally, cells are assumed to behave normally after transfection if their morphology remains unchanged, but this assumption has not been rigorously tested.
We investigated whether the transfection with fluorescently tagged cytoskeletal constructs (EGFP-actin and LifeAct) alters the mechanical behavior of invasive MDA-MB 231 breast carcinoma cells. Transfected cells showed unaltered morphology and spreading area, but their

ability to invade porous 3D collagen gels was strongly impaired. Cell stiffness measured with magnetically applied forces to 4.5 µm RGD-coated beads was reduced by 2-3 fold in transfected cells. Moreover, beads detached from transfected cells at pulling forces that were 5-fold smaller compared to control cells. These data demonstrate that transfection with fluorescently tagged cytoskeletal proteins strongly impairs the mechanical behavior of cells, without any visible changes in morphology. This behavior can only partly be attributed to the transfection procedure, as cell transfection with single tag pEGFP and to a lesser degree treatment with the transfection agent lipofectamine alone also resulted in a somewhat reduced cell stiffness and reduced bead binding strength. In summary, our data show that transfection in particular with fluorescently tagged cytoskeletal proteins may result in artefactual cell behavior and may strongly bias mechanical measurements.

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Design and engineering of genetically encoded, high performance FRET biosensors of Rho GTPases.

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Genetically encoded, forster resonance energy transfer (FRET)-based biosensors are proven extremely valuable in elucidating the spatiotemporal dynamics of signaling proteins in living cells. However, current design strategies rely on empirical sampling of fluorescent proteins and linker sequences, resulting in suboptimal dynamic range of the sensors and thus limited application. Through rational design, we sought to improve the existing biosensors of Rho GTPases, in which interaction between Rho GTPases with their effectors is coupled to changes in FRET between two fluorescent proteins in a single polypeptide. Firstly, the high FRET state is improved by taking advantage of the dimeric nature of fluorescent proteins and their circular permutants. Secondly, a further decreased low FRET state is achieved via inserting repeats of a stable α-helix from the ribosomal protein L9, rather than an unstructured linker, between the fluorescent proteins to disrupt dimerization and therefore diminish FRET efficiency. Lastly, switching between these two FRET states is coupled to the interaction between GTPases and their effectors through modeling of the crystal structures of the fluorescent protein dimer and the GTPase/effector domain complexes. These engineering steps resulted in a set of genetically encoded, FRET-based biosensors for Rac, Cdc42 and RhoA that have markedly improved dynamic range. FRET/CFP emission ratios were increased by 4-6 folds in HEK293 cells expressing mutant sensors mimicking the bound state, comparing to ratios in cells expressing mutants mimicking the unbound state. Live cell imaging using these sensors revealed dynamic and distinct activation profiles of the individual Rho family GTPases in migrating cells. Because the high FRET efficiency is intrinsic to the engineered FRET module, we expect such design can be generalized to produce high performance biosensors for other signaling proteins.

Integrins and Cell-Extracellular Matrix Interactions

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Cell Fate and Elasticity Dictates Cell-ECM Attachment Strength and Detachment Mechanism.

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Cell attachment strength has been used as a metric to assess cell behaviors ranging from cell-ECM binding forces to matrix fiber strength. Shear force-induced detachment in these assays has typically been shown to be binary with the cell either attached with relatively normal morphology or absent at physiological or higher forces, but these assays have been limited to a relatively small number of cell types, e.g. fibroblasts, fibrosarcoma cells, etc. Using a spinning disc device that applies a radially-dependent shear force over a short duration, e.g. 5 minutes, here we investigate the attachment strength and detachment mechanism for a number of species and cell sources that vary in their degree of senescence from pluripotent and multipotent stem cells to cancer cells to common fibroblasts. While data from murine fibroblasts confirms binary adhesion states with relatively little cytoskeletal remodeling if any before detachment, tremendous remodeling was observed in mouse embryonic stem cells (mESCs) during this short shear exposure. Cells oriented themselves parallel to the applied shear and elongated up to aspect ratios of 4:1. Human bone marrow derived stem cells (BMSCs) also aligned with a shear up to an aspect ratio of 6:1. However after prolonged culture, BMSCs lost their ability to remodel under shear and detached in a binary fashion, perhaps indicating that a certain degree of pluripotency is required for the cytoskeleton to remain elastic and remodel. Since differentiation scales with cell stiffness, the ability of cells to rearrange under shear depends mostly on their elasticity. Softer cells such as undifferentiated BMSCs have an advantage to quickly adopt to the direction of shear and align themselves parallel to the force, reducing their total drag and consequently are able to withstand a higher shear force than their integrin density would let expect. Cell softness also correlates with cancer progression, an subsequent analysis could show that applying super-physiological shear could be used to select for cells with higher metastasis potential. Regardless, these data indicate that previous consideration for cell attachment strength, namely that it correlates to integrin density, may in fact be also dependent on cell state and its elasticity.

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GEF-H1 and LARG regulate adhesion reinforcement in response to mechanical tension.

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Cells can “sense” and convert mechanical forces into specific biochemical signals that ultimately regulate cellular processes. Among all the structures involved in mechano-sensing, focal adhesion (FA) proteins and their matrix anchor, integrins, have emerged as important actors in mechanotransduction. Application of force on integrins triggers cytoskeletal rearrangements and growth of the associated adhesion complex, resulting in increased cellular stiffness, also known as reinforcement. Since this ability of FAs to respond to force is involved in various aspects of biology, from development to physiology and disease, many efforts have been directed recently to understand how mechanical forces regulate FA components and how they assemble.
We found that application of force on integrin triggered activation of the Rho GEFs LARG and GEF-H1 and their recruitment to the FA. Interestingly, we observed that LARG and GEF-H1 were activated by independent signaling pathways. We found that Fyn was required for LARG

force-dependent activation, while GEF-H1 was activated by Erk downstream of FAK. Using magnetic tweezers, we observed that depletion of LARG or GEF-H1 prevented the cellular stiffening in response to force, indicating that both Rho GEFs are necessary for the cellular response to force. More recently, we observed that GEF-H1 and LARG were activated when cells were grown on stiff substrates, suggesting that cell-generated contractility regulates LARG and GEF-H1 activity.

These data show that external force, as well as cell-generated tension, activates the Rho GEF LARG and GEF-H1, which in turn regulate adhesion reinforcement. Identification of Rho GEFs and/or GAPs modulated by force on other cell surface receptors is under investigation.

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Mechanical tension promotes mast cell degranulation through an RGD-integrin dependent pathway.

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Mechanical forces applied to the extracellular (ECM) matrices of various tissues are essential in maintaining tissue homeostasis and physiology. Alterations in mechanical tension can result in a maladaptive response that includes induced cellular stress and activation of pathological signaling pathways. Mechanical forces applied to the extracellular matrix are largely transmitted to the cell via receptors of the integrin family. Specifically, RGD-specific integrins have been shown to act as mechanotransducers converting mechanical stresses into biological signals. Mast cells are immune cells that participate in several biological functions including wound healing, fibrosis, cell recruitment, asthma, inflammation and mediation of vascular permeability. In several pathological diseases where changes in the mechanical environment stimulate changes in the ECM, mast cell density and activation are increased significantly. The biological changes that occur to mast cells during mechanical tension have not been investigated. Our objective was to 1) examine the mechanosensitivity of the mast cell by subjecting it to cyclic mechanical load, and 2) determine the role of RGD-specific integrins in mast cell response to mechanical force. We applied 5% and 10% cyclic, uniaxial tension to three dimensional fibrin constructs seeded with RBL-2H3 cells (a mast cell line) and monitored the cellular response over a period of 24 hrs. Our results showed that at 5% and 10% cyclic mechanical load, RBL-2H3 degranulation, as measured by secretion of β-hexosaminidase, significantly increased (2.1 to 2.3 fold, respectively) in a load- and time-dependent manner when compared to the non-stretched controls ($p<0.003$). Furthermore, mechanical loading did not compromise cell viability or increase cell proliferation. To determine if RGD-dependent integrins mediated mast cell degranulation in response to mechanical tension, we inhibited cell-matrix interactions by treating the RBL-2H3 cells with Echistatin, a disintegrin that tightly binds RGD-specific integrins. Treatment with Echistatin attenuated load-induced degranulation (by ~50%, $p<0.0004$) without compromising cell viability. These results suggest a novel mechanism by which mechanical stretch induces mast cell activation via RGD-dependent mechanotransduction.

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Substratum stiffness regulates switch control of cell proliferation and differentiation in primary cultures of mice proximal tubular cells.

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Differentiated renal tubular epithelial cells *in vivo* normally reside on soft basement membrane containing laminin-rich extracellular matrix and remain quiescent status. Accumulated studies indicate that substratum stiffness might regulate cell behavior, i.e. migration, proliferation,

differentiation, apoptosis and tumorigenesis. Our studies showed that mouse proximal tubular (mPT) cells in primary culture on stiffer substrate gradually lost their tubular morphology and differentiated properties with the increase of cell proliferation which could be alleviated when cells were cultured on softer substrate. Here we investigated how substratum stiffness regulates the switch of cell proliferation and differentiation in primary mPT cells. One of the possible candidates involved in mechanosensing machinery is β1 integrin. We showed that low substratum stiffness not only suppressed β1 integrin activation but also protein level via post-translational regulation. In addition, low substratum stiffness also downregulated Smad3, ERK and P38MAPK. High substratum stiffness-induced cell spreading, proliferation and dedifferentiation were alleviated by 4B4 (β1 integrin neutralized antibody) and UO126 (ERK1/2 inhibitor) treatment, but not affected by inhibition of Smad3 and P38MAPK pathways. Combination of the oligo-microarray data and bioinformatic prediction database revealed that hepatocyte nuclear factor 4α(HNF4α), a member of the nuclear receptor family of transcription factors, might play a critical role in regulation of a set of genes related to cell proliferation and differentiation in mPT cells. Interestingly, inhibition of ERK preserved-cell differentiation and inhibited cell proliferation induced by high substratum stiffness might due to the maintenance of HNF4αlevel. This study provides basic understanding of how substratum stiffness regulates the switch of cell proliferation and differentiation in proximal tubule by modulation of transcription factor HNF4α.

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Cell volume decreases with increasing substrate stiffness.

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Cell morphology and stiffness have been observed to change with their growing substrate stiffness. But whether they change their size due to substrate stiffness is not known. We measured the volume of cells that are grown on substrate with different stiffness with confocal microscopy. We find that both cell volume and cell nuclear volume decrease with the increasing substrate stiffness, and the volume changing is cell cortex contraction dependent.

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Isolation and characterization of human dermal papillar and reticular fibroblasts.

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The dermis of human skin is partitioned into histological and physiological distinct zones. The most superficial one, the papillar dermis and the deeper one, the reticular dermis contain papillar (Fp) and reticular fibroblasts (Fr) respectively. Little is known about these two sub-populations of dermal mesenchymal cells, their specific role and biological markers

Objectives: To isolate and characterize these two sub-populations of cells in terms of migration (healing), proliferation and clonogenic potential, differentiation, collagen remodeling and tensile strengths expression in response to neurotrophins (NTs), and genes expression profile.

Methods: dermis was sectioned with a dermatome (Fp 0-0.3mm and Fr >0.7mm) and cells obtained by explant culture and collagenase I digestion. Migration was evaluated by the ability of cell to colonize an articicial scar in vitro (healing) and by the number of explants producing cells. Growth potential of cells was determined by counting cells over 7 days and the ability of single cells to form colonies. Differentiation into myofibroblasts was analysed by the expression of alpha-SMA (Smooth Muscle Actin). Collagen remodeling ability of cells was evaluated by the

retraction of floating gels and tensile strengths by a GlaSbox device with and without treatment with neurotrophins (NTs). Transcriptional analysis of genes was performed by Taqman Low Density Array methods (TLDA).
Results: Fp have a higher migration potential than Fr according to the explant culture and healing test. We observed that the rate of growth and the clonogenic potential were higher for Fp than for Fr. Alpha-SMA expression was higher in Fr compared to Fp. At basal rate, collagen remodeling was more pronounced in Fr than Fp the later developing higher tensile strengths than Fr. Regarding NTs, and increase of tensile strengths was observed with NTs in both subpopulations of cells. The transcriptional analysis of genes expression showed that types I, III, V collagen are up-regulated in Fr as well as MMP-14, TIMP-1 and TIMP-2 whereas MMP-1 and MMP-4 are up-regulated in Fp.
Conclusion: Taken together these preliminary results showed a specific genes expression signature between papillary and reticular dermal fibroblasts. Papillary fibroblasts have a more proliferation and migration profile, since reticular ones are more involved in a tensile and differentiation process, both subpopulations of cells responding to neuroptrophins.

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Differential Response of Arterial and Venous Endothelial Cells to Extracellular Matrix is Modulated by Oxygen.
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Adhesion of endothelial cell (EC) integrins to extracellular-matrix (ECM) components is one of the key events to trigger intracellular signaling that will ultimately result in proper vascular development. Even within one tissue the endothelial phenotype differ between arteries and veins. Here we tested the hypothesis that anchorage-dependent processes such as proliferation, viability, survival and actin organization of venous (VEC) and arterial EC (AEC) differently depend on ECM proteins. Moreover, because of different oxygen tension in AEC and VEC, we tested oxygen as a co-modulator of ECM effects. Primary human placental VEC and AEC were grown in collagen I and IV, fibronectin, laminin, gelatin and uncoated plates and exposed to 12% and 21% oxygen. Our main findings revealed that VEC are more sensitive than AEC to changes in the ECM composition. Proliferation and survival of VEC, in contrast to AEC, were profoundly increased by the presence of collagen I and fibronectin as compared to gelatin or uncoated plates. Focal adhesion kinase (Fak) inhibition studies demonstrated that these effects were dependent on Fak. They were also modulated by oxygen. VEC were more susceptible to the oxygen-dependent ECM effects than AEC. However, no differential ECM effect on actin organization was observed between the two cell types. These data provide first evidence that AEC and VEC from the same vascular loop respond differently to ECM and oxygen and this may be Fak dependent.

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HIM-4/Hemicentin Links the Uterine and Vulval Basement Membranes during Anchor Cell Invasion in C. elegans.
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Cell invasion across basement membrane (BM) occurs frequently during normal development and disease processes, such as cancer, but has been difficult to model *in vitro* and dissect *in vivo*. Anchor cell (AC) invasion is a genetically and visually tractable invasion process during *C. elegans* vulval development in which the uterine AC breaches two distinct BMs (uterine and vulval BM) to contact the underlying vulval epithelium. How the breaching of two BMs is coordinated during the invasion process is unknown. Previous work from the Sherwood Lab has

shown that mutations in a matricellular protein, HIM-4, the *C. elegans* ortholog of hemicentin, result in a 17% AC invasion defect. Transgenic worms expressing HIM-4::GFPdisplay distinct HIM-4 punctae specifically in the BM beneath the AC prior to invasion. As the AC invades, the HIM-4 punctae are pushed aside and accumulate around the edge of the BM hole. By imaging worms expressing LAM-1::GFP in the *rol-6(su1006)* background, we were able to obtain a ventral view of BM dynamics during AC invasion at a higher resolution than previous studies. Our results reveal that in 90% of wild type animals the AC invades simultaneously through the uterine and vulval BM, creating a single hole in both BMs. Strikingly, only 10% of *him-4(rh319)* worms breach the two BMs simultaneously, with 90% first opening up a hole in the uterine BM and subsequently penetrating the vulval BM in a delayed manner. By physically separating the uterine and vulval tissues, we have observed that the uterine and vulval BM become tightly linked specifically under the AC before invasion. Further, after AC invasion, the distinct vulval and uterine BM are fused into a continuous sheet at the edge of the AC. We hypothesize that HIM-4 functions in a novel process, adhering two distinct BM, to facilitate AC invasion. Interestingly, BM adherence and fusion have been observed in other developmental contexts, such as glomeruli formation and alveolar development, but the mechanisms underlying these BM changes remain unknown. BM remodeling during AC invasion may provide a tractable model to understand the molecular mechanism of BM adherence and fusion.

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Fibroblast clustering on three-dimensional collagen matrices: Role of α5 integrin.

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Cell organization and plasticity respond to diverse stimuli in the cellular microenvironment. Fibroblasts cultured in medium containing fetal bovine serum (FBS) or lysophosphatidic acid (LPA) on three-dimensional (3D) collagen matrices establish a procontractile phenotype that leads to the formation of cell clusters. The formation of clusters did not occur when cells were incubated with platelet-derived growth factor (PDGF), which activates a promigratory phenotype. The switch between clustering and dispersion phenotypes is reversible, indicating that signaling pathways can be turned on and off. To learn about the mechanism responsible for stabilizing cell-cell interactions when cells form clusters, we studied cluster formation by BR-5 human foreskin fibroblasts under conditions in which we used siRNA technology to modulate cell-cell and cell-matrix junctional complexes. Silencing N-cadherin expression interfered with formation of adherens junctions as detected by localization of N-cadherin and beta catenin. However, under these conditions cell cluster formation was unaffected. On the other hand, silencing alpha-5-integrin not only blocked cell adhesion to fibronectin (FN)-coated culture dishes, but also interfered with cell clustering on collagen matrices. Subsequent studies supported the idea that cell clusters were stabilized by integrin interaction with cell secreted fibronectin matrix. In cell clusters, alpha-5 integrin and fibronectin were observed in close proximity in cell clusters. Fibronectin matrix in the clusters was cell-derived since the FN distribution appeared similar regardless whether clustering took place in FBS-containing medium (which contains plasma FN) or in LPA (which lacks plasma FN). Once formed, the FN matrix appeared to be stable because addition of PDGF to clustered cells caused cell dispersion without causing disruption of the FN matrix. Our studies provide new insights in to the mechanism by which the growth factor microenvironment of cells on 3D collagen matrices can modulate changes in cell and matrix organization.

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SHARPIN is an endogenous inhibitor of beta1-integrin activation.

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Regulated activation of integrins is critical for cell adhesion, motility and tissue homeostasis. Talin and Kindlins activate β1-integrins, but the counteracting inhibiting mechanisms are poorly defined. Here we identified SHARPIN as an important inactivator of β1-integrins in an RNAi-screen. SHARPIN inhibited β1-integrin functions (affecting cell adhesion and migration) in human cancer cells and primary leukocytes. Fibroblasts, leukocytes and keratinocytes from SHARPIN-deficient mice exhibited increased β1-integrin activity (and increased cell migration) which was fully rescued by re-expression of SHARPIN. Mechanistically, SHARPIN directly bound to a conserved cytoplasmic region of integrin α-subunits and inhibited recruitment of Talin and Kindlin to the integrin. Therefore, SHARPIN binding to the cytoplasmic tail of α-integrins inhibits the critical switching of β1-integrins from inactive to active conformations.

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Fibronectin promotes mesenchymal cell condensation and regulates Sox9 during chondrogenic differentiation.

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Mesenchymal stem cells (MSCs) have potential as a therapeutic tool for joint repair due to their ability to differentiate into chondrocytes and deposit cartilaginous extracellular matrix. Fibronectin is abundant in growth plates at joints suggesting that this matrix protein may have a role in cartilage formation. Bone marrow-derived MSCs were induced to undergo chondrogenic differentiation in high-density micromass or pellet culture by addition of TGFβ3 and other factors. Cells condense into a compact aggregate during the first 24 hours followed by differentiation of condensed cells into chondrocytes, a process that requires changes in cell organization, gene expression, and matrix composition. Our results show that fibronectin production increased by day 3, remained elevated through day 9, and then decreased to a moderate level through day 21. Fibronectin matrix within the high-density culture also increased over time as detected by immunofluorescence. Type I collagen had a similar profile of secretion and matrix incorporation as fibronectin. We observed transient upregulation of the condensation marker N-cadherin at day 1. mRNA expression for Sox9, a major transcription factor required for chondrogenic differentiation, was increased after day 2 and remained elevated for an extended period during differentiation. Thus the increase in fibronectin level at day 3 corresponds with the increase in Sox9 which led us to hypothesize that fibronectin facilitates early chondrogenic differentiation. To test the requirement for fibronectin, we used siRNAs to knock down its expression in MSCs. Interestingly, we observed an early effect of reduced fibronectin, namely, impaired cell condensation, which was accompanied by a transient decrease in N-cadherin and a prolonged reduction in Sox9 mRNAs. Addition of exogenous fibronectin was sufficient to partially restore Sox9 mRNA expression. These results indicate that fibronectin-cell interactions facilitate condensation and regulate expression of an essential differentiation marker. To investigate potential mechanisms for the fibronectin effects, chondrogenesis was induced in the presence of excess fibronectin cell binding domain (CBD) fragment to disrupt integrin binding to fibronectin. Our preliminary results show that addition of the CBD fragment inhibited

condensation and reduced expression of Sox9. We propose that fibronectin has a dual role in chondrogenesis, participating in cell condensation and signaling through integrins to induce Sox9 expression.

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An Antibody to the Lutheran Glycoprotein (Lu) Recognizing the LU4 Blood Type Variant Prevents LU Binding to Laminin α5.

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The Lutheran blood group glycoprotein (Lu), an Ig superfamily (IgSF) transmembrane receptor, is also known as basal cell adhesion molecule (B-CAM). Lu/B-CAM is a specific receptor for laminin α5, a major component of basement membranes in various tissues. Previous reports have also shown that Lu/B-CAM binding to laminin α5 contributes to sickle cell vaso-occlusion. However, since there are no useful tools such as function-blocking antibody and drug, it is still unclear how epithelial and sickled red blood cells adhere to laminin α5 through Lu/B-CAM. By studying several monoclonal antibodies that recognize Lu/B-CAM, we discovered a function-blocking antibody that inhibits the binding of Lu/B-CAM to laminin α5. It also suggested that the function-blocking antibody recognized an epitope of Lu/B-CAM involved in binding laminin α5. Therefore, to characterize the function-blocking antibody, we first identified the site on Lu/B-CAM recognized by this antibody. The extracellular domain of Lu/B-CAM contains five IgSF domains, D1-D2-D3-D4-D5. To narrow the region recognized by the antibody, we produced a series of truncated proteins with sequentially deleted Ig-like domains and chimeric proteins replaced with the analogous domain of melanoma cell adhesion molecule (Mel-CAM). The antibody epitope was localized to D2, but not to the D3 domain containing the major part of the laminin α5 binding site. Furthermore, mutagenesis studies showed that Arg^{175} was crucial for forming the epitope. Arg^{175} was also located at the LU4 blood group antigenic site. To examine if Arg^{175} was essential for forming the epitope, we also produced mutant protein substituted with Ala. However, although Arg^{175} was a part of the epitope recognized by the function-blocking antibody, it was not involved in the binding of laminin α5. The results suggested that the antibody bound sufficiently close to sterically hinder the interaction with α5. Inhibition assay using the antibody also showed that Lu/B-CAM served as a secondary receptor in the adhesion of carcinoma cells to laminin α5. This function-blocking antibody against Lu/B-CAM would be useful for not only investigating cell adhesion to laminin α5 but also developing drugs to inhibit sickle cell vaso-occlusion.

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Role of IRSp53 in Cell-Extracellular Matrix Adhesion and Filopodia Formation in C2C12 Cells.

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IRSp53 (Insulin Receptor Substrate) is an adaptor molecule which couples membrane-deforming activity with the actin cytoskeleton remodelling. Actin cytoskeleton plays a critical role in cell adhesion. Cell-extracellular matrix (ECM) adhesion is vital for many processes such as proliferation, differentiation and cell migration. Binding of integrin receptors to their respective ligands in the ECM lead to integrin clustering and activation that triggers the formation of large multiprotein complexes on the cytosolic face of the plasma membrane, referred to as Focal adhesions (FA). Vinculin is a component of cell adhesion complex that links actin cytoskeleton to integrins. Fibronectin, a major extra-cellular matrix protein, is one of the major constituents of endomysium (a layer of connective tissue that ensheaths a muscle fiber). Thus we sought to

determine the role of IRSp53 in adhesion to fibronectin in mouse muscle progenitor cells C2C12. We found a significant reduction in binding and spreading of C2C12 expressing IRSp53 as compared to control cells on fibronectin coated wells whereas knockdown of endogenous IRSp53 in C2C12 cells slightly increased the binding and cell spreading on fibronectin coated wells. We also investigated the effect of IRSp53 expression on localization of focal adhesion protein vinculin. Prominent vinculin patches were observed in control and knockdown cells, whereas IRSp53 expressing cells showed reduced vinculin patches. These results suggest that expression of IRSp53 inhibits adhesion of C2C12 cells on fibronectin and vinculin patches assembly. IRSp53 consists of three well-characterized domains an N-terminal IMD (IRSp53 and Missing in metastasis Domain) followed by a GBD (GTPase Binding Domain) motif and a SH3 domain. We further identified the role of the three domains of IRSp53 (IMD, GBD and SH3 domains) in localization and filopodia induction in C2C12 cells. We observed membrane localization and filopodia formation in cells transfected with IRSp53-EGFP expressing plasmid. Mutations in any of the three domains (IMD, GBD or SH3) impaired filopodia formation ability of IRSp53. Significant membrane localization was observed in IRSp53267N-EGFP (GBD mutant) expressing cells and less membrane localization in IRSp532A-EGFP (SH3 mutant) expressing cells while no membrane localization was observed in IRSp534A-EGFP (IMD mutant) expressing cells. This implies that IRSp53IMD domain is important for recruiting IRSp53 to the plasma membrane as well as for inducing membrane protrusions by IRSp53 in C2C12 cells.

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Galectin-8 medium and long isoforms have opposite effects upon apoptosis in T cells and differential expression in lupus.

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Galectins are a family of carbohydrate binding proteins (lectins) that are secreted through a non-conventional pathway and interact with beta-galactosides of cell surface and extracellular matrix glycoproteins, contributing to modulate a variety of cellular processes, including T cell homeostasis. Galectin-8 (Gal-8) consists of two different carbohydrate recognition domains (CRDs) joined by a linker peptide whose length variation gives rise to Medium (Gal-8M) and Long (Gal-8L) isoforms. Gal-8M has been the most studied. Previously, we showed that Gal-8M induces apoptosis on T cells through a pathway involving phosphatidic acid mediated ERK activation. Here we compared the effects of Gal-8L and Gal-8M on apoptosis and ERK1/2 and AKT signaling in Jurkat T cells and also assessed the expression levels in peripheral blood mononuclear cells (PBMC; 70% T cells) from normal individuals and patients with systemic lupus erythematosus (SLE), the prototypic autoimmune disease. Gal-8L does not induce apoptosis but instead counteracts the apoptosis, as well as the activation of beta1-integrin and ERK1/2, induced by Gal-8M. ERK1/2 activation is proapoptotic in T cells. Gal-8L also increases activation of anti-apoptotic AKT pathway. Strikingly, CRDs alone have similar anti-apoptotic effects, suggesting that Gal-8L might act as a monomeric CRD. RT-PCR shows an increased expression of Gal-8M in PBMC from SLE patients. These results are the first indication that isoforms of Gal-8 exert opposing effects on T cell homeostasis, with Gal-8L counteracting the immunosuppressor action of Gal-8M, and the changes in expression levels suggest a role in the pathogenesis of SLE.

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Cell-Cell Junctions II

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Regulation of cell-cell and cell-ECM forces during the initial stages of epithelial cell scattering.

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Transition of cells from an epithelial to a mesenchymal phenotype is important during various stages of development and during disease progression. However, the role played by physical forces in orchestrating such a transition has been largely unclear. We have previously shown that cells exert significant tension at the cell-cell contact in an epithelial cell pair and that this tension directly increases with the level of cell-ECM force. This suggests that significant force redistribution must occur at the cell-microenvironment interface during epithelial to mesenchymal transition (EMT). Here, we use a classical model of EMT – the hepatocyte growth factor (HGF) induced scattering of MDCK cells – to determine the effect of growth factor induction on cell-cell and cell-ECM forces. In an epithelial cell pair with a single cell-cell interface, HGF induction leads to enhanced protrusive activity and marked expansion of cell area. Increased spread area, in turn, leads to a substantial increase in the total cell-ECM traction exerted on the substrate. Importantly, the tension at the cell-cell contact also increases in proportion to the total cell-ECM force. This suggests that enhanced protrusive activity dynamically modulates cell-ECM as well as cell-cell forces. Understanding how spatio-temporal regulation of cell protrusive activity impacts cell-cell and cell-ECM forces has direct implications for understanding morphogenesis and tumor metastasis.

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Rigidity-dependent T Cell Costimulation.

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T cells play a key role in mediating adaptive immunity. Activation of these cells occurs through engagement of the T Cell Receptor (TCR) complex with peptide-loaded MHC on the Antigen Presenting Cell (APC) surface, within a small area of contact termed the immune synapse. Costimulation through CD28, also a membrane-bound receptor, augments the TCR response and is needed for activation of naive T cells. The immune synapse is also characterized by an active and dynamic cytoskeleton which transports TCR and CD28 microclusters along the cell surface, posing the possibility that subsequnt signaling may be modified by the mechanical response of the extracellular environment. To explore such a possibility, this study examines activation of mouse CD4+ T cells by polyacrylamide gels presenting ligands to CD3 (activating component of the TCR) and CD28. IL-2 secretion, a high-level measure of costimulation, correlates directly with elastic modulus over a range of 10 – 200 kPa. Furthermore, this response can be divided into two regimes; on the stiffest surfaces, cell exhibit strong attachment and modulation of IL-2 secretion, while on the softest surfaces, cytokine secretion and attachment are both reduced. Phosphorylation of Lck and Zap70 were similarly reduced on the softest surfaces, suggesting that changes in early TCR signaling differentiate the two regimes. By presenting CD3 and CD28 ligands on different surfaces, we further demonstrated that mechanosensing is associated primarily with TCR signaling, although a minor and contrasting rigidity response was observed for CD28 signaling. This study presents the first evidence of cellular-level mechanosensing in T lymphocytes. Importantly, physical connections between the

cytoskeleton and TCR or CD28 have not been clearly delineated. Signaling through these receptors is thus a new realm of mechanobiology, and highly complementary to current understanding of similar responses to integrin and cadherin systems.

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Fine mapping of the plakophilin-1 interaction with desmoplakin: Implications for desmosome plaque assembly.

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Desmosomes are prominent cell-cell adhesive junctions in stratified squamous epithelial tissues, such as the skin and oral cavity, and are known to provide a point of continuity for the intermediate filament cytoskeleton throughout these epithelial tissues. The transmembrane core of the desmosome consists of the desmosomal cadherins (desmogleins and desmocollins) and is linked to the intermediate filament cytoskeleton through interactions with cytoplasmic desmosomal plaque proteins. These include plakoglobin, plakophilins and plakin family members. Plakophilins are armadillo repeat containing proteins that serve as scaffold proteins to cluster the desmosomal cadherins and recruit the IF cytoskeleton through interactions with desmoplakin. Over expression of pkp-1 in A431 cells resulted in increased desmoplakin recruitment to cell borders. We hypothesized that Pkp-1 interactions with desmoplakin play an important regulatory role in desmosome dynamics and disruption of this interaction results in decreased desmosome assembly. In order to study this interaction more closely, we sought to map the sequences of plakophilin-1/desmoplakin interaction. Previous studies have shown that the amino terminal domain of pkp-1 is important for binding to desmoplakin. Using a yeast two-hybrid approach; we mapped the interaction to a highly conserved sequence shared by other plakophilin isoforms. Point mutants were generated and shown to disrupt the plakophilin-1/desmoplakin interaction. Additionally, we demonstrated that plakophilin-1 mutants unable to interact with desmoplakin failed to promote desmosome assembly in A431D cells. Further characterization of plakophilin interactions reveal the ability of plakophilins to interact with one another, raising the possibility that plakophilins dimerize during desmosome plaque assembly. These studies extend our understanding of the role of plakophilin in desmosomal plaque assembly.

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Plakophilin-3 interacts with 14-3-3 sigma (stratifin) independent of desmosome assembly.

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Desmosomes are prominent adhesive junctions present in all epithelial tissues. Assembly and linkage of the desmosome to the intermediate filament cytoskeleton is dependent on a family of cytoplasmic proteins that constitute the desmosomal plaque. Plakophilin-3, a member of the armadillo family of proteins, is a component of the inner dense plaque of the desmosome and loss of plakophilin expression is known to affect junction assembly. Mechanisms regulating plakophilin-3 incorporation into the desmosomal plaque and overall junction assembly are poorly understood. In the current study we sought to identify novel plakophilin-3 interacting partners in an effort to identify mechanisms controlling desmosome assembly in epithelial cells. We identified stratifin (14-3-3 sigma) as a plakophilin-3 interacting protein using an affinity purification and mass spectroscopy approach. We confirmed this interaction in vivo by immunoprecipitation and we show this interaction is direct by yeast two-hybrid analysis. We mapped the domain of plakophilin-3 that is responsible for this interaction and found the

interaction to require a sequence not conserved in plakophilin-1 or plakophilin-2. Interestingly, the plakophilin-3/stratifin complex resides in the cytosol and is not incorporated into the desmosomal plaque. Interactions with stratifin in the cytosol may sequester a pool of plakophilin-3 in the cytosol and limit the pool of plakophilin-3 available to assemble the desmosome. These finding suggest that plakopihilin-3 may have unique and uncharacterized roles in the cytosol that may be distinct from its role in desmosomal adhesion.

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Scribble and the Met receptor tyrosine kinase.

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The activity or protein levels of Met, a receptor tyrosine kinase activated by hepatocyte growth factor (HGF), are frequently elevated in human cancers. In epithelial cells in culture, activation of the Met receptor induces a phenotype reminiscent of epithelial to mesenchymal transition (EMT), a process implicated in the formation of metastases. This includes the loss of cell-cell adhesion, remodeling of cell polarity, cell migration and invasion through matrix. However, how Met signaling elicits the changes in cellular morphology required for this metastasis-like behavior of cells is still largely unknown. We identified Scribble, a conserved scaffold protein previously shown to be required for cell-cell adhesion, cell migration and cell polarity as a physical interactor of the Met receptor. Scribble acts as a tumor suppressor in flies and mammalian systems and altered levels or mislocalization of the Scribble protein are observed in many human cancers. This makes it a promising candidate to execute Met-dependent remodeling of cellular architecture in order to facilitate cell migration or invasion and contribute to the oncogenic potential of the Met receptor.
By co-immunoprecipitations, we were able to show that Scribble and Met interact. Consistent with this finding, we observe partial co-localization of Scribble and the Met receptor at the basolateral cortex of polarized epithelial cells using immunofluorencence. Biochemical analysis indicates that the interaction between Scribble and Met is independent of maturation or kinase activation status of the receptor and does not require the Met scaffold protein Gab1. Importantly, preliminary data further suggest that abrogation of Scribble function leads to changes in cell migration in response to HGF, suggesting that Scribble modulates this process downstream of the Met receptor.
Given the role of Scribble in the organization of cell polarity, cell migration and as a tumor suppressor, we propose a role for Scribble in Met signaling, contributing to the changes in cellular organization observed during Met-induced signal transduction. We are currently analyzing whether the interaction between Scribble and Met is required for the cellular response to Met signaling, which subcellular compartments are important for the interaction, and which protein domains of Scribble and Met are involved in this process. Progress towards answering these questions this will be presented.

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Regulation of Anoikis by the Discs Large 1 Tumour Suppressor.

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Human Papillomaviruses (HPVs) are the causative agents of cervical cancer, the second major cause of cancer related death in women worldwide. Most cervical cancers are caused by two HPV types, HPV-16 and HPV-18. These viruses encode two oncoproteins, E6 and E7, both of

which are required for the development and maintenance of the transformed phenotype. The oncogenic E6 proteins all possess PDZ binding motifs (PBMs) that confer interaction with cellular PDZ domain containing proteins. Through this PBM, HPV-16 and HPV-18 E6 both target two critical regulators of cell polarity, with HPV-16 E6 interacting with Scribble, whilst HPV-18 E6 interact with Discs Large 1 (Dlg1). We have been interested in understanding what aspects of the functions of Scribble and Dlg1 are relevant for the ability of HPV-16 and HPV-18 E6 to induce malignancy.
Using epithelial cells in which the expression of Scribble and Dlg1 have been ablated we demonstrate that loss of Scribble enhances cell invasion, reduces cell-cell contact and increases cell proliferation, characteristics that are favourable for HPV induced malignancy. In contrast, loss of Dlg reduces cell invasion potential and slows cell proliferation, effects that would seem at odds with a tumour suppressor potential. However we have found that Dlg is a powerful regulator of the apoptotic process of anoikis with loss of Dlg greatly increasing a cells resistance to this apoptotic response. Furthermore, HPV-18 containing cervical tumour derived cells have a high degree of resistance to anoikis: this is independent of the p53 status but dependent upon the presence of HPV-18 E6 and the absence of Dlg1. Using proteomic and microarray approaches we are now assessing the impact of the loss of Dlg1 upon gene expression profiles and identifying the relevant interacting partners of Dlg that might explain its anoikis regulating activities.
Taken together these results demonstrate that HPV E6 targeting of the Scribble/Dlg cell polarity regulating complex, can effect multiple levels of tumourigenesis, including, invasion, cell proliferation and anoikis.

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Cell Adhesion Molecules Selection as Candidates for Differential Manipulation.

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Nanotechnology is a promising field that opens the possibility of minimally invasive in vivo manipulation in order to accomplish neuronal restoration. We propose that Cell Adhesion Molecules (CAM’s) can be used as targets for differential manipulation. CAM´s are essential at all developmental stages including those leading to a functional neuromuscular junction (NMJ). However, knowledge of how CAM´s organize spatiotemporally to facilitate assembly the NMJ network is limited. Differential manipulation can be achieved only with prior understanding of how CAM’s are normally organized in synaptogenesis through development. Immunocytochemistry assays and confocal microscopy enables three-dimensional visualization of CAM’s in the ventral somatic musculature of Drosophila embryos. Selected CAM´s (such as FasciclinII (FasII), Integrin and Dscam) are visualized from embryonic stages 12 to 18 in order to observe how they organize through development. This allows us to discern which CAM is ideal for manipulation. FasII, known for its role in axon guidance, is a candidate cell adhesion molecule for manipulation because it is expressed in axon bundles in the pattern that makes up ventral somatic musculature. Three dimensional localization of FasII in the anterior and posterior commisures of the ventral cord and the axon bundles in the ventral somatic musculature of stage 15 embryos has led it to be considered as a candidate for differential manipulation. My results, together with nanomaterial toxic assessment will bring us closer to achieve neuronal differential manipulation.

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Effects of Cigarette Smoke Exposure on Expression and Function of the Adherence Junction Molecule CD146 in Pulmonary Endothelial Cells.

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Cell adhesion molecule CD146 is a transmembrane glycoprotein constitutively expressed in all types of endothelial cells (EC). Two forms of CD146 have been found: the membrane-anchored form and the soluble form (sCD146). The plasma concentration of sCD146 is modulated in inflammatory diseases associated with endothelial alterations. We have investigated the role of endothelial CD146 in the cigarette smoke-induced emphysema *in vivo* and in pulmonary endothelial cells (EC) *in vitro*. Sprague Dawley rats exposed to cigarette smoke for 2 months developed significant emphysematous changes (as measured by mean linear intercept) and had increased levels of circulating and bronchoalveolar lavage fluid sCD146. CD146 is highly expressed in rat pulmonary microvascular endothelial cells (RPMVEC) and to a much lower extend in pulmonary macrovascular endothelial cells. Treatment with cigarette smoke extract (CSE) *in vitro* resulted in a decreased membrane-bound CD146 expression as well as reduced gene expression and increased sCD146 levels in the medium. Moreover, CSE-induced downregulation of CD146 expression increased vascular permeability of RPMVEC measured by EVANs Blue and macrophage migration assay. Similar results were found upon silencing of CD146 suggesting a role of CD146 in controlling tissue inflammation. Circulating levels of sCD146 were significantly increased in plasma of patients with COPD and correlated with the disease severity. In summary, CD146 plays an important role in pulmonary vascular EC function and its circulating form might reflect vascular endothelial injury.

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Hydrogen Peroxide Produces A Selective Increase In Renal Epithelial Cell Paracellular Permeability To Large Solutes Via src Family Kinase Activation.

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Renal ischemia/reperfusion (I/R) injury is a significant cause of acute kidney injury and contributes substantially to patient morbidity and mortality, e.g., in renal transplant. Tubular backleak, due to breakdown of the renal epithelial cell paracellular permeability barrier, is a major component of renal I/R injury. Renal I/R injury is associated with increased tissue levels of hydrogen peroxide (H_2O_2). We have begun an analysis of the mechanism by which H_2O_2 produces increased renal epithelial cell paracellular permeability and the role of src Family Kinases (SFKs) in mediating the H_2O_2-induced increase. H_2O_2 produced a concentration-dependent increase in paracellular permeability of renal epithelial cell lines derived from both proximal (LLC-PK1) and distal (mIMCD3, and MDCK) nephron segments. “Leak pathway” permeability (low capacity, large solute size; measured using calcein and fluorescein-dextran4000) was selectively increased at H_2O_2 concentrations that did not produce cell death (Trypan Blue-positive nuclei). Data suggest an enhanced response with increasing solute size. In contrast, “pore pathway” permeability (high capacity, small solute size; measured as TransEpithelial Resistance (TER)) was only increased at H_2O_2 concentrations that also increased cell death. H_2O_2 produced a time-dependent, transient activation of SFKs. The H_2O_2-induced increase in leak pathway permeability was attenuated by SFK inhibitors, PP1 and PP2, but not by the inactive analog, PP3. H_2O_2 treatment did not produce changes in the cellular

contents of the tight junction (TJ) proteins occludin, claudin-1, ZO-1, or ZO-2. Under our extraction conditions (1% Triton X-100), the majority of all four TJ proteins was in the Triton X-100-soluble fraction. Moreover, our data indicate that the distribution of occludin and claudin-1 between Triton X-100-soluble and -insoluble fractions was unaffected by hydrogen peroxide treatment. However, the subcellular localization of occludin, but not claudin-1, to the TJ region was disrupted by H_2O_2 treatment. These data demonstrate that H_2O_2 selectively increases paracellular permeability via the leak pathway and that this effect is mediated, at least in part, by SFKs, possibly through modulation of occludin association with the tight junction structure.

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Mitochondrial Translocation in Lymphocytes of Human Autologous Macrophage-Lymphocyte Rosettes.

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The phenomenon of multiple immunological synapses on macrophage-lymphocyte rosette (MLR) (Cabral and Novak, 1992, 99; Novak and Cabral 2008, 09) refers to cellular association between human blood monocyte-derived macrophages and lymphocytes from autologous cultures total leukocytes extracted from the blood, which bind selectively forming rosettes with central macrophage and lymphocytes adhered (MLRs). The contact area between a T cell and antigen-presenting cell is known as "immunological synapse" (Grakoui et al, 1999) and the multiple interactions that occur leading to a "signal" for T cell activation. The cells interact in the MLR phenomenon may present special features in their areas of contact or cell-cell interaction, and in the surrounding cytoplasm. Has focused attention on the organization of proteins in the contact area of the IS. Objectives: ultrastructural study to observe the location of mitochondria in the cells of the MLRs, taking into account the energy requirements for activation. Materials and methods: We used healthy human blood samples, anticoagulated with heparin (n = 10) (donated by the Blood Bank, UNC, anonymity, data serology). Autologous cultures in TC199 medium (SIGMA, St. Louis, MO). Samples: 48 and 144 h. MLR technique (Cabral y Novak, 1992, 99). Cell sediment samples of MLRs underwent electron microscopy. MET: Zeiss LEO-906E. Results: We observed redistribution of mitochondria in lymphocytes to the immune synapse area in MLRs. Conclusion: This distribution coincides with mitochondrial energy needs and opening channels of entry of Ca (2 +) for cellular activation.

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Regulation of Intercalated Disc Formation during Postnatal Cardiac Development by the E3 Ubiquitin Ligase Wwp1.

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The developing postnatal heart maintains electrical and mechanical function while undergoing substantial changes in cardiomyocyte *architecture*. The electro-mechanical coupling between adjacent cardiomyocytes is tightly controlled by intercellular junctions localized at the intercalated discs (ICD) to ensure healthy and synchronized mechanical coupling (mediated by the fascia adherens and by desmosomes) and electrical conduction (via gap junctions). The spatiotemporal expression patterns of intercellular junction proteins are closely coordinated during postnatal cardiac development. During this period, which lasts about 8 weeks in mice, 90 days in rats, and 6 years in humans, there is a progressive and sequential accumulation of the intercellular junction proteins at the ICD. Several intercellular junction proteins have been shown

to be regulated via ubiquitination. Our recent data show that the E3 ubiquitin ligase Wwp1 is highly expressed in cardiomyocytes throughout this developmental window, and that Wwp1 can ubiquitylate the gap junction protein connexin 43 (Cx43). The objective of this study was to examine how modulating Wwp1 expression affects the structure and function of ICD during postnatal cardiac development. To achieve this objective, we created mice which either ubiquitously overexpress Wwp1 or completely lack Wwp1 activity. We found that all of the Wwp1 overexpressers died very suddenly around 2 months of age, while *Wwp1* knockouts were viable and fertile. Cardiac tissue derived from Wwp1 overexpressers was assessed by confocal microscopy and western blotting and showed mislocalization as well as diminished abundance of Cx43 at 4, 6 and 8 weeks of age. Consistent with loss of myocyte electrocoupling due to altered Cx43 expression, electrocardiogram abnormalities were noted in the Wwp1 overexpressers. The effect of Wwp1 on ICD formation seemed to be specific to Cx43 as the expression and localization of other ICD proteins such as N-Cadherin and Desmoplakin were not affected at any of the time points examined. In contrast to the transgenic mice, the *Wwp1* knockout animals showed an increase in the abundance of Cx43 protein in cardiac tissue at 2, 4, 6 and 8 weeks of age. Thus, this study highlights a new role of Wwp1 in regulating Cx43 protein levels during cardiac postnatal development.

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Preparation of claudin-4 binder using C-terminal fragment of *Clostridium perfringens* enterotoxin and its application to mucosal vaccination.

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Objective: Mucosal vaccines activate both mucosal and systemic immune responses, and are less painful, as they are needle free. Thus, mucosal vaccination appears to be an ideal vaccination strategy. The effective delivery of antigens to mucosa-associated lymphoid tissues (MALT) is a critical issue for the development of potent mucosal vaccines. Claudin-4, a tetra-transmembrane protein, is expressed in epithelial cells covering MALT (Tamagawa et al., Lab Invest, 2003). We previously showed that claudin-4-targeting is a promising strategy for mucosal vaccination using a claudin-4 ligand, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) (Kakutani et al., Biomaterials, 2010). In the present study, we determined the functional domain of C-CPE, and we subsequently attempted to prepare a novel claudin binder with higher affinity for claudin. We also applied the novel claudin binder to mucosal vaccination.

Methods: We genetically prepared alanine-substituted C-CPE mutants, and we determined their affinity for claudin-4 by BIAcore analysis. We also prepared a fusion protein of ovalbumin (OVA) as a model antigen with C-CPE mutants. Interaction of C-CPE mutant-fused OVA with claudin-4 was examined by ELISA using an immunoplate coated with claudin-displaying baculovirus. The titers of OVA-specific antibody in serum and mucosal washes were determined by ELISA. Production of interferon-γ and interleukin-13 in the splenocytes isolated from the immunized mice were measured using a commercially available ELISA kit. In an anti-tumor assay, female C57BL/6 mice (6-8 weeks) were nasally immunized with C-CPE mutant-fused OVA once a week for 3 weeks. Seven days after the last immunization, mice were subcutaneously inoculated with 1×10^6 EG7-OVA cells, and tumor growth was monitored.

Results: Substitution of some amino acids with alanine increased affinity of C-CPE for claudin-4, with KD values increasing from 455 pM to 46 pM. When mice were immunized with the C-CPE mutant-fused OVA, OVA-specific serum IgG and nasal IgA increased 10-fold when compared to mice immunized with C-CPE-fused OVA. Immunization with C-CPE mutant-fused OVA activated stronger Th1- and Th2-type responses than in mice immunized with C-CPE-

fused OVA. C-CPE mutant-fused OVA exhibited greater anti-tumor activity than C-CPE-fused OVA.
Conclusion: These results indicate that the alanine-substituted C-CPE mutant is a promising lead for claudin-targeted mucosal vaccines.

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The Functional and Mechanical Consequences of Age in *Drosophila* Myocardium.
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Drosophila melanogaster is a genetically malleable organism with a short life span, making it a tractable system in which to study mechanical effects of aging and genetic modification on tissue, such as impaired heart function. We performed an micromechanical analysis on the heart tubes of juvenile and geriatric flies to investigate how heart stiffness may correlate with cardiac myopathies and explain the origin of dysfunction based on genetic screening with RNA-interference. Female *yellow-white* (*yw*) flies experience decreased diastolic diameter between 1 and 5 weeks of age (>20%) which we attributed to an increase in passive tension. Mechanical analysis revealed that cardiomyocytes stiffen more than two-fold between 1 and 5 weeks of age (1.0± 0.12 vs. 3.8 ± 0.30 kiloPascal, kPa, a unit of stiffness), but only at cell-cell junctions. Conversely, *white 1118* (*w1118*) flies showed no change in stiffness with position along the transverse axis of the tube (p = 0.7165 and 0.1035 between center and edge of tube in 1 and 5 week hearts, respectively) or age (p = 0.1923 and 0.8829 between 1 and 5 weeks at center and edge of heart tube, respectively) despite a drastic change in function; aged *w1118* were significantly more arrhythmic but had less diastolic dysfunction (<10% decrease in diastolic diameter between 1 and 5 weeks) than *yw* flies. Heterozygous deletion of the membrane-localized Integrin-linked kinase (ILK) dramatically reduces arrhythmia in *w1118* flies (>35% reduction in arrhythmia index, a measure of the standard deviation of heart period) though no stiffness change was observed. This may indicate that arrhythmogenesis more directly influences *active* tension produced during cross-bridge cycling rather than myofibrillar structure. To that end, *yw* flies were treated with RNA-interference against active tension producing myosin heavy chain (MHC), which resulted in severely impaired contraction and reduced stiffness after 1 week (1.0± 0.12 vs. 1.8 ± 0.13 kPa). These first *in situ* mechanical analyses of living myocardium have resolved differences in passive cardiac mechanics due to age and genetic modification and suggest that the aging phenotype and its mechanism differs significantly between *Drosophila* strains.

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Effects of hematite nanoparticles on human intestinal cells.
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The human body is constantly exposed to various nanoparticles used in commercial consumer products. One such nanoparticle is hematite which is used in commercial products such as pigments, catalysts, medical devices, sensors, and recording media. This may accumulate in the environment which can have a potential impact on the human intestinal system. In our study, we hypothesize that hematite nanoparticles at the sizes and concentrations tested will negatively impact Caco-2 cells the human intestinal epithelial cells, with increasing time. We have used various techniques such as trans-epithelial electrical resistance (TEER) measurements, immunocytochemistry and microscopic analysis (confocal and electron

microscopy) to support our hypothesis. Three sizes of nanohematite were used for the study namely, diameters of 17nm, 53nm, 100nm. Each of these nanoparticles was used at three concentrations namely, 1ppm, 10ppm, and 100ppm and the study was conducted at increasing time intervals. The results from our study show that the smallest and the largest nanohematite sizes affect the Caco-2 cells more than the intermediate sizes with increasing time. Also, our results show that nanohematite particles affect the cells at both physiological and non-physiological concentrations. Our results thus support our hypothesis that nanohematite at certain sizes and concentrations is detrimental to the human intestinal Caco-2 cells with increasing time.

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Evaluation of Atrazine Permeation in Caco-2 Cells.

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Atrazine is a widely used and commercially available herbicide in the United States. Some studies have reported that human exposure to atrazine does not pose a health risk; while other animal model studies suggest that exposure can have profound effects, such as feminizing male frogs. Studies have been done on the impact of atrazine on intestinal epithelial cells focusing on changes in proliferative ability (*Environ Res,* 75: 85, 1997). If atrazine from farms and other agricultural locations is washing into our lakes, streams and aquifers, what impact could this have on drinking water and thus on freshwater aquatic species and on human health? Some studies have indicated that in heavily farmed regions of the United States significant levels of atrazine are present in drinking water. But is this drinking water laced with atrazine posing a health risk to those who drink it? Can the herbicide, atrazine, cross the epithelial monolayer of the human gastrointestinal tract? Our hypothesis was that atrazine will permeate across an epithelial monolayer of cells. We used the human intestinal cell line, Caco-2 cells, to determine the transepithelial transport of atrazine from the apical or luminal side to the basolateral or serosal side. Caco-2 cells are an established model system for measuring the permeability of chemicals that can be ingested and adversely impact human health.

Science Education

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NSF IGERT at UIUC: Training the Next Generation of Leaders in Cellular and Molecular Mechanics and BioNanotechnology (CMMB IGERT).

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The goal of the CMMB IGERT is to produce the next generation of leaders who will define the new frontiers of Cellular and Molecular Mechanics and BioNanotechnology. The living cell is the most fundamental building block of all biological systems. Recent discoveries show that mechanical signals play a critical role in cell functionality. How cells transduce mechanical signals to functionalities at different length scales presents a scientific grand challenge of our time. We have responded to this timely need and assembled a preeminent interdisciplinary

team of researchers across UIUC and partner institutions to train the next generation of engineers and biologists to address this grand challenge.

We are improving on traditional models of graduate education by (a) creating an integrated educational and research environment and support mechanisms to provide interdisciplinary experiences in biology, engineering, and bionanotechnology; (b) infusing critical thinking, leadership, communication, team building, ethics training, and other essential topics into the program; (c) recruiting and retaining a diverse set of trainees; (d) enabling the Student Leadership Council to shape strategic directions of the IGERT; (e) supporting international experiences; (f) assessing the value-add of the IGERT experience; and (g) broadening impact by permeating the IGERT's educational system to other programs at UIUC, UC Merced, NCCU, and other institutions. Our goal is to not only transform graduate education on our campus, but also to be an empirically validated model for other programs in this area.

We are bridging biology and engineering via three intellectual themes: (a) cellular-scale bio-mechanical processes driven by molecular motors in cells; (b) critical controlling mechanical phenomena at the micro- and nano-scale that underpin cellular-scale processes; and (c) mechanical and biochemical communication between cells and the extra-cellular matrix. The mechanochemical transduction processes behind these interconnected themes are poorly understood. Recent advances in micro/nanotechnology and molecular scale computational methodologies will catalyze this quantitative biological revolution through the unique capabilities of patterning, probing, imaging, and interpreting biological phenomena at a cellular and molecular scale. A real integration of these domains can make a revolutionary impact in many areas such as tissue/regenerative engineering, mechanobiology, biological energy harvesting, sensing/actuation, cells-as-a-machine, synthetic biology, etc. Cross-disciplinary training of a new generation of scientists is crucial to realizing the potential in these arenas.

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Teaching critical analysis of scientific papers and scientific writing in a Molecular Biology for Master's students class.

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Ability to analyze primary scientific literature and ability to communicate scientific ideas clearly are important skills for biology students. This report describes methods and preliminary results of practicing these skills in a Graduate Molecular Biology course taught to a small class of students enrolled in the contiguous BS/MS program in Biology at UCSD. During the course, figure-by-figure analyses of five primary research papers were conducted in small groups. In preparation to each discussion, students wrote a one-page report that contained their own analysis of the paper. At the end of the course, 70% of the students characterized their gains in scientific paper analysis as "high" or "very high". The average learning gain, calculated from average scores for Paper 1 and Paper 4 was 0.45. In another module of the same course, students practiced scientific writing by completing an Annotated Bibliography assignment and writing an Introduction to their own thesis. Close to 90% of the students evaluated the Introduction assignment as "Very useful" or "Useful" for such aspects as critical analysis of papers and practicing scientific writing.

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Using Information and Science Literacy Instruction to Improve Student Performance on Laboratory Research Writing Assignments.

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We tested the hypothesis that increasing students' understanding of how to ask scientific research questions, to use library resources for science, and to find and evaluate scholarly information on science topics would enhance students' understanding of the habits of mind of scientists and how they communicate scientific results. In this upper division microbiology course team taught by a science librarian and a biology faculty member, students were taught information literacy skills such as finding and evaluating primary literature during a beginning of the course library session. Students were asked to construct an annotated bibliography in which they found, summarized, and evaluated ten scientific sources related to an assigned microbiological topic. In a seemingly unrelated assignment, the students performed laboratory research in which they isolated and identified a microbiological unknown and reported their results in a manuscript format. In this study that included courses taught in 2008, 2009, and 2010, we tested our original hypothesis by evaluating whether instruction in information and science literacy enhanced students' abilities to write laboratory manuscripts in the style and sophistication of research scientists. We found increases in student confidence levels in the information literacy skills over the course of the semester. We also found that as we have improved our information literacy instructional methods over the three successive years of the course, students' scientific manuscript writing has improved and moved from a generic "lab report" style to a publishable research manuscript style. In summary, we have found that teaching information and scientific literacy skills and engaging students with the primary literature can help students become better scientific communicators. This technique is likely applicable across a broad range of scientific disciplines.

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Using Graphic Novels to Engage the Modern Student.

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Bio100 at San Diego State University is a large introductory biology class for non-science majors, consisting of lectures given by NIH IRACDA postdoctoral fellows and supplemented by online reading materials. We are investigating the use of an alternative medium, an online graphic novel, to supplement the biological principles presented in the course. We hypothesize that an accessible storyline will allow students to better understand biological principles through connections to real-life situations. The graphic novel focuses on an independent story of a Mt. Everest climbing expedition. Hyperlinks embedded within the graphic novel connect students to online modules describing the biological bases of hypothermia, dehydration, muscle contraction, bacterial infection, and other relevant real-life processes. The graphic novel and the corresponding modules highlight important concepts students need to master to perform well on summative assessments. A pilot experiment showed students were engaged by this unique approach to learning biology, and we are currently analyzing student performance on exams to assess the effectiveness of this approach both as a supplemental teaching method and as an alternative method for presenting course material.

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Communicating cell-biology research to the non-specialist: The “POPULAR” style poster.

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Miami University’s NSF-funded Undergraduate Research and Mentoring (URM) program is aimed at retaining underrepresented student groups in the Biological Sciences and to prepare them for graduate study. An activity that the students engage in during the Spring term is to develop a “popular-style” poster. The OBJECTIVE of this exercise is to enable undergraduates and their graduate student mentors to communicate cell biology research to a non-specialist audience, which we defined as high school students, their teachers and first year University majors. APPROACH: Students attended 3-4 workshops over a 4-week period to learn about effective communication of research projects and experimental outcomes to non-specialists, develop and present poster drafts, and peer-review of poster drafts. All students were asked to consider and articulate the following: (1) The biological process and why it is interesting. (2) The model organism used in the research and its relevance. (3) An overview of the research, including the questions addressed. (4) The techniques used, with a line or two describing the usefulness of the techniques. (5) Some visual depiction of results, e.g., pictures/graphs. (6) A short statement about the source of funding and why the agency is interested in the research. (7) A sampling of journals that the lab’s work has been published in, indicating undergraduate authors. (8) Photos of undergraduates at work in the lab. RESULTS: Seventeen undergraduate students and five graduate students developed posters in the past two years. They have been presented at multiple venues that include Miami University’s Annual Undergraduate Research Forum (held in April each year), Recruitment events held each Fall by the Office of Admissions, and in Teacher Professional Development venues. CONCLUSION: The process of creating popular posters enabled undergraduates as well as graduate students to better understand their projects, to become aware of the need to communicate science/research to multiple audiences, and to become confident in their ability to do so as a community of researchers.

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Interdisciplinary Communication Laboratory for Undergraduate Biology (iCLUB).

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There is a growing awareness that major advances in research can be made through collaborations across traditional disciplines. Students and faculty involved in interdisciplinary research activities often confront obstacles to healthy collaboration. These obstacles may include: (1) Differences in personal and research values, (2) Differences in research practices and (3) Overall discomfort with the close interpersonal communication that is critical to any collaboration.

In the summer of 2010, two assistant professors (one from biology and one from theater) at Old Dominion University offered an eight-hour foundational communications workshop entitled "Let's Talk Research: Learning to Communicate in Collaboration.” The workshop provided hands-on training specifically focused on helping researchers better collaborate at the intersection of Math and Biology. Faculty and student participants were introduced to tools that actors use to foster healthy communication. The workshops were action-oriented. Participants immediately practiced the communication and collaboration skills that they learned by exploring existing problems in their research collaborations. Survey evaluations revealed that students and faculty

gained skill in (1) building rapport and (2) listening well. Participants most valued the development of (3) an increased comfort with communicating within conflict and (4) skills in identifying and communicating goals and values with potential collaborators. The original workshop groups now form the founding members of iCLUB, a recently NSF-funded RCN UBE-Incubator (DBI-1061935) for the development of communication skills in research scientists, graduate students, and undergraduates working at disciplinary intersections. We will present the structure of the workshops, the relationship of theater to communication and collaborative practice and overall structure of the iCLUB network.

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Student Attitudes Toward Using Facebook as a Tool for the Dissemination of Laboratory Results in a Cell Biology Course.

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Facebook was used as a medium for disseminating laboratory results in a sophomore-level undergraduate cell biology course. Each week, at the conclusion of the laboratory period, digital photographs of laboratory results were taken and displayed as an album in Facebook. Students in the course were given access to all of the laboratory results, so that they could compare their results with other groups' results and use this information to write their laboratory reports. Students were expected to include a photograph of their data in their report. At the conclusion of the course, 40 of 52 total students enrolled (76.9%) participated in a voluntary survey which assessed their attitudes toward using Facebook for this purpose. Of the students surveyed, 100% selected “agree” or “strongly agree” in response to the following statements: “Having lab data on Facebook was convenient”, “Being able to look at data from the entire class was useful to me in writing my lab report”, and “I recommend that Facebook continue to be used for data dissemination in future cell biology courses, based on my experience this semester”. These data suggest that students perceive Facebook to be a useful tool for the dissemination of student laboratory results.

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A structured approach to reading the primary literature that increases student critical thinking.

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Reading primary literature increases students' confidence, scientific literacy, and scientific process skills. Different approaches exist for analyzing primary literature: peer teaching, reporting on and writing journal-style articles, and linking articles to research projects. While previous studies highlight the importance of reading the primary literature in engaging students' interest in science and increasing their confidence, there have been few studies of how specific ways of reading the primary literature affect students' critical thinking (CT) skills. In this study, we compared the CT gains of students who follow a structured method for reading the primary literature to students who read and discuss the same articles after answering instructor-generated questions. We hypothesize that using the structured method will increase student CT skills beyond what is achieved through traditional discussion of research articles. The structured method we used compels students to analyze how and why a set of results is presented in a publication. We defined CT as student performance at the analysis, synthesis, and evaluation levels of Bloom's taxonomy. To determine gains in CT skills, we compared student performance at the start and the end of the semester in two different assessments: 1) exam questions at the analysis, synthesis and evaluation levels, and 2) an individual, in-class article critique exercise, which we scored by determining student performance at the analysis, synthesis and evaluation

levels. Mixed 3-way ANOVA of exam scores shows that CT skills of students who engaged in the structured method for reading primary literature increased significantly when compared to students who used the non-structured method (interaction of teaching method and pretest/posttest, $p = 0.034$). Mixed 3-way ANOVA of student performance in the article critique exercise showed that, while both groups had gains in CT skills, students who followed a structured approach for analyzing the literature had significant gains over those of students in the other group (interaction of teaching method and pretest/posttest, $p = 0.014$). Our results suggest that a structured method for reading the primary literature that compels students to dissect the "what, why and how" of scientific research leads to greater CT gains than merely having students answer discussion-generating questions prior to talking about a research article.

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Journal Club: A four-year class to improve scientific literacy and outreach among undergraduate biology majors.

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Journal Club: A four-year class to improve scientific literacy and outreach among undergraduate biology majors

Leading institutions on education reform have called for the development of pedagogical practices that significantly improve scientific literacy of undergraduate biology majors. The QBIC (Quantifying Biology In the Classroom) program is an NSF and NIH funded program in the Department of Biological Sciences at Florida International University (FIU) developed specifically to address this call and other areas of biology instruction. As part of a larger pedagogical strategy called 'The Teaching Pentagon', we developed a seminar-styled readings class called 'Journal Club' which is a required class for all QBIC students during their tenure at FIU. This class has both horizontal and vertical structures. Horizontally, it contextualizes concepts introduced in lecture and laboratory classes by discussing scientific literature on the same topic. Vertically, each successive matriculation year represents a different stage in how students are taught to digest and communicate scientific literature. Students are introduced to literature in their freshman year using primarily review papers, but supplemented also by popular science writing. As sophomores students are taught to digest complex analytical articles and then as juniors, critique these papers and design new experiments based on the questions exposed by the conclusions. The seniors in our program learn to then communicate seemingly complicated scientific topics to non-scientific audiences.

We have found that Journal Club acts as a solid reinforcement to the major lecture with some students reporting that this class is their primary mechanism for truly understanding the worldly application of biological concepts. Exit surveys of our first graduating class also indicate that this class is a major platform for student training in science understanding and communication. This is a relatively new course and its goals are tied to the overarching instructional scheme as well as the more general goals of the program. Therefore, we have only just begun to apply more stringent assessment tools for this course and others. Student evaluations over the semesters have shown steady increases in both reported understanding of scientific literature, as well as the intellectual advantage provided by this class for their performances in lecture.

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Information Literacy in a General Biology Laboratory: Developing Life-Long Skills.

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In today's complex, information-rich society, it is vital to assist incoming biology freshmen to develop the necessary set of abilities and skills required to become effective users and producers of information. These skills are essential in their academic development and later in their professional life. Information literacy is most effective when it is embedded into the discipline-specific learning outcomes that tie into course goals and activities. Through a Howard Hughes Medical Institute initiative, the Biology Department at the University of Puerto Rico at Mayaguez in collaboration with library faculty designed an information literacy module to guide students in the use of library resources and teach them how to effectively search and evaluate appropriate information while emphasizing the importance of scientific primary literature in their field. The module was implemented in a laboratory exercise of the first General Biology course. It integrated an online tutorial and a complementary exercise for a lab research assignment. Students searched for references for their lab project and after viewing the online module evaluated their references. The reference discussion form was graded and evaluated by the lab teaching assistant. The module was initially pilot tested in a single laboratory section and was thoroughly assessed and modified prior to full implementation to the entire course population. Through this exercise, students improved their information literacy competency as evidenced by their reference evaluation exercise and other assessment results. They were able to better distinguish primary from secondary information as well as to better search the web and evaluate internet resources. Students evaluated the module as very effective, precise and easy to follow. Likewise, teaching assistants reaffirmed that this information was necessary and valuable. The module has now been modified to clarify misconceptions regarding reliable internet scientific resources and to include a citation instruction segment as requested by the students. Furthermore, students now have the opportunity to reinforce these skills throughout exercises in other courses that have also included information literacy activities. This repeated and progressive exposure to reliable scientific literacy will strengthen our students' skills and instruct them in up-to-date methods to obtaining scientific information and, thus, contribute to their becoming proficient scientists and lifelong learners.

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Using a Model Systems Workshop to Teach Cell Biology.

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Numerous schools have integrated active learning approaches into their biology classes to replace or enhance the traditional setting where the professor stands in front of the room and lectures. These new approaches are more student than faculty centered and have been shown to be more effective than passive learning. One such example of this type of approach is in having students participate in laboratory research experiences outside the classroom. In many cases, this can occur early on in the student career. These experiences enhance the development of critical thinking skills and communication skills. In addition, courses in laboratory research may stimulate students to become interested in science. These courses try to link the knowledge that students gain in traditional classes with the practice of performing laboratory research. At many schools, it is difficult to accommodate many students doing research due to a limited budget and resources of the school as well as faculty time. To overcome this obstacle, schools have been trying to incorporate laboratory research or the methods that researchers

use into the curriculum. In the present study, we have organized and held a workshop on model systems in cell biology. Our goals were to introduce students, even those in the beginning levels of their careers, to model systems. We hoped that this experience would build critical thinking skills in the students and we hoped to interest students in pursuing a career in cell biology research. To improve their communication skills, we taught the student how to present their work through posters and explained that this is how scientists present their work. The effectiveness of the workshop was assessed using pre- and post-tests. There were 17 participants in the 2-week workshop and although the average attendance was only four days, there was a 40% gain in knowledge.

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Developing laboratory activities using primary cultures that consolidate cell biology concepts and quantitative reasoning.

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The use of mathematics is central to cell biology, but talented undergraduates sometimes shut down when encountering quantitative tasks in the context of a biology laboratory. They insist that they do not have the tools they need despite having had the relevant skills since high school and practice as undergraduates, such as work with concentrations in general chemistry. Also central to cell biology is primary cell culture, providing students opportunities to see how cells can be separated from the tissues in which they resided, how they respond to isolation, and what they need to survive. In an advanced undergraduate cell biology course, students have been isolating cells from chicken embryos and goldfish scales in experiments that incorporate practice in mathematics into activities that are novel and exciting to them, are relatively inexpensive to perform at a basic level, and can be expanded to incorporate available resources. In the chicken embryo laboratory, they are given powdered forms of chemicals that may influence the growth of dorsal root ganglion neurons they have dissected from embryos, choose concentrations to test and how to make them, and assess the treatments' effects. In the goldfish laboratory, they monitor scale keratocyte movement using ImageJ, compare methods of defining cell position, and compare rates of movement under different conditions. Protocols and opportunities for collaboration in further developing these activities will be shared.

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Achieving true teamwork in student course teams.

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The optimal value of team-based learning or other pedagogies that involve work by student teams can only be realized if teams function well. To avoid student frustration and the inefficiencies associated with laggards or overbearing or irresponsible team members, we have employed several strategies to set up effective teams and hold them accountable throughout the term. A key element of our approach is to create permanent (semester-long) teams with high diversity, including diversity of personal strengths. Each week team members list their specific contributions to all team activities of the week, from preparation for team quizzes and in-class activities to thoughtful contributions to elements of the semester-long major team project, and all team members must agree that the claims are accurate. Twice a semester each student evaluates the strengths and weaknesses of all team members and grades their contributions relative to each other; these evaluations are returned to students. The first time the exercise serves as a non-graded formative assessment, while at the end of the semester, these peer reviews by teammates account for about 6% of a student's final course grade. The vast majority

of students report that their team worked effectively with this framework for accountability and that they appreciated the varying talents that team members brought to their work as a team.

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Teaching Introductory Biology from a Historical Perspective.

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Introductory biology courses usually consist of a survey of biological concepts from a compartmentalized point of view. Very often this approach focuses on levels of organization: molecules, cells, organisms, etc. We find this approach limited in at least two significant ways: 1) Often evolution and ecology are taught as distinct sections near the end of textbooks, which implies that they are only significant for the multicellular organisms covered near the end of the course. 2) This compartmentalized approach makes it difficult for students to develop an integrated understanding of biology. Here we describe a novel method of delivering a year-long introductory biology sequence. Our approach surveys the origin of biological processes, cells, and multicellular organisms in the historical sequence in which they actually occurred. Thus, the course begins with the chemistry of the early earth and moves to the origins of metabolism and the appearance of RNA as a catalyst and a hereditary molecule. This leads naturally into the first cells and the structural and metabolic characteristics of prokaryotes. The next step in the course is the endosymbiotic events that led to the eukaryotes, allowing us to explore for a second time cellular mechanisms such as replication, transcription, translation, and metabolism, now in eukaryotic cells. After discussion of sexual reproduction and phylogenetic analysis, we explore the diversity of single-celled and then multicellular eukaryotes, their physiological processes, and their interactions with the environment. Throughout each of these stages we emphasize how ecological niche and natural selection are the twin drivers of evolution on earth. We find that this method allows students to more effectively connect the vast amount of information presented in an introductory survey course. We present here our initial successes and challenges with this approach.

1003

Integrating Concepts in Biology for Introductory College Biology.

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We redesigned the first year college course (called *Integrating Concepts in Biology*) using the first principles of learning: students learn best when they construct their own knowledge and when new material is related to what they already know. Our redesigned approach emphasized data interpretation, the utility of mathematics in biology and deemphasized memorization. We divide biology into five big ideas (information, evolution, cells, emergent properties, and homeostasis) and address each of these big ideas at five levels of organization (molecules, cells, organisms, populations, and ecological systems). We compared our new course with two other traditional sections taught by two different instructors. Class time was traditional in format, similar to the other two sections. We hypothesized our students would: 1) score at least as well as the traditional students on content questions; 2) perform better on data interpretation questions; and 3) show significant changes in their perception of biology as a scientific discipline. Students participated in pre- and post-semester assessment of student perceptions. Our students performed significantly better on the data interpretation assessment than those in the traditional sections ($p = 0.04$) and demonstrated no significant difference in performance on the factual content assessment ($p = 0.91$). Our students exhibited significantly greater improvement in their ability to interpret experimental data over the course of the semester (p <

0.01). Several aspects of student perceptions were significantly different, indicating that students acquired a more realistic perception of biology as a discipline and may have developed a more accurate evaluation of their own scientific abilities ($p < 0.05$). *Integrating Concepts in Biology* improved critical thinking and disciplinary perceptions without compromising content knowledge.

1004

Exams Requiring Higher Order Thinking Skills Encourage Greater Conceptual Understanding in Introductory Biology.

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With the limited amount of time that instructors are afforded to spend with students during class time in an introductory biology course, instructors must often rely on individualized preparations done by students in preparation for exams. In order to encourage study processes that will enhance and solidify deep conceptual understanding, rather than rote memorization, the exams must be designed appropriately. In this study, students' biology conceptual understanding were compared for two exam formats: low-level regurgitation type exams demanding only recall of course materials verses high-level exams which test content knowledge, associated reasoning abilities, and the ability to transfer knowledge to novel situations. Subjects were taken from two introductory, non-majors biology courses taught in a guided-inquiry fashion by the same instructor. One course was given only low-level exams while the other course was given only high-level exams, based on Bloom's Taxonomy. Results show that students who prepared for high-level exams outperformed those who prepared for low-level exams on both knowledge/recall questions as well as advanced conceptual understanding and transfer questions. The effect was especially robust for students of lower reasoning ability and less prior content knowledge. Thus, it appears that exam design has a large impact on student learning despite identical in-class and homework experiences, with exams aimed at testing higher-order thinking skills encouraging greater gains in biological conceptual understanding.

1005

Bridging the Gap Between Concrete and Theoretical to Teach Gene Expression.

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Gene expression is a highly theoretical concept that is often difficult for students to understand because many lack the necessary reasoning skills to comprehend it. As a consequence, teaching gene expression is difficult and misconceptions abound. In order to test the hypothesis that concrete analogies can help students overcome reasoning barriers and achieve theoretical conceptual understanding, a novel method of teaching gene expression utilizing concrete intermediates was designed and assessed. Results showed that this active learning approach was more successful than traditional lecture in achieving conceptual gains. In addition, students with lower reasoning ability and less prior conceptions experienced higher learning gains. Theoretical implications about the effects of reasoning ability and prior conceptions on learning as well as instructional implications for teaching theoretical concepts are discussed.

1006

Journey into HeLa Cells – Integrating Courses to Provide an Inquiry-Based Learning Experience.

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In the traditional classroom, the emphasis is *memory* of the material rather than *understanding* of how that material fits within the world. Content is important, but critical thinking, problem solving, and application of concepts, transforms facts into “usable knowledge” for the student, which leads to increased interest in the sciences [*How People Learn, pg 9*]. By demonstrating the interplay between several scientific tools, we hoped to provide students with a more holistic view of science. During the spring 2011 semester, we implemented a project to ascertain the effectiveness of an embedded cell biology focused research module in coordinated upper-division Biotechnology Lab and Bioinformatics course. The objective was to give a practical foundation in basic molecular biological techniques and a relevant scenario for exploring the power of bioinformatics. Another objective was to have students read and discuss the bestseller, *The Immortal Life of Henrietta Lacks* by Rebecca Skloot.

Twenty senior Biology majors concurrently enrolled in both courses participated in this research experience revolving around HeLa cells. This HeLa cell project formed the beginnings of an interdisciplinary experience for students, which clearly illustrated that science includes the ethical, legal and socioeconomic aspects. We assessed the effectiveness of the integration of the courses on student engagement, enthusiasm and interest using the Classroom Undergraduate Research Experience (CURE) survey as well as our own end of course questionnaire along with course evaluations and course assessments**.** Students enjoyed the collaborative work and felt a sense of meaning and purpose to their research projects. They also indicated that the coordination provided deeper comprehension of course material.

Typically in undergraduate Biology major courses, students study cell division using pre-made slides of onion root tip with the learning objective to be able to identify the key stages in mitosis. This traditional method of learning mitosis does not provide students with skills related to process-level thinking beyond memorization of the steps. Toward that end, this experience was designed to allow students to master several laboratory techniques and provided a platform for exposure to bioinformatics tools in context. The longer term vision for this project is to generate the reagents which would allow students to follow their gene of choice throughout cell division in real-time.

1007

Measuring Outcomes of Summer Research Experiences for Future Science & Math Teachers.

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Summer programs have been put in place at a number of universities to provide research experiences for teachers so that they may gain an understanding of and comfort level with the process of research by engaging in in-depth, authentic research projects and subsequently engage their K-12 students in meaningful inquiry and problem-solving. We describe a summer research institute that we have designed for pre-service teachers in the Cal Teach secondary science and mathematics teacher credential program, and the instruments we developed for measuring outcomes of this program. A Knowledge Integration measurement tool specific for

the research process effectively measures conceptual understanding of research by analyzing responses from pre/post surveys and teacher interviews. We show that the opportunity to engage in research experiences provides participants with a deeper understanding of research methods, the nature of science and math, the process of experimentation, and science and math content. Moreover, such experiences also help future teachers develop their ability to guide their future K-12 students to develop skills in scientific thinking, designing and critiquing experiments, and solving problems.

1008

Fellowships in Research and Science Teaching (FIRST): A non-traditional post-doctoral training that generates effective researchers and educators.

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Fellowships in Research and Science Teaching (FIRST) is a three-year post-doctoral program that combines traditional post-doctoral research training with intensive instruction in pedagogy. FIRST is supported by an Institutional Research and Career Development Award (IRACDA) grant from the National Institute of General Medical Sciences (NIGMS), a division of NIH. The goals of the FIRST program are 1) to generate faculty who are successful, independent researchers, as well as effective educators, and 2) to strengthen research-oriented teaching at Minority Serving Institutions (MSIs). To achieve these goals, FIRST fellows work under the direction of research mentors from Emory University, a nationally recognized biomedical research institute in Atlanta, GA. Additionally, fellows receive training in innovative pedagogical methods such as technology-based teaching and active learning and apply these skills at one of three top national MSIs within the Atlanta University Center (AUC): Morehouse College, Clark-Atlanta University, or Spelman College. A recent study indicated that FIRST fellows have the same publication rates and obtain the same amount of external funding as traditionally trained post-doctoral fellows. Furthermore, FIRST fellows were more successful than their peers at obtaining academic positions, demonstrating that FIRST effectively provides the training necessary for fellows to become successful faculty. Recently, in an endeavor to promote careers in the sciences among underrepresented minorities, FIRST has established a new undergraduate research program for AUC students. Undergraduate program participants spend one semester at Emory working directly with a FIRST fellow on an independent research project. These students receive a stipend, experience working in a top-tier research environment, and have opportunities to share their findings at national scientific meetings. FIRST fellows serving as mentors in the program gain experience mentoring undergraduates and managing research projects. Overall, this undergraduate research program offers both career development training for FIRST fellows and provides MSI students with high-quality, hands-on research experience.

1009

Pathways over Time: An Adaptable Project in Functional Genomics for Large Introductory Laboratory Classes.

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The explosion of genomic sequence information, particularly for microbial organisms, presents opportunities to engage large numbers of undergraduate students in authentic research projects within regular laboratory classes. The importance of undergraduate research on student learning and retention in science is already well-documented. The Boston College Biology Department has redesigned its introductory laboratory classes in molecular cell biology and genetics into a single-semester 3-credit course that incorporates original research on the

functional evolution of enzymes involved in methionine biosynthesis. Approximately 150 students, distributed between 10 sections, enroll in the course each semester. The project uses the budding yeast *Saccharomyces cerevisiae* to analyze gene function, because it is inexpensive to maintain, is easily cultured by undergraduate students, and has been extensively studied by yeast geneticists. Another key element in the selection of *S. cerevisiae* was the availability of deletion strains and overexpression plasmids for all of the *S. cerevisiae* ORFs. During two pilot semesters, students identified orthologs of S. cerevisiae Met proteins from the fission yeast, *Schizosaccharomyces pombe*. The two species are separated by close to a billion years of evolution, yet students were able to demonstrate the functional conservation of several genes by complementation of S. cerevisiae deletion mutants. Methionine biosynthesis in yeast involves several multi-enzyme pathways, which are subject to a variety of regulatory controls. This project topic was selected because it incorporates departmental learning goals, including student understanding of evolution, structure/function relationships, information transfer, energy transformation and systems biology. Other course goals addressed in the project include the application of the scientific process, quantitative analysis, the use of databases and bioinformatics tools and the ethical conduct of research. Students become "experts" on one *MET* gene and collaborate in 3-membered teams on wet bench experiments. Students working in parallel in the ten different sections communicate their results on a website hosted at Drupal Gardens. Assessment data from two pilot semesters indicate significant gains in student ability to conduct scientific research, to communicate research results, to use biological databases and to work with peers on experiments. The basic design of the Pathways over Time project can be easily adapted to other organisms and enzyme systems. The three elements required for course design are a multi-gene system to study, a genetically-tractable organism for complementation analyses, and a test organism with a sequenced genome.

1010

Implementation of inquiry-based plant tissue culture laboratory modules in minority undergraduate teaching laboratory.

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Recent studies have led to the conclusion that hands-on inquiry based research activities allow undergraduate students to feel the excitement and self-investment that is related to discovery of new knowledge and thus enhances their interest in pursuing a research career in the future. Here, we present a model for large-scale implementation of guided-research on a topic of broad interest, plant tissue culture, to increase interest in research at a 99% minority institution. Plant tissue culture is based upon the theory of totipotency, that is, the genetically based ability of a cell or a nonembryonic organ to form all the cell types in the adult organism. Multi-week laboratory module was implemented in the introductory botany course impacting 140 minority students per year by providing hands-on experience in using tissue culture tools. In this module the students use African violets (Gloxinia sp., Fam. Gesneriaceae) to gain experience in plant tissue culture techniques. The objective was for the students to learn how to take part of the plant from in vivo to in vitro by the establishment of aseptic techniques as well as to multiply plants under in vitro conditions by utilizing different media components. Assessment of the gain of content knowledge and learning perceptions revealed that our novel approach allowed the students to learn while increasing their self-perception of scientific methodology. In three semesters, at the completion of the lab module, the students reported an approximately 2.5-fold overall increase in the post-module assessment for content knowledge gained compared to pre-module assessment as well as an enhanced understanding of scientific process, increased proficiency of plant tissue culture methods and increase in confidence as researchers. Prior to

full-implementation, the laboratory module was pilot tested in a lab section and compared to a control lab section, with both section being taught by the same teaching assistant.

1011

Implementing an inquiry-based research module in a microbiology laboratory for minority undergraduate students.

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Participating in undergraduate research has been noted as one of the key elements for a successful career in science in the future. The Biology Department at the University of Puerto in Mayaguez has a population of 1,600 minority undergraduate students most of who do not have a chance to participate in research during their undergraduate years. In an effort to provide hands-on experience, an inquiry-based research module divided in three sections was designed and implemented in the general microbiology laboratory course. The impacted students were exposed to fundamental and modern molecular microbiology techniques for the characterization of bacterial strains that were isolated from extreme environments in Puerto Rico as part of a research project currently under way. These modules complement phenotype-based taxonomy with basic molecular methods and reinforced a research oriented approach to the characterization of unknown isolates and microbial communities. The assessment performed to the pilot sections reflected that the module had an impact on the students compared to the control section taught by the same teaching assistant. Answers to specific questions about techniques and scientific knowledge contained in the module were significantly improved in the post test. In questions about perception and scientific procedures, the students also showed that the module helped them to reinsure their confidence in developing an experimental approach through scientific methodology. The module also helped the students develop techniques in observing and collecting data. Eighty three percent of them were confident to perform this task after the module and 68% of them said that the module helped to develop this skill. Finally, 90% of the students in the pilot section felt confident in performing bacterial characterization experiments using molecular techniques compared to 39% of confidence reflected in the control section. Similar assessment results were obtained when the module was fully implemented impacting 217 undergraduate students, 7 teaching assistants and 1 laboratory coordinator. Overall, students demonstrated that they learned basic principles of molecular characterization of bacterial isolates by molecular techniques while increasing their confidence in learning scientific methodology.

1012

Teaching English Scientific Writing at the Undergraduate Level in Taiwan.

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Taiwanese undergraduate students in the biological and biomedical sciences have limited opportunities to practice English scientific writing. This leads to a great disadvantage when these knowledgeable, thoughtful, and talented students try to communicate about science in writing. A course that focuses on the structure and practice of writing a meeting abstract and a scientific paper has been designed and taught. The course is organized around the principle that practicing, coupled with feedback from the instructor, is the best way to improve a student's writing skills. The class emphasizes that writing is a process, and that sufficient thought and time must be invested in order to obtain a quality result. The concept that plagiarism is unacceptable and strictly forbidden is also noted throughout the course. Weekly lectures provide students with instructions about the specific grammar rules used in manuscripts and about the structures and organization of paragraphs, with an emphasis on executing the next writing

assignment. During the semester, students engage in several writing exercises. These assignments include writing a title and abstract, the first paragraph of an introduction, a paragraph of methods, a paragraph of results, and a paragraph for a discussion. Assignments are submitted via e-mail to the instructor as Microsoft Word documents. Feedback is provided to the students using the “track changes” feature in Microsoft Word. Initial experience revealed that several of the writing assignments required passage through multiple rounds of the “practice and feedback” process before the writing displayed significant improvement. During the semester, it was also observed that using examples from the student’s own work to highlight how improvements in the writing assignments could be made was very useful, as many students struggled with the same or similar problems. Colleagues in Taiwan have provided me positive feedback on the course, and the rating for the initial class by the students was 86.5%, which is considered very positive.

1013

Using video assignments in a 200-level Cell Biology class to promote student engagement.

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Students in a 200-level Cell Biology class developed a series of video- assignments based on research being conducted in the biological science departments. The OBJECTIVE of this assignment was to promote student engagement with the course material using a non-traditional assignment. APPROACH: Each video assignment had two sections. One part focused on communicating an overview of each lab’s research topic and its relevance, emphasizing basic cell biology concepts and any relationship to health/disease. Students were also expected to include information about the model organism used by the lab. Interviews with the faculty member/graduate student and undergraduates were included in these videos, as were lab tours. The second section of the video assignment was designed to provide information about undergraduate research opportunities- how to find them, how to get started, what are the expectations and the benefits for students. Prior to making the videos, students had to complete “background” assignments to better understand the research topic. They then developed scripts which were edited, before the videos were made. Staff from the Office of New Student programs was consulted to provide a “real-world” perspective for students about the usefulness of these videos for incoming freshman. RESULTS: Surveys that assessed effectiveness of the project indicated that 80% of the class (n=62) identified the project as being beneficial. The perceived benefits included: awareness of ongoing research activities, better understanding/connection of course concepts, confidence in using scientific language, the importance of logical flow of information, and an opportunity to “teach” science. In the following semester, some students returned to serve as undergraduate assistants, and worked with the Office of New Student programs to develop videos for the incoming class of 2015. CONCLUSIONS: The video assignment in a cell biology class allowed students to develop an understanding of course material in the context of ongoing research programs at the institution.

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Undergraduate Associates as Peer Leaders in a 200-level Cell Biology Class.

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BACKGROUND: In a 200-level Cell Biology course offered during the Fall 2010 semester, all students participated in a semester-long group assignment, Model Organisms in Research, which provided opportunities for students to understand basic cell biology concepts in the

context of ongoing research programs at Miami University. Students were required to read scientific literature, synthesize material from multiple sources, interview Miami University researchers who use model organisms, and collaborate with group members to compile a final paper and video. This integrative approach to learning cellular biology was facilitated by the use of Undergraduate Associates (UAs), students who had taken and excelled in the course the previous semester. The OBJECTIVE of this approach was to provide a class of 75 students with one-on-one instruction on the assignment, and for the UAs to develop peer mentoring skills. APPROACH: Each of the eight UAs worked with 2 groups of 4-5 students. They met with the instructor every other week to discuss the instruction, report progress, and to develop grading rubrics. RESULTS: UAs worked with students to accomplish the group assignment's major outcomes, which included: (1) Gain familiarity with research literature to explore the role of model organisms in health and disease, (2) Understand common themes in cell biology revealed through comparative analysis across organisms, (3) Gain an understanding of model organism research at Miami, and (4) Communicate model organism research to K-12 students and non-science majors. CONCLUSIONS: This integrative approach yielded many beneficial results for the instructor, UAs, and students. For the Instructor, the UAs' input into designing the assignment and providing one-on-one instruction in a large class was valuable. For the Undergraduate Associates, the project offered the unique opportunity to get first-hand curricular experience in designing, leading, and executing a collaborative group project from beginning to end. Students in the class had an opportunity to work with UAs who had taken the class before and to apply their cell biology concepts to develop a video assignment.

1015

Evaluation of Different Nutrient Medias on Population Growth of *Tetrahymena pyriformis.*

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In the literature are reported various nutrient medias in which *Tetrahymena pyriformis* is grown; this study sought to determine which medium was most effective at supporting the most population growth while also resisting contamination. Multiple cultures were grown in four different media formulations with varying quantities of proteose peptone, yeast, glucose, iron chloride, and salt. Their populations were counted by hemocytometer over a period of 4 days. Three of the 4 medias were able to support a growing population with little or no contamination; the salt-containing media (PPYS, n=5) did not support culture growth by any measure. The media of proteose peptone, yeast, and iron chloride (PPY, n=5) appeared to support the most rapid growth, but there was no significant difference between populations when compared to populations grown in media that contained glucose (PPYG, n=5) instead of iron chloride (p=0.46) and populations grown in media (SSP, n=5) that contained both glucose and iron chloride (p=0.33). However, when the PPY media was compared to PPYS media, a highly significant difference was found (p=0.007). No media showed signs of contamination in the time frame studied. Therefore, it was concluded that the PPY, PPYG, and SSP medias each would be sufficient for supporting a growing population of *T. pyriformis* while also resisting bacterial contamination for acute or other short-term studies.

1016

Development of a monolayer cell-binding assay to quantify the time-dependent strengthening of cell-cell adhesion.

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Cell adhesion plays a critical role in early development (e.g., the sculpting of tissues and organs), in daily maintenance of the body (e.g., wound healing), as well as in a number of aberrant conditions (e.g., tumor metastasis). A thorough understanding of cellular adhesion is thus a theme of broad importance in biomedical research. Results obtained using the dual pipette assay in the Dufour laboratory at Institut Curie demonstrate clearly that adhesions strengthen with time[1], and work continues there to determine how. The goal of the work presented here is to design a less expensive assay, readily employable at Colby-Sawyer, that yields data comparable to those obtained with the dual pipette assay, allowing us to contribute to the resolution of that basic question. We have chosen to explore the utility of a monolayer cell binding assay. The dual pipette assay measures separation forces between one pair of cells at a time; the monolayer assay provides less quantitative precision, but in addition to its considerably lower cost, allows us to observe the binding behavior of a much greater number of cells. Unlabeled cells are centrifuged in 4–well plates (Nunc) at 5000 rpm for 15 min to form a confluent monolayer, fluorescent probe cells are brought into contact with the monolayer, also by centrifugation (1000 rpm for 1 min), and after a fixed period of interaction (variable), the adhesion between the probe cells and the monolayer is quantified (% cells remaining bound) following disruption by a gyratory shear (100-200 rpm). This poster details the establishment of the parameters to make the assay functional. This project is supported by the New Hampshire IDeA Network of Biological Research Excellence (NH-INBRE) NIH Grant Number 1P20RR030360-01 from the INBRE Program of the National Center for Research Resources.

[1]Chu, Y-S, et al. (2004) Force measurements in E-cadherin–mediated cell doublets reveal rapid adhesion strengthened by actin cytoskeleton remodeling through Rac and Cdc42, JCB vol. 167 no. 6 1183-1194

1017

The Genomics Education Partnership (GEP): Undergraduate Student Analysis of the Evolution of Dot Chromosomes in Drosophila.

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An effective method for teaching science is to engage students in doing science. The GEP, a group of faculty from over 80 primarily undergraduate institutions, is using comparative genomics to engage students in research within regular academic-year laboratory courses. Using a versatile curriculum thathas been adapted to many different class settings (as the core of a laboratory course, within a lab for a broader course, or as an independent research project), GEP undergraduatesundertake projects to improve draft genomic sequences and/or participate in the gene annotation of these improvedsequences. GEP undergraduates have improved more than 2.4 millionbases of draft genomic sequence from several species of *Drosophila*and have produced hundreds of gene models using evidence-basedmanual annotation. Analyses of the Müller F elements (the dot chromosomes) of four Drosophila species (*D. melanogaster, D. erecta, D. grimshawi* and *D. mojavensis*) show that these genomic regions, which exhibit heterochromatic properties, are similar to each other and distinct from euchromatic reference regions. All dot chromosomes exhibit a high repeat density; the ~80 genes present show a

larger size and lower codon bias compared to genes in euchromatic domains. While there are major changes in the relative order and orientation of the genes, ~90% remain on the Müller F element. A subset of dot genes can be found in a euchromatic domain on another Müller element in at least one species; these "wanderer" genes exhibit the properties of the local environment in which they reside. A recent anonymous survey of GEP faculty showed that they are generally very happy with the core program and found teaching in this way to be a source of satisfaction. Institutional support has been positive. We find this approach rewarding for both faculty andstudents, and invite additional faculty to join us (see our website http://gep.wustl.edu; next workshop June 2012). Supported by HHMI grant # 52005780 and NIH grant R01 GM068388 to SCRE.

1018

Cloning and characterization of a maize zmbrd1 gene.

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Brassinosteroids (BRs) are a group of plant steroid hormones that regulate growth and development. Plants defective in brassinosteroid biosynthesis and regulation exhibit dwarf phenotype and alteration of the cell structure and growth. To date, the role of brassinosteroids has not been investigated in maize. A maize mutant with severe dwarfing was identified and used for positional cloning of the causative mutation. A single base nonsense substitution in the Zea mais brassinosteroid-deficient (zmbrd1) gene was identified. This gene is a homolog of the rice OsDwarf and tomato Dwarf gene and encodes a BR6 oxidase enzyme that is responsible for the last step in the brassinosteroid biosynthesis. The maize genome contains only one homolog for BR6 oxidase. Maize plants homozygous for the mutation in zmbrd1 gene are sterile, exhibit severe dwarf phenotypes, no etiolation response, alterations in the structure of the leaf, and abnormalities of leaf cell morphology. The expression of zmbrd1 gene is negatively regulated by brassinosteroids such that low levels of brassinosteroids result in high gene expression. Supplementing of growth media with 10-6M brassinolide partially rescues the mutant phenotype and lowers down the expression of zmbrd1 in the homozygous mutant plants. Characterizing maize mutants defective in brassinosteroid biosynthesis will lead to better understanding of the brassinosteroid biosynthesis and biosignaling pathways might help in developing new semi-dwarf maize varieties.

1019

The Internet Fosters Collaborations among College and Pre-College Students and Research Scientists.

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Students and scientists from four institutions--Lincoln K-8 Choice School, high school students from Sidwell Friends School, college students and a scientist from St. Joseph's University, and scientists from the Mayo Clinic--have worked together to develop research experiments that can be performed using the internet. In this collaboration, we utilized Fish Cam to develop a detailed shoaling analysis of the common goldfish. Fish Cam is an on-line tool that enables students to observe and collect data in real time from a shoaling test tank in the Biodiversity Lab at SJU. Shoaling, an aggregation behavior, provides fish with numerous benefits, including enhanced foraging success, access to potential mates, and protection from predators. Goldfish (Carassius auratus) have been utilized in shoaling studies (see Shinozuka and Watanabe, 2004), and it is known that they shoal; however, a detailed examination of shoaling behavior and shoalmate

choice has not yet been performed. In the laboratory, shoaling behavior is easily demonstrated in three-chambered tanks in which a solitary fish in the center chamber displays shoaling behavior by swimming near the side chamber containing a group of fish, as opposed to swimming near an empty chamber. At SFS, students in Introductory Accelerated Biology conduct Independent Research Projects in which they develop and conduct original scientific experiments, many involving zebrafish. Zebrafish are extremely helpful in classrooms because they provide an approachable method of learning science and cultivate a growing interest in young scientists. Previous experiments have included examining the effects of PBDEs on the development of zebrafish embryos, fin regeneration at different developmental stages, the inheritance and expression on the transgenic Glo gene, and the effects of light on sex differentiation in relation to pigment levels. We plan to expand this work to study shoaling behaviors in GloFish™ (transgenic zebrafish that express the Glo gene) of different colors. We have performed simple Mendelian crosses to learn the fundamentals of genetics and inheritance. Further studies will include genotypic analysis from these fish to determine which copy of the Glo gene that the fish have and how many copies of the gene are present. The identity of the different fluorescent genes present in GloFish and variations in the gene copy number can be detected by PCR and sequencing. Future experiments will be proposed by those students actively involved in this collaboration, allowing students from all levels of education to work together to conduct scientific research. Scientists could also utilize such on-line tools to perform behavior experiments, including genetic analyses, using the manpower provided by engaging other students in this process.

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AP Biology Students Make Significant Gains vs. Controls in the SUN (Students Understanding eNergy) Pilot Study.

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The SUN (Students Understanding eNergy) Project is a new method for learning about biological energy transfer that depends upon: 1) the rationale for the directionality of spontaneous electron movement, 2) a one-to-one comparison of the hydrogen fuel cell and cellular respiration, and 3) physical models within nested trays that allow learners to configure and manipulate key components of the chloroplast and mitochondrion. Last year at this meeting we reported that regular and AP biology teachers who attended a SUN Workshop made significant gains in understanding photosynthesis, cellular respiration and the underlying energy principles; and that those significant gains were maintained a year later for the regular biology teacher group vs. their original scores and vs. cluster, randomized controls who had not yet attended the SUN Workshop. Here we report knowledge gains made by the students of teachers who participated in a concomitant AP pilot study. Seven AP teachers at seven different schools used “business as usual” methods during the 2009-2010 school year with one class of students. In the summer of 2010, they attended the SUN Workshop. Then six of these teachers used the SUN teaching materials in their classrooms the following school year (one was no longer assigned to teach AP biology). By necessity, these were different student groups. Comparison of student achievement during both years shows that students during the “control” year made a statistically moderate significant gain (.55 effect size) when measured with a 15-item multiple-choice exam at the beginning and end of the AP biology course. However, after the teachers attended the SUN Workshop and then used these materials in their classrooms, students made a statistically large significant gain (0.84 effect size) in content understanding on this assessment pre/post course. These results along with preliminary data regarding significant

student effects in regular biology classrooms vs. controls in the cluster, randomized controlled trial suggest that this program of professional development coupled with new teaching tools has promise for helping students understand these complex topics.

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Cell Biology is Learned Better when Combined with Art.

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Is student learning enhanced through cross-disciplinary teaching of artistic expression along with cell biology? The Cell Motion BioBus, a mobile microscope lab housed on a retrofitted 1974 transit bus, has teamed up with the Indianapolis Museum of Art (IMA) to find out.

The pilot experiment was conducted in October 2010, when the BioBus and its team of cell biologists joined art educators at IMA. Students from four Indianapolis schools came to IMA's 100 Acres Art and Nature Park to participate in three activities over the course of 90 minutes: (1) a tour of the park, led by a museum docent, in which students were encouraged to look closely and articulate their observations about different pieces of art in the park; (2) an art-making activity, led by an IMA artist, using materials from the park; (3) hands-on science lessons aboard the BioBus, in which we collected samples from the park's lake and analyzed the physiology of single-celled organisms using on-board microscopes.

In post-visit evaluations, teachers described the program as "a great experience for [their] kids," and were especially positive about how combining "scientific, artistic and communication skills;" helped make science become less intimidating and more accessible to their students, because it showed them "that their observations are valid and everyday language can be used as the beginning of the scientific process."

The main complaint reported by teachers was not being well-prepared, and, as a result, it did not fit with their classroom teaching. To address this issue, we organized a three-day seminar in July 2011 to prepare for the October 2011 version of the program. At the end of this workshop, teacher's reported an increased confidence in their ability to use visual thinking strategies to encourage engagement with art as well as scientific exploration and discovery.

Here we present these results along with the results collected during the October 2011 school visits, revealing both the benefits of and challenges in designing a cross-disciplinary activity combining artistic and scientific expression.

Membrane Fission and Coat Proteins

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Functional studies of the retromer and WASH complexes.

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The retromer complex is a conserved endosomal protein sorting complex that sorts membrane proteins into nascent endosomal tubules. The recognition of membrane proteins is mediated by the cargo-selective retromer complex, a stable trimer of the VPS35, VPS29 and VPS26

proteins. We have recently reported that the cargo-selective retromer complex associates with the WASH complex, a multimeric protein complex that regulates tubule dynamics at endosomes. Here we show that the retromer-WASH complex interaction occurs through the long unstructured “tail” domain of the WASH complex-Fam21 protein binding to VPS35, an interaction that is necessary and sufficient to target the WASH complex to endosomes. Additionally the Fam21-tail interacts with FKBP15, a protein associated with ulcerative colitis, to mediate the membrane association of FKBP15. Inhibition of V-ATPase activity inhibits the endosomal association of FKBP15 and abolishes Snx1-tubule formation suggesting a mechanistic link between endosomal acidification and protein sorting. Overexpression of the Fam21-tail results in cell-spreading defects implicating the activity of the WASH-complex in regulating the mobilization of membrane into the endosome-to-cell surface pathway

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Clathrin-mediated endocytosis is required for biogenesis of the contractile vacuole in *Dictyostelium.*

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Clathrin-mediated endocytosis (CME) is the process by which eukaryotes selectively internalize receptors from the plasma membrane. Disruption of clathrin is lethal in multicellular organisms but unicellular organisms can survive indicating that CME may have evolved a more specialized function in higher eukaryotes. We have found that in the unicellular organism *Dictyostelium* the major components of CME, clathrin and AP-2, behave in a similar way to their counterparts in higher eukaryotes. Firstly, we labeled the *Dictyostelium* homolog of the alpha subunit of AP-2 with GFP and showed that it is incorporated into a heterotetrameric AP-2 complex homologous to that in mammals. Next, we observed the behavior of fluorescently tagged clathrin or AP-2 by total internal reflection fluorescence microscopy. Simultaneous imaging of clathrin and AP-2 showed that these proteins co-localize to membrane puncta and that they disappear together. We investigated the nature of the disappearing AP-2 and clathrin puncta by alternating between wide-field and evanescent field illumination. Using this technique we showed that AP-2 and clathrin disappearing puncta leave the plasma membrane and move into the cell, characteristic of CME. Furthermore, we show a plasma membrane cargo is internalized by CME. Dajumin is an integral membrane protein that is localized to the contractile vacuole in *Dictyostelium*. We show that dajumin also localizes to the plasma membrane where it is internalized by CME. In cells that lack clathrin no contractile vacuole is present and dajumin is trapped on the plasma membrane. We propose a role for CME in organelle biogenesis whereby membranes and proteins are trafficked via the plasma membrane. Furthermore these findings indicate that CME may play different roles in unicellular and multicellular organisms, explaining the different phenotypes seen with clathrin perturbations. Since *Dictyostelium* can transition between unicellular and multicellular states our findings can be extended to study the different physiological roles of CME during these stages of development.

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Regulation of clathrin coat assembly by Eps15 homology domain-mediated interactions during endocytosis.

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Clathrin-mediated endocytosis involves a coordinated series of molecular events regulated by interactions between a variety of proteins and lipids through specific modules. One such module is the Eps15 homology (EH) domain, a highly conserved protein-protein interaction domain

present in a number of proteins distributed from yeast to mammals. Several lines of evidence suggest that the yeast EH domain-containing proteins (EHDPs), Pan1p, End3p and Ede1p, play important roles during endocytosis. Although genetic and cell-biological studies of these proteins have suggested a role for the EH domains in clathrin-mediated endocytosis, it is still unclear how they regulate clathrin coat assembly. To explore the role of the EH domain in yeast endocytosis, we mutated those of Pan1p, End3p or Ede1p, respectively, and examined the effects of single, double or triple mutation on clathrin coat assembly. We found that mutations of the EH domain caused a defect of cargo internalization and a delay of clathrin coat assembly, but had no effect on assembly of the actin patch. We also demonstrated functional redundancy between the EH domains of Pan1p, End3p and Ede1p for endocytosis. Interestingly, the dynamics of several endocytic proteins were differentially affected by various EH domain mutations, suggesting functional diversity of each EH domain.

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Clathrin Light Chain Regulates Endocytosis by Restricting the Attachment of the Yeast Hip1R Homologue, Sla2, to the Actin Cytoskeleton.

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Endocytosis in *Saccharomyces cerevisiae* occurs in distinct stages: (1) an immobile phase, which is marked by accumulation of clathrin and clathrin adaptors at the site of invagination; (2) a slow mobile phase where additional factors are recruited, as actin assembly and membrane invagination occur; and (3) a fast mobile phase after scission when the nascent vesicle with associated actin moves deeper into the cell. We previously demonstrated that the disruption of clathrin causes delayed internalization and aberrant actin events, and found that overexpression of the N-terminus of clathrin LC, which binds Sla2 (the Hip1R homologue), suppresses the endocytic defects of clathrin deficient yeast. To understand the role of LC, we performed the first Synthetic Genetic Array analysis of clathrin, utilizing an N-terminal deletion of Clc1 (*clc1-Δ19-76*). This clathrin mutant was systematically crossed to the genome wide viable null collection and double mutant progeny were selected and scored for exacerbation or suppression of the growth phenotype. We identified the *clc1Δ19-76* allele as alleviating to the null mutation of verprolin (*vrp1Δ*). Vrp1 is known to interact with the Arp2/3 complex activators Las17 (WASp) and Myo3/5 (type-1 myosins) during the mobile phase of endocytosis. *vrp1*Δ yeast are endocytic deficient, display prolonged fluorescent lifetimes of early endocytic factors (Sla1 & Sla2), and have reduced inward movement of endocytic patches and these defects were suppressed by *clc1Δ19-76.* This suppression was dependent upon the talin-like domain of Sla2 and these LC residues are important for displacing Sla2 from actin, *in vitro*. We further tested the Vrp1 interacting actin nucleation promoting factors (NPFs), by combining each *las17*Δ or *myo3/5*Δ with *clc1-*Δ*19-76*. As expected, the LC-NT mutant suppressed both the endocytic and growth defects caused by loss of these major NPFs. Our work suggests that *clc1-Δ19-76* prolongs actin engagement by Sla2 leading stabilization of the membrane tubule over a longer period and concluding in eventual internalization despite suboptimal actin assembly. In wildtype cells LC binding Sla2 likely acts to maintaining directional tension during endocytosis.

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Measuring and modeling the lifetime of clathrin-coated structures.

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During clathrin-mediated endocytosis (CME), the assembly of clathrin-coated pits (CCPs) is a complex process regulated by interactions between structural and sorting proteins, receptors, and cargo molecules. Live-cell imaging of fluorescently-tagged CME-associated proteins via total internal reflection fluorescence microscopy has revealed a remarkable heterogeneity in CCP dynamics. Although many variables that contribute to this heterogeneity have been identified (i.e., cargo type/size, receptor concentration, local concentration of various accessory proteins), the hierarchy of molecular events that regulate CCP assembly is for the most part unknown. The distribution of CCP lifetimes is a powerful and widely used descriptive metric for the assessment of CME dynamics and identification of, or differentiation between, effects of molecular perturbation. Lifetimes are determined by detecting and tracking CCPs labeled with overexpressed fluorescent fusion proteins. High precision of the automated methods employed to this end is crucial, as any systematic error will introduce a shift towards shorter lifetimes in the estimated distributions. Here, we show that lifetime estimation is highly sensitive to signal-to-noise ratio (SNR) and, as a consequence, to the level of expression of the fluorescent fusion protein used as a fiduciary for CCP detection and tracking. In this context, we introduce a new set of tools designed for high sensitivity even at low SNR conditions, and show how the resulting lifetime distributions relate to the kinetics of CCP maturation. Finally, using a state-based kinetic model, we identify kinetically distinct subpopulations of CCPs based on the measured lifetimes.

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Dynamics of Clathrin Coated Pits.

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The early state of receptor mediated endocytosis involves the formation of transient structures known as clathrin coated pits (CCPs). Depending on the detailed energetics of protein binding and associated membrane transformations, the pits either mature into clathrin coated vesicles (CCVs) or regress and vanish from the cell surface. The former are referred to as “productive” CCPs and the latter as “abortive” CCPs. We build a simple model for CCP dynamics which includes the free energy of CCP formation and a related kinetic scheme, and demonstrate how the model can be used to describe various aspects of CCP dynamics. In particular, we carry out Monte Carlo simulations to investigate the time development of CCP size and explain the origin of abortive pits and features of their lifetime distribution. By fitting the results of the simulations to experimental data, we are able to estimate values of the free energy changes involved in formation of the clathrin-associated protein complexes that comprise the coat. We also show how the binding of cargo might modify the coat parameters and thereby facilitate CCV formation. Finally, we observe how variations in the lifetimes of pits that result in CCVs are linked to the stochastic associations and disassociations of the coat components.

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Regulating AP-2 interactions to control vesicle size and number during clathrin-mediated endocytosis.

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The clathrin adaptor protein AP-2 is the core organizing element in clathrin-mediated endocytosis; it concentrates cargo and clathrin at the site of vesicle formation and it orchestrates a myriad of endocytic accessory proteins to control aspects of vesicle formation ranging from membrane deformation to vesicle fission. Thus, clathrin and accessory proteins need to interact with AP-2 in a temporally and spatially controlled manner, yet it remains elusive as to how access of these binding partners to AP-2 is regulated. Using knock down and functional rescue studies, we now demonstrate that the AP-2-binding protein NECAP 1 is a critical regulator of AP-2 interactions. In fact, NECAP 1 needs to be present in a complex with AP-2 to enhance the stability of FCHo2-marked vesicle nucleation sites, thereby controlling the number of clathrin-coated vesicles that form. During vesicle formation, NECAP 1 and AP-2 function cooperatively in the efficient recruitment of other accessory proteins that in turn control vesicle size. Moreover, we use binding assays and nuclear magnetic resonance studies to reveal NECAP 1 as the first known factor that competes with clathrin for access to the AP-2 beta2-linker, positioning NECAP 1 to regulate the ability of AP-2 to recruit and polymerize clathrin. Thus, our data demonstrate that NECAP 1 is a key modulator of the AP-2 interactome needed for productive vesicle formation and reveal a new layer of organizational control within the endocytic protein machinery.

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Regulation of dynamin-mediated fission by endocytic accessory proteins.

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The large GTPase dynamin plays a dual role during CME: at early stages it monitors the maturation of clathrin coated pits (CCPs), whereas at late stages it assembles at the neck of deeply invaginated CCPs and mediates membrane fission. Dynamin consists of five functionally-defined domains. These include, the G domain, which mediates GTP hydrolysis, and a middle domain and GTPase effector domain that together are involved in self-assembly. The pleckstrin homology domain mediates lipid interactions, and the Pro/Arg domain (PRD) mediates interactions with dynamin's SH3 domain-containing partners. Structure-function analyses of a series of mutants have revealed that dynamin's ability to catalyze membrane fission depends on its interdependent abilities to a) bind and self-assemble onto membranes, b) generate high curvature on these membranes, and c) hydrolyze GTP.
Over the past 15 years a myriad of endocytic accessory proteins have been identified based on their interaction with clathrin, AP2 or dynamin. Here, we studied the effect of dynamin binding partners and their regulation of dynamin-mediated fission. We tested the major dynamin binding partners: SNX9, Endophilin and Amphiphysin, all of which contain membrane remodeling BAR domains.
Using an in vitro fission assay, we found that these effectors regulate dynamin mediated fission at multiple levels. 1) The interaction between the PRD and the SH3 domain of all three binding partners negatively regulates fission. 2) In the absence of this interaction fission is facilitated by amphiphysin and endophilin most likely supported by the BAR domain. 3) Our results imply that the SH3 domain in addition negatively regulates the BAR domain, perhaps in a manner similar to that recently identified for syndapin (Rao et al., 2010, PNAS 107:8213).
Fission is a stochastic process and requires multiple rounds of dynamin assembly, membrane squeezing, GTP hydrolysis and disassembly. Therefore we tested the GTPase activities in the

presence of these effectors. Our results imply that under conditions that facilitate fission, GTPase activities were reduced. This unexpected inverse relationship of GTPase activity and fission was not observed with dynamin mutants displaying decreased GTPase hydrolysis. We speculate that dynamin binding partners prolong the residence time of dynamin at the membrane by promoting self-assembly. This results in fewer rounds of assembly and disassembly and therefore in lower GTPase activity and increased membrane squeezing. To test our hypothesis, further assessment of the effect of dynamin binding partners on dynamin's multiple biochemical activities will be necessary.

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Dynamin's powerstroke.

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The dynamin family of proteins are large GTPases involved in membrane fission and fusion events throughout the cell. Our goal is to understand the dynamic structural properties of these proteins and correlate them with their diverse cellular functions. The founding member, dynamin, is crucial for endocytosis, synaptic membrane recycling, membrane trafficking within the cell, and associates with filamentous actin. Purified dynamin readily self-assembles into rings and spirals and forms similar structures on liposomes, generating dynamin-lipid tubes that constrict and twist upon GTP hydrolysis. Previously, we solved the three-dimensional structure of ΔPRD-dynamin tubules both in the non-constricted and constricted states to a resolution of ~20 Å. Recently, we have improved the resolution of ΔPRD-dynamin to 12 Å. The new map reveals additional density in the GTPase domain that would accommodate the bundle-signaling-element (BSE), which consists of the N- and C- terminal α helices of the GTPase domain and the C-terminal α helix of the GED domain. In addition, the new map resolves the middle radial density into two segments that twist and tightly interact. We have docked the GDP.AlF_4^- GG domain (GTPase domain-GED fragment) crystal structure and a new GMP-PCP GG crystal structure into our 3D map. Comparison between the GTP-bound state (GMP-PCP) and transition state (GDP.AlF_4^-) within the 3D EM map suggests that the conformational change induced by the GTP hydrolysis is driving a large swing of the BSE. We predict that the BSE movement is dynamin's power stroke that results in a significant twist and constriction of the underlying lipid bilayer leading to membrane fission. In addition to the ΔPRD-dynamin 3D map, we have calculated a 3D map of full-length dynamin in a further constricted state, with a resolution of ~15Å. The inner lumenal diameter of this structure is ~2-4 nm, a range that is compatible with spontaneous lipid fusion. Currently we are docking the GG domain crystal structures into our K44A-dyn map to identify changes within dynamin domains that leads to maximum constriction and ultimately membrane fission.

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The Structures of COPI-coated Vesicles.

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We used novel cryo-electron tomography methods to resolve the structure of the COPI coat *in situ* on COPI-coated vesicles derived from a budding reaction. We found that the repetitive unit of the coat is a triad of three coatomer complexes. We determined the location and arrangement of the triads on individual vesicles an discovered that the protein coat is not complete but

contains gaps. Structures of COPII and clathrin coats assembled *in vitro*, without membranes, have led to the current model where coat proteins assemble using essentially identical local contacts to form regular, geometrical cages. In this model, structural flexibility of the components allow formation of cages of different size. In striking contrast to this model, we revealed that COPI-coated vesicles are assembled through non-identical local contacts. By adopting different conformations and interacting with different stoichiometries, the coat forms vesicles with variable sizes and shapes. Regulating the relative frequencies of conformations in the same vesicle (for example stabilizing one interaction) would provide an avenue for cellular regulation of trafficking vesicle size.

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The role of cPLA2á in vesiculation of GPI-anchored protein-containing endosomes.

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The lipid modifier phospholipase A2 (PLA2) catalyzes the hydrolysis of phospholipids to inverted-cone shaped lysophospholipids that contribute to membrane curvature. Here we demonstrate a role for cytosolic PLA2-alpha (cPLA2á) in the vesiculation of glycosylphosphatidylinositol anchored protein (GPI-AP)-containing membranes. In this study, we used endogenous CD59 as a model for GPI-APs. Upon depletion of cPLA2á, CD59-containing endosomes become hypertubular. We further showed that accumulation of lysophospholipids, following the inhibition with lysophospholipid acyltransferase (LPAT) inhibitor, vesiculates CD59-containing endosomes. However, over-expression of cPLA2á does not increase the vesiculation of these endosomes, implicating other factors required in the fission of CD59-containing membranes. Indeed, we found that depletion of EHD1, a C-terminal Eps15 homology domain (EHD) protein, predicted to act as a “pinchase”, also induces the hypertubulation of CD59-containing endosomes. Furthermore, EHD1 interacts with cPLA2á. The results presented here provide evidence that the lipid modifier cPLA2á and EHD1 are involved in the vesiculation of CD59-containing endosomes. We speculate that cPLA2á induces membrane curvature, allowing recruitment of EHD1 to sever the curved membranes.

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ER export of GPI-anchored proteins requires COPII coat rigidity conferred by Sec13p.

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Eukaryotic secretory proteins are exported from their site of synthesis in the endoplasmic reticulum (ER) via transport vesicles that form when the proteins of the COPII coat assemble on the cytosolic face of the ER membrane. The hierarchical assembly of the five COPII subunits couples cargo selection with membrane deformation to generate and populate transport carriers. Despite a wealth of biochemical and structural information on the COPII coat, we still lack a mechanistic understanding of the biophysical processes that shape membrane deformation during genesis of these diverse transport carriers. We sought to probe this process by examining the molecular function of the outer coat component, Sec13. Although first characterized as a COPII coat protein, Sec13 is also a component of the nuclear pore complex, where its interaction with Nup145 is structurally analogous to its partnership with Sec31 and is thought to stabilize the curvature of the nuclear envelope much like Sec13-Sec31 imparts or stabilizes membrane curvature associated with COPII vesicles. Sec13 also binds in a structurally similar manner to Sec16, an essential but enigmatic regulator of COPII transport. It is therefore surprising that Sec13 is dispensable in the context of yeast mutants known as bypass-of-sec-thirteen (bst) mutants. The mechanism by which bst mutations permit secretion in

the absence of an otherwise essential coat component remains unknown. We sought to exploit the bst phenotype to further probe the function of Sec13. We undertook an exhaustive survey of the yeast genome to define novel bst mutants, identifying additional components with roles in biogenesis of GPI-anchored proteins. We propose that exclusion of an entire class of lipid-anchored proteins from COPII vesicles creates a locally altered membrane that is somewhat permissive in terms of membrane curvature and can therefore be bent into a transport vesicle in the absence of the rigidifying effect of Sec13. In support of our model, we demonstrate that a rigidified version of Sec31 supports viability in the absence of an additional bst mutation.

ER and Golgi Transport II

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Sedlin binds Sar1p and controls type II procollagen trafficking by modulating the cycle of COPII.

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Genetic defects of sedlin, a conserved component of the TRAPP complex, cause spondyloepiphyseal dysplasia tarda (SEDT), a condition characterised by impaired chondrogenesis that results in short stature, flattening of the vertebrae and premature osteoarthritis. Prompted by the consideration that sedlin is ubiquitously expressed but that sedlin mutations cause cartilaginous-restricted dysfunctions, we hypothesized that sedlin might exert a role in the transport of chondrocyte-specific cargoes, such as type II procollagen (PCII). This hypothesis was reinforced by the fact that certain mutations in PCII give rise to autosomal dominant forms of spondyloepiphyseal dysplasia. We tested this hypothesis by analyzing the involvement of sedlin in the transport of different classes of secretory cargoes and found that sedlin is selectively required for PCII to exit the ER, while it is not essential for the ER exit of small soluble and membrane-associated cargoes. We have also identified the molecular mechanism underlying this role of sedlin in its ability to bind the GTPase Sar1 and to control the membrane-cytosol cycle of Sar1 itself and of the COPII coat complex at the level of the ER exit sites. Sedlin depletion and/or mutation in SEDT patients slows down the Sar1 cycle and prolongs the membrane association of Sar1-GTP at ER exit sites, thus inducing constriction and premature fission of nascent carriers which fail to incorporate the large PC protofibrils but are still competent for smaller secretory cargoes.

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The Role of Mammalian COPII Component SEC24D.

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Approximately one-third of all eukaryotic proteins transit the intracellular secretory pathway, exiting the ER in COPII-coated vesicles. Sec24 is the COPII component thought to be primarily responsible for the recruitment of transmembrane cargoes or cargo adaptors into newly forming vesicles. Mammalian genomes encode four Sec24 paralogs (*Sec24a-d)*, though little is known about their comparative functions and cargo-specificities. Co-immunoprecipitation experiments indicate that all four SEC24 paralogs interact with both paralogs of the SEC24 binding partner SEC23 (SEC23a/b) at relatively equivalent ratios. To address the *in vivo* function of the

SEC24D paralog, we generated two independent lines of SEC24D-deficient mice (carrying two distinct *Sec24d* gene-trap alleles). Mouse *Sec24d* is located on chromosome 3 and contains 23 exons. The first allele, carrying a gene-trap cassette insertion in intron 8, results in very early embryonic lethality (prior to the blastocyst stage of development) in homozygotes. Mice heterozygous for this *Sec24d* gene-trap allele have no apparent phenotype. The second allele contains a gene-trap in the more distal intron 20, with mice homozygous for this allele demonstrating delayed embryonic lethality (close to the expected number of *Sec24d-/-* embryos at E10.5, but none surviving to E18.5). The discrepancy between the two gene-trap alleles is likely explained by the ~1% residual normal splicing around the gene-trap in the second line. Finally, embryonic lethality in homozygotes for the first Sec24d allele is rescued by the introduction of a BAC transgene spanning the *Sec24d* gene. Current studies are examining the phenotype of SEC24C deficiency and the potential overlap in function between SEC24C and D by transgenic rescue experiments.

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COPII components SEC23A and SEC23B are required for normal murine development and survival.

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Approximately one third of all eukaryotic proteins transit the intracellular secretory pathway. COPII vesicles play a key role in transporting proteins from the ER to the Golgi. SEC23 is an essential component of the COPII vesicle coat, and two paralogs (SEC23A and SEC23B) are known in mammals. Human mutations in SEC23A and SEC23B are associated with cranio-lenticulo-sutural dysplasia and congenital dyserythropoietic anemia type II (CDAII), respectively. We recently generated mice deficient for SEC23A and SEC23B, as described in more detail by Zhang et al. Mice deficient for SEC23A die in mid-embryogenesis, and mice deficient for SEC23B exhibit massive pancreatic degeneration and perinatal lethality, but no anemia. Intercrosses between Sec23a+/- Sec23b+/- and Sec23a+/- Sec23b +/+ mice resulted in a statistically significant underrepresentation of Sec23a-/- Sec23b+/- embryos at E9.5 compared to all other genotypes (p=0.019), suggesting a significant genetic interaction between Sec23a and Sec23b. These findings indicate that humans and mice with mutations in Sec23a and Sec23b have disparate but tissue-specific phenotypes. Two independent Sec23a bacterial artificial chromosomes (BACs) and two independent Sec23b BACs were used to generate C57B6/SJL transgenic founder mice, which were crossed to generate a total of 8 Sec23a -/-, Tg+ and a total of 19 Sec23b -/-, Tg+ “rescue” mice. “Rescued” Sec23a-/-, Tg+ and Sec23b-/-, Tg+ mice are observed in expected Mendelian ratios; they are viable and grossly normal on autopsy and histological examination. RT-PCR and western blot analysis revealed differential endogenous tissue expression patterns for Sec23a and Sec23b in wildtype mice. Sec23b is expressed at a significantly higher level in the mouse pancreas as compared to bone marrow. These results confirm that the phenotypes we have observed are due solely to SEC23A and SEC23B deficiency, and not unintended effects of the gene targeting on a nearby gene. These findings also demonstrate that the Sec23a and Sec23b BACs contain the required regulatory sequences for sufficient physiologic expression of their corresponding genes in vivo. Ongoing studies utilizing BAC recombineering will determine whether Sec23a and Sec23b can functionally substitute for one another when expressed in the appropriate context in the mouse.

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Mutations that allow formation of COPII vesicles in the absence of Sec13p.

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Secretory proteins are exported from the ER via COPII vesicles, which form when the proteins of the COPII coat assemble on the ER membrane. The outer layer of the COPII coat is thought to consist of a cage assembled from Sec31p-Sec13p heterotetramers. Whereas Sec31p and all other members of the COPII coat are absolutely essential, Sec13p is dispensable in certain yeast mutants, termed *bypass-of-sec13 (bst)* mutants (Elrod-Ericson and Kaiser, 1996, Mol Biol Cell 7: 1043). We conducted genome-wide screens in order to identify a full array of *bst* mutations in yeast. We find that the strongest *bst* mutants interfere with maturation of GPI-anchored proteins (GPI-APs) or disrupt the p24 complex, which is required for efficient export of GPI-APs from the ER. We hypothesize that because of their highly asymmetric distribution within the ER membrane, GPI-APs may make the membrane more resistant to introduction of positive curvature required for vesicle formation. Thus, a full COPII coat may be needed for deformation of a membrane enriched in GPI-APs, but under more permissive conditions Sec31p may be able to form the outer layer of the coat on its own.

An interesting new *BST* gene is *ERV29*, which encodes a receptor for soluble COPII cargo proteins. Whereas *erv29Δ* is a very weak suppressor on its own, its bst phenotype is greatly enhanced when we combine it with other weak *bst* mutants *erp1Δ* and *erp2Δ*. In addition, we identified many ribosomal and translational mutants as weak bst's. These
observations lead us to suggest that changing the bulk concentration of COPII cargo proteins at the ER may also influence the ease of COPII vesicle production.

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Regulation of COPII-dependent secretion of select extracellular matrix proteins in zebrafish skeletal morphogenesis.

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The COPII machinery is the primary mediator of ER-to-Golgi transport and appears to be dynamically regulated, although the mechanisms of this regulation are only beginning to be elucidated. Using a positional cloning strategy, we identified a zebrafish mutant with a point mutation in the DNA binding domain of ER localized transcription factor creb3l2 that leads to a malformed head skeleton. The creb3l2 mutant phenotype is similar to those of previously identified zebrafish COPII mutants, and Creb3l2 directly regulates the expression of genes encoding select COPII proteins, suggesting that primary role of Creb3l2 is to modulate the availability of COPII components and the secretory capacity of the cell. The creb3l2 mutation is a hypomorphic allele that retains approximately 50% of its transcriptional activity, indicating that collagen secretion is sensitive to even modest changes in Creb3l2. Early patterning of creb3l2 mutant cartilage elements appears normal, and mutant chondrocytes begin producing type II collagen. However, mutant chondrocytes fail to secrete all of the collagen they produce, resulting in a backlog of collagen within the ER, a failure to maintain the ECM collagen, and aberrant chondrocyte maturation. Similarly, loss of Creb3l2 in the notochord leads to failure to secrete type II/IV collagens, while secretion of other cargos, including glycoaminoglycans and large ECM component laminin, is unperturbed. Creb3l2 is the first identified transcription factor to couple the secretory activity of cells to the progression of developmental programs. These

findings are likely to lead to better understanding of the etiology of human skeletal birth defects and adult-onset diseases linked to dysregulated ECM deposition.

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Luminal calcium regulates ER/ Golgi anterograde transport efficiency through ALG-2/SEC31 interactions.

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Luminal calcium released from secretory organelles has been suggested to play a regulatory role in vesicle transport early in the secretory pathway. In ER-to-Golgi transport, specific depletion of luminal calcium leads to unidentified enlarged clusters of intermediate compartment markers at ER exit sites. In vitro, blocking luminal calcium egress leads to release of residual (post-budding) COPII subunit sec31 and concomitant increased COPII vesicle fusogenicity, suggesting a role for residual coat retention in the calcium regulation of transport. The calcium sensor apoptosis-linked gene 2 (ALG-2) stabilizes residual sec31 on COPII vesicles in response to calcium and slows fusion, suggesting that it may be part of the signaling cascade by which luminal calcium exerts its effects on ER-to-Golgi transport (Bentley et al., 2010. Molecular Biology of the Cell 21:1033). Here we demonstrate using a kinetic morphological transport assay that specific luminal calcium depletion leads to a significant decrease in ER-to-Golgi transport rates for transmembrane cargo proteins. Furthermore, detailed ultrastructural analysis revealed that luminal calcium depletion is accompanied by an increase in numbers of COPII buds and tethered but unfused COPII vesicles at ER exit sites. Importantly, no accumulation of enlarged fusion structures was observed. Finally, in over-expression studies, we demonstrate that disruption of interactions between calcium sensor ALG-2 and sec31 cause severe defects in ER-to-Golgi transport. This work identifies for the first time a functional requirement for luminal calcium at a demonstrably post-protein-folding step in ER/Golgi transport in intact cells. The kinetic and ultrastructural analyses combined indicate that luminal calcium may be involved in promoting efficient post-ER sorting of transmembrane cargo between anterograde and retrograde directions of movement; in the absence of luminal calcium, the overall efficiency of anterograde transport decreases while anterograde vesicle formation is stimulated. Other possible interpretations are discussed. For the first time, ALG-2 interactions with sec31 are shown to posses a functionally critical role in anterograde transport, indicating that ALG-2 is at least one of the calcium sensors that effect luminal calcium's regulatory role in vesicle trafficking.

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Sec31p as a sole component of the COPII cage: Sec13p is dispensible under conditions influencing membrane rigidity.

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Nascent proteins that enter the secretory pathway via the endoplasmic reticulum (ER) are packaged into vesicles for forward transport. Vesicles are sculpted from the ER membrane by the cytosolic COPII protein coat - the G-protein, Sar1p, Sec23/Sec24p and Sec13/Sec31p - in a process that is highly conserved in eukaryotes. In budding yeast, these COPII proteins are essential for viability, however, in mutants called bypass of Sec thirteen (*bst*), cells can survive without Sec13p. All strong *bst* mutations affect biogenesis or trafficking of GPI-anchored proteins (GPI-AP). We are exploring a model whereby that Sec31p can form the outer coat without Sec13p in *bst* mutants. This is supported by structural studies suggesting that Sec31p forms both the critical intermolecular contacts of the outer cage and the interactions with the

inner coat complex (Bi et al, 2007, Dev Cell, 13:635; Stagg et al, 2008, Cell 134:474). We have purified monomeric Sec31p and demonstrated that Sec31p or a Sec31p-β-blade mutant (where the Sec13p-interacting loop is replaced with an inert linker) can be specifically recruited to the COPII coat on synthetic liposomes in the absence of Sec13p. We have also demonstrated that Sec31p and the Sec31p mutant can form vesicles in the absence of Sec13p using an in vitro budding assay from microsomal membranes. We are also currently investigating the role of Sec13p in limiting the flexibility of the Sec13/31p outer coat during vesicle formation in wild type and *bst* mutant cells, where rigid GPI-APs could influence generation of membrane curvature. We have already defined one mutant, Sec31p-Δhinge, that lacks the entire inter-domain flexible region in order to mimic flexibility of the complex in the presence of Sec13p. Surprisingly, even though Sec31p-Δhinge does not bind to Sec13p, it allows for growth of yeast cells in the absence of *bst* mutations where the Sec13/31p complex is otherwise required.

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Sorting of ER-Golgi SNAREs by the COPII Coat in Saccharomyces cerevisiae.

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Proteins that leave the ER via the secretory pathway are transported to the Golgi in COPII-coated vesicles. The selective and efficient recruitment of proteins into COPII vesicles is mainly achieved via signal-mediated or receptor-dependent sorting. In yeast cells, Sed5p (Qa-SNARE), Sec22p (R-SNARE), Bet1p (Qc-SNARE) and Bos1p (Qb-SNARE) form a SNARE complex that is essential for ER-Golgi transport and these SNAREs are known to be efficiently packaged into newly forming COPII-coated vesicles. Although Sec24p-binding sequences have been identified for Sed5p, Sec22p and Bet1p, via structural and *in vitro* biochemical approaches (Bos1p appears not to directly bind to COPII proteins), the physiological consequences of deficiencies in SNARE-COPII interactions have not been extensively explored. We have examined the physiological consequences of amino acid substitutions to SNAREs that knockdown their binding to the COPII coat in *in vitro* assays. We find that in the absence of a Sec24p-binding motif, Sed5p, Sec22p or Bet1p are inefficiently exported from the ER. Nonetheless, despite the profound transport delay of these SNAREs, cell growth and protein transport (in general) was not significantly affected in cells singly expressing these COPII binding-deficient SNAREs. Loss of both COPII binding motifs on Sed5p was, however, lethal to cells. In addition, we establish that a deficiency in the ER export of one member of the SNARE complex does not affect the transport of any other member, suggesting that the sorting and transport of Sed5p, Sec22p, Bet1p and Bos1p via COPII-coated vesicles is not co-dependent.

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Chlamydia trachomatis hijacks intra-Golgi COG complex-dependent vesicle trafficking pathway.

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Chlamydia spp. are obligate intracellular bacteria that replicate inside the host cell in a bacterial-modified-unique compartment called the Inclusion. As other intracellular pathogens, chlamydiae exploit host membrane trafficking pathways to prevent lysosomal fusion and to acquire energy and nutrients essential for their survival and replication. The Conserved Oligomeric Golgi (COG) complex is a ubiquitously expressed membrane-associated protein complex that functions in a retrograde intra-Golgi trafficking through associations with coiled-coil tethers, SNAREs, Rabs and COPI proteins. Several COG complex-interacting proteins, including Rab6, Rab14 and Syntaxin 6 are implicated in chlamydial development. In this study, we analyzed the recruitment

of the COG complex and GS15-positive COG complex-dependent (CCD) vesicles to Chlamydia trachomatis inclusion and their participation in chlamydial growth. Immunofluorescent analysis revealed that both GFP-tagged and endogenous COG complex subunits associated with inclusions in a serovar-independent manner by 8 h post infection and were maintained throughout the entire developmental cycle. Golgi v-SNARE GS15 was associated with inclusions 24 h post infection, but was absent on the mid-cycle (8 h) inclusions, indicating that this Golgi SNARE is directed to inclusions after COG complex recruitment. Silencing of COG8 and GS15 by siRNA significantly decreased infectious yield of chlamydiae. Further, aberrant bacteria and membranous structures were observed inside inclusions by electron microscopy in cells depleted of COG8 or GS15. Our results showed that C. trachomatis hijacks the COG complex to re-direct the population of Golgi-derived retrograde vesicles to inclusions. These vesicles likely deliver nutrients that are required for bacterial development and replication.

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The Conserved Oligomeric Golgi (COG) Complex exists in multiple subcomplexes in HeLa Cells.

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The Conserved Oligomeric Golgi (COG) complex is a hetero-octameric peripheral protein complex that is proposed to function as a tether during intra-Golgi retrograde vesicle transport. The COG complex is composed of 8 proteins (COG's 1-8) that are predicted to assemble into two subcomplexes; lobe A, consisting of COG's 1-4, and lobe B, consisting of COG's 5-8. While the exact configuration of the COG complex has yet to be elucidated, the bi-lobed model appears to be an over simplification of the possible arrangements of the subcomplexes. We have shown that individual siRNA knockdown (KD) of COG subunits illustrates distinct phenotypes between lobe A and lobe B subunits, suggesting the two lobes to have separate jobs as trafficking machinery. In correlation with this theory, fluorescence recovery after photobleaching (FRAP), and fluorescence loss in photobleaching (FLIP) exhibited different kinetic profiles for lobe A and lobe B subunits. The lobe B subunit COG8-GFP cycled on and off Golgi membranes faster than the lobe A subunit YFP-COG3, suggesting a population of the COG8 containing lobe B that is absent of COG3 containing lobe A. Finally, gel filtration of separated HeLa cytosol and membrane illustrated a co-elution of COG's 3, 5, 6, and 8 in both the cytosol and membrane, which corresponded in size to an octameric protein complex. Furthermore, all tested COG subunits in the membrane fraction also co-eluted in a protein complex which corresponded to the tetramer. Uniquely, COG's 5 and 6 eluted in the cytosol in a slightly later fraction then the membrane bound subcomplex, suggesting that COG's 5 and 6 might exist in a cytosolic subcomplex that is smaller than the COG8 containing lobe B. Based on these data, we propose a model wherein the full octameric COG complex is present on both Golgi membranes and in the cytosol. Also in this model, we propose the existence of a previously uncharacterized third subcomplex containing COG's 5 and 6.

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Golgin-160 recruits the dynein motor to position the Golgi Apparatus.

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Intracellular motility and organelle positioning depend on the recruitment of molecular motors by specific membrane receptors. Although ER-to-Golgi trafficking and Golgi positioning are the best-known examples of inward motility mediated by the microtubule minus-end-directed motor dynein, the dynein membrane receptor is unknown. Here, we show that the Golgi protein golgin-

160 is required and sufficient for recruiting dynein and for conferring centripetal motility to secretory membranes. Further, golgin-160 directly bound the intermediate chain of the dynein motor and this dynein/golgin-160 complex dissociated from membranes during mitosis allowing Golgi dispersal. These results identify the first physiologically regulated motor receptor in the early secretory pathway, and suggest a molecular basis for achieving dramatic changes in trafficking and organelle positioning during cell division.

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Intracellular Trafficking of the Amyloid β-Protein Precursor Regulated by PDZ domains of X11-Like.

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Amyloid β(Aβ), a causative peptide of Alzheimer's disease, is generated by intracellular metabolism of amyloid β-protein precursor (APP). In general, mature APP (mAPP, N- and O-glycosylated form) is subject to successive cleavages by α- or β-, and γ-secretases in the late protein secretory pathway and/or at plasma membrane, while immature APP (imAPP, N-glycosylated form) locates in the early secretory pathway such as endoplasmic reticulum or cis-Golgi, in which imAPP is not subject to metabolic cleavages. X11-like (X11L) is a neural adaptor protein composed of a phosphotyrosine-binding (PTB) and two C-terminal PDZ domains. X11L suppresses amyloidogenic cleavage of mAPP by direct binding of X11L through its PTB domain, thereby generation of Aβ lowers. However, how these functional domains of X11L do regulate APP trafficking and/or metabolism remains unclear. Here we performed a functional dissection of X11L. Using cells expressing various domain-deleted X11L mutants, intracellular APP trafficking and localization were examined along with analysis of APP metabolism including maturation (O-glycosylation). We revealed that X11L accumulates imAPP into the early secretory pathway by mediation of its C-terminal PDZ domains, without being bound to imAPP directly. With this novel function, X11L suppresses overall APP metabolism and results in further suppression of Aβ generation. Interestingly some of the accumulated imAPP in the early secretory pathway are likely to appear on plasma membrane by unidentified novel mechanism. Trafficking of imAPP to plasma membrane is observed in other X11 family proteins, X11 and X11L2, but not in other APP-binding partners such as FE65 and JIP1. We also revealed that conformational changes of X11L regulated APP metabolism and intracellular trafficking of APP. It is herein clear that PDZ domains and its conformation of X11L protein regulate APP metabolism at multiple steps in intracellular protein secretory pathways.

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Detection of in situ cleaved p115 in rapid protein inactivation.

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Gene perturbation methods are commonly used in the study of gene and protein function. Therefore, we had recently developed a rapid protein inactivation technique utilizing the tobacco etch virus (TEV)-derived protease. The TEV protease recognizes the ENLYFQG amino acid sequence and specifically cleaves between Q and G. We developed antibodies that recognize the cleaved TEV (ENLYFQ) sequence, both in vitro and in vivo, but do not bind to uncleaved TEV (ENLYFQG). Using these antibodies, we successfully detected in situ protein cleavage of a vesicle tethering protein, p115 during its inactivation by the TEV protease. These antibodies used in combination with the TEV protease may be a useful complement to other perturbation methods.

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Cellulase Production in *Neurospora crassa.*

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The filamentous fungus *Neurospora crassa* is a well-studied model organism that is frequently isolated from the environment in association with burned vegetation. Enzyme activities related to the metabolism of plant cell wall material have been observed in *N. crassa*, but the overall cellular response of the organism to such a recalcitrant carbon source remains poorly defined. In order to investigate the elements involved in fungal growth on cellulose, we utilized the near full genome collection of single gene knockout strains that has been generated under the auspices of the Neurospora Genome Project. Strains with deletions in loci thought likely to play a role in cellulase activity or production – including the known secretome of *N. crassa* when cultured on plant biomass, homologs of the *Saccharomyces cerevisiae* secretion apparatus, and proteins predicted to traverse the secretory pathway – were singled out for analysis. This subset of the *N. crassa* deletion collection was cultured on a cellulosic substrate and the levels of secreted protein and cellulase activity were compared to wild-type. A number of hyper- and hypo-secretion mutants have been identified. Sequence analysis suggests that while some of the deleted genes likely act in transcription, protein synthesis, and intracellular trafficking, many of the loci encode fungal-specific proteins of undetermined functions. The on-going characterization of these secretion mutants will enhance our understanding of the ability of *N. crassa* to utilize the complex carbon sources present in its natural environment.

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Characterization of Cellulase Secretion in the Filamentous Fungus *Neurospora crassa.*

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Many filamentous fungi survive in nature by secreting large amounts of hydrolytic enzymes that allow them to degrade and utilize lignocellulosic substrates for growth. Because of their inherent ability to secrete large amounts of proteins, filamentous fungi are used for high-level protein production in various industries, such as the textile, pharmaceutical, and biofuels industries. Although the basic conserved components of the eukaryotic secretion pathway characterized in yeast and higher organisms are also conserved in filamentous fungi, the highly polarized and compartmentalized growth mode of filamentous fungal hyphae mandates pathways of secretion that are specific to these fungi. Although certain aspects of filamentous fungal secretion are under active study, such as the terminal steps of polarized secretion at the hyphal tip, a basic characterization of the entire pathway from start to finish remains to be performed. Such a characterization may provide insights into how filamentous fungi are able to secrete large amounts of enzymes and how these fungi can be engineered to produce even more enzymes in the future. This is particularly of interest to the process of biofuels production, in which the inexpensive production of large amounts of cellulases is a major bottleneck to the efficient and cost-effective production of cellulosic biofuels. In nature the model fungus *Neurospora crassa* secretes a host of cellulases to allow it to grow on burnt vegetation. The tractability of *N. crassa* makes it an excellent model to characterize protein secretion in filamentous fungi, particularly the secretion of industrially relevant cellulases. To achieve this goal we are characterizing the pathway of cellulase secretion in *N. crassa* by following the trafficking of the fluorescently tagged endoglucanse 2 (EG-2) enzyme, a major secreted endocellulase. To determine the compartments through which cellulases traffic we are co-localizing EG-2-GFP with fluorescently-tagged markers of the ER, Golgi, endosomes, and the Spitzenkorper (a specialized secretion structure at the tips of filamentous hyphae). We are additionally assaying the consequences to EG-2-GFP trafficking of blocks to secretion imposed by pharmacological

or mutational insults. Our initial results suggest partial co-localization of EG-2-GFP with a marker of Golgi bodies, as well as the absence of obvious localization to the Spitzenkorper. These data suggest that cellulases traffic through the classical secretory pathway, but may be secreted at lateral regions instead of to the tips, which is traditionally considered to be the main site of secretion in filamentous fungi.

Establishing and Maintaining Organelle Structure

1050

The B9 Complex Protects the Cilia as a Privileged Membrane Domain.

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The primary cilium functions as cellular antenna that transduces extracellular signals to intracellular responses, such as Shh signaling1. The failure of cilia function leads to a multitude of human diseases, together termed ciliopathies2. How cilia are formed and how signaling proteins are selectively localized to cilia still remains unclear. Through the use of RNAi screening, proteomics, cell biological and mouse genetics approaches, we have identified a protein network at the base of the cilia that forms a barrier to membrane protein diffusion and thereby, promote cilia maintenance and selective receptor localization in the cilium membrane. The network contains all of the B9 domain proteins present in the mammalian genome, 4 proteins that are disrupted in human ciliopathies, including B9D1, MKS1, AHI1, CC2D2A and 3 previously uncharacterized proteins. The assembly of the B9 network at the basal body occurs before and independently of intraflagellar transport, demonstrating the fundamental role that it plays in formation and function of the primary cilia. siRNA disruption of the complex causes a reduction in cilia formation and a loss of signaling receptors from the remaining cilia. Strikingly, knockdown cells have increased amounts of plasma membrane proteins in the ciliary membrane and increased diffusion into the ciliary membrane. KO of either of two distinct components of the complex have identical defects in Shh signaling and ciliogenesis, providing the basis for understanding human ciliopathies with disruptions of the B9 network. Therefore, we propose that the B9-network is an essential complex for normal cilia function and acts as a diffusional barrier between the cilia and plasma membranes to maintain cilia as a compartmentalized signaling organelle.

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Reversibly changing directional flow in the plant endoplasmic reticulum.

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Luminal and membrane proteins of the endoplasmic reticulum (ER) of plants flow directionally through the network. There is a possibility that the ER acts as a delivery nanopipe between organelles with which the ER is associated in plant cells. Flow at one particular ER-organelle subdomain, the chloroplast/ER nexus, is analyzed. This subdomain is of interest because it is the putative site of convergence of lipid traffic coming from the chloroplast's prokaryotic and the ER's eukaryotic lipid biosynthetic pathways. Photostimulation with a 405 nm laser at this site disrupts directional flow of proteins in the ER, producing aggregates (boluses) of luminal and ER membrane protein in the vicinity of the chloroplast/ER nexus. For several minutes following photostimulation, bolus formation propagates throughout the ER network and the rate and directionality of fluorescent ER protein diffusion at the nexus changes, as shown by quantitative assymetric fluorescence recovery after photobleaching (AFRAP). During recovery from

photostimulation, as boluses dissipate, directional diffusion is re-established, and the ER network is remodeled surrounding the chloroplast and at the nexus site.

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Proteasome-mediated degradation of ER membrane-derived cytoplasmic inclusions generated by the Amyotrophic Lateral Sclerosis-linked mutant form of VAPB.

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VAP-B (Vesicle-Associated Membrane Protein-Associated Protein B) is a ubiquitously expressed, ER-resident tail-anchored protein that mainly functions as adaptor for lipid-exchange proteins. Its mutant form, P56S-VAPB, has been linked to a dominantly inherited form of amyotrophic lateral sclerosis (ALS8). P56S-VAPB forms intracellular inclusions, whose role in ALS pathogenesis has not yet been elucidated. We recently clarified the ultrastructure of these inclusions and their precise relationship with the ER, demonstrating that they are formed by profoundly remodeled stacked ER cisternae that appear to be held together by the mutated cytosolic domain of VAPB (Fasana E. et al., FASEB J. 24:1419, 2010). In the present study, we used stable HeLa TetOff cell lines inducibly expressing wild type and P56S-VAPB to investigate the dynamics of inclusion generation and degradation. Our results indicate that P56S-VAPB inclusions undergo turnover and that the rate of degradation of the aggregated mutant is faster than that of the wild type protein. At variance with other inclusion-forming mutant proteins, which are first ubiquitinated and then aggregate, P56S-VAPB becomes ubiquitinated after sequestration in inclusions. Degradation of the mutant protein involves the proteasome with no apparent participation of macro-autophagy. Involvement of the p97 ATPase in the extraction of poly-ubiquitinated P56S-VAPB from the ER membrane is suggested by the observation that transfection of a dominant negative p97 stabilizes mutant VAPB. Our results reveal surprisingly efficient poly-ubiquitination, extraction from the ER, and proteasomal degradation of a severely aggregated mutant protein. Furthermore, they suggest that the slow onset of P56S-linked familial ALS is not a consequence of the progressive accumulation of the mutant protein over time, and that the dominant inheritance of the mutant allele may rather be related to haploinsufficiency.

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Comparative Analysis of GOLPH3 Family Member Proteins.

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GOLPH3 is a Golgi-localized protein that is conserved from yeast to mammals. Previously, our lab has shown that GOLPH3 binds the phosphoinositide lipid phospatidyl inositol-4-phosphate (PI4P), connects the Golgi to the actin cytoskeleton through an interaction with Myosin 18A, and is required for anterograde trafficking functions as well as for maintenance of the characteristic Golgi ribbon morphology (H.C. Dippold, et al. Cell. 139(2):337-51. 2009). In higher vertebrate species there are 2 genes in the GOLPH3 family, GOLPH3 and GOLPH3L. Although GOLPH3 is known to have important roles at the Golgi and has also recently been identified as an oncogene, (K.L. Scott, et al. Nature. 459(7250):1085-90. 2009.) relatively little is known about GOLPH3L. Thus our objective was to characterize the relative functions of GOLPH3 versus GOLPH3L proteins in mammalian cells. The predicted sequence of GOLPH3L protein possesses a high level of homology with GOLPH3, particularly in the PI4P binding region, thus

we hypothesized that GOLPH3L may have similar activities in the cell to GOLPH3. First, we determined the relative expression levels of GOLPH3 versus GOLPH3L in various mouse tissues and various cell lines. Next, using an in vitro protein-lipid overlay assay, we compared binding of GOLPH3 and GOLPH3L to PI4P in vitro. Additionally, we expressed fluorescently-tagged versions of GOLPH3 and GOLPH3L in cells and determined whether they colocalized with each other or known Golgi markers using immunofluorescence microscopy. Finally we are knocking down GOLPH3 versus GOLPH3L to investigate their specific roles in cells. We expect to be able to discuss similarities and differences between the functions of GOLPH3 and GOLPH3L.

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Rapamycin-induced increase in vacuole size scaling in budding yeast is not due to macroautophagy.

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The vacuole of *S. cerevisiae* is responsible for critical cellular functions including waste processing, and the morphology of this organelle is closely associated with these functions. For example, rapamycin treatment of cells induces many responses due to the inhibition of TOR signaling. Among these responses are a dramatic expansion of vacuole size, which we have quantified using novel size analysis methods based on 3-D fluorescence microscopy. Our measurements show that upon addition of rapamycin, vacuole surface area and volume scaling relationships with respect to cell size increase over a timescale of hours. Interestingly, these effects are not due to macroautophagy as has been suggested. To determine what is the cause, we have begun measuring vacuole size scaling in various known mutants of membrane trafficking. One such mutant is *fab1Δ* which affects lipid kinase activity, has enlarged vacuoles, and has previously been shown to affect TOR signaling. Vacuole size scaling in the *fab1Δ* mutant both in the absence and presence of rapamycin is very similar to that of rapamycin-treated wild-type cells, indicating that the *fab1Δ* phenotype is related to TOR signaling pathways. Furthermore, measurements on the *apl5Δ* mutant suggest that vacuole size scaling depends upon the flux of membrane through various trafficking pathways. One possible model to explain these observations, then, is that rapamycin treatment leads to increased trafficking to the vacuole independent of macroautophagy and/or decreased retrograde trafficking from the vacuole. Future experiments will continue to explore the role of membrane trafficking pathways in establishing vacuole size.

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Eisosome filaments organize the plasma membrane in fission yeast.

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The cortical cytoskeleton interacts with the plasma membrane to mediate a range of cellular activities such as endocytosis, cell motility, and the maintenance of cell rigidity. Traditional polymers including actin, microtubules, and septins contribute to the cortical cytoskeleton, but additional filament systems may also exist. In yeast cells, cortical structures called eisosomes generate specialized domains to cluster specific proteins at sites of membrane invaginations. The BAR domain protein Pil1 organizes eisosomes, but the assembly mechanism and organizing principles have been unclear. Here, we show that purified Pil1 assembles into filaments in vitro and forms linear cortical filaments in fission yeast cells. Pil1 cortical filaments are excluded from regions of cell growth, and are independent of the actin and microtubule cytoskeletons. Upon increased expression, Pil1 forms cytoplasmic rods that are stable and span

the length of cylindrical fission yeast cells. In cells, Pil1 filaments assemble slowly at the cell cortex and appear stable by timelapse microscopy and FRAP. This stability does not require the cell wall, but Pil1 and the transmembrane protein Fhn1 co-localize and are interdependent for localization to cortical filaments. Consistent with its formation of cortical filaments, we find a role for Pil1 in generating spatial order and cortical rigidity of the plasma membrane. Our combined results indicate that Pil1 filaments form a cortical cytoskeleton in yeast, with implications for the role of filament assembly in the spatial organization and mechanical rigidity of cells.

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New structural features of endoplasmic reticulum revealed by serial section electron microscopy.

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New methodology for serial section electron microscopy using ATUM (automated tape-collecting microtome) and scanning electron microscopy has been developed for tracing neural connections but is also potentially very useful for cell biology. This technique allows reliable collection of very thin sections (~30 nm) through large volumes with well-defined staining of membranes. The structure of the endoplasmic reticulum (ER) was investigated in the neuron cell bodies, axons and dendrites in a 50 μm cube of mouse cerebral cortex layer 4/5.

The ER in the cytoplasm of neuronal somata is a mix of loosely organized sheets and tubules. The sheets have a sharp edge in cross section. This edge is likely to be enriched in curvature generating proteins such as reticulon (Shibata et al., 2010). Some regions of the ER consist only of interconnecting sheets. We believe that we have identified the fundamental structural motif of this type of ER. In series of serial sections through this structure, we could often find a single 30 nm thick section containing three sheets (e.g. #1, #2, #3) meeting at ~120°. As one views the serial sections on one side of this junction, only two of the sheets are connected (e.g. #1, #2) whereas the third sheet (#1) is separate, with its edge converging towards the junction. The situation is the same when approached from the other side, except that two other sheets are connected (e.g. #2, #3) and the third is separate and converging (e.g. #1). We propose that there is a single continuous sharp edge that runs through the junction (e.g. passing from #3 to #1) and that this is the key structural component of this three dimensional structure. Modeling using the concepts of membrane curvature elasticity shows that an initial U shape indentation of an edge of a two dimensional sheet transitions to this three dimensional structure in order to minimize the membrane bending energy.

In addition to this sheet structure, we also found very narrow (~20 nm), unbranched, and sometimes long (>1 μm) ER tubules which were very abundant in axons but are also present in cell bodies and dendrites. These may correspond to the 17 nm diameter tubules induced by recombinant YOP1 or reticulon added to artificial planar membranes (Hu et al., 2008).

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A screen for microtubule-membrane linkers identifies LOM1, an endoplasmic reticulum protein required for nuclear envelope architecture.

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Membrane-bound organelles exhibit a characteristic morphology and distribution within cells, and their regulated positioning and movement is key to the successful execution of fundamental cellular processes such as mitosis and cell polarization. The spatial internal organization of cells

is determined in part by interactions of organelle membranes with the microtubule cytoskeleton and concomitant with microtubule reorganization during mitosis, organelles undergo drastic morphological changes, suggesting that organelle-cytoskeleton contacts are also remodeled. However, a complete inventory of the molecular linkers between cell organelles and microtubules and an understanding of their regulation, e.g. during the cell cycle, are lacking. We developed an unbiased biochemical approach to isolate candidates for organelle-microtubule linker proteins from *Xenopus laevis* egg extracts based on their ability to bind both, cellular membranes and microtubules. Mass spectrometry of recovered proteins revealed several known organelle-microtubule linker proteins as well as a number of candidate LOM (**L**inker of **O**rganelles and **M**icrotubules) proteins that we are now investigating in tissue culture cells. We have found that LOM1, a previously uncharacterized endoplasmic reticulum- and microtubule-associated protein, plays an unexpected and critical role in nuclear envelope homeostasis. LOM1 depletion from HeLa cells by RNA interference (RNAi) resulted in irregularly shaped nuclei, apparently displaying deep invaginations of the nuclear envelope, defects that could be rescued by overexpressing an RNAi-resistant LOM1 construct. Remarkably, during mitosis in LOM1(RNAi) cells the endoplasmic reticulum displayed an aberrant association with the mitotic chromatin. We hypothesize that LOM1 functions to prevent premature access of the endoplasmic reticulum to the mitotic chromatin and thereby ensures correct post-mitotic nuclear envelope formation while in the absence of LOM1, endoplasmic reticulum membrane can get trapped between decondensing chromosomes leading to the irregularly shaped nuclei observed after LOM1 RNAi. These observations suggest that previously unappreciated microtubule-membrane interactions contribute to the generation of proper intracellular architecture during the cell cycle.

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Assembly of an Algal Eyespot.

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The eyespot of *Chlamydomonas* is a photosensory organelle required for phototaxis of the motile, single-celled green alga. The eyespot is assembled near the plus end of one of four bundled microtubule rootlets (D4) at the equator of the cell and comprises both plasma membrane and chloroplast components. Fluorescence microscopy with antibodies directed against acetylated microtubules, the rhodopsin photoreceptor ChR1, a chloroplast envelope protein EYE2, and a chloroplast pigment granule protein EYE3, in wild-type and a variety of mutant cells has led to the model that ChR1 is transported through the endomembrane system to the minus end of the D4 rootlet, and trafficked in the plasma membrane along the acetylated microtubules to the equator of the cell where it promotes assembly of the chloroplast envelope (EYE2) and pigment granule (EYE3) proteins to form the eyespot. The order of protein assembly into the eyespot is being investigated during cell division. In wild-type cells, the nascent D4 rootlet extends from the region of the daughter basal body toward the equator of the cell. Then, a small, oval patch of ChR1 accumulates near the plus end of the rootlet. The ChR1 patch remains near the equator of the cell while the acetylated rootlet extends temporarily to the posterior end of the cell, then regresses back toward the equator. In wild-type cells, EYE2 protein co-localizes with ChR1, but in *mlt1* and *cop3* (ChR1) mutant cells, EYE2 is observed in an oval shape at the proper location in the absence of ChR1. Experiments are under way to more precisely define the timeline of eyespot protein assembly, and to knock out or knock down both ChR1 and the highly similar ChR2 photoreceptor to test the hypothesis that the photoreceptors provide the master cue for eyespot assembly and location.

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GTP binding, but not hydrolysis, is required for the atlastin 'pre-fusion' to 'post-fusion' transition.

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Formation of the branched endoplasmic reticulum (ER) network requires homotypic tethering and fusion of membrane tubules mediated by the membrane-anchored GTPase atlastin. Recent structural studies reveal two dimeric conformers of atlastin proposed to correspond to the tethered, ‘pre-fusion’ state and a ‘post-fusion state’. Though the structures suggest a fusion mechanism, how the ‘pre-fusion’ conformer transitions to the ‘post-fusion’ conformer and how the transition is coupled to GTP binding and hydrolysis are unknown. Here we identify a salt bridge specific to the ‘post-fusion’ conformer to be required. Charge reversal of each individual residue of the interaction pair blocked atlastin-mediated ER network branching, while a compensatory charge reversal to re-establish electrostatic attraction restored function. In vitro assays confirmed that GTP binding and formation of the identified salt bridge is essential for achieving the ‘post-fusion’ state. Surprisingly, nucleotide hydrolysis was not required. Together, these results suggest that GTP hydrolysis may drive the disassembly, rather than assembly, of the atlastin ‘post-fusion’ complex.

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Electron Tomography Reveals that Rab6 Limits Golgi Cisternal Proliferation and the Accumulation of TGN Proximal Coated Vesicles.

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We have shown previously that Rab6, a small trans Golgi localized GTPase, is critical to the maintenance of the juxtanuclear Golgi ribbon in mammalian cells. Rab6 knockdown or overexpression of GDP-Rab6 suppresses Golgi ribbon disruption in response to depletion of either ZW10/RINT1 or COG, structurally related tether complexes (Sun et al., MBoC, 2007). Based on the multiplicity of identified Rab6 effectors, e.g., TMF/ARA160 (cisternal adhesion), myosin IIA (Golgi tubule release), and Bicaudal D (vesicle transport), we hypothesized that an effector-by-effector, candidate protein approach was unlikely to reveal critical, upstream, suppressive effects of Rab6 on Golgi organization. Hence, we took the unbiased, high-resolution technique of electron tomography as a readout for the role of Rab6 in Golgi structure. We found that Rab6-depletion produced profound changes in HeLa cell Golgi cisternal organization including a 60% increase in cisternal number and a >3-fold increase in cisternal length. Consistent with previous observations that Rab6 facilitates anterograde trafficking (Grigoriev et al., 2009; Miserey-Linke et al., 2010), we found that the trans Golgi/trans Golgi network (TGN) was dilated. Unexpectedly, there was a pronounced TGN proximal accumulation of both coated vesicles and arrested budding/fusion structures that we term omega figures. Based on coat appearance and object size, the coated structures, be they free vesicles or omega figures, appeared to fall into two classes, clathrin- and COPI-coated. Omega figures were often located laterally along regions of non-adherent cisternae. The co-accumulation of coated omega figures and coated vesicles may have a common origin in delayed vesicle fusion. We failed to observe any accumulation of uncoated vesicles with Rab6 depletion. In striking contrast when cells were depleted of the Rab6-effector, myosin IIA, we did observe a marked accumulation of uncoated vesicles that were often trans Golgi linked. This outcome is important because it suggests that the role of Rab6 in Golgi organization is dominated by a critical subset of effectors. In addition to Golgi-specific effects, there was a significant accumulation of

multivesicular bodies, apparent autophagosomes, in Rab6 depleted cells. This may be an indirect effect; the S. pombe Rab6 orthologue has been linked to lysosomal stress responses through modulation of the TOR complex 2 (Tatebe et al., 2010). (Supported by a grant from the US National Science Foundation to B. Storrie and a grant from the Australian Medical Research Council to B. J. Marsh and B. Storrie)

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Calsequestrin-1 is required for association but not for targeting of triadin to the junctional sarcoplasmic reticulum membranes.

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Junctional membrane complexes (JMCs) of skeletal muscle cells are formed by two membrane compartments: the sarcolemma / transverse tubules (T-tubules) and the junctional region of the sarcoplasmic reticulum (jSR). These specialized domains, also called triads, are important for allowing the functional coupling between the voltage-operated Ca2+ channels, dihydropyridine receptors (DHPRs), on the sarcolemma / T-tubules and the ryanodine receptors / Ca2+ release channels on the jSR, in order to ensure proper execution of the excitation-contraction coupling mechanism. Different proteins are selectively localized at JMCs, however the specific molecular determinants leading to protein targeting and retention at JMCs are still unknown. To address these questions we expressed wild type and deleted GFP-tagged versions of triadin, a single transmembrane protein of the jSR, in rat primary myotubes. Three regions (named A, B and C) were identified as required for triadin targeting to the jSR. Analysis of protein mobility through FRAP experiments revealed that assembly of triadin into the jSR was accompanied by a significant decrease in protein mobility that was mediated by region C. GST-pull down experiments showed that region C was mainly able to interact with calsequestrin 1 (CSQ1), the major Ca2+ binding protein of the SR. These interactions were also supported by results from FRET experiments in myotubes and in non-muscle cells. Interestingly, experiments with myotubes from CSQ1 knockout mice revealed that, in the absence of CSQ1, triadin was properly localized to the jSR, although its mobile fraction was dramatically increased. These results suggest that CSQ1 is directly involved in retaining triadin to the jSR complexes, whereas protein targeting to the jSR is likely to be mediated by additional interactions with other proteins.

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Trophoblast Cell-Cell Fusion and Dysferlin Expression is Mediated by the PKC Pathway.

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Cell-cell fusion is a cardinal event in the formation of multinucleate syncytia and is part of the normal biology of the syncytiotrophoblast (STB) layer of the human placenta. The STB forms the interface between maternal blood and the placenta and is crucial for fetal development. The STB is formed by fusion of progenitor cells, the cytotrophoblasts (CTB). Dysferlin (DYSF) a putitive plasma membrane repair protein is expressed at high levels at the apical plasma membrane of the STB but not by the CTB. We have utilized the BeWo cell line as a surrogate for the CTB. These cells can be induced to fuse and form syncytia by forskolin (FK), which causes elevation of cyclic AMP (cAMP) and activation of cAMP/protein kinase (PKA). BeWo cells mimic the *in vivo* situation where the CTB does not express DYSF while the STB expresses DYSF. Thus control BeWo cells have little, if any, DYSF, while FK-treated cells fuse and express DYSF. We now show that treatment of BeWo cells with 4β-phorbol 12 myristate 13-acetate (PMA), an activator of protein kinase C (PKC) but not PKA, also induced BeWo cells

to fuse and up-regulated DYSF in a dose and time-dependent manner. These effects were largely blocked by the PKC inhibitor bisindolymaleimide I. An inactive analog of PMA, 4α-phorbol 12-myristate 13-acetate, did not induce BeWo cell fusion or DYSF expression. PMA induced BeWo cell fusion was not dependent upon DYSF expressions, since BeWo cells that do not express DYSF upon cell fusion (DYSF knock down cells) fused when treated with PMA. Interestingly, when BeWo cells were treated with PMA and FK simultaneously there was a synergistic enhancement of DYSF expression. These results indicate that multiple signaling pathways may be involved in regulating both cytotrophoblast fusion and the expression of DYSF in the syncytiotrophoblast.

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Serial Block Face Scanning Electron Microscopy is a novel approach that extends 3D reconstruction of the endoplasmic reticulum to the cellular level.

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In addition to electron tomography (ET) that is effective on subcellular level, other alternative methods that extend the imaging to the cellular level have been developed. New scanning electron microscopy (SEM) techniques are based on automated sequential imaging of a freshly cut block face of resin-embedded specimens using a back scatter detector yielding images resembling TEM images of thin sections. A fresh block face is created by an ultramicrotome inserted in the imaging chamber (SBF-SEM) or by focused ion beam (FIB-SEM). In this work we utilized SBF-SEM technology to study the ER of interphase and naturally mitotic metaphase Huh-7 cells. The use of SBF-SEM allowed us to image and model large portions, up to the whole cell, of ER with the slice thickness of 25-50 nm, which is more than sufficient to resolve all morphological entities of the ER including 60-80 nm fenestrations on sheets and branch points connecting ER sheets and tubules. Progression of early mitotic cells to metaphase showed both dramatic rearrangement of the ER network and structural transformation of ER sheets to smaller and more fenestrated units. As result the mitotic ER forms numeric fenestrated sheets and short tubules arranged as concentric ER layers in the cell cortex. In addition, modeling of the middle parts of the cell revealed the existence of long tubular ER profiles originating from the cortical ER layers and extending towards the center of the cell in alignment with the mitotic spindle.

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A role for Rab10 in Endoplasmic Reticulum network assembly.

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The Endoplasmic Reticulum (ER) is a continuous membrane bound organelle that composes the nuclear envelope and the peripheral ER. The structure of the ER is quite dynamic; during interphase, the ER continuously rearranges its structure while maintaining continuity in a process that requires an active fusion machinery. Very little is known about how homotypic ER fusion is regulated. Recently, the atlastin family of proteins was shown to regulate ER fusion and integrity. Our studies aim to identify additional components that regulate ER fusion and dynamics.

To isolate novel ER fusion machinery components, we used an ER rich *in vitro* system derived from *Xenopus laevis* egg extract. In this system, ER vesicles fuse to form tubular networks in a GTP-dependent manner. We then used this system as a starting material to identify ER derived

GTP-binding proteins that may regulate ER fusion. The purified GTP-binding proteins were identified by Mass Spectrometry. Of the thirteen GTP-binding proteins identified, eleven were Rab proteins. Rab proteins are known to have GTPase functions and have been implicated in membrane fusion of vesicles and organelles. Indeed, previous studies have shown that *in vitro* ER formation is disrupted by the addition of a Rab GDP-dissociation inhibitor (GDI); indicating a Rab could be involved in the GTP-dependent fusion mechanism required for ER network formation. Of the eleven Rab GTPases identified, only one, Rab8/10, had no previously identified function.

We determined that Rab8/10 is tightly associated with ER membranes *in vitro* and can be dissociated from ER membranes by addition of purified RabGDI. In humans there are two *Xenopus* Rab8/10 homologs, Rab8 and Rab10. Rab10 has 99% identity to *Xenopus* Rab8/10, whereas Rab8 has 95% identity. We localized fluorescently tagged Rab8 and Rab10 in mammalian COS cells, and found that Rab10 is unique in its ability to localize strongly to ER membranes. To test a role for Rab10 in ER morphology, we overexpressed mutant constructs of Rab10 in Cos-7 cells. A dominant negative mutant of Rab10 (T23N) changes peripheral ER morphology by converting the tubular ER into sheets. We observed a similar altered ER morphology when endogenous Rab10 was depleted by siRNA from Cos-7 cells. Together, these data suggest a role for Rab10 in the maintenance of tubular ER morphology. Current studies are aimed at identifying the factors that regulate Rab10 binding to the ER.

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Reticulon 4b modulates mammalian Endoplasmic Reticulum.

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Endoplasmic Reticulum (ER) hosts multiple essential cell functions including protein and lipid synthesis, regulation of intracellular calcium concentration and cellular trafficking. Morphologically ER can be divided into peripheral tubules and sheet-like nuclear envelope and perinuclear ER.

Reticulons are a large family of membrane associated proteins sharing a common C-terminal Reticulon Homology Domain (RHD). Previously it has been shown that Reticulon proteins are required to maintain the tubular morphology of ER. Reticulons are inducing membrane curvature by insertion of wedge-shaped RHD to outer ER membrane leaflet and by forming oligomers with each others and several other membrane proteins (DP1/Yop1p/Atlastins). The earliest results demonstrating these events were obtained by expressing neuro-specific exogenous Reticulon 4a in cultured mammalian cells.

Here we show that ubiquitously expressed Reticulon 4 splice variant Rtn4b has similar role in mammalian fibroblast cells. We have used both light microscopy and high resolution electron microscopy to study these morphological changes of ER upon manipulation of Rtn4b expression levels. Depletion of the endogenous Rtn4b via siRNA treatment leads to formation of large peripheral ER sheet structures in Huh-7 cells whereas overexpression of the protein produces long, unbranched tubules. Notably the siRNA treatment does not affect the fenestrations typical for the Huh-7 ER sheets. To better outline these subtle morphological changes we have used electron microscopic 3D data acquisition methods electron tomography and serial blockface scanning electron microscopy.

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Role of Triadin in the Organization of Reticulum Membrane at the Muscle Triad.

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Calcium release from the sarcoplasmic reticulum (SR) triggers skeletal muscle cell contraction and takes place in highly organized membrane structures, called triads, distributed along the muscle fiber. A triad is composed of two SR terminal cisternae surrounding a plasma membrane transverse-tubule. This architecture is essential to sustain the activity of the calcium channel RyR1, which is located in the membrane of SR cisternae. However, little is known about the molecular mechanisms allowing the formation and maintenance of SR terminal cisternae. We have previously shown that ablation of triadin, a SR transmembrane protein interacting with RyR1, induces a modification of the shape of the triads in KO mice. Here we explore the intrinsic molecular properties of the triadin Trisk 95. *We show that when ectopically expressed, Trisk 95 is able to modulate reticulum membrane morphology. The membrane deformations that are induced by Trisk 95 are accompanied by modifications of the microtubule network organization. We provide evidences that the multimerization of Trisk 95 via disulfide bridges, and an indirect interaction with microtubules, are responsible for the ability of Trisk 95 to structure reticulum membrane. When domains responsible for these molecular properties are deleted from Trisk 95, its anchoring to the triads in muscle cells is strongly decreased. Overall, our data suggest that multimerization of Trisk 95 and binding to microtubules contribute to the organization of membranes of the SR in a triad.*

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DNA replication and nuclear membrane growth are coupled to nuclear migration in budding yeast.

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During mitosis of budding yeast, astral microtubule-dependent forces facilitate migration and insertion of the nucleus into the bud neck, the future site of cytokinesis. Subsequently, chromosomes and the nuclear membrane are partitioned between daughters during anaphase spindle elongation. We show here that efficient insertion of chromosomes and the nuclear envelope into the bud neck requires one hand DNA replication and on the other hand nuclear membrane growth. Upon block in DNA replication chromosomes do not migrate into the bud neck. Mutations that cause untethering of chromosomes from the nuclear envelope (NE) facilitate chromosome insertion into the bud in cells with unreplicated chromosomes. In addition, as the nucleus enters the bud prior to anaphase, a NE subdomain becomes anchored to the bud cortex in an actin-dependent manner. Generation of this subdomain requires lipid synthesis, the secretory pathway and the exocyst complex. Exocyst mutations cause nuclear migration defects and display genetic interactions with mutations that deactivate astral microtubule-dependent nuclear migration. Thus, detachment of the chromosomes from the NE and exocyst-dependent anchoring of the NE at the cortex prior to anaphase facilitate correct positioning of the chromosomes and nucleus relative to the cleavage apparatus.

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Yip1A Cycling Between the ER and Golgi is Not Required for its ER Structuring Function.

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The endoplasmic reticulum (ER) in animal cells is a dynamic organelle whose structure is characterized by an interconnecting network of membrane sheets and branching tubules that extends from the nuclear envelope to the cell periphery. We have previously identified Yip1A as a key mediator of ER organization and dispersal. Yip1A depletion in HeLa cells led to dramatic restructuring of the network into multiple, micrometer-sized concentric whorls. Interestingly, Yip1A is not strictly localized to the ER, but rather is rapidly cycling between the ER and cis-Golgi and has been implicated in retrograde trafficking (Kano et al., 2009). In order to dissect Yip1A's ER structuring function away from other proposed roles for the protein in membrane trafficking, we created a Yip1A construct that was localized strictly to the ER. We determined that the cytoplasmic domain of Yip1A was sufficient for export of the protein out of the ER and that the first 83 amino acids were required, with their truncation leading to an ER restricted Yip1A construct. Through a gene replacement assay, we found this ER restricted Yip1A construct to be fully functional in maintaining ER network dispersal. This suggests that Yip1A cycling between the ER and Golgi is not required and that Yip1A is playing a direct role in structuring the ER. Current work includes alanine scanning the rest of the protein to identify critical residues in the transmembrane domain.

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ARL4A acts with GCC185 and CLASPs to modulate Golgi-derived microtubules and Golgi organization.

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Golgi apparatus is essential for fundamental cellular functions. The structural integrity of the Golgi complex is known to be dependent upon microtubule-Golgi interaction and microtubule polymerization. The trans-Golgi network golgin GCC185 has been demonstrated to interact with the CLASP family of microtubule-binding proteins to recruit them to the Golgi, providing a microtubule-Golgi interaction and enhancing the elongation of Golgi-derived microtubules for Golgi maintenance. We show here that a less characterized ARL protein, ARL4A, acts with GCC185 to modulate Golgi-derived microtubules and Golgi organization. ARL4A directly interacts with GCC185 in a GTP-dependent manner. Sub-coiled-coil regions of GCC185's CC2 domain are required for the interaction between GCC185 and ARL4A. Depletion of ARL4A reproduces the GCC185-depleted phenotype, causing fragmentation of the Golgi compartment. GCC185 and ARL4A localize to the Golgi independent of each other. Deletion of the ARL4A-interacting region of GCC185 results in inability to maintain Golgi structure. Depletion of ARL4A impairs GCC185-CLASP interaction in vivo and abolishes the GCC185-mediated Golgi recruitment of CLASPs. Nocodazole washout experiments indicated that depletion of ARL4A, GCC185, or CLASPs impair the elongation of Golgi-derived microtubules and Golgi reassembly. In summary, we infer that ARL4A may alter integrity of Golgi structure by facilitating interaction of GCC185 with CLASPs, elongation of Golgi-derived microtubules, and Golgi reassembly.

Nucleocytoplasmic Transport

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Function of mRNA binding by TREX2 components Sac3 and Thp1 in integrating transcription, processing and nuclear export.

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Nuclear export of messenger RNA requires formation of transport-competent mRNPs. TREX-2, a conserved TRanscription-EXport complex, coordinates transcriptional processes with the recruitment of export factors to nascent mRNA. Here, we report the crystal structure of a key TREX-2 segment, consisting of Sac3 residues 253-551 bound to Thp1 and Sem1. Both Sac3 and Thp1 have PCI folds based on a stack of α-helices, capped by a winged helix domain. Importantly, the juxtaposition of the Sac3 and Thp1 winged helix domains within TREX-2 generates a platform that mediates binding of nucleic acids. Structure-guided mutations in Sac3 and Thp1 underline the importance of the Thp1-Sac3 interaction and nucleic acid binding in coupling transcription with mRNP assembly/export. Overall, the structure of the Sac3-Thp1-Sem1 complex provides a structural basis for understanding how the TREX-2 complex facilitates the formation of export-competent mRNPs at the nuclear face of nuclear pores, and thereby provides a “fast track” for the nuclear export of a subset of transcripts such as those from the GAL system. The close juxtaposition of the winged helix domains of Sac3 and Thp1 is critical for binding mRNA and thereby bringing it in close apposition to the Mex67-Mtr2 export factor. This, in turn, facilitates a remodeling of the mRNP that displaces Yra1 while attaching Mex67-Mtr2 more strongly. Sem1 functions to stabilize Thp1 and probably functions in an analogous way in other complexes that contain PCI folds.

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Etoposide-induced protein Ei24 is a novel negative regulator of nuclear import.

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Etoposide-induced mRNA 2.4kb (Ei24) was initially identified as a p53-induced pro-apoptotic gene, but a function for the encoded protein has yet to be described. Intriguingly, amino acid residues 44-94 of Ei24 share significant homology with the region of Importin (IMP)-α2 that binds IMPβ1, the IMP-β binding (IBB) domain, which we designated the IBB-like (IBBL) domain. Pulldowns from transfected HeLa cells indicate that GFP-Ei24 can bind specifically to endogenously expressed IMPα2 and IMPβ1, but not IMPα3. Additionally, Ei24 is able to reduce the nuclear accumulation of proteins dependent on IMPα2/β1 (e.g. p53) or IMPβ1 (e.g. telomeric-repeat binding factor 1) for nuclear import in cotransfected HeLa cells (overexpressing BclXL), as determined by quantitative confocal laser scanning microscopic analysis. In contrast, mutation of specific arginine residues to alanine within Ei24’s IBBL domain relieves Ei24’s inhibitory activity and does not affect the nuclear import of IMP-dependent cargoes. This is the first report that an endoplasmic reticulum (ER)-localised protein, Ei24, can negatively regulate nuclear transport globally by sequestering IMPβ1 and possibly IMPαs at the ER and thereby reduce the import of nuclear localizing proteins, with implications for critical cellular processes such as proliferation, differentiation and development. Since mutations in Ei24 are linked to

breast cancer, deregulation of Ei24 localization and/or activity clearly may be detrimental to normal cellular function and thus contribute significantly to oncogenesis.

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Simulating nuclear pore transport: bridging the gap between qualitative theories and high precision experiments.

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Various theories attempt to explain selective transport through the nuclear pore complex. These theories lag in biophysical detail when compared with the current state of experiment that includes measurements of cargo transport at the single molecule level. Thus, progress in resolving between them is limited by the qualitative way in which these ideas have been formulated.
Our approach is to develop a computational simulation that is based on known structural elements rather than a particular transport theory. The results agree with a variety of experimental data including size cutoff for cargo transport with (30-nm diameter) and without (<10 nm) nuclear localization signals (NLS), macroscopic transport rates (hundreds per second), and single cargo transit times (milliseconds). The recently observed bimodal cargo distribution is predicted, as is the relative invariance of single cargo transit times out to large size (even as macroscopic transport rate decreases). Additional predictions concern the effects of the number of NLS tags, the RanGTP gradient, and phenylalanine-glycine nucleopore protein (FG-Nup) structure, flexibility, and cross-linking.
Significantly, results are consistent with and elucidate the molecular mechanisms of some existing theories (selective phase, virtual gate, and selective gate models). In effect, these results begin to bridge the gap between the current qualitative state of existing ideas of transport and the high precision experiments that elucidate transport at the single molecule level. Through our simulations, a model emerges that is a hybrid of a number of preexisting models as well as a Brownian ratchet model, in which a cargo-karyopherin complex remains bound to the same FG-Nups for its entire trajectory through the nuclear pore complex until RanGTP severs the cargo-Nup bonds to effect release into the nucleus.

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CRM1-independent Nuclear Export of the Thyroid Hormone Receptor is Mediated by Exportin 5.

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Thyroid hormone receptor α (TRα) and thyroid hormone receptor β (TRβ) are nuclear receptors that bind to thyroid hormone to activate or repress target genes involved in metabolism, growth, and development. Although primarily found in the nucleus, TRα and TRβ rapidly shuttle in and out of the nucleus through the nuclear pore complex. Previously, we showed that TR nuclear export is not completely blocked when cells are treated with leptomycin B to inhibit the export factor CRM1, suggesting that TR can also exit the nucleus by a CRM1-independent pathway. To determine which export factors are involved in the CRM1-independent pathway, RNA interference was used to knockdown gene expression of transportin 1, transportin 2, exportin 5, and exportin 6. The effect of knockdown on the nucleocytoplasmic distribution of GFP-tagged TRα and TRβ was assessed in live HeLa cells using fluorescence recovery after photobleaching (FRAP). Knockdown of exportin 5 altered TRs nuclear export dynamics; recovery was markedly slower in photobleached nuclei, indicating that nuclear export was inhibited. To determine whether increased nuclear export had an impact on TR-mediated gene expression, we co-

expressed TRα and TRβ, exportin 5, and a thyroid hormone response element (TRE)-mediated CAT reporter gene. CAT ELISA showed a decrease of TRE-mediated CAT reporter gene expression when increased amounts of exportin 5 were present. Further, we showed that when exportin 5 is over-expressed, the distribution of TR shifts to a more cytoplasmic localization. Taken together, our data suggest that TR nuclear export is mediated, in part, by exportin 5, and that disrupting the fine balance between nuclear import and export can lead to changes in TR-mediated gene expression.

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NLSs of Influenza nucleoprotein as novel targets for antiviral.

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Influenza A virus poses a serious threat to world public health, particularly the pandemic H1N1 and avian H5N1. Currently, the major anti-influenza strategies are vaccination and antiviral drugs. However, both of these approaches target structural components of the virus which undergo mutation and become resistant to these antiviral approaches. An alternative way to inhibiting influenza infection is to interfere with the viral entry into the nucleus, since this process is necessary for viral replication. So far, there are at least two stretches of amino acid sequences located on influenza nucleoprotein (NP) that are know to mediate the nuclear import of the viral ribonucleoproteins (vRNPs) containing the viral genome: NLS1 at the N-terminus of NP, and NLS2 spanning residues 198-216. Sequence alignment shows both NLSs on NP are highly conserved between different strains of influenza A, suggesting that NLSs are ideal candidate for novel antiviral approaches. To assess this, we are first defining the contribution of the NLSs of NP to nuclear import. The functional role of NLS1 has been very well characterized in previous studies. The specific role of the NLS2 is, however, ill-defined. In our study, we generated chimeric protein by fusing NLS1 or NLS2 to a heterologous protein and characterize the contribution of these NLSs to nuclear import. Our results showed that NLS2 renders a weak nuclear import behavior compared to NLS1. Interestingly, with only one basic amino acid difference (lysine to arginine) the NLS2 of seasonal flu (H1N1 and H5N1) contributes stronger to the nuclear import of the chimera protein than the NLS2 from pandemic H1N1 and avian H5N1. Using site-directed mutagenesis we further analyzed the contribution of the NLS2's basic amino acids to the nuclear import of the chimera protein. Our results suggest that NLS2 from pandemic H1N1 and avian H5N1 function as a classical bipartite NLS. However, NLS2 from seasonal flu (H1N1 and H5N1) behaves as a monopartite NLS instead of a bipartite NLS. By performing competition assays using NLS1 and NLS2 chimeras in infected cells, we found that the competing NLSs were able to successfully delayed infection of influenza A virus. This finding clearly indicates that a good strategy to employ in the development of new influenza antiviral drugs is to interfere with the function of NLSs of influenza NP.

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Remodeling of ribonucleoprotein complexes during export through the nuclear pore complex.

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Export of ribonucleoprotein (RNP) complexes from the nucleus requires multiple essential factors. Specific transport factors recognize the cargo and facilitate docking and translocation through the nuclear pore complex (NPC). These transport factors require interaction with a class of NPC proteins termed FG Nups for export. In addition, release of the transport factor from the RNP and changes in the RNP protein composition are key to conferring directional exit. For mRNA export, stimulation of the RNA-dependent ATPase Dbp5 directs transport directionality.

A key step in the Dbp5 cycle is the remodeling of the mRNP to release the general mRNA transport factor Mex67-Mtr2.

Ribosomal subunits are among the largest cargo transported through the NPC. Others have shown that the fully-assembled 60S subunit binds multiple different transport factors to allow efficient nuclear export. This includes a role for Mex67-Mtr2 and several others – Arx1, Ecm1, and the karyopherin Crm1. To determine whether Dbp5 is also responsible for removing Mex67-Mtr2 from 60S subunits, we generated and tested a series of double mutants with *dbp5* and alleles of genes encoding other reported ribosomal export factors. Strikingly, genetic interactions were observed between a *dbp5* mutant and mutants of ribosome export factors. Additionally, the *dbp5*^{ts} mutant accumulates 60S subunits in the nucleus. *In vitro* reconstitution experiments to assess remodeling are currently underway. These experiments suggest that Dbp5-mediated removal of Mex67-Mtr2 at terminal steps of NPC translocation is conserved between mRNA and 60S ribosomal export.

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Karyopherin-independent nuclear transport of large cytoskeletal proteins.

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The nucleocytoplasmic shuttling of molecules is an essential mechanism for eukaryotic cells to maintain proper cellular functions. The nucleoplasm is separated from the cytoplasm by the nuclear envelope, which harbors the nuclear pore complex (NPC) as a selective channel/barrier for macromolecules. In recent years, the presence and the functions of cytoskeletal proteins in the nucleus is becoming one of the active areas of research. In this study, we report the followings: (i) Some of the nuclear-localizing cytoskeletal proteins are much larger than the size limitation of the passive diffusion through the NPC and do not contain any classical nuclear localization signals (NLS). (ii) Analyses of the nuclear transport mechanisms using digitonin-permeabilized HeLa cells revealed that several large cytoskeletal proteins, including actinin-4, beta-I spectrin, and beta-catenin, can pass through the NPC in a karyopherin-independent manner. (iii) In contrast, a hydrophilic molecule, 70kDa dextran, was completely excluded from the nucleus. Considering their amphiphilic molecular structures, these cytoskeletal proteins may adapt their structure to the hydrophilic environment inside the NPC and spontaneously migrate into the nucleus without a help of karyopherins. (iv) The potential role of amphiphilic structural motif in nuclear targeting was revealed both *in vivo* and *in vitro*. When GFP-fused fragments containing several tandem arrays of spectrin-repeat, a structural motif found in actinin, spectrin, and dystrophin, were expressed in HeLa cells, the GFP signals were found in the nucleus. (v) The same GFP-fragments also exhibited the nuclear targeting properties by *in vitro* nuclear transport assay, suggesting the role of amphiphilic motif in nuclear targeting.

These findings demonstrate the dynamic nucleocytoplasmic shuttling of cytoskeletal giant proteins in a karyopherin-independent manner and suggest that size, shape, and hydrophobicity of the molecule are the possible determinants for the molecular transport through the NPC. We propose such spontaneous migration of large non-NLS proteins into the nucleus as an alternative mechanism of the nuclear transport which is mediated by amphiphilic structural motifs in the molecule.

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Investigating the mechanism and function of nuclear size regulation during *Xenopus laevis* development.

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The size of the nucleus varies with cell type, developmental stage, and organism, yet the ratio of nuclear to cytoplasmic volume is tightly controlled in most systems. Altered nuclear size, nucleocytoplasmic ratio, or morphology is characteristic of many cancers, but the significance of nuclear size regulation for cell physiology is not understood. We are using the frog *Xenopus laevis* to study mechanisms of nuclear size control during development, and to determine the functional consequences of altering nuclear size in vivo. Nuclear size scales smaller during the rapid cleavages of early *X. laevis* embryos, both prior to the midblastula transition (MBT) and post-MBT. We showed previously that reductions in nuclear protein import regulate pre-MBT nuclear size, but how post-MBT nuclear size is determined is unknown. Interestingly, the MBT is marked by many changes including extension of cell cycle length, loss of cell division synchrony, and upregulation of zygotic transcription. To test whether changes occurring at the MBT alter nuclear size, we examined the role of nuclear export, which is upregulated at the MBT. Treating embryos or embryo extracts with a small molecule inhibitor of the major nuclear export factor Crm1 had no effect on nuclear size. However, large pre-MBT embryo nuclei shrank when incubated in post-MBT embryo extract, concomitant with loss of nuclear lamin B but no change in the number of nuclear pore complexes, suggesting that active remodeling of the nuclear lamina contributes to post-MBT nuclear size changes. We are currently testing whether changes in the composition or structure of nuclear and ER membranes regulate post-MBT nuclear size. Elegant work has shown that the timing of MBT correlates with the nucleocytoplasmic ratio. To test whether changing nuclear size alters the MBT, we injected one blastomere of a two-cell embryo with mRNA encoding importin α or Ntf2, factors we have shown to increase or decrease nuclear size, respectively. Embryo halves injected with importin α exhibited an apparently accelerated MBT as evidenced by premature slowing of cell divisions relative to the uninjected side, while Ntf2 injection delayed MBT. Nuclear size differences within the embryo persisted in tadpoles, whose tails consistently bent toward the side of the embryo with smaller nuclei. These results indicate that nuclear size influences cell division timing and patterning in the developing embryo. We are currently investigating how nuclear size correlates with the expression timing of different developmental markers and devising more assays to correlate nuclear size and cell function.

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Defining selective mRNA export mechanisms in response to stress in Saccharomyces cerevisiae.

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Eukaryotic cells respond to major cytotoxic environmental stress through dramatic alterations in gene expression. An essential process that regulates these changes in the gene expression pathway is the export of messenger RNA (mRNA) from the nucleus to the cytoplasm. As mRNA is synthesized, the transcript is packaged with proteins to form messenger ribonucleoprotein particles (mRNPs). The protein composition of these mRNPs is highly dynamic, characterized by the association and dissociation of specific proteins that mediate efficient mRNA translocation through nuclear pore complexes (NPCs) and proper gene expression under changing environmental stimuli. One fundamental cellular response pathway in the budding yeast *Saccharomyces cerevisiae* is the heat shock stress response. During the heat shock

response, transcription and export of specific heat shock-induced mRNAs are upregulated while non-heat shock-induced mRNAs are retained in the nucleus, liberating the translation machinery for synthesis of heat shock proteins. The precise molecular determinants that control the selective mRNA export of heat shock mRNPs remain largely uncharacterized.
Several key factors implicated in non-heat shock mRNA export are also essential for the export of heat shock mRNAs. One of these factors, mRNA export receptor Mex67, requires adaptor proteins to facilitate its efficient binding to mRNA; however, each of its previously identified adaptors (Yra1, Nab2, and Npl3) is dispensable for the export of heat shock mRNAs. To identify potential adaptors for Mex67 function in the heat shock mRNA export pathway, we isolated Mex67-associated mRNPs under heat shock and non-heat shock conditions. The composition of these purified complexes was examined by mass spectrometry and Pab1 was identified as a potential heat shock-specific Mex67 interaction partner. Using an *in vivo* [^{35}S]methionine-labeling assay, no defect in the production of heat shock proteins was observed in a panel of *pab1* deletion mutants encoding changes within the RNA binding domains. Postulating that Nab2 may compensate for the loss of Pab1 function, we generated *nab2 pab1* double mutants. Indeed, enhanced temperature sensitivity was observed, indicating a strong genetic interaction between Nab2 and Pab1. Our current work is focused on further characterizing the heat shock response by examining the roles of Nab2 and Pab1 in heat shock mRNA export as well as by isolating heat shock specific mRNPs. Together, these strategies aim to reveal the mechanism for Mex67-mediated export of heat shock mRNA.

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Nuclear trafficking of Dystrophin Dp71.

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Dystrophin Dp71 is the major product of the Duchenne muscular dystrophy gene (DMD); however, its neuronal function is poorly understood. We have previously adopted the PC12 cell line as in vitro neuronal model for studying Dp71 function. These cells express two Dp71 splicing isoforms; both of them spliced out for exon 71 and with exon 78 either present (Dp71d) or spliced out (Dp71f). While the lack of exon 71 do not change the reading frame, the loss of exon 78 does, resulting in the replacement of the last 13 C-terminal hydrophilic amino acids of dystrophin with 31 new hydrophobic amino acids in the Dp71 protein. It seems that alternative splicing regulates the localization of Dp71; while Dp71f is distributed solely in the cytoplasm, Dp71d exhibits a predominant nuclear localization. In this study we revealed that the ZZ domain of Dp71d is sufficient to drive the nuclear import of Dp71d, suggesting that this motif might function as nuclear localization signal (NLS). Interestingly, we found that leptomycin B (LMB)-mediated nuclear export inhibition resulted in accumulation of both Dp71d and Dp71f, which indicates that Dp71f, previously regarded as exclusive cytoplasmic protein, undergoes indeed a transient nuclear import. Considering that Dp71f differs from Dp71d by the presence of 31 hydrophobic amino acids in the carboxy-terminus, we hypothesized that this motif contains a nuclear export signal that export Dp71f efficiently from the nucleus to the cytoplasm. Further studies are required to test this hypothesis.

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Structural insights into the function of Yrb2, a cofactor that facilitates CRM1-mediated nuclear export.

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The karyopherin CRM1 mediates nuclear export of cargo macromolecules containing a leucine-rich nuclear export signal (NES). CRM1 forms a ternary complex with cargo and RanGTP in the

nucleus. This complex translocates across the nuclear pores and dissociates in the cytoplasm. The yeast protein Yrb2 is a nuclear protein containing nucleoporin-like FG repeats and a Ran-binding domain (RanBD) that is homologous to RanBP1. However, Yrb2 binds RanGTP only very weakly, unlike RanBP1 that binds RanGTP with high affinity and functions as a cytoplasmic disassembly factor for the CRM1-cargo-RanGTP complex. The null allele of Yrb2 is cold sensitive and deficient in NES-dependent nuclear export. Here we show that Yrb2 dramatically increases the rate of association of CRM1 and cargo in the presence of RanGTP. Both FG-repeats and RanBD of Yrb2 are required to increase the on-rate of cargo. Furthermore, we determined a 2.3 angstrom-resolution crystal structure of CRM1-Yrb2-RanGTP complex. The structure revealed FG-repeats binding sites on the outer surface of CRM1, and structure-based mutagenesis indicated that these sites are also the binding sites for FG-nucleoporins. The structure of CRM1-Yrb2-RanGTP complex is similar to the previously determined structure of CRM1-RanBP1-RanGTP complex in that the HEAT9 loop is bound to the inner surface of CRM1, keeping the NES-binding cleft closed. However, because the RanGTP-RanBD interactions are very weak, the dissociation of RanBD coupled with the opening up of the NES-binding cleft would be expected to occur easily. Once NES is bound to CRM1, re-binding of RanBD is inhibited, and this would in turn facilitate dissociation of FG-repeats of Yrb2. Based on the biochemical and structural data, we propose a two-step model in which Yrb2 functions catalytically as a coordinating scaffold to increase the efficiency of CRM1-mediated nuclear export: (i) The FG-repeats and RanBD of Yrb2 recruit CRM1 and RanGTP, forming a ternary CRM1-Yrb2-RanGTP complex. By occluding the FG-repeats binding sites, Yrb2 could prevent cargo-free CRM1 from docking at and moving through the nuclear pores, inhibiting futile export of cargo-free CRM1. (ii) The binding of NES to CRM1 is associated with the dissociation of Yrb2, forming a CRM1-cargo-RanGTP complex that has FG-repeats binding sites exposed and so can translocate across the nuclear pores. The dissociated Yrb2 would then participate in another round of the rapid assembly of export-competent complex in the nucleus.

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Characterization of the nuclear import pathway for the baculovirus AcMNPV.

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Some animal viruses have been engineered to deliver genes into an individual's cells and tissues in the process of gene therapy to help treat diseases. For genes to be expressed, the viral vector containing the gene must successfully enter the nucleus of the recipient's cell. This process is often limited by cellular proteins and structures that block the accessibility of the nucleus. Baculovirus is a great example of an animal virus that is being developed for gene therapy, yet the mechanism this virus uses to enter the nucleus remains uncharacterized. Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the archetype of the Baculoviridae family, is an arthropod-specific, enveloped, rod-shaped virus with circular double-stranded DNA genome that replicates in the nucleus of its host insect cells. Using electron microscopy we demonstrated that baculovirus nucleocapsids remain fully intact while entering the nucleus through nuclear pore complexes (NPCs). We have used the well-established digitonin permeabilized cell nuclear import assay to characterize in more detail the nuclear import of the baculovirus nucleocapsid. With this assay, we used fluorescently-tagged viral nucleocapsids and anti-nucleocapsid antibody, to determine the fate of the imported nuclocapsids visualized by fluorescence microscopy under different conditions (with and without energy, with and without cytosolic components, low temperature). Using this system, we observed that nuclear import of AcMNPV nucleocapsids in HeLa cells occur with or without energy and cytosolic components. Interestingly, nuclear import in its host insect cells took place

only in the presence of cytosolic components extracted from insect cells and an energy regenerating source. Our data suggests that the ability and efficiency of nuclear import of AcMNPV nucleocapsids differs depending on the cell type. Furthermore, we aim to deplete proteins on NPCs using siRNA to determine the role of these proteins in the nuclear import of AcMNPV nucleocapsids. Our study will elucidate cellular proteins vital to the nuclear import of baculovirus, thereby promoting different factors that can enhance the delivery of this virus into the nucleus. These results will aid in designing baculovirus as an efficient viral vector for gene delivery.

Nuclear Lamins

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Role of the ZMPSTE24 Protease in Lamin A Processing and Progeroid Disorders.

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The post-translational processing of the nuclear scaffold protein lamin A plays a critical role in human health and disease. Lamin A is first synthesized as a precursor protein that undergoes a set of sequential modifications, including farnesylation, proteolysis and carboxylmethylation of the C-terminal CAAX motif. The prenylated and methylated tail is then cleaved by the Zn^{2+} metalloprotease ZMPSTE24 to yield mature lamin A. While the purpose of removing the elaborately modified lamin A tail remains unclear, failure to do so underlies several human progeroid disorders, including the premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS), and may be relevant to normal aging as well. Eighteen distinct human ZMPSTE24 mutations have currently been identified that result in the progeroid disorders MAD-B, atypical progeria, and restrictive dermopathy (RD). Interestingly, diseases of the greatest severity correlate with higher amounts of uncleaved, persistently modified prelamin A. We have begun to characterize this critical enzymatic event by determining the features of both the ZMPSTE24 enzyme, and its substrate prelamin A, that are important for efficient cleavage of the lamin A tail. We have utilized activity assays to characterize human disease alleles of ZMPSTE24 that are predicted to retain partial activity, and we are developing an *in vitro* cleavage assay to better characterize the enzymatic mechanism. With regard to the lamin A substrate, we have determined that ZMPSTE24 recognition elements are completely contained within a small 41 amino acid C-terminal region. We have identified several contextual requirements within this region that include, in addition to the farnesylated cysteine, an amino acid preference in the region surrounding the cleavage site. Interestingly, increasing the distance between the farnesyl-cysteine and the cleavage site reduces the ability of ZMPSTE24 to cleave, suggesting a spatial requirement for enzyme binding and/or cleavage to occur. Our studies are uncovering important features of an enzyme whose activity is vital to human health and longevity.

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Mitotic Defects and Pervasive Aneuploidy Accompany Loss of RB1 Activity in Mouse *Lmna^{Dhe}* Dermal Fibroblasts.

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Lamin A (*LMNA*) is a component of the nuclear lamina and is mutated in several human diseases, including Emery-Dreifuss muscular dystrophy and the premature aging syndrome Hutchinson-Gilford progeria syndrome. Cells from progeria patients exhibit cell cycle defects in

both interphase and mitosis. Mouse models with loss of LMNA function have reduced Retinoblastoma protein (RB1) activity, leading to aberrant cell cycle control in interphase, but how mitosis is affected by LMNA is not well understood. Cell cycle and structural phenotypes were examined in cells from mice with the *Lmna* allele, Disheveled hair and ears (*Lmna*^{Dhe}). Dermal fibroblasts from heterozygous *Lmna*^{Dhe} (*Lmna*$^{Dhe/+}$) mice exhibited many phenotypes of human laminopathy cells. These included severe perturbations to the nuclear shape and lamina, increased DNA damage, and slow growth rates due to mitotic delay. Interestingly, *Lmna*$^{Dhe/+}$ fibroblasts had reduced levels of hypophosphorylated RB1 and the non-SMC condensin II-subunit D3 (NCAP-D3), a mitosis specific centromere condensin subunit that depends on RB1 activity. Mitotic check point control by mitotic arrest deficient-like 1 (MAD2L1) also was perturbed in *Lmna*$^{Dhe/+}$ cells. *Lmna*$^{Dhe/+}$ fibroblasts were consistently aneuploid and had higher levels of micronuclei and anaphase bridges than normal fibroblasts, consistent with chromosome segregation defects. These data suggest that the effects of LMNA on RB1 include both interphase and mitotic cell cycle control, supporting a key role for RB1 in the phenotypes of laminopathy-related cells.

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Lamin-A/C is a nuclear rheostat that scales with tissue rigidity and modulates cell lineage.

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Lamin proteins of the nuclear envelope are expressed throughout the body, but lamin mutants such as those causing progeria syndrome often affect specific solid tissues. Here, the relative quantities of A/C and B-type lamins are measured as components of the nuclear proteome in a range of normal adult tissues using novel, label-free tandem mass spectrometry quantitation corroborated by immunoblotting. Cells derived from soft and stiff tissues are also micromanipulated and shown to have soft or stiff nuclei that reflect the levels of Lamin-A/C. Brain tissue, which is unaffected in progeria, has an elasticity about 10-fold softer than striated muscle and has proportionately lower levels of A-type lamins – about 40% of the level of B-type lamins that are uniformly expressed among tissues. Defects in muscle, cartilage and bone feature more prominently in progeria, and nuclei from these increasingly stiff tissues have increasing levels of A-type lamin. Manipulating Lamin-A/C expression in stem cells also directs cell lineage with knockdown favoring softer tissue cells. Stress-driven changes in nuclear protein structures are further revealed by differences in the covalent tagging of cysteines during application of mechanical shear stress. Thus we show that the nucleus is capable of physically transducing stress.

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Lamin mutations disturb actin organization and nuclear translocation of MRTF-A.

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Mutations in the LMNA gene encoding the ubiquitously expressed nuclear envelope protein lamins A and C cause Emery-Dreifuss muscular dystrophy (EDMD), dilated cardiomyopathy, and a broad spectrum of other diseases (laminopathies). The disease mechanism remains unclear, but the muscle-specific phenotypes in many of the laminopathies suggest a mechanical and/or tissue-specific transcriptional component to the disease mechanism. Myocardin-related transcription factor A (MRTF-A) was recently identified as an important player in cellular mechanosensing, responding to changes in actin polymerization by translocating to the nucleus, where it acts as co-activator for serum response factor (SRF).

OBJECTIVES: To test the hypothesis that lamin A mutations associated with muscular laminopathies can impair MRTF-A signalling and thereby contribute to muscle dysfunction in EDMD and dilated cardiomyopathy and to identify possible mechanisms.

METHODS: Using time lapse imaging, immunostaining, and other biochemical approaches, we examined MRTF-localization and signalling in mouse embryo fibroblasts (MEFs) from lamin A/C–null mice, animals carrying a lamin mutation causing dilated cardiomyopathy (Lmna N195K), and wild-type littermates. Immunostaining of skeletal and cardiac tissue sections were used to confirm MRTF-A localization in vivo.

RESULTS: Nuclear translocation of MRTF-A in response to serum stimulation was almost completely abrogated in lamin A/C–null and Lmna N195K mutant MEFs, and these cells had impaired activation of SRF-target genes. Consistent with the in vitro findings, nuclear localization of MRTF-A was significantly reduced in skeletal and cardiac muscle sections of lamin A/C–null and Lmna N195K mutant mice. The nuclear translocation defect was associated with altered actin polymerization dynamics in the lamin mutant cells. Since emerin, a nuclear envelope protein, was recently shown to promote actin polymerization and is mislocalized in lamin A/C mutant cells, we examined whether emerin could be involved in the observed MRTF-A translocation defect by altering nuclear or cytoskeletal actin dynamics. Importantly, emerin-null MEFs displayed similar defects in actin organization and MRTF-A translocation as the lamin mutant cells, and ectopic expression of emerin rescued the defects in emerin-null and lamin mutant MEFs.

CONCLUSIONS: We have identified a new mechanism by which lamins A and C, likely mediated by emerin, can modulate actin polymerization and thereby alter activity of MRTF-A and SRF-dependent genes, providing a possible explanation for the muscle-specific phenotypes in several laminopathies.

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The Roles of Nuclear Lamin B1 in Cell Proliferation, Longevity and Senescence.

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The A- and B-type nuclear lamins (LA/C, LB1 and LB2) are the major components of the nuclear lamina, forming separate but interconnecting meshwork structures (Shimi et al., 2008). Numerous mutations in the human LA/C gene cause the rare premature aging disease Hutchinson–Gilford progeria syndrome (HGPS). Interestingly, fibroblasts from several HGPS patients had an early onset of replicative senescence in culture, and LB1 expression in these cells was significantly decreased during senescence (Taimen et al., 2009). We have investigated the physiological significance of this loss of LB1 expression in normal diploid WI-38 cells during normal senescence by immunofluorescence, quantitative immunoblotting and qRT-PCR. The expression of LA/C and LB2 remains unchanged during spontaneous senescence progression, but LB1 protein expression and LB1 mRNA decrease dramatically as cells approach and become senescent. The inactivation of the p53 and pRb pathways in LB1 silenced cells have revealed that premature senescence induced by oncogenic stress also decreases LB1 expression through a pRb-dependent mechanism. Furthermore, silencing LB1 expression using shRNA slowed cell proliferation and induced rapid premature senescence. We have studied the p53 and pRb pathways for this LB1 silencing effect by the inactivation of these

pathways and showed that the proliferation defects induced by LB1 silencing require the activation of p53 but not pRb. These results indicate that LB1 functions in proliferation are upstream of the p53 and pRb pathways. Interestingly, mitochondrial ROS production is reduced by LB1 silencing. This reduction is p53-dependent and can be rescued by growth under hypoxic conditions. Furthermore, our analyses of qRT-PCR arrays have indicated that several genes upstream of p53 and some p53 target genes are altered in expression. In contrast to the effects of LB1 silencing, the over-expression of LB1 increases both the rate of cell proliferation and longevity. This over-expression of LB1 causes cell cycle arrest at the G1/S boundary and hyperphosphorylation of pRb. These results demonstrate the importance of LB1 in regulating the proliferation and longevity of human diploid cells through a p53-ROS signaling pathway. Supported by the NCI and the Progeria Research Foundation.

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Cardiac consequences of ERK1/2 phosphorylation in the Lmna-/- mouse model.

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Mice lacking the *Lmna* gene (*Lmna*$^{-/-}$), a nuclear scaffold protein, display multiple tissue defects and die by 8 weeks of age from dilated cardiomyopathy with associated conduction defects. We sought to further characterize the cardiomyopathic phenotype and determine whether restoration of a cardiac-specific *Lmna* transgene (Tg) in a *Lmna*$^{-/-}$ mouse would specifically rescue its cardiac pathology. In order to achieve this, we crossed *Lmna*$^{+/-}$ mice (Sullivan et al., 1999) with mice overexpressing human wild-type lamin A driven by the heart-specific α-myosin heavy chain promoter (Wang et al., 2006). We measured the cardiac performance of the resulting *Lmna*$^{-/-}$; Tg mice by echocardiogram and electrocardiogram as well as performed histopathological and biochemical analyses on heart tissue.

We observed increased activating phosphorylation of ERK1/2 in hearts of *Lmna*$^{-/-}$ mice consistent with previous studies using the *Lmna*$^{H222P/H222P}$ knock-in mouse model for Emery-Dreifuss muscular dystrophy. We also observed an increased heart weight to body weight ratio in *Lmna*$^{-/-}$ mice consistent with findings that show ERK1/2 to be an important regulator of cardiac hypertrophy. *Lmna* transgene expression resulted in attenuated ERK1/2 phosphorylation relative to *Lmna*$^{-/-}$ and a corresponding partial rescue of the hypertrophy. *Lmna*$^{-/-}$; Tg hearts also display significantly increased contractility and preservation of myocardial performance compared to *Lmna*$^{-/-}$ hearts as measured by echocardiogram.

Connexin43 (Cx43), the protein primarily comprising gap junctions in the heart, is a known target for phosphorylation by ERK1/2 resulting in decreased permeability and function of the gap junction. We observed that Cx43 levels at the intercalated disc were significantly decreased in *Lmna*$^{-/-}$ hearts and partially improved in *Lmna*$^{-/-}$; Tg mice. Electrocardiography revealed arrhythmic events and increased frequency of PR interval prolongation in *Lmna*$^{-/-}$ mice, which was also partially rescued in *Lmna*$^{-/-}$; Tg mice. Ultimately, we achieved a 12% median extension in lifespan in *Lmna*$^{-/-}$; Tg mice compared to *Lmna*$^{-/-}$ mice.

We have shown that the absence of lamin A in the heart is lifespan-limiting and results in a cardiomyopathic phenotype likely due to downstream effects of ERK1/2 phosphorylation causing both hypertrophy and conduction defects. We plan to further investigate the conduction aspect of this phenotype by determining the phosphorylation state of three ERK1/2-specific serine residues of Cx43 (S255, S279, S282), and potentially others, in *Lmna*$^{-/-}$ hearts and

cardiomyocytes. In addition, we hope to determine whether the expression of a Cx43 knock-in mutant (S255A/S279A/S282A) will rescue the defects observed in the conduction system.

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Compression-induced changes in chromatin movement and nucleoskeletal organization.

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Cells experience a wide-range of physiological forces and biochemical cues that can regulate gene expression. These stimulants may change the placement of chromatin territories from peripheral, gene silencing, to central, active transcription regions. Defining the subnuclear architecture in a cell during external stimulation will lead to improved understanding of gene regulation. In order to measure general nuclear organizational change, we monitored the subnuclear space using particle tracking. HeLa and osteosarcoma (Saos-2) cells were transfected with gfp-tagged subnuclear fiducial particles and exposed to a compressive force assay. During compression, cells showed increased subnuclear movement in comparison to uncompressed cells and the magnitude of change suggests a shift from passive to active subnuclear order.

Alternatively, the disease Hutchinson Gilford progeria syndrome (HGPS) presents a model system in which a localization of lamins to the periphery of the nucleus causes a stiffening of the nuclear lamina and blebbing morphology. It is also believed that gene expression is inhibited by the expression of progerin and may cause a barrier during genome organizational response to mechanostransduction. HeLa and Saos-2 cells were co-transfected for expression of the progerin morphology and gfp-tagged subnuclear fiducial particles. In progerin-expressing cells the subnuclear movement shows no clear passive to active organizational response during compression. This lack of regime change may suggest that a stiffening of the lamina shell causes a discoordination during mechanotransduction and altered subnuclear order. Progerin-expressing cells also show unique crack propagation from points of lamina concentration. Altered nuclear structure in progerin causes differences in subnuclear order during compression and may correlate to unique changes in chromatin migration and gene expression.

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Molecular dynamics stimulations of nuclear lamin A and progerin tail domains confirmed by in vitro stability studies: implications for the nuclear lamina.

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Studying the biophysical properties of proteins can provide insights into their *in vivo* behavior. Defects in lamin A, a nucleoskeletal intermediate filament protein, occur at many points in the protein and the relation of a particular mutation to the resulting clinical pathology is not understood. Secondary structure analysis of the tail domain indicates that it is a mainly intrinsically disordered protein. Replica exchange molecular dynamics simulations of the tail domain indicate that the tail domain exists as an ensemble of semi-stable structures. We confirm our simulated structures by comparing the thermodynamic properties of the ensemble structures to *in vitro* stability measurements. Interestingly, all the protein conformations show that the ZMPSTE 24 cleavage site of the precursor form of the protein orients itself in such a way as to facilitate cleavage during the maturation process. Furthermore, Δ50 lamin A tail domain, the mutant protein responsible for Hutchinson-Gilford progeria syndrome, was simulated in order to understand the effect of the characteristic 50 amino acid deletion on

protein conformation. Both experimentally and *in silico*, the Δ50 lamin A tail domain shows increased stability, appears more compact and displays less heterogeneity than the mature lamin A tail domain. Δ50 lamin A tail domain's compactness suggests a more general resistance to turnover and more stable filament structures, as previously observed, potentially leading to the toxic accumulation observed in disease. Altogether these results suggest that the altered structure and stability of the tail domain can explain changed protein-protein and protein-DNA interactions and may represent an etiology of the disease.

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B-type lamins regulates neither their bound genes nor embryonic stem cells but are essential for organogenesis.

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B-type lamins are believed to regulate gene silencing of their tethered genes. They are also thought to be essential for several basic functions including cell survival, DNA replication, and cell proliferation. Using genome-wide chromatin-lamin B interaction and gene expression analyses, we show that chromatin-lamin-B interaction correlates with reduction of gene expression during embryonic stem cells (ESC) differentiation toward the trophectoderm (TE) lineage. To further investigate the significance of the chromatin-lamin-B interaction, we created lamin-B1 and -B2 double knock out (DKO) ESCs. These lamin-B DKO ESCs do not express any type of lamins including lamin-A/C, -B1, -B2 and -B3. Unexpectedly, their nuclear morphology, growth rates, ploidy, expression of pluripotent markers, and potential to differentiate to TE cells are indistinguishable from wild-type ESCs. Gene expression array analysis of these DKO ESCs and TE cells conclusively demonstrates that B-type lamins are not required for the silencing of their bound genes during ESC differentiation toward TE cells. Furthermore we report that the lamin-B DKO mice can develop to term. However they die soon after birth with a small body size and defects in multiple organs including the lung, diaphragm, phrenic nerves, and the brain. We further define the requirement for B-type lamins in regulating spindle orientation in neural progenitor cells (NPC), migration of neurons, and survival of both NPCs and neurons. While refuting several general requirements for B-type lamins, our findings redefine the essential role of these nuclear proteins in organogenesis.

Chromatin and Chromosome Organization

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Characterization of Transcriptional Regulation in iPS Cells.

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Background: Induced pluripotent stem (iPS) cells generated from somatic cells by direct reprogramming have pluripotency potential similar to that observed for embryonic stem cells. It is essential to fully understand transcriptional regulation of the genome in pluripotency and differentiation. Transcription factors bind to specific DNA response elements to regulate target genes. Identification of any given transcription factor binding sites can be partially assessed by Chromatin Immunoprecipitation (ChIP) combined with deep sequencing (ChIP-seq). However, ChIP and ChIP-seq provide only a narrow view of one specific target. Our laboratory has

pioneered analysis of DNase I hypersensitive sites (DHS) combined with deep sequencing (DHS-seq) to interrogate the landscape of all sites in the genome accessible to transcriptional regulation (John et al, Nature Genetics, 2010). We recently characterized chromatin changes and identified critical transcriptional regulatory events involved during adipogenesis in cultured cells (Siersbæk et , EMBO J, 2011). *Objective:* Unbiased analysis of genome-wide accessible sites provides a global view of chromatin organization and uncovers novel regulatory elements that determine transcriptional regulation in pluripotency and differentiation. *Methods:* iPS cells derived from normal human donors were maintained on irradiated mouse embryonic fibroblast feeder layer (MEF) or on Matrigel, and differentiated into embryonic bodies or mesenchymal stem cells. *Results:* Pluripotent cells were characterized by DHS-seq, gene expression by qPCR and flow cytometry. Genome-wide chromatin accessibility was compared to ENCODE data for human ES cells. *Conclusions*: Our study is the first genome-wide analysis of transcription factor accessibility in human iPS cells. These data will provide a baseline for characterization of normal and disease-associated differentiation of human iPS cells.

Keywords: chromatin organization, differentiation, DNAse I hypersensitive sites, microarray, osteoblast, transcriptional regulation

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Investigating linker histone H1 function and dynamics in Xenopus.

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Linker histone H1 is a fundamental chromatin protein thought to compact nucleosomes into a 30 nm fiber, but its role in higher order chromosome architecture is poorly understood. In *Xenopus* egg extracts, H1 depletion causes a 2-fold lengthwise decondensation of mitotic chromosomes, while addition of exogenous H1 causes hypercompaction, indicating its important role in the establishing and maintaining proper mitotic chromosome structure in this system. Unlike core histones, H1′s association with chromatin is dynamic. We showed previously that rapid H1 turnover measured by fluorescence recovery after photobleaching (FRAP) requires cytoplasmic factors. Here we establish an assay to identify cellular factors that modify H1 dynamics during mitosis. Sperm chromatin in metaphase-arrested egg extract diluted 1:3 in buffer and supplemented with GFP-H1 is subjected to FRAP. In control reactions, fluorescence recovers with a half time of 2.3 seconds. Depletion of ATP from the system with Apyrase treatment abolishes dynamics. We are using this assay to evaluate the effects of known H1 chaperones, including importin beta and RanBP7, and nucleoplasmin. To identify other H1 interacting proteins and post-translational modifications that contribute to cell cycle-specific H1 dynamics, we are utilizing mass spectrometry to characterize the embryonic isoform H1M (B4). Our long term goal is to understand the mechanisms that regulate H1's dynamic behavior, particularly during mitosis, and how this translates into condensation of the mitotic chromosome.

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Rapid De Novo Centromere Formation Occurs Independently of Heterochromatin Protein 1 in C. elegans Embryos.

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Naked DNA injected into the *C. elegans* germline concatemerizes to form extrachromosomal arrays that are inherited through meiosis and mitosis with variable fidelity. The mechanisms underlying array formation and segregation are not known. Here, we show that extrachromosomal arrays form *de novo* centromeres at high frequency. This provides a unique

opportunity to study *de novo* centromere formation, which has proven challenging in most systems because of its extremely low frequency. *De novo* centromerized arrays recruit the centromeric histone variant CeCENP-A and its assembly factor KNL-2, build a kinetochore that includes microtubule binding and checkpoint signaling proteins, and autonomously segregate on the mitotic spindle. The DNA injected into the germline does not require any homology to *C. elegans* sequences to support array formation and segregation. Live imaging following injection of DNA into the syncytial germline revealed that arrays form after fertilization in the 1-cell embryo cytoplasm through a process that involves both homologous recombination and non-homologous end joining. Acquisition of segregation competency by the arrays lags behind their formation—individual arrays gradually transition from passive inheritance to active segregation during the early embryonic cell divisions. We used the rapid *de novo* centromerization of extrachromosomal arrays to investigate the role of heterochromatin in *de novo* centromere formation. Arrays become strongly enriched for the heterochromatin-associated H3K9me3 mark over time, indicating that centromerization is compatible with heterochromatin assembly. Using null mutants, we found that that the two Heterochromatin Protein 1 (HP1) family proteins HPL-1 and HPL-2 are dispensable for *de novo* centromerization. In addition, partial inhibition of the HP1 family proteins accelerated the acquisition of segregation competence, suggesting that HP1-dependent heterochromatin may antagonize *de novo* centromerization. These findings report the first direct visualization of new centromere formation in living cells, establish that naked DNA injected into the *C. elegans* germline rapidly builds *de novo* centromeres, and reveal that HP1-dependent heterochromatin assembly is not essential for *de novo* centromerization. Extrachromosomal arrays in *C. elegans* thus provide a robust model to investigate the initial assembly and self-organization of centromeric chromatin, which has the potential to advance artificial chromosome engineering in human cells.

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Systematic analysis of the dynamics of single chromosome territories in live eukaryotic cells.

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Our picture of general principles and specific mechanisms of how higher order chromatin organization in eukaryotic cells is established and maintained is still very incomplete. This gives a strong incentive for studying interphase chromosome structure and dynamics in vivo. In the past live cell studies have relied on the analysis of single fluorescently labeled loci, which has yielded a variety of interesting dynamic behaviors depending on the chromosomal context. However, we are currently lacking a global quantitative analysis of the dynamics of interphase chromatin.

Here, we established an approach that allows us to quantitatively analyze the dynamics of single chromosome territories in human cells in interphase. After replication labeling the DNA backbone by a pulse of fluorescent nucleotides, cells are cultured through successive cell divisions until only a single chromosome remains labeled in a speckled pattern. The dynamics of this territory is then recorded by high-resolution confocal time-lapse microscopy with up to 10 Hz sampling, followed by tracking of the labeled sub-territory positions and statistical analysis of the resulting trajectories. In a data set of 200 chromosomes and 977 foci trajectories, we characterized the diffusional behavior of interphase chromosomes at different time scales to obtain a global view of interphase chromatin dynamics and examine effects of subnuclear position and cell cycle progression from G1 via S to G2 phase. Our data reveals that interphase chromatin dynamics is highly constrained and is characterized by slow anomalous diffusion, with only very few loci showing rare more dynamic behavior. Interestingly, short-range diffusion

was significantly faster for euchromatic/early- compared to heterochromatic/late-replicating chromatin and euchromatic/early replicating chromatin dynamics changed with cell cycle progression. Our approach allows the quantitative comparison of the diffusional behavior of chromatin state between physiological and perturbed chromatin states.

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Differential gene expression of nuclear label-retaining cells in the normal mouse mammary gland.

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Introduction-Somatic stem cells are hypothesized to protect themselves from mutation and subsequent cancer risk through a process of selective segregation of template DNA strands during cell division. In the developing mouse mammary gland these cells can be identified through the incorporation and retention of nucleic acid label during stem cell synthesis associated with pubertal expansion of the gland. These labeled cells are termed label-retaining cells (LRCs) and are present after extended chase periods. The objective of these studies was to identify genes specific to LRCs in the developing mouse mammary gland.
Methods- Immunohistochemistry and PCR arrays were performed on tissue sections and cells respectively collected from murine mammary glands that had been labeled with 5-ethynl-2'-deoxyuridine (EdU) for 2 weeks during allometric ductal growth. The cells that incorporate the nuclear label and maintain the label following a 10-week chase period are termed label-retaining cells (LRCs). A second label, 5-bromodeoxyuridine (5BrdU), was administered 2 hrs before euthanasia to identify cells traversing the cell cycle. Just prior to euthanasia the mice received 5BrdU for 2 hours. Mammary glands were collected at 3 and 6 months of age, dissociated, stained for EdU and 5BrdU and sorted into 4 populations (double negative, EdU-positive, 5BrdU-positive, and double-positive). The RNA was isolated from the 4 populations and PCR-arrays were performed that target know stem cell genes and stem cell pathways.
Results-As determined previously a population of mouse mammary epithelial take up nuclear label and retain this label over an extended chase period with a certain number of these cells continuing to cycle. Our results indicate that genes associated with the Notch and Hedgehog signaling pathways are differentially regulated between LRCs and non-LRCs. Other genes found to be differentially expressed are involved in histone modification, DNA modification, and growth factor cleavage.

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Genome-wide Analysis of the Binding Sites of the JIL-1 H3S10 Kinase and its Contribution to Modulation of Gene Expression.

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The JIL-1 kinase localizes specifically to euchromatin interband regions of polytene chromosomes and is the kinase responsible for histone H3S10 phosphorylation at interphase in *Drosophila*. Mutational analyses have shown that *JIL-1* is essential for viability and genetic interaction assays with *JIL-1* hypomorphic and null allelic combinations demonstrated that JIL-1 can counterbalance the gene-silencing effect of the three major heterochromatin components Su(var)3-9, Su(var)3-7, and HP1a on position-effect variegation. In order to further determine the interplay between epigenetic chromatin modifications and gene expression, we conducted a genome-wide analysis of JIL-1 kinase binding sites by ChIP-seq and combined it with an analysis of whole genome transcription level changes by RNA-seq in the absence of JIL-1. In

order to have the ability to specifically map and correlate the location of JIL-1 binding sites with the locations of the epigenetic histone H3S10 phosphorylation mark, salivary gland cells from third instar larvae were analyzed. Salivary gland nuclei are all at interphase excluding contributions from mitotic histone H3S10 phosphorylation. We found that most of 1675 identified JIL-1 binding peaks locate around 200 bp upstream of transcription start sites. Furthermore, we compared the transcriptome profiles of salivary glands from wild type and *JIL-1* null mutants by next generation sequencing. Interestingly, in the absence of H3S10 phosphorylation by JIL-1 the expression of 68% of normally active genes (1057 out of 1556) was repressed, whereas the expression of most normally inactive genes (177 out of 181) was activated. Taken together, these observations suggest a model where histone H3S10 phosphorylation may play a dual role in modulating gene expression depending on the state and context of other epigenetic marks, but that H3S10 phosphorylation mainly facilitates gene expression of active genes by maintaining an open chromatin structure at promoter regions by counteracting heterochromatization. Supported by NIH grant GM62916.

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A Balance Between the Epigenetic Marks H3S10ph and H3K9me2 Modulates Gene Expression at the w^{m4} Allele in *Drosophila.*

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The JIL-1 kinase is a multidomain protein that localizes specifically to euchromatin interband regions of polytene chromosomes and is the kinase responsible for histone H3S10 phosphorylation at interphase. We have previously presented evidence that a function of the epigenetic histone H3S10ph mark is to antagonize heterochromatization by participating in a dynamic balance between factors promoting repression and activation of gene expression as measured by position-effect variegation (PEV) assays. Here we have tested this model by transgenically expressing various truncated versions of JIL-1, with or without kinase activity, and correlating their effect on PEV with the levels of the H3S10ph and H3K9me2 marks at two reporter genes as determined by ChIP assays. In the pericentric insertion line *118E-10* the level of the H3K9me2 mark at the *hsp70-white* reporter was inversely proportional to the H3S10ph level - with increased levels in the absence of H3S10 phosphorylation and decreased levels with increased levels of the H3S10ph mark. Interestingly, however, *JIL-1* can act either as an enhancer or indirectly as a suppressor of w^{m4} PEV, depending on the precise levels of JIL-1 and H3S10 phosphorylation and the concomitant effects on H3K9me2 distribution. The w^{m4} X chromosome contains an inversion that juxtaposes the euchromatic *white* gene and pericentric heterochromatic sequences. Consequently, Lerach et al. (Genetics, 173:2403, 2006) proposed a model where the suppression of PEV of w^{m4} in strong *JIL-1* hypomorphic backgrounds is due to a reduction in the level of heterochromatic factors at the pericentromeric heterochromatin near the inversion breakpoint site that reduces their potential for heterochromatic spreading and silencing. As predicted by this model the results show that compared to wild-type levels suppression of PEV of w^{m4} is directly correlated with a decrease in the H3K9me2 mark at the *white* gene. Supported by NIH grant GM62916.

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The budding yeast Cse4 nucleosome is precisely positioned within the 80-bp Centromere DNA Element II.

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The centromere is the genetic locus that organizes the proteinaceous kinetochore and is responsible for attachment of the chromosome to the spindle at mitosis and meiosis. In most eukaryotes, the centromere consists of highly repetitive DNA sequences that are occupied by nucleosomes containing the CenH3 histone variant, whereas in budding yeast, an ~120-bp Centromere DNA Element (CDE) that is sufficient for centromere function is occupied by a single CenH3 (Cse4) nucleosome. However, the precise location, structure and composition of this nucleosome has been contested. To address this issue, we mapped yeast centromeric chromatin at single base-pair resolution using micrococcal nuclease (MNase) mapping, chromatin immunoprecipitation and paired-end sequencing. Intact particles containing both Cse4 and H2A are precisely protected from MNase over the entire CDE of all 16 yeast centromeres in both solubilized chromatin and the insoluble kinetochore. Small DNA binding proteins protect CDEI and CDEIII and delimit the centromeric nucleosome particle to the ~80-bp CDEII, enough for only a single DNA wrap. Functional centromeres are immediately flanked by subnucleosome-sized particles, and beyond by well-positioned conventional nucleosomes. Surprisingly, Cse4 overproduction caused genome-wide incorporation of Cse4-containing nucleosomes that wrap as much DNA as octameric particles produced in vitro. Preferential enrichment of these misincorporated nucleosomes at sites of high histone turnover suggests that stabilization of the Cse4 particle with a single DNA wrap at the CDE is necessary for kinetochore maintenance. Our findings reconcile seemingly conflicting observations in previous studies of centromeric nucleosomes.

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Nuclear architecture in Caenorhabditis elegans embryos.

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Most of the studies in the field of nuclear architecture have been performed either on blood cells or cells in culture. As a result, data on in vivo nuclear architecture is generally lacking. We have therefore initiated a study of nuclear architecture in a widely used model organism, the Caenorhabditis elegans nematode. This small roundworm presents several advantages for such a study, the most significant of which being that it develops according to an invariant cell lineage. The developmental constancy of C.elegans allows 1) to compare nuclear architecture in cells that are rigorously equivalent in terms of history, developmental potential and gene expression profile; and 2) to identify changes in the nucleus associated with lineage commitment and cellular differentiation. Our initial experiments have been aimed at providing a detailed description of nuclear organization and structure in early embryonic cells of C. elegans. Since growth does not occur during the development of this organism, nuclear size diminishes as embryogenesis proceeds. Our investigation focuses on the consequence of this size reduction on the organization of the interchromatin compartment. Preliminary resuls are reported here.

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Homologous chromosome interaction after induction of double-strand DNA breakage in G0/G1-phase cells.

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Background: The main mechanisms of DNA double strand break repair in vertebrates are believed to be nonhomologous end-joining (NHEJ) in G0/G1-phase cells and homologous recombination in S/G2 cells, where a sister chromatid provides a template for repair. **Methods**: We explored a hypothesis that homologous recombination is more common than previously expected and can occur during the G0/G1 phase, where a homologous chromosome may be used as a template for repair. We used multicolor 3D-FISH with arm-specific paints and cenrtromeric probes and confocal microscopy to analyze the interaction between pairs of homologous chromosomes in both untreated and irradiated (5 Gy) primary cultured human epithelial thyroid cells and fibroblasts. PCNA and cyclin A immunostains were used to define the cell cycle stage. **Results**: The immuno-FISH analysis of six chromosomes (chr. 2, 3, 10, 11, 16, and 17) revealed that 15-26% (mean, 19%) of homologous chromosome pairs in G0/G1-phase cells had spatial contact between reciprocal arms in untreated epithelial cells and fibroblasts. Following exposure to ionizing radiation, the frequency of arm-specific contact between homologous chromosomes doubled to 28-45% (mean, 38%). For comparison, the contact between opposite arms of homologous chromosome (p:q) was close to 1% (range, 0.6-1.6%) for all chromosomes in untreated cells and it remained at the same level after cell irradiation, likely representing a level of random contact. **Conclusions**: The results of this study indicate that in G0/G1-phase human cells homologous chromosomes can make contact which is increased after induction of double strand DNA breaks by radiation. It is conceivable that this process offers homology for homologous recombination to occur during G0/G1 phase of the cell cycle.

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Identifying the First Telomerase RNA in a Filamentous Fungus.

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The ends of eukaryotic chromosomes are protected by nucleoprotein structures termed telomeres, which are synthesized by the essential enzyme, telomerase. We are studying the telomeric components of the *Aspergilli*, as these filamentous fungi have exceptionally short telomeres. A key component of telomerase, the telomerase RNA, has not been identified in any filamentous fungus. The telomerase RNAs from even closely related organisms are highly divergent and thus are difficult to identify through bioinformatics. Since *Aspergillus oryzae* has a particularly long telomeric repeat of 5' TTAGGGTCAACA 3', and we knew the telomerase RNA must contain this sequence as a template to synthesize the telomeres, we were able to identify roughly 20 candidate sequences in the *A. oryzae* genome. Experiments utilizing RT-PCR and PCR have shown that one candidate located on chromosome III is transcribed whereas all other candidates in the genome were not transcribed. The 5' end of this RNA sequence has been identified using RLM-RACE to be at most 301 bp from the start of the template, but the 3' end remains undetermined. Successive experiments using 3' RACE have proved inconclusive indicating that the 3' end of the telomerase RNA could lack a poly(A) tail. Using RT-PCR, we determined that the 3' end is between 1567 and 1775 bp from the template. These results

suggest that the total telomerase RNA length in *A. oryzae* is between 1868 and 2076 bp. The length is plausible as it is similar to the telomerase RNA lengths of yeasts. This suggests that we may have discovered the first telomerase RNA in a filamentous fungus.

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Potential Recombinogenic Genes Interact With Each Other At Nanometer Distances In Human Interphase Nuclei.

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Background: It has been well established that loci which participate in oncogenic rearrangements are non-randomly positioned and close to each other in human cell nuclei. However the actual distance between these loci has never been determined. The phenomenon of Fluorescence Resonance Energy Transfer (FRET) is observed when a donor flourophore is close enough to transfer some of it energy to an acceptor flourophore. The objective of this study was to perform FRET on directly labeled DNA molecules in order to determine the spatial distance between potentially recombinogenic loci. **Methods**: Touch preparations of cells from freshly excised normal human thyroid tissue and TPC1 cell lines were subjected to 3-D fixation to preserve the nuclear architecture and assayed using FISH and confocal microscopy. FRET-SE (sensitized emission) analysis was carried out in 2 donors with 500 nuclei analyzed for each case using Alexa 488-594 donor-acceptor combination. **Results**: To validate the use of FRET-SE in combination with DNA-FISH, TPC1 cell line that harbors the *RET/PTC1* rearrangement with fusion of *RET* and *H4* was used as a positive control. FRET between *RET* and *H4* probes was observed in 96.1% of the analyzed nuclei with mean FRET efficiency value (Eapp) of 14.40% (Donor-Acceptor (DA) distance = 8.15 nm). For the experimental set, touch preparations of normal thyroid cells were subjected to DNA-FISH with probes for *RET* and *H4*, *RET* and *NCOA4,* and *BRAF* and *AKAP9*, which are partners of recurrent chromosomal rearrangements found with different incidence in thyroid cancer. The frequency of probe pairs that showed FRET were as follows: *RET-H4* – 5.31 ± 1.09%, *RET-NCOA4* – 3.57 ± 0.28%, *BRAF-AKAP9* – 1.77 ± 0.01%. Based on the mean Eapp values for each of the probe pairs the DA distances for these probe pairs ranged from 8.61nm to 9.77nm. The FRET frequencies for the probe pairs did not correlate with genomic separation between the loci (r = 0.5355) but correlated with the incidence of respective rearrangements in thyroid tumors (r = 0.9877). **Conclusion**: Using FRET analysis of DNA-FISH preparations, we found that potential recombinogenic genes can be positioned in human interphase nuclei within nanometer distances of each other and the frequency of such a close positioning correlates with the prevalence of respective chromosomal rearrangements in human tumors.

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Investigation of the *Drosophila* MAGE gene.

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MAGE (Melanoma antigen) is an expanded gene family in placental mammals. MAGE proteins play important roles in cell cycle regulation and neuronal differentiation. Two MAGE genes, Necdin and MAGEL2, are inactivated in the neurodevelopmental disorder Prader-Willi syndrome, along with other genes. Non-mammalian organisms, including the fruit fly *Drosophila melanogaster*, have a single MAGE gene in their genome. The *Drosophila* MAGE gene (DMAGE) may play a role in cell cycle regulation and cell survival in postembryonic neurogenesis. MAGE proteins are part of a chromosome maintenance complex (SMC5/6) in yeast and human cells. We hypothesize that the *Drosophila* MAGE protein is also part of the *Drosophila* SMC5/6 protein complex that plays a role in maintaining genome stability and

regulating cell cycle. Co-immunoprecipitation and *in vitro* pull-down experiments demonstrate that the DMAGE protein interacts with the NSE4 and the NSE1 subunits of the SMC5/6 complex. Over-expression of DMAGE in flies and a *Drosophila* cell line indicate that DMAGE may arrest cell proliferation. We also generated a null DMAGE *Drosophila* mutant which we tested for sensitivity to selected genotoxic agents. In conclusion, it appears that DMAGE cooperates with the SMC5/6 complex to maintain genome stability and it may also play a role in regulating the cell cycle. We would like to acknowledge the funding from the Cancer Research Society (Canada).

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Single molecule dynamics of chromatin fibers.

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The first level of genome organization within the eukaryotic nucleus is the wrapping of DNA around histones to form chromatin. Higher order organization and compaction of the chromatin fiber is required to package the genome into the nucleus in interphase and to condense chromosomes for mitotic segregation. Although condensed within the cell, chromatin must remain accessible to the enzymatic machinery that replicates, transcribes, repairs and segregates the genome. Despite extensive work on chromatin and chromosome organization we have an incomplete understanding of the basic physical properties of the chromatin fiber, how those properties influence the dynamics and organization of chromosomes, and how they may be modulated epigenetically. In order to measure the dynamics of chromatin fibers we are applying a new method that enables us track the diffusion of single fluorescently labeled molecules of chromatin in solution. Our tracking method utilizes spatial modulation of a focused laser beam used to excite Cy3b attached covalently to a chromatin fiber, analog demodulation to extract an error signal, and real-time feedback to piezoelectric positioning stages to compensate for Brownian motion. We have been able to monitor the changes in compaction of single chromatin fibers in response to changes in the ionic environment by measuring changes in the diffusion coefficient. Using chromatin fibers with single molecule fluorescent labels we have monitored the association and dissociation kinetics of individual chromatin fibers in solution via quantitative fluorescence measurements, and have begun to study association morphology by studying the dependence of the diffusion coefficient of an aggregate on the number of fibers contained. We are working to characterize the interaction of chromatin molecules in response to changes in ionic strength or chromatin binding proteins, and have begun to explore the use of tracking Fluorescence Correlation Spectroscopy (tFCS) as an improved approach to characterizing mechanical properties of individual aggregates. We are exploring the use of tFCS to characterize conformational fluctuations of individual chromatin fibers and fiber-aggregates on timescales ranging from microseconds to seconds. Unlike traditional methods such as analytical ultracentrifugation that measure the ensemble properties of chromatin in solution, we are able to measure heterogeneous distributions with single-fiber resolution and to characterize the response of individual chromatin fibers to environmental changes. These methods enable us to measure changes in the properties of chromatin in response to DNA sequence, chromatin composition and modification, macromolecular crowding and specific chromatin binding proteins.

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Scaling Chromosome Condensation to Cell Size.

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During development or differentiation, cells often change in size. Mechanisms describing how organelles scale their size to the continuously changing size of cells are poorly understood. This problem is well exemplified during embryonic development of organisms like *Caenorhabditis elegans* or *Xenopus laevis*. As a consequence of multiple rounds of cell divisions without changing the size, cells decrease in length by two orders of magnitude (from 1,2 mm to 12 ìm) in *X. laevis*. Using *X. laevis* or *C. elegans* embryos as model organisms, it has been shown that mitotic structures including mitotic spindle length, centrosome size and nucleus size all scale with cell size. Despite these dramatic changes, the genome size is constant in all diploid cells in an organism. In order to facilitate cell division in smaller cells, mitotic chromosomes must increase the degree of condensation throughout development. In *C. elegans* adult worms, the mitotic chromosomes are more condensed in smaller cell then in larger cells. We are using the invariant developmental program of *C. elegans* embryos to investigate how cells adapt the level of chromosome condensation as cell decreases in size. Preliminary results indicate that metaphase plate length scales with cell size. This change in chromosomes condensation could be an outcome of a cellular size control or a programmed developmental change. To test the first hypothesis, we aim to assess the implication of known DNA condensation and cell size scaling factors on scaling of the metaphase plate length to cell size. Key regulators of mitotic chromosome assembly include DNA topoisomerase II, the condensin complexes, histone H1 or post-translational modification of histones such has H3 S10 phosphorylation. A differential recruitment of those factors on mitotic chromosomes could adjust the level of condensation according to cell size. Quantitative immunofluorescence of those factors will be use to evaluate their recruitment on mitotic chromosomes. Previous works have shown that cell size, spindle length and centrosomes size are internally controlled and interdependent. We will test if the size of those structures can affect the length of the metaphase plate by use of RNAi depletion of known proteins required to set the size of those structures. To test the second hypothesis, we are currently optimizing a large-scale screen to identify new genes that would affect the metaphase plate length during development. We aim to deplete known embryonic lethal gene and use time-lapse microscopy to observe mitosis during the development of *C. elegans* embryos. The overall goal of this study is to identify new mechanisms regulating size of organelles.

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The Unusual Features of Active Genes on Drosophila melanogaster Chromosome Four: Reinterpreting the Roles of Chromatin Modifications.

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The small fourth (dot) chromosome of *Drosophila melanogaster* exhibits overall characteristics of a heterochromatic domain, including high levels of H3K9me2/3 and HP1a, but contains ~80 genes in its 1.2 Mb distal portion. Mapping of histone modifications and chromosomal proteins in S2 and BG3 cells, as well as several life stages, shows that these genes have unique characteristics, distinctive from active genes in either euchromatin or pericentric heterochromatin. H3K9me2/3 and HP1a are depleted at the TSSs (transcription start sites) of active fourth chromosome genes, which, as expected, are occupied by H3K4me2/3 and RNA pol II. However, POF, HP1a, and H3K9me3 are found at high levels across the gene body. There is no evidence of euchromatin “islands” supporting expression. Reporter sites permissive

for expression appear to be those regulated (in at least some cell types) by the Polycomb system. While fourth chromosome genes exhibit the same range of expression levels as do euchromatic genes, there are few if any associated with a paused polymerase. The continued presence of HP1a and H3K9me3 at active genes results in a shift in association patterns, with H3K9me2 now being unique in its stronger correlation with inactive genes. Depletion of POF results in loss of HP1a from fourth gene bodies, but HP1a continues to be present in the pericentric heterochromatin and at repeat clusters on the fourth, indicating a dual targeting mechanism for HP1a association with the fourth chromosome. These findings challenge our prior interpretations of the role of chromatin structure in gene regulation and focus attention on the modifications at the TSS. Supported by NIH grants U01HG0004258 to GH Karpen and R01 GM068388 to SCRE.

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The role of Sir4 in de novo assembly of heterochromatin in budding yeast.

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Changes in the locations and boundaries of heterochromatin are critical during development of multicellular organisms, and *de novo* establishment of heterochromatin in budding yeast, *Saccharomyces cerevisiae*, is the best understood example of how these dynamic changes occur in the context of cell cycle progression. Heterochromatin in budding yeast is composed of polymers of the SIR (Silent Information Regulator) complex bound to nucleosomal DNA. Assembly of heterochromatin requires all three proteins of the Sir complex: the histone deacetylase, Sir2 and the histone binding proteins Sir3 and Sir4.

De novo assembly of heterochromatin in budding yeast requires at least one to five cellular divisions. Previous studies have shown that this assembly is not regulated by active DNA replication but by another event that occurs in the S-phase of the cell cycle. Recent work has suggested that the cell cycle inhibition of chromatin modifying enzymes may be the mechanism for how cells limit when they can assemble new sites of heterochromatin.

Halving the levels of Sir4 in cells causes a dramatic loss of silencing at telomeres, and these data have led us to test a simple hypothesis: the abundance of Sir4 protein regulates the assembly of new regions of heterochromatin, and its regulated destruction may prevent this assembly during the G1 phase of the cell cycle.

We are examining *de novo* assembly of heterochromatin using an assay for establishment of a site of heterochromatin in individual cells. Introduction of additional copies of *SIR4* on low copy plasmids leads to a marked increase in the number of cells that are able to establish heterochromatin after only one division. Addition of *SIR4* at high copy number leads to ablation of this effect, while overexpression of Sir4 prevents establishment of heterochromatin. Additionally, halving the amount of *SIR4* decreases the ability of cells to establish new sites of heterochromatin. We are currently testing if the stability of Sir4 changes in G1 and whether changes in Sir4 levels are epistatic to mutations in histone modifying enzymes.

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Deciphering the CENP-A nucleosomal structure.

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Chromosome segregation during mitosis is mediated by the formation of a linkage between centrometic DNA and microtubules that generates force to faithfully distribute them into daughter cells. Defects in centromeres result in aneuploidy, which is considered as a hallmark of cancer and a number of devastating human pathologies such as embryonic implantation

failure. Centromeric regions are defined by nucleosomes containing the histone H3 variant, CENP-A. CENP-A is proposed to act as an epigenetic mark specifying and maintaining centromere identity in each cell division, thus maintaining ploidy. In dividing cells, incorporation of new CENP-A into nucleosomes is not coupled to DNA replication. During S-phase, the CENP-A population is distributed equally between the newly synthesized centromeres of the daughter chromomsomes. After cell division, centromeres are repopulated with CENP-A during G1 in preparation for the subsequent S-phase. During the past few years, there has been much debate over the molecular composition of the CENP-A nucleosomes in respect to the canonical octameric nucleosomes. Our goal is to visualize components of single CENP-A nucleosomes and thereby define structural elements constituting the centromeric nucleosomes. To do this, we developed a total internal reflection fluorescence microscopy (TIRFM) assay coupled to a nucleosome-immunoprecipitation technique to visualize single CENP-A nucleosomes. Nuclei were extracted from HeLa cells stably expressing a CENP-A-YFP fusion, sonicated and perfused into our custom-designed flow chambers. The resultant single-molecule images were analysed by an automated detection software developed by our group and confirmed by manual analysis. Our preliminary observations, confirmed by the two methods, suggest that there is a mixed population of one- and two-CENP-A containing nucleosomes in HeLa cells. By visualizing other key components of centromeric nucleosomes and generating cell cycle staged extracts, our approach will thus help shed light on fundamental aspects of centromere biology.

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CENP-A Nucleosome Structure Transits Between Tetramers and Octamers Throughout the Cell Cycle.

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Controversy over the structure of the centromeric CENP-A nucleosome has ignited debate that spans organisms from plants, yeast, flies, and humans. The range of reported structures include octamers, hexamers, and both hetero- and homo-typic tetramers. To reconcile these differences, several have proposed that the centromeric nucleosome transitions between octamers, tetramers, and intermediates (Probst et al, 2009; Dalal and Bui, 2010). We sought to test this hypothesis by synchronizing human cells at Early G1, G1/S, S, and G2/M phases. Utilizing biophysical Atomic Force Microscopy (AFM) and biochemical purifications of CENP-A nucleosomes, we were able to track structural and kinetochore protein dynamics across the cell cycle. Further studies are currently underway to elucidate the mechanistic properties involved in structural transitions, and whether this mechanism is conserved in other organisms.

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The Chl1 helicase, Fen1 flap endonuclease and the establishment factor Ctf7/Eco1 interact together to perform a role in cohesion establishment.

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The accurate segregation of chromosomes to each daughter cell requires sister chromatid cohesion to maintain the identity of sister chromatids from the time of DNA replication to anaphase onset. The establishment of cohesion involves the acetylation of a cohesion subunit (Smc3) by the establishment factor Ctf7/Eco1 (Unal et al. 2008; Ben-Shahar et al. 2008). Ctf7/Eco1 interacts with a number of replication proteins (RFC complexes, PCNA) and DNA helicases (Chl1) (Kenna and SKibbens, 2003, Moldovan et al. 2003, Skibbens, 2004, Maradeo and Skibbens, 2010); all of which play individual roles in sister chromatid cohesion. The actual mechanism of cohesion establishment is still largely unknown. Here, we provide new evidence from Saccharomyces cerevisiae that Chl1 promotes cohesion establishment through a post-replicative model. Our results show that Chl1 interacts with chromatin under normal conditions,

contrary to previous studies which suggest that Chl1 chromatin recruitment is dependent on DNA damage (Ogiwara et al. 2007). We also show that Chl1 chromatin recruitment is cell cycle regulated. Next, we document novel genetic interactions between CHL1 and FEN1 and also between CTF7/ECO1 and FEN1. Subsequent co-immunoprecipitation studies further reveal that Chl1 physically interacts with Fen1. Preliminary results also show that Fen1 physically interacts with Ctf7/Eco1. These observations provide strong evidence in support of a post replicative model of cohesion establishment, wherein lagging strand processing events are coupled to the process of cohesion establishment. We speculate that the post replicative events help establish cohesion by either enhancing the acetyl-transferase activity of Ctf7/Eco1 or making the cohesin subunits more accessible to Ctf7/Eco1 dependent acetylation. Taken together, our study reveals important information that for the first time directly links the process of cohesion establishment to post replicative events.

Mitosis and Meiosis II

1112

Consequences of a prolonged mitosis.

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During mitosis, the replicated genome is segregated into the two daughter cells. Microtubules emanating from opposing spindle poles attach to the DNA through kinetochores, allowing sister chromatids to be pulled to opposite sides of the cell. Attachment occurs at random such that many incorrect microtubule-kinetochore attachments can occur during the early stages of mitosis that can potentially lead to incorrect segregation of sister chromatids. The spindle assembly checkpoint senses these incorrect attachments and provides time for the correction of these attachments by preventing mitotic progression until all attachments are corrected. Nonetheless, some incorrect attachments may not be sensed by the SAC, causing sister chromatids to segregate incorrectly. Recently, evidence was presented for the existence of an additional checkpoint that could restrict the proliferative capacity of cells that were generated from a “problematic” mitosis as the duration of mitosis itself determines the proliferative capacity of the daughter cells (Uetake and Sluder, 2010). This could provide a means by which cells that have encountered problems during mitosis and therefore might have spent more time in mitosis than normal, can be excluded from the cell cycle.

The aim of our research is to elucidate the mechanisms involved in the timing of mitosis that dictate the proliferative capacity of daughter cells. In order to biochemically assess the mechanisms involved in the G1 arrest we developed an experimental setup that allows us to investigate the events during mitosis and the subsequent G1 phase that are involved in arresting the cells. We have observed an increase in mitotic p53 protein level, when cells are trapped in mitosis for a prolonged period of time. Moreover, daughter cells originating from a prolonged mitosis show increased p53 protein levels whereas daughter cells originating from a normal mitosis do not. The increase of mitotic p53 levels seems to be translation dependent.

Reference

Uetake, Y., and Sluder, G. (2010). Prolonged prometaphase blocks daughter cell proliferation despite normal completion of mitosis. Curr Biol *20*, 1666-1671.

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Analysis of sororin phosphorylation and dephosphorylation.

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Cell cycle control mechanisms are altered in cancer cells, often leading to chromosomal instability; however, these mechanisms are not fully characterized. Cohesin, a protein complex consisting of Smc1, Smc3, Scc1, and Scc3, protects sister chromatids from separating at inappropriate times. In addition, certain accessory proteins to cohesin, such as sororin, are required for cohesion maintenance. Cdk1-mediated phosphorylation of sororin regulates sister chromatid cohesion. These results were obtained by mutating nine Cdk1 consensus sites to alanine to create sororin-9A. Through co-immunoprecipitations, chromosome drops, and localization studies, we were able to show that sororin-9A stays bound to chromatin throughout mitosis, while sororin-WT is removed at prometaphase. Next, we generated glutamic acid mutations to create sororin-9E. We co-transfected HeLa M cells with shRNA sororin and sororin-9E and quantified the mitotic index. As expected, knocking down sororin increased the mitotic index, likely as a result of activation of the spindle assembly checkpoint. Surprisingly, sororin-9E was able to rescue the mitotic block caused by shRNA sororin. Interestingly, Nishiyama et al. (2010) demonstrated that sororin binds to PDS5, an accessory protein to cohesin, through an FGF motif. We therefore mutated the FGF motif in sororin-9A to AGA, co-transfected HeLa M cells with shRNA sororin and the sororin-9A AGA mutant and quantified the mitotic index. We expected cells to be blocked in mitosis because sororin is theoretically no longer able to bind to PDS5, thereby activating the spindle assembly checkpoint; however, the sororin-9A AGA mutant behaved like wild-type sororin. These results suggest that sororin has an additional binding partner to PDS5. Even though we have identified the kinase responsible for phosphorylating sororin, the phosphatase that dephosphorylates sororin remains elusive. Sororin is known to be dephosphorylated by four hours after nocodazole release (Rankin et al., 2005). Okadaic acid, an inhibitor of PP2A, delayed the dephosphorylation of Sororin-WT upon nocodazole release; suggesting that PP2A or another okadaic acid-sensitive phosphatase targets sororin for dephosphorylation upon mitotic exit. Future experiments will be to determine the other protein or proteins that bind to sororin and confirm that the phosphatase is PP2A. From these experiments we hope to fully characterize the roles of phosphorylation and dephosphorylation in sororin regulation.

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Structural Basis of Interaction between Human Survivin and N-terminus Histone H3 Phosphorylated on Threonine 3.

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A four-protein complex known as chromosome passenger complex (CPC) is a central regulator of mitosis. The proper regulation of chromosome segregation requires the positioning of the CPC to the inner centromere, which is a region on mitotic chromosomes between kinetochores. We have recently shown that this inner centromere localization is mediated by the survivin subunit of the CPC complex, which binds to the N-terminal tail of histone H3 that has been phosphorylated on Thr3 by the Haspin kinase.
Here we used X-ray crystallography to characterize molecular-level interactions between human survivin and the N-terminus histone H3 phosphorylated on Thr3. Our crystal structure of wild-type survivin complexed with the N-terminus of histone H3 phosphorylated on Thr3 revealed that the histone tail binds to the surface of the BIR domain and the phosphorous group of H3-

Thr3 is bound by His80 and Lys62 from this domain. Survivin Glu65 binds the side chain of Arg2 of the tail and Asp71 recognizes the N-terminal Ala1 in a hydrophobic pocket formed by Leu64, Glu76 and Trp67. Survivin point mutants at either His80Ala, Glu65Ala and Lys62Ala bind the phosphorylated histone tail poorly as measured by isothermal titration calorimetry, which confirms the importance of these amino acids to histone tail binding. When a 12mer histone tail peptide was co-crystalized, only the N-terminal four residues were ordered.
The determined structure of wild-type survivin with bound histone H3 demonstrates how the phosphorylation mark on Thr3 can be recognized by survivin and is an another example of how BIR domains recognize the N-terminal residues of proteins.

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Localization determinants in the carboxy-terminal region of human Topoisomerase IIα.

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It is not known how the essential enzyme DNA topoisomerase IIα becomes localized to condensed chromosomes in mitosis. Here we define primary sequence features in the enzyme's carboxy terminal region (CTR) that are critical for this function. Consistent with previous reports we find that two regions of the CTR have nuclear localization signal (NLS) activity required for nuclear import of TopoIIα. Unexpectedly, we find that NLS elements are required for the localization of TopoIIα to mitotic chromosomes. Along with the hKid chromokinesin, this is a second example of NLS-dependent chromosome targeting, indicating that this may be a general mechanism in mitotic cells. We show that neither nuclear localization during interphase nor passage through nuclear pores is required for the targeting of TopoIIα to mitotic chromosomes. Therefore, nuclear import *per se* is not strictly coupled to the mechanism of mitotic chromosome targeting. Furthermore, the NLS elements are not sufficient for chromosome targeting: we identify a third essential domain in the CTR of the enzyme (here named the TDT domain: TopoIIα DNA Tether) that binds directly to DNA and without which TopoIIα chromosome localization is compromised. Unlike the NLS elements, the TDT in isolation is sufficient for chromosome localization. Finally, we have examined the significance of these features using a novel knockdown/rescue assay in HeLa cells. The data presented characterize an unexpected relationship between nuclear localization sequences and chromosome binding which is entirely dependent on the C-terminus of TopoIIα, and provides mechanistic insight into how the enzyme is localized to mitotic chromosome where it performs its essential activity in sister chromatid resolution.

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Investigating the Role of Spindle-Associated F-actin in Mitotic Spindle Structure and Function.

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Mitotic spindles are microtubule-based structures responsible for partitioning genetic information into each new daughter cell. The role of microtubules and microtubule-associated proteins in this process has been well characterized, however whether or not F-actin is required—or is even present--within the spindle has long been controversial. In this work we examine spindle-associated F-actin in the intact Xenopus laevis epithelium. Immunofluorence of labeled phalloidin revealed F-actin at spindle poles, F-actin cables extending from poles towards the cortex, and F-actin cables spanning between poles within the spindle. Similar localization patterns are detected with gamma-actin antibodies. Live imaging experiments corroborated these results and highlighted the dynamic nature of these actin cables as they move within and

around the spindle throughout mitosis. In addition, treatment with the Arp2/3 complex inhibitor CK-666 resulted in a loss of cortical F-actin, while spindle-associated F-actin and mitotic spindle structure remained intact. Collectively, these results show that a) mitotic spindles in intact vertebrate epithelia contain a substantial amount of F-actin cables; b) these cables form coincident with spindle assembly, and; c) spindle F-actin, rather than cortical F-actin, may be essential for proper mitotic spindle structure and function.

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Microtubules search for lost kinetochores by pivoting around the spindle pole.

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During cell division, proper segregation of genetic material between the two daughter cells requires that the spindle microtubules attach to the chromosomes via kinetochores, protein complexes on the chromosome. In fission yeast, kinetochore capture by microtubules can be observed when kinetochores are lost in the nucleoplasm, which can be induced by spindle disassembly during metaphase. It is, however, unknown how microtubules find lost kinetochores. We observed that lost kinetochores are captured by microtubules pivoting around the spindle pole body, instead of extending towards the kinetochores. By introducing a theoretical model, we show that the observed random movement of microtubules is sufficient to explain the process of kinetochore capture. We thus reveal a mechanism where microtubules explore space by pivoting, as they search for intracellular targets.

1118

Checkpoint-independent stabilization of kinetochore-microtubule attachments by Mad2 in human cells.

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Faithful chromosome segregation is required for cell and organism viability, and is lost in solid tumors with aneuploid karyotypes that display chromosomal instability (CIN). Mitotic fidelity in normal cells in ensured through two mechanisms: the spindle assembly checkpoint (SAC) and the cellular mechanism that corrects errors in kinetochore microtubule attachments (k-MT). The SAC prevents anaphase onset until all chromosomes attach to spindle microtubules, and provides time for the correction of k-MT attachment errors that arise in prometaphase. Mad2 is essential for SAC function and is frequently overexpressed in tumor cells where it increases the frequency of lagging chromosomes in anaphase. We have previously shown that cancers cells with CIN have hyperstable k-MT attachments and fail to efficiently correct k-MT attachment errors elevating the frequency of lagging chromosomes in anaphase. This suggests a role for Mad2 in regulating the stability of k-MT attachments. To address this we manipulated the cellular levels of Mad2 and used fluorescence dissipation after photoactivation to measure k-MT stability in human normal diploid RPE-1 cells. We show that k-MT attachments are destabilized by Mad2 depletion and hyperstabilized by Mad2 overexpression in both prometaphase and metaphase cells. The consequence of hyperstabilized k-MT attachments induced by Mad2 overexpression is the persistence of k-MT attachment errors manifest by lagging chromosomes in anaphase, an effect that can be rescued by increasing the k-MT turnover rate by expression of the kinesin-13 MCAK. These effects on k-MT attachment stability are independent of the mitotic checkpoint because k-MT attachment stability is unaltered upon Mad1 depletion and Mad2 overexpression hyperstabilizes k-MT attachments in Mad1-deficient cells. Mad2 mediates these effects by altering the centromeric quantity and activity of Aurora B, a kinase known to regulate k-MT attachment stability. Thus, Mad2 acts independently of the checkpoint to stabilize

k-MT attachments generating a positive feedback loop for checkpoint satisfaction by driving microtubule occupancy at kinetochores.

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The Kif18A chromosome alignment mechanism differentially affects mitotic progression among human cells.

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During cell division, the kinesin-8 Kif18A regulates kinetochore-microtubule plus-end dynamics to drive metaphase chromosome alignment. Studies thus far suggest that Kif18A is essential in human cells: HeLa cells depleted of Kif18A exhibit a robust, spindle assembly checkpoint (SAC)-dependent arrest. Strikingly, we found that non-transformed euploid RPE-1 cells do not arrest in mitosis when siRNA-depleted of Kif18A despite exhibiting a defect in chromosome positioning prior to anaphase onset. To determine the extent to which this interline variation occurs, we used RNAi to deplete Kif18A from a panel of twelve transformed and nontransformed human cell lines, and gauged mitotic arrest by an increase in mitotic index. Cell lines showed a range of responses, from strong arrest to no arrest despite a sharp decrease in Kif18A levels as gauged by immunofluorescence. Arrest correlated with SAC signaling: HeLa cells showed an increase in kinetochore-localized Mad1 puncta when depleted of Kif18A, whereas RPE-1 cells did not. Consistent with this observation, the intrakinetochore distance (delta) in Kif18A-depleted HeLa cells was systematically reduced compared to Kif18A-depleted RPE-1 cells. Kif18A is required for normal metaphase chromosome alignment in cells which do not depend on it for mitotic exit: Kif18A-depleted RPE-1 cells initiate anaphase without first achieving tight metaphase chromosome alignment. Surprisingly, despite a defect in chromosome mis-alignment, we did not observe a strong increase in kinetochore oscillation amplitude (DAP, deviation from the average position) in Kif18A-depleted RPE-1 cells. In conclusion, we have found that some tissue culture cell lines satisfy the SAC and exit mitosis despite depletion of Kif18A and a lack of proper metaphase chromosome alignment. The cellular characteristics that underlie a dependence on Kif18A are currently under study.

1120

P21- activated kinase 4 (PAK4) is required for metaphase spindle positioning and anchoring.

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The oncogenic kinase PAK4 was recently involved in the regulation of G1 and G2/M phases of the cell cycle. We also identify that PAK4 regulates Ran GTPase activity during mitosis. Here, we show that, PAK4 depleted cells enter mitosis and maintain a prolonged metaphase like state. In these cells, chromosome congression to the metaphase plate occurs with normal kinetics but is followed by an extended period during which membrane blebbing and spindle rotation are observed. We find that bipolar PAK4 depleted metaphase like spindles have a defective astral MTs network that likely prevent efficient spindle anchoring and proper spindle tension. As a result, the mitotic spindles are not centered in the cell but in close contact with the cell cortex, centrosome fragmentation occurs, a subset of chromosomes detach from the metaphase plate and move toward the spindle poles inducing a scattered chromosome phenotype with active spindle checkpoint (SAC).

PAK4 regulates the acto-myosin cytoskeleton dynamics and we report that PAK4 depletion results in the induction of cortical membrane blebbing during prometaphase arrest. However, we show that membrane blebs that are strongly enriched in phospho-cofilin are not responsible for

the poor anchoring of the spindle. Since PAK4 depletion interferes with proper localization of components of the dynein dynactin complexes, we propose that PAK4 deletion mediated mitotic defects could reflect a loss of the activity of the motor protein.

1121

Function of protein 4.1R in mitotic spindle orientation.

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Protein 4.1R, originally characterized as a crucial membrane skeletal protein in mature red cells, plays an important role in cell division during mitosis. Biochemical studies have revealed that 4.1R binds to microtubules, stabilizes the spectrin-actin network and anchors it to the plasma membrane, and directly interacts with the large nuclear mitotic apparatus NuMA protein, suggesting that 4.1R plays diverse roles within the cytoskeleton. However, the importance of the interaction between 4.1R and cytoskeletal proteins is still unclear. Here, we show that 4.1R contributes to kinetochore-microtubule attachments, spindle organization and orientation. During mitosis, 4.1R is accumulates in the cell cortex at the end of retraction fibers, and is localized throughout the spindles and spindle midzone. Depletion of 4.1R by RNA interference induced mitotic delay and defects in retraction fiber formation, chromosome alignment, spindle assembly and spindle orientation. Spindle misorientation is linked to unstable astral microtubules and suppressed by the restoration of 4.1R in 4.1R-depleted cells. 4.1R depletion mislocalized the p150Glued subunit of dynactin and NuMA. Our data support a model in which 4.1R recruits NuMA at cell cortex, which provides link between cell cortex and the force generator dynein, to regulate spindle orientation during mitosis.

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The spindle assembly checkpoint fails to monitor the lack of tension on few kinetochores in meiosis I mouse oocytes.

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Chromosome segregation in female meiosis I is unusual in that the acentrosomal meiotic spindle poles are not anchored via astral microtubules to the cortex. By Cre recombinase-mediated removal in oocytes of the portion of the gene encoding the microtubule binding site of NuMA, implicated previously in anchoring microtubules at poles in concert with cytoplasmic dynein, we determine that without functional NuMA microtubules lose connection to meiosis I spindle poles, resulting in highly disorganized early spindle assembly. Subsequently, very long spindles form with hyperfocused poles. The kinetochores of homologs make stable attachments to microtubules in these spindles, but with reduced tension and accompanied by alignment defects. Despite this, the spindle assembly checkpoint is not activated and advance to anaphase I and first polar body extrusion take place without delay. Females without functional NuMA in oocytes are hypofertile, producing aneuploid eggs with altered chromosome number for one or more chromosomes. These findings establish that in mammalian meiosis I the spindle assembly checkpoint is weaker than in mitosis, with one or a few misattached kinetochores unable to sustain meiotic arrest and offering an explanation for why meiosis I in mammals is so error prone.

1123

Dalek: A mammalian outer nuclear envelope protein required for early meiotic progression in mice.

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The nuclear envelope has a specialized role in early meiotic prophase I. Chromosomes attach to the surface of the nuclear envelope (NE) through interactions with inner nuclear membrane SUN-domain proteins. These SUN-domain proteins interact lumenally with outer nuclear membrane KASH domain proteins that associate with cytoskeletal constituents, including the dynein motor complex. The primary purpose of this specialized structure appears to involve the translocation of meiotic chromosomes during the process of homolog pairing, synapsis and/or recombination. Meiotic KASH and SUN constituents have been functionally characterized in a variety of model organisms; however, in mammals only the SUN-domain protein Sun1 has been identified. Here we describe the identification and characterization of Dalek, a mammalian KASH domain protein expressed in early meiotic germ cells where it is detected at the NE-chromosome attachment site, coincident with telomeres and Sun1. We can demonstrate that Dalek interacts with the cytoplasmic dynein motor complex, which is colocalized with Dalek at the NE-chromosome attachment sites during early meiosis. Loss of Dalek in homozygous gene-deleted mice leads to sterility, diminished gonad size, and meiotic germ cell arrest. These studies support our overall hypothesis that Dalek is the mammalian KASH-domain protein required for active movement of chromosomes during early meiotic prophase I.

1124

Maternally-recruited Aurora C kinase is more stable than Aurora B to support mouse oocyte maturation and early development.

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Oocytes express meiosis-specific forms of many proteins required for maturation, including proteins that function in cell division such as WEE1B, REC8, and Aurora kinase C (AURKC). AURKC is a germ cell specific member of the conserved Aurora kinase family, and is most similar to the ubiquitously expressed AURKB. Because of their similarity, the significance of AURKC is unclear. AURKB is degraded in mitotic cell divisions and contains destruction motifs that AURKC lacks, suggesting that differential stability underlies the requirement for AURKC. Here we show that endogenous AURKB protein decreases during maturation while AURKC increases. Using fluorescently-tagged reporter proteins, we find that AURKB is gradually degraded while AURKC is more stable. The increase in AURKC reflects a maturation-associated recruitment of *Aurkc* mRNA. *Aurkb* is not transcribed until the blastocyst stage, which suggests that AURKC compensates for loss of AURKB during maturation and early development. Consistent with this model, we find that female *Aurkc*$^{-/-}$ mice are subfertile. Oocytes from these mice take longer to undergo anaphase I and have a higher incidence of abnormal chromosome configurations. Moreover, when isolated from knockout mice, fewer *in vitro*-cultured embryos develop to the blastocyst stage. In contrast to its centromere localization in wild-type oocytes, AURKB mimics AURKC localization on chromatin in mutant oocytes, suggesting that AURKB and AURKC have similar functions. Taken together, our results provide an explanation for the existence of AURKC in oocytes based on both message recruitment and protein stability.

1125

MPF and MAPK are required for inhibiting premature destruction of the APC D-box substrates in mouse oocytes.

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In meiosis and mitosis, APC^{cdc20} ubiquitinates securin and cyclin B1 to induce anaphase onset and exit from M-phase. More recently, it has been shown that APC^{cdh1} is required for maintaining prophase I arrest by suppressing the levels of cyclin B1. Moreover, during prometaphase I, APC^{cdh1} degrades Cdc20 thereby delaying the onset of APC^{cdc20} activity and the onset of anaphase. However, in contrast to mitosis, the meiotic mechanisms of APCregulation are as yet unexplored, especially during the prolonged prometaphase I.

Here we show that inhibition of MPF and MAPK, but not either one alone, using roscovitine and UO126, respectively, induces premature decrease in the levels of the APC D-box substrates during prometaphase of meiosis I. This effect could be rescued using the proteasome inhibitor MG132, indicating that the decrease is due to a premature targeted destruction. These results suggest that the activity of MPF and MAPK is required for the stability and accumulation of the APC D-box substrates during prometaphase I. Ongoing work is set to explore the mechanisms behind such premature instability upon the inhibition of MPF and MAPK activities.

1126

Efficient siRNA-mediated gene silencing in mouse oocytes by long-term follicle culture.

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Accurate chromosome segregation during oocyte meiosis is fundamental for fertility and normal development of the early embryo. In most animals, and importantly mammals and humans, the first meiotic division is extremely asymmetric to retain the maternally deposited storage proteins and RNA required by the early embryo in the oocyte. The mechanisms of asymmetric mammalian meiosis and chromosome segregation are molecularly not understood and require robust molecular loss-of-function approaches. Mouse oocytes are the experimentally accessible model system closest to human physiology. Although female germ line specific conditional knockout strategies exist and have been employed successfully in the past to identify regulators of meiosis, this approach is very time consuming and thus effectively limits loss-of-function analysis to a few genes.

Therefore, we established a new and simple approach to study gene function in mouse oocytes, by knocking down genes during oocyte growth in long-term follicle culture. siRNA microinjection into early preantral follicle-enclosed oocytes isolated from 12-day old mice followed by eight day in vitro culture leads to efficient knock down of the targeted genes in fully grown maturation competent oocytes. Importantly, this includes genes with abundant and stable maternally deposited protein products that can no longer be silenced in fully grown oocytes. We demonstrate the validity of our method with three genes involved in meiotic cell division: Polo like kinase 1 (Plk1), Cenp-c and Formin2 (Fmn-2). Using our rapid silencing method, we observed defects in asymmetric spindle migration (Fmn2) or chromosome segregation (Plk1 and Cenp-c) that are in agreement with previous reports from knock-out mice. Our robust method allows us to rapidly inactivate even highly expressed and stable gene products and thereby provides us with a tool to study gene function in mammalian oocyte maturation in an efficient and reliable manner. This method will be invaluable to unravel the molecular mechanism of the first asymmetric meiotic division in mouse oocytes.

1127

Mitotic cell rounding is essential for proper spindle assembly.

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In the vast majority of described cases, animal somatic cells round up as they get in mitosis. When cells are in a dense tissue, like an epithelium or a tumor, they are able to deform their neighbors to achieve a nearly perfect spherical shape. Several studies have investigated the molecular and structural changes related to mitotic rounding, but the function of this drastic change in shape is still unknown. Many cultured animal cells display a similar mitotic rounding behavior, interphase cells being often only a few microns high (typically 5 to 7 µm), while spherical mitotic cells can reach up to 20 µm above the culture substrate. We developed a device to precisely control the distance between two surfaces, one bearing cultured cells and the other being made of a material of controlled stiffness. Cells were able to deform ceilings of up to a few kPa while rounding up, which is a typical tissue stiffness. But for harder ceiling (above 10 kPa), they were prevented from rounding and displayed spindle orientation and spindle assembly defects. Using hard ceilings, we precisely varied the spacing. When the ceiling was at 10 µm, spindle orientation was lost but division was normal. Leaving even less place for cell rounding lead to formation of abnormal spindles: normal bipolar mitotic spindles would first form but spindle poles would eventually break before all chromosome had congressed, leading to mutlipolar spindles, mitotic catastrophe and eventually death of cells attempting to divide. We then tried to understand why mitotic spindle assembly is affected by space limitation. Studying in more details chromosome congression in mitotic cell prevented from rounding, we observed that chromosome capture and bi-orientation was strongly affected, leading to chromosomes detaching from the metaphase plate, eventually forming ectopic poles. A model for efficient kinetochore bi-orientation was recently proposed by A. Khodjakov and A. Mogilner, stating that initial chromosome organization in a 'rable star' conformation was essential. Lack of space would thus prevent such a spatial organization of prometaphase chromosomes, leading to mono-oriented kinetochores, or even synthelic attachments. When the delay in kinetochore capture was too long, unattached kinetochores would nucleate their own microtubule fibers, eventually leading to formation of ectopic spindle poles. These results lead us to propose the following working model: mitotic cell rounding would allow cells to push their neighbors and thus to protect the space in which the mitotic spindle is assembled, in order to accurately segregate their chromosomes. In addition, our assay might allow us to discover the precise mechanism by which cells are able to round up when confined by a soft material. We could show that affecting cell rounding mechanism would induce death of cells attempting to divide under a soft material, while either control cells under similar constrain, or cells treated but not confined would divide normally. Cancer cells might be even more sensitive than normal cells to defects in rounding as they often have more chromosomes and are growing in a stiffer environment, suggesting that targeting mitotic cell rounding could be a potent anti –tumor approach.

1128

Reorganization of spatial-temporal dynamics of Eg5 in the mitotic spindle requires TPX2 and dynein.

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The homotetrameric plus-end directed kinesin-5, Eg5, cross-links and slides antiparallel microtubules and is required for centrosome separation during spindle formation. Eg5 localizes to spindle microtubules and is enriched at spindle poles. How the spatial and temporal distribution of Eg5 is regulated throughout mitosis is not established. To address this, we used fluorescence recovery after photobleaching (FRAP) and total internal reflection fluorescence (TIRF) microscopy. GFP^{LAP} tagged Eg5 was expressed from a mouse bacterial artificial chromosome (BAC) under the control of the endogenous promoter. Using the S-peptide in the LAP tag we show that GFP^{LAP} -Eg5 and endogenous Eg5 interact in cells, and that GFP^{LAP}-Eg5 co-localizes with endogenous Eg5 throughout mitosis. FRAP experiments show that GFP^{LAP}-Eg5 is highly dynamic with a recovery half-time < 10 sec in the half-spindle of prometaphase, metaphase and anaphase cells. To gain additional insight into the behavior of Eg5, we used TIRF microscopy. Fluorescent punctae of GFP^{LAP}-Eg5 were highly dynamic and maximum intensity projections of time series of images showed that the punctae aligned along linear tracks. Automated particle tracking showed that most punctae had dwell times less than 0.2 sec. Plots of mean displacement for punctae on astral microtubules showed minus-end directed motion during metaphase; in anaphase cells motion was directed toward microtubule plus-ends. On interzonal microtubules, punctae showed diffusive or transiently directed behavior, and the mean displacement was less than that observed on astral microtubules. Following inhibition of dynein with CC1, minus-end directed motion of Eg5 on astral microtubules was reduced; depletion of TPX2, which interacts with Eg5, resulted in plus-end directed motion on astral microtubules in metaphase cells. However, Eg5 motion on interzonal microtubules was not changed by these treatments. Our results show that Eg5 is highly dynamic on spindle microtubules; that poleward transport requires dynein and TPX2.

1129

How Physical Confinement Regulates Spindle Assembly and Size.

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A fundamental question in biology is how large intracellular structures, such as the mitotic spindle, can assemble and function across the broad range of cell sizes in the embryo . This problem has been difficult to address since no single tissue culture system exists that can generate large quantities of cells spanning the range of eukaryotic cell sizes (from 10 to 1000 microns in length). Our objective was to create cell-like compartments in vitro to probe whether physical confinement can directly control steady-state spindle length. We used a combination of *Xenopus* egg extract systems, which can cycle and self-assemble spindles in vitro, and a number of techniques for encapsulation (extract droplets, giant unilamellar vesicles filled with cytoplasm, and PDMS chambers), to create artificial cells of varying size, each with the ability to generate a functional spindle. Preliminary results demonstrate that the *Xenopus* spindles can sense and respond to the size of the environment they assemble in. These findings suggest that at least partial spindle scaling can occur independently from developmental regulation.

1130

Kif18A Accumulation at the Microtubule Plus-End Modulates Destabilization Activity.

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The mitotic spindle maintains a constant length and size despite the constant switching of the spindle microtubules (MTs) between states of polymerization and depolymerization. Members of the Kinesin-8 family are important for chromosome positioning and for regulation of spindle length. The human Kinesin-8, Kif18A, is a plus-end directed motor that could act either as a plus-end MT depolymerase or as a plus-end capping protein. To determine the mechanism that Kif18A uses to regulate spindle length, we expressed a series of GFP-tagged Kif18A domain constructs in HeLa cells. We found that Kif18A has an ATP-independent MT binding site at the C-terminal end of the tail domain that is important for proper localization of the protein. Overexpression of a construct lacking this region abolishes plus end accumulation of Kif18A and its ability to regulate spindle length. To explore how a depolymerase or a capping protein could promote MT destabilization, we used a Monte Carlo simulation to ask how changing the motor's motility or binding properties would affect MT length distributions. Our modeling predicts that both fast motility and retention at the MT plus end are important for MT destabilization activity. In addition, our simulations suggest that a depolymerase and a capping protein can use very different underlying mechanisms to generate nearly identical effects on MT length distribution. We are currently testing our model-based hypothesis for how the additional MT binding site in the Kif18A tail affects its ability to destabilize a dynamic population of MTs *in vitro*. Together our data highlight the complex control of MT dynamics necessary for controlling proper spindle morphology.

1131

Nucleation and Transport Organize Microtubules in the Meiotic Spindle.

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Spindles are arrays of microtubules that segregate chromosomes during cell division. It has been difficult to distinguish between different models of spindle assembly due to a lack of information on the organization of microtubules in these structures. Here we present a novel method, based on localized laser ablation and high resolution imaging, capable of measuring the detailed architecture of spindles and find that microtubules are shortest near poles and become progressively longer towards the center of the spindle. These data, in combination with biochemical perturbations and mathematical modeling, is sufficient to reject previously proposed models of spindle assembly. Our results support a new model of spindle assembly in which microtubule polymerization dynamics are not spatially regulated, microtubule transport locally sorts microtubules — determining their proper organization in the spindle without moving them appreciable distances —, and the length of the spindle is largely controlled by the profile of microtubule nucleation.

1132

Tropomyosin 4, actin binding protein, contributes to the proper spindle orientation during mitosis and meiosis.

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The network of actin filaments participates in the dynamic regulation of mitotic cellular functions, including centrosome migration, spindle orientation and cytokinesis. The actin cytoskeleton is reorganized during mitosis to form rounded cells with increased cortical rigidity, suggesting that

the formation and function of actin filaments are regulated through mitotic progression. Polo-like kinase 1 (Plk1) is a key regulator of cell cycle progression, especially relevant to the events at mitosis. We identified an actin-filament associated protein tropomyosin 4 (Tm4) as a possible substrate of mitotic kinase Plk1. Tropomyosins are α-helial dimers that bind and stabilize actin microfilaments by regulating their accessibility to other actin-associated proteins. To address the function of Tm4 during mitosis, we performed immunofluorescence microscopic analysis using anti-Tm4. The results showed that Tm4 localized to the centrosome, cell cortex and cytoplasm through prophase to metaphase. Then, we analyzed the effect of siRNA-mediated Tm4-depletion by live-cell imaging and immunofluorescence analysis. We found that Tm4 siRNA treatment destabilized cortical actin organization. This resulted in irregular centrosome movement and misorientation of the spindle, which ended up with a delay of anaphase onset. We further showed that Tm4-depletion by siRNA causes the defect of myosin II cortical recruitment, which contributes to centrosome movement. Expression of wild-type Tm4, but not unphospholylated Tm4 mutant, restored the proper spindle orientation, confirming the requirement of phosphorylated Tm4 for proper spindle orientation. Our results suggest that Plk1-mediated Tm4 phosphorylation stabilizes cortical actin networks during mitosis and thus is required for accurate spindle orientation. Further experiments with mouse oocytes revealed that Tm4 depletion inhibited meiotic maturation. Taken together, we conclude that Tm4 plays important roles in spindle orientation and chromosome migration in both mitosis and meiosis.

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Plk1 regulates the kinesin-13 Kif2b to promote accurate chromosome segregation.

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The polo-like kinase Plk1 is overexpressed in many human cancers and is associated with tumorigenesis. Plk1 is a key regulator of mitosis through its phosphorylation of substrates with roles in numerous processes including spindle assembly, centrosome maturation, checkpoint signaling, and cytokinesis. Recent evidence has revealed that Plk1 also plays a role in chromosome segregation through regulation of kinetochore components such as dynein and CLIP-170. Here we identify a novel kinetochore substrate of Plk1, the human kinesin-13 Kif2b, which promotes faithful chromosome segregation through correction of improper kinetochore-microtubule (k-MT) attachments. Using selective Plk1 inhibitors and in vitro phosphorylation assays coupled with mass spectrometry, we show that Plk1 directly phosphorylates Kif2b at two residues, and that these phosphorylation events differentially regulate its cellular function. Phosphorylation of Serine 204 is required for the kinetochore localization of Kif2b, and phosphorylation of Threonine 125 is required for Kif2b activity. A non-phosphorylatable Serine 204 mutant fails to localize to kinetochores and cannot correct k-MT attachment errors, while a non-phosphorylatable Threonine 125 mutant localizes normally but also fails to correct k-MT attachment errors. These data demonstrate a novel mechanism for Plk1 in ensuring accurate chromosome segregation through regulation of k-MT dynamics and correction of k-MT attachment errors.

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Unpaired chromosomes in Caenorhabditis elegans female meiosis are associated with abnormal segregation.

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Attachment between homologous chromosomes during meiosis I is essential for accurate segregation of chromosomes. Surprisingly, humans with three X chromosomes (triploX) have

normal fertility and give birth predominantly to children with a normal chromosome complement (Reference). If the unpaired X segregated randomly at meiosis I, triploX mothers would produce 50% eggs with one X and 50% eggs with two X chromosomes. The low frequency of XXX and XXY offspring observed suggests that human female meiosis possesses a mechanism that prevents the inheritance of the unpaired X chromosome. In the *C. elegans him-8* mutant, which possesses two unpaired univalent X chromosomes at meiosis I, a similar situation occurs. If the two univalent X chromosomes segregated randomly at meiosis I, him-8 mutants should produce 25% XO male, 50% XX hermaphrodite and 25% XXX progeny. Instead Hodgkin et al. (REFERENCE) reported 38% XO male, 56% XX hermaphrodite and 8% XXX progeny, suggesting that female meiosis in *C. elegans* also possesses a mechanism of selective removal of unpaired X chromosomes. We are currently testing the hypothesis that univalent X chromosomes are selectively extruded into the first polar body. Using live imaging and fixed immunofluorescence, we found that 96% of *him-8* metaphase I spindles have 7 chromosomes at meiosis I whereas X% of wild-type metaphase I spindles had 6 chromosomes. This result demonstrates that both univalent X chromosomes in him-8 worms are still present at metaphase I. 100% of wild-type metaphase II spindles had 6 chromosomes whereas 41% of him-8 metaphase II spindles had 5 chromosomes, 55% had 6 chromosomes and 4% had 7 chromosomes. These numbers suggest that univalent chromosomes are lost between metaphase I and metaphase II. 86% of him-8 anaphase I spindles had 1 or 2 lagging chromosomes whereas only 2% of wild-type anaphase I spindles had lagging chromosomes. Quantification the fates of lagging chromosomes reveals that 60% of these are expelled into the polar body and 33% them are retained in the embryo. Our results are consistent with a model in which univalent X chromosomes biorient at metaphase I but lag at anaphase I because cohesin between sister chromatids is not cleaved. The delayed, poleward movement of these bioriented univalents is biased toward the polar body end of the spindle.

1135

Activation of Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) Repair on Double-Strand Breaks of Mitotic Telomeres.

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Telomeres are essential for protecting chromosome ends from degradation and chromosome fusion. Uncapping of telomeres during interphase elicits a DNA damage response mechanism that results in chromosomal end fusion, chromosome instability and eventually tumorigenesis. However, information about how telomeres detect and repair the damage during mitosis is not well known. In the present study we investigate the repair of HR and NHEJ in laser microirradiation-induced DSBs of telomeres that are induced during the mitotic phase. To determine the nature of DSB repair in mitosis, we irradiated individual telomeres of asynchronous cells with a laser dose that allows cell survival. Microirradiation was used in combination with GFP live-cell imaging and dual-labeling to study DSB repair by monitoring the co-localization of yH2AX with factors involved in either HR or HNEJ. We found that DNA repair proteins (Ku, BRCA1, FANCD2, WRN) and checkpoint sensors (p-Chk1, p-p53) are recruited to microirradiated telomeres. In addition, the phosphorylation status of the Chk1 protein kinase, a well-characterized checkpoint target protein was observed. Consistent with the presence of recognition factors and amplification kinase ATM, phosphorylated Chk1 was present in microirradiated telomeres, and was able to activate chk1-dependent cell cycle arrest. Despite this, telomeres fail to accumulate the checkpoint mediator 53BP1 protein. In addition, we have determined that the kinetics of NBS1 localization at DSBs on telomeres appears to be faster compared to non-telomere areas. In contrast, Ctip, which mediates the first step of resection during HR, is recruited later than the NHEJ and HR repair proteins. These results may provide

information about the accessibility of damaging agents and repair processes in telomere DNA, thus contributing to a better understanding of aging and cancer.

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Survivin-Independent Chromosome Biorientation and Segregation.

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The Chromosomal Passenger Complex (CPC) participates in multiple events during cell division. The four subunits of this complex are the kinase Aurora B, its activating scaffold INCENP, and the targeting factors Survivin and Borealin. The C-terminus of INCENP binds and activates Aurora B kinase. The N-terminus of INCENP trimerizes with Survivin and Borealin to form the CPC. One of the CPC's major functions is during chromosome segregation, where it corrects improper attachments between the centromere regions of chromosomes and spindle microtubules. Recent work has shown that CPC inner-centromere targeting involves recognition by the Survivin subunit of two distinct histone modifications: one modification is directly recognized by a domain of Survivin and the other modification is recognized by Shugoshin, which in turn binds to Survivin. Thus, the current paradigm for CPC function in chromosome segregation is based on Survivin-dependent recognition of specific chromatin features near centromeres. We have generated mutants in the budding yeast INCENP (Sli15) subunit of the CPC that no longer bind to Survivin (Bir1) or Borealin (Nbl1). Surprisingly, cells where the only source of INCENP is this mutant protein exhibit no chromosome segregation defects or growth defects, even under conditions that enhance the consequences of mild chromosome segregation errors. Even more unexpectedly, these INCENP mutants completely alleviated the severe growth defects and cell lethality observed in genetic deletions of either Survivin or Borealin. These INCENP mutants also completely suppressed the severe growth and chromosome segregation defects observed in mutants of proteins that function upstream of Survivin recruitment to chromatin. However, a strong synthetic negative growth defect is observed when INCENP mutants are combined with mutants of the inner-kinetochore COMA complex. These data, which reveal normal chromosome biorientation and segregation in the absence of Survivin, challenge the current paradigm that CPC function in chromosome segregation is based on Survivin-dependent recognition of specific chromatin features at centromeres and indicate that there are additional pathways mediating CPC targeting and function during chromosome segregation.

1137

Interkinetic nuclear migration in the vertebrate neural tube.

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Neural progenitor cells in the pseudostratified neuroepithelium in vertebrates undergo interkinetic nuclear migration, which results in mitotic cells localized to the apical surface whereas interphase nuclei are distributed throughout the rest of the epithelium. Using time-lapse confocal microscopy on chicken neural tube and mouse cerebral cortex slices, we show that the speed of nuclear migration toward the apical surface increases at the start of G2 phase, and this is a microtubule-dependent process. Late in G2 phase, centrosomes leave the apical surface after cilia are lost. Nuclear envelope breakdown initiates away from the apical surface at the time that centrosomes reach the nucleus. The mitotic cell then rounds up to the apical surface, which is an actin-dependent process. Centrosome detachment from the apical surface is correlated with ciliary breakdown. When ciliary breakdown is inhibited, nuclear envelope breakdown occurs closer to the apical surface.

1138

Aneuploidy causes chromosome mis-segregation and karyotype-dependent phenotypic differences in cancer cells.

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Cancers display distinct aneuploid karyotypes and typically mis-segregate chromosomes at high rates. While chromosome mis-segregation results in the generation of aneuploidy by definition, the effect aneuploidy has on chromosome segregation is unclear. To test the effects of aneuploidy on chromosome segregation we utilized the pseudo-diploid colorectal cancer cell line DLD1 (2n=46) and variants of this line containing defined artificial trisomies for chromosomes 7 and 13, termed DLD1+7 and DLD1+13 respectively. We found that the two aneuploid lines exhibited higher rates of chromosome mis-segregation compared to the pseudo-diploid cell line. These results were confirmed by metaphase spread analysis, which showed that the proportion of cells with abnormal chromosome number is larger in the aneuploid cell lines compared to the pseudo-diploid one. Time-lapse analysis of mitosis showed that DLD1+7 cells progressed through mitosis (nuclear envelope breakdown – anaphase onset) more slowly whereas DLD1+13 cells progressed with similar timing compared to DLD1 cells. Interestingly, however, DLD1+13 cells frequently exhibited cytokinesis failure or delay. Accordingly, we observed high rates of polyploidization in DLD1+13 cells as detected by metaphase spread analysis. Microarray analysis of genes overexpressed on chromosome 13 reveal spastic paraplegia 20 (SPG20) overexpressed 9.27 fold (Upender et al., *Can. Res.*, 2004). The protein Spartin encoded by SPG20 has been previously implicated in the progression of cytokinesis (Renvoise et al., *MBoC*, 2010), suggesting that overexpression of this protein may explain the phenotypes observed in DLD1+13 cells. Experiments aimed at testing this hypothesis are currently ongoing. Overall, our study shows that aneuploidy is sufficient to induce chromosome mis-segregation in cancer cells. Moreover, our data indicate that different aneuploidies are associated with distinct chromosome segregation defects and distinct cellular phenotypes.

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Discovery of Novel Anti-Mitotic Compounds Targeting Hec1.

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Hec1 (also called Ndc80) contributes to proper microtubule-kinetochore interactions and spindle assembly checkpoint (SAC) signalling, two functions that are required for normal mitotic progression. Perturbation of Hec1 results in SAC signalling defects, chromosome misalignment, and structural spindle abnormalities. High expression levels of Hec1 are linked to poor prognosis in many cancers making Hec1 a potential mitotic target for cancer intervention by small compounds. We have conducted a combination of virtual and cell-based high-throughput screens to identify small molecules that target the microtubule-binding domain of Hec1. Three million commercially available compounds were screened virtually using FRED and SurFlex softwares. The top 150 compounds were tested in cell-based assays for their ability to induce mitotic arrest. This led to the discovery of several hit compounds that cause chromosome misalignment and mitotic arrest. All hit compounds affect the formation of correct kinetochore-microtubule attachments and, importantly, some of them do not interfere with tubulin polymerization in vitro. The two most promising compounds selected for further studies show binding to Hec1 in preliminary protein-ligand interaction assays. These compounds also inhibit the growth of various cancer cell lines and reduce the size of prostate cancer cell spheroids in 3D cultures. We have recently identified effective structural analogs of the original hit compounds. Interestingly, one hit and its analog exhibit cancer cell selectivity as they potently

kill the tumorigenic MCF7 breast cancer cells but do not affect the growth of the non-tumorigenic MCF10A breast epithelial cells. Currently we are running pilot animal experiments with the lead compounds.

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A 3-M syndrome associated complex in regulating mitosis and cytokinesis and maintaining genome integrity.

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Cullins are a family of evolutionarily conserved proteins that bind to the small RING finger protein, ROC1, to constitute potentially a large number of distinct E3 ubiquitin ligases. Disruption of cullin 7 (Cul7) in mice causes intrauterine growth retardation and perinatal death, and germline mutations in human CUL7 are associated with 3-M syndrome and short stature in Yakuts characterized by pre- and postnatal growth retardation. We found that CUL7 is localized on the mitotic apparatus, and CUL7 depletion causes chromatids alignment delay, prometaphase arrest, mid-body premature disassembly, tetraploidy, and wide spread mitotic cell death. We showed that the function of CUL7 is essential for normal microtubule dynamics and CUL7 depletion sensitizes cells to microtubule stress. These mitotic, cytokinesis and microtubule defects caused by CUL7 depletion can be rescued by wild type, but not CUL7 mutants derived from 3-M patients and were recaptured in CUL7 mutated 3-M skin fibroblasts. We further demonstrate that the product of the other two 3-M associated genes, OBSL1 and CCDC8, bind CUL7 and, when depleted, destabilizes CUL7 or compromises CUL7 localization to the mitotic apparatus, leading to similar defects in microtubule dynamics, mitosis and cytokinesis. We propose a 3-M associated complex in maintaining the microtubule and genome integrity that is essential for the normal development.

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Analysis of cell division defects during the progression of the atherosclerosis.

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Smooth muscle cells (SMCs) proliferation in atherogenesis leads to neointimal thickening. We set to analyze the division rate, the frequency of division defects, and key regulators of division fidelity during progression of in neointimal layer as well as the medial layer beneath it and comparison with adjacent medial layers and uninjured media.
Using a balloon injury rat carotid artery model we studied the division rate as well as karyokinesis and cytokinesis defects in rat carotid neointimal layer at 4 days after balloon injury, the time when the first SMCs start to migrate and form the neointimal layer. The division rate and division defects were higher in the neointimal layer compared to uninjured controls and medial layers where migration of SMCs was not initiated. The division rate in the neointimal layer and in the medial layer beneath it were comparable, but the defect frequency was higher in the neointimal layer. The division rate in neointimal layer increase to 7 days, then it decreased slightly, while apoptosis increased from 4 days to 14 days, suggesting that at 7 days there is a maximal neointimal thickening due to proliferation. PKCα and RHAMM inhibition in a wounded injury cultured neointimal SMCs model caused an increase in multipolar division spindles, centrosome fragmentation and detachment from spindles, binucleate cells, and micronuclei, suggesting that they are required for karyokinesis and cytokinesis fidelity.

RHAMM centrosomal targeting sequence is necessary for normal cellular localization of RHAMM and for spindle pole organization in division and for rear polarization of MTOC of interphase migratory neointimal SMCs.
Neointimal SMCs from injured rat carotid arteries and underlying medial SMCs exhibit elevated rates of proliferation, and the neointimal cell exhibit spindle and cell division defects. PKCαand RHAMM regulate the fidelity of centrosome based division events in neointimal SMCs, and represent valuable targets for possible control of progression of atherogeneis. The centrosomal targeting sequence of RHAMM is necessary for its localization and for spindle pole organization and rear polarization of MTOC.

Novel Imaging Reagents

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PAmKate is a novel far-red probe of the PARFP family for multicolor super-resolution FPALM cell imaging.

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We have developed a far-red shifted photoactivatable red fluorescent protein (PARFP), named PAmKate. Before photoactivation PAmKate absorbs at 440 nm and does not fluoresce. After photoactivation with UV-violet light it becomes fluorescent. As compared to PAmCherry1, PAmKate has ~20 red-shifted excitation and ~30 nm red-shifted fluorescence. PAmKate was applied to a super-resolution 3-color ratiometric fluorescence photoactivation localization microscopy (FPALM) simultaneously with Dendra2 and PAmCherry1 PARFPs, which were spectrally resolved based on photon counts in two imaging channels. The 3-color FPALM imaging of fibroblasts revealed the specific interactions between the membrane proteins and membrane-associated actin. Based on experimental data for our dark-to-red PARFPs, including PAmTagRFP, PAmCherry1, and PAmKate, we propose three types of the photoactivation mechanisms. PARFPs of the first type (PAmCherry1-like type) stop its chromophore maturation at a dark B form (Subach et al., 2010a). Illumination of the B form with UV-violet light results in its oxidation and formation of a red fluorescent R form (B->R). PARFPs of the second type (PATagRFP-like type) stop its chromophore maturation at a dark V form (Subach et al., 2010b). During subsequent illumination with UV-violet light it is oxidized via the B form to the fluorescent R form (V->B->R). PARFPs of the third type (PAmKate-like type) mature with the formation of a dark protonated far-red H-FR form. Illumination of the H-FR form with UV-violet light results in its deprotonation with the formation of a far-red fluorescent FR form (H-FR->FR). Exploring different photoactivation mechanisms will allow design of future advanced photoactivatable probes for super-resolution cell imaging such as orange-shifted PARFPs and PARFPs with large Stokes shifts. Multicolor FPALM, such as demonstrated using PAmKate, will allow addressing mechanistic questions regarding biological functions including mapping of several molecules within a molecular machine, stoichiometric arrangement of several molecules within a subcellular structure, and motion of several molecules within a subcellular environment.

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Photoswitchable orange fluorescent protein forms novel far-red chromophore after irradiation with visible light.

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Photoswitchable fluorescent proteins (PSFPs) with red-shifted illumination are in high demand. In this work based on mOrange we have developed a monomeric PSmOrange protein that is initially orange (ex/em at 548/565 nm) and becomes far-red (ex/em at 636/662 nm) after irradiation with blue-green light. Compared to orange proteins, PSmOrange has greater brightness, better photostability, higher photoswitching contrast, and faster maturation.
PSmOrange has several advantages over green-to-red PSFPs. First, red-shifted characteristics of PSmOrange enable its utilization for multicolor imaging with a cyan-to-green PSCFP2. Second, a good spectral resolution between forms of PSmOrange and red fluorescence of mCherry allows use of these two proteins in the same cell for multicolor imaging. Third, photon statistics of the photoconverted PSmOrange molecules showed that PSmOrange can be used in photoactivation localization microscopy (PALM) experiments and provides a signal considerably far-red shifted compared to other PSFPs. Fourth, when excited using 633 nm or 638 nm lasers or 640/30 nm excitation filter, the photoconverted PSmOrange has higher brightness than conventional far-red fluorescent proteins. Fifth, PSmOrange can be photoconverted with blue light in tissues of a living mouse directly through the skin.
Mass spectrometry of PSmOrange showed that fluorescence of photoconverted PSmOrange results from a novel chromophore containing N-acylimine with a carbon-oxygen double bond coplanar with other double bonds of the chromophore. Thus, our work has resulted in development of the new probe that extends cell and tissue imaging capabilities and has provided insight into photoconversion mechanism of the orange to the far-red state.

1144

A bacteriophytochrome-based near infra-red fluorescent protein for in vivo imaging.

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Non-invasive imaging of dynamic processes in animal tissues using fluorescent proteins (FPs) is a powerful emerging technique due to its simplicity and versatility. However, such application of FPs in mammals has long been challenging since hemoglobin and melanin absorb light in the same spectral region where the majority of FPs are excited and fluoresce. Molecular evolution of the RpBphP2 bacteriophytochrome allowed us to develop a near infra-red fluorescent protein named iRFP, with the excitation/emission maxima at 690 nm/713 nm, inside of so called "near-infrared optical window" where mammalian tissues are relatively transparent. The high brightness and stability in cells, high photostability, and low cytotoxicity suggest iRFP as a probe of choice for the long-term whole-body imaging of small animals. We demonstrated visualization of mouse internal organs by targeting iRFP encoding adenoviruses into liver. Bright iRFP signal was observed for 10 days after virus injection, indicating the protein is stable in vivo. Another important application of iRFP that was shown was fluorescence-based monitoring of the growth of a mouse tumor xenograft. iRFP signal from the developing tumor was observed starting one week after cancer cell injection. To become fluorescent, iRFP requires the endogenous chromophore biliverdin, a product of heme metabolism in all mammalian tissues. Since iRFP has high affinity for biliverdin, the relatively low endogenous concentration of the latter is enough to make iRFP fluorescent, which eliminates the need for the exogenous biliverdin injection. Thus, iRFP is as easy to use as conventional GFP-like proteins. Spectral properties suggest iRFP as a probe for FACS sorting using common 633-640 nm red lasers. Compared to previously developed phytochrome-based probes, iRFP demonstrated superior in vitro and in

vivo properties. Comparing iRFP with far-red GFP-like FPs showed that iRFP provided substantially higher a signal-to-background ratio in a mouse model. Currently, iRFP is the brightest bacteriophytochrome-based and the most far-red shifted FP. Future iRFP applications include imaging of internal organs, monitoring of tumor growth, spreading of infection in body, and tissue development and regeneration.

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The GAF only domain TePixJ from Cyanobacteriochrome of Thermosynechococcus elongatus BP-1 transfected into mammalian cells for enhanced microscopy.

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Phytochromes are widely occurring photoreceptors that mainly respond to red/far-red light in the visible spectrum. Thermophilic cyanobacteria such as *Thermosynechococcus elongatus BP-1* has a phytochrome homologue that demonstrates it's ability to fluoresce red after a mutation in its GAF domain sequence. In this study we characterized isolated GAF domain of TePixJ (Tll0569) from class II cyanobacteriochromes. The project explores use of the GAF only domain of this protein with and without a mutation, which modifies cyanobacteriochromes into a short, bright red fluorescent biliprotein. The truncated GAF domain of TePixJ is 174 amino acids long which is much shorter than previously characterized Infrared Fluorescent Proteins (IFPs). We are engineering a plasmid that can be transfected into mammalian cells using DsRed-Monomer-Actin and AcGFP1-Tubulin vectors as models. Once our protein is expressed in mammalian cells after transfection, it will help with the visualization of the actin or tubulin cytoskeleton under enhanced microscopes. Transfected lymphocyte cells expressing the red-tagged cytoskeleton will be imaged during infection with GFP-labeled HIV in collaboration with partners at the Center for Biophotonics Science and Technology (CBST-UC Davis).

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Optimization of amino acid length of the GAF domain of Tlr0924 cyanobacteriochrome from Thermosynechococcus elongatus BP-1 for bright-red fluorescence.

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Cyanobacteriochromes are photoreceptor proteins found in cyanobacteria and structurally related to plant phytochromes. Cyanobacteriochromes covalently bind a linear tetrapyrrole to the GAF domain and are blue/green photoreversible. Analysis of the Tlr0924 cyanobacteriochrome from the thermophilic cyanobacterium Thermosynechococcus elongatus has shown that the protein exhibits photoconversion between blue and green-absorbing states across a wide range of temperatures. Furthermore, the mutant C499D protein no longer retains the ability to interconvert between the blue and green light-absorbing states and instead fluoresces red. This red fluorescent property has the potential to be useful as a tag for microscopic visualization in living cells. We have fully characterized the spectral properties of the full length mutant protein and have designed four truncations of the GAF domain. Our goal is to determine the smallest possible amino acid sequence that retains or enhances the spectral properties of the full-length mutant. We have altered the amino acid sequence at both ends to determine the most appropriate boundaries for the GAF domain. Both ends of the GAF domain are part of alpha helices which are presumed to contribute to the structural stability of the molecule. The binding pocket into which the tetrapyrrole is nestled is made up of beta-pleated sheets which are contiguous with these alpha helices. Our work seeks to elucidate the role of

the length of the alpha helices in preserving structure and function of the small bright red fluorescent tags derived from Tlr0924.

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Elongation of the Tll0899 GAF domain from the Thermosynechoccus elongatus cyanobacterium for enhanced red-fluorescent proteins.

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Cyanobacteriochromes derive from a family of bilin photoreceptors commonly found in plant phytochromes. These photoreceptive proteins are blue/green photoreversible and function by covalently binding to a linear tetrapyrrole with the GAF domain. Our study involves the cyanobacteriochrome Tll0899 from Thermosynechococcus elongatus, a thermophilic cyanobacterium. We have shown that a mutation in the Tll0899 (152 amino acids) results in a red fluorescent protein that no longer interconverts between blue/green photoreversibility. This mutated red fluorescent protein has an absorbance peak that is not as bright as we desire. If the brightness is enhanced, we plan to utilize this property as a tag to visually observe microscopic cellular events. Tll0899 is unique among this class with a motif of DXDW; all other class II cyanobacteriochromes contain the motif DXDF. The ends of the GAF domain are part of the alpha helices of the protein. These helices are alleged to be the structural support of the molecule. We suggest that the previous truncations of the GAF domain of Tll0899 were too short and hypothesize that the elongation at the ends of the GAF domain will be a stronger structure allowing the protein to fluoresce more brightly. Currently we have four new sequences, each of which vary in number of extended amino acid lengths to the GAF domain. Our goal is to determine if one of these extended GAF domains can enhance the red fluorescence of the mutated protein.

1148

Monomeric Orange Fluorescent Protein with a Large Stokes Shift for Advanced FCS and FRET Microscopy.

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Multicolor imaging using fluorescent proteins (FPs) is a powerful approach to study processes in live cells. Of special interest are the methods that utilize a single excitation wavelength such as the multicolor single-laser fluorescence cross-correlation spectroscopy (FCCS) and simultaneous imaging of several biosensors, which are based on the fluorescence resonance energy transfer (FRET). These techniques require probes with new spectral properties to complement the existing FP toolbox. We have developed a monomeric orange FP with a large Stokes shift (LSS), named LSSmOrange. With excitation/emission maxima at 437/572 nm, respectively, it fills up a gap in the palette of LSS FPs between the green T-Sapphire, yellow mAmetrine and red LSSmKate1, LSSmKate2, and mKeima. LSSmOrange has the brightness comparable to that of T-Sapphire and mAmetrine, pKa value of 5.7, and half-time of its chromophore maturation of 2.3 h at 37°C. Photostability of purified LSSmOrange is similar to that of T-Sapphire, mAmetrine, and mKeima. Due to a lack of other excitation peaks except 437 nm, LSSmOrange can be used as an additional orange color for imaging with conventional orange and red FPs. We demonstrated the applicability of LSSmOrange for multicolor applications using a single-wavelength excitation of several FPs with distinct emission spectra. LSSmOrange is well spectrally resolved with red LSSmKate1, green T-Sapphire and blue TagBFP in a flow cytometry with a single excitation laser. We also applied LSSmOrange to a four-color single-laser FCCS approach using four different FPs. We demonstrated that the four-

color FCCS can be utilized to study co-migrating and single-migrating particles of different stoichiometry. In combination with far-red mKate2 protein as an acceptor, LSSmOrange makes an efficient FRET pair. It can be used together with common CFP-YFP pair in a dual-FRET imaging using a single excitation wavelength. We applied this strategy to simultaneously monitor two biosensors in a live cell.

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Optical recording of action potentials in mammalian neurons with a voltage indicating protein.

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Reliable optical detection of single action potentials in mammalian neurons has been one of the longest-standing open challenges in neuroscience. Here we achieve this goal in cultured neurons using a new voltage indicating protein (VIP1) which has approximately 10-fold improvements in sensitivity and speed over other genetically encoded voltage indicators. The endogenous fluorescence of our microbial rhodopsin-based VIP1 showed 2-fold variation in brightness over physiological voltage range, with a sub-millisecond response time. Single action potentials yielded bursts of fluorescence with optical signal-to-noise ratio > 10. The mutant VIP2 showed 50% greater sensitivity than VIP1 and lacked endogenous proton pumping, but had a slower response (41 ms). VIP2 was still capable of resolving individual action potentials. Microbial rhodopsin-based voltage indicators promise to enable optical interrogation of complex neural circuits, and electrophysiology in systems for which electrode-based techniques are challenging.

1150

Expanding the scope of bioluminescence imaging: synthetic luciferins and mutant luciferases.

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Firefly luciferase-catalyzed light emission from D-luciferin is widely used as a reporter of gene expression and enzymatic activity both in vitro and in vivo. Despite the power of bioluminescence for non-invasive tumor imaging and anti-cancer drug discovery, light emission from luciferase is fundamentally limited by the photochemistry of the luciferin substrate and its ability to access the luciferase (modulated by affinity, cell permeability, and cellular efflux). We have recently described synthetic aminoluciferin derivatives such as CycLuc1 and CycLuc2 which exhibit light emission at longer wavelengths than D-luciferin (> 600 nm) and have increased affinity for luciferase. However, these desirable properties come with the caveat that aminoluciferin substrates exhibit marked burst kinetics with wild-type firefly luciferase due to product inhibition. To help overcome this limitation, we have screened for and identified mutant luciferases that can efficiently emit light with aminoluciferins, both in vitro and in live mammalian cells. We have also synthesized new aminoluciferins that are substrates for these mutant luciferases and yield bioluminescent light emission at even longer wavelengths that are more suitable for whole animal imaging (> 630 nm). Together, these synthetic luciferin substrates and mutant luciferases have the potential to expand the scope of bioluminescent detection, allowing bioluminescence at wavelengths where tissue is more transparent to light, and the construction of orthogonal luciferase-luciferin pairs for multiplexed bioluminescent imaging.

1151

Directed Evolution of a Probe Ligase and Application to Imaging of Intra- and Inter-cellular Protein-Protein Interactions.

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Chemical fluorophores such as Cy dyes and AlexaFluors have superior photophysical properties to fluorescent proteins and are much smaller. To use such fluorophores for protein imaging in living cells, we have developed a site-specific protein labeling method based on engineered enzymes that catalyze fluorophore ligation onto peptide recognition sequences within living cells. Here, we will describe two efforts to re-engineer the *E. coli* enzyme, lipoic acid ligase (LplA), to catalyze ligation of fluorescent probes onto recombinant proteins, instead of its natural substrate lipoic acid. First we will describe the structure-guided mutagenesis of LplA to accept a blue coumarin fluorophore. The resulting “coumarin ligase” can be used to image a wide variety of cellular proteins with high signal/noise ratio and minimal steric perturbation. Second, we will describe the use of yeast display evolution and fluorescence activated cell sorting to evolve LplA into a probe ligase with high activity in the secretory pathway. Selections from a 10^7 library of LplA variants produced a mutant with dramatically improved activity compared to the hand-engineered ligase, and could be used to image cell surface protein-protein interactions within the same cell, as well as between contacting cells. These probe ligases should be useful tools for cell biologists interested in studying protein function in living cells with minimal steric perturbation.

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Wash-Free Fluorescence Imaging in Living Cells using SNAP-tag Fluorogenic Probes.

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The ability to study the dynamic functions of proteins in living cells has been greatly aided by the development and application of tagging tools. An emerging technique for live cell imaging and proteomics applications is the site-specific labeling of cellular proteins with chemical probes. In this approach, small organic molecules are coupled to the protein being studied via a fusion tag, either by self-labeling or enzymatic ligation. Among the most prominent fusion tags is the SNAP-tag, an engineered variant of the human repair protein O6-alkylguanine-DNA alkyltransferase (hAGT) that covalently reacts with O6-benzylguanine (BG) derivatives bearing a chemical or optical probe. During the reaction with a substrate, a stable thioether bond is formed between the reactive cysteine of the tag and the label. SNAP-tag reactions proceed with a well-defined mechanism, predictable stoichiometry and rapid kinetics, irrespective of the fusion protein attached to the tag. However, as for the vast majority of chemical labeling approaches, a thorough wash step is required to reduce fluorescence background signals due to the presence of unreacted probes. Besides being a tedious and time-consuming process, this requirement may potentially limit some applications, such as direct quantification of protein concentration in cell lysates or real-time monitoring of molecular events like receptor-ligand binding, endocytosis, trafficking, and expression of newly synthesized proteins. Thus a strong need remains for efficient molecular imaging methods that enable researchers to assess real-time detection and high-contrast imaging. Herein, we report the design and application of intramolecularly quenched fluorogenic probes that become highly fluorescent upon reaction with SNAP-tag. We demonstrated the feasibility of wash-free labeling protocol for plasma membrane localization and internalization of the epidermal growth factor receptor (EGFR) in living HEK293 cells. We successfully obtained wash-free images of intracellular SNAP tagged histone H2B or β-tubulin in U2OS cells using cytoplasmic delivery techniques, such as glass beads or transfection

reagents, to deliver cell-impermeant fluorogenic probes. In summary, the labeling system described here opens new avenues for for real-time monitoring of highly dynamic processes in living cells, and for high throughput screening of proteins in complex biosystems and drug discovery.

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A chemical strategy for the delivery of sulfonated fluorophores into live cells.

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Live cell fluorescence imaging permits real-time observation of cellular activity. However, most cell-permeable organic fluorophores are very hydrophobic, localizing to cellular membranes and organelles rather than the cytoplasm. One method to increase the water-solubility of a molecule is through the use of sulfonate groups. Unfortunately, this highly polar negatively-charged group generally prevents passage of the molecule through the lipid membrane.

Our lab has developed a method to block the negative charge of sulfonated dyes by disguising them as chemically-stable, esterase-labile sulfonate esters. We have shown that these protecting groups are cleaved by pig liver esterase (PLE) in vitro. Moreover, this strategy can be used to deliver dansyl sulfonate dyes into the cytoplasm of live cells. We next sought to determine whether this approach could be applied to the delivery of near-IR fluorophores, which are more synthetically challenging but superior for live cell imaging.

Living tissue is most transparent to near-IR light (650-900 nm), the ideal wavelength range for the imaging of living systems. However, near-IR fluorophores necessarily present a larger planar hydrophobic surface area than visible-wavelength dyes, increasing their propensity to aggregate. Furthermore, most unsulfonated near-IR dyes are cationic and readily accumulate in mitochondrial membranes. By applying our delivery method to these molecules, we hope to be able to image cells in real-time without the damage incurred by shorter wavelength radiation.

Here we report the synthesis of highly photostable near-IR oxazine fluorophores that incorporate esterase-labile sulfonate esters. We have found that the sulfonate protecting groups in these near-IR dyes, like those of dansyl sulfonates, are selectively unmasked by pig liver esterase. These dyes have potential applications for imaging of anion efflux by drug resistance pumps and for the labeling of cellular proteins with near-IR fluorophores.

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Mixed-surface, Lipid-tethered Quantum Dots for Targeting Cells and Tissues.

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Quantum dots (QDs), with their variable luminescent properties, are rapidly transcending traditional labeling techniques in biological imaging and hold vast potential for biosensing applications. An obstacle in any biosensor development is targeted specificity. Here we report a facile procedure for creating QDs targeted to the cell membrane with the goal of cell-surface protease biosensing. This procedure generates water-soluble QDs with variable coverage of lipid functional groups. The resulting hydrophobicity is quantitatively controlled by the molar ratio of lipids per QD. Appropriate tuning of the hydrophobicity ensures solubility in common aqueous cell culture media and while providing affinity to the lipid bilayer of cell membranes. The reaction and exchange process was directly evaluated by measuring UV-vis absorption spectra associated with dithiocarbamate formation. Cell membrane binding and endocytosis rates were

assessed using flow cytometry and total internal reflection fluorescence imaging with live cells. Tissue affinity was measured using histochemical staining and fluorescence imaging of frozen tissue sections. Increases in cell and tissue binding were found to be regulated by both QD hydrophobicity and surface charge, underlying the importance of QD surface properties in the optimization of both luminescence and targeting capability.

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Two-color in vivo Single mRNA Counting in Yeast for Measuring Intrinsic Noise.

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The development of a method for live cell imaging of mRNA has yielded important information concerning transcription, mRNA export and localization. However, it has been generally limited to tagging of a single gene and has failed to generate resolution of single mRNAs in yeast. The ability to image mRNA in two colors with equal specificity and sensitivity drastically increases the experimental range of the system, making it possible to observe two mRNAs simultaneously, or to tag different regions of the same transcript. Here, we describe a method using a new RNA-tag, derived from the PP7 bacteriophage, for visualizing single *MDN1* transcripts. Single molecule counting using this PP7-system in combination with the MS2-system is used to quantify expression of endogenous MDN1 alleles. Time-dependent expression analysis in diploid yeast revealed that both alleles were expressed independently of one another, with uncorrelated steady-state fluctuations that provide the first measurement of intrinsic noise in mRNA expression over time. Supported by grant GM 57071 to RHS.

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Cell Biology Assays Using Improved DNA Detection Reagents.

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Symmetrical homodimers of cyanine dyes are known to have exceptional sensitivity for detection of nucleic acids with very high fluorescence enhancement upon binding to DNA. In addition, they exhibit increased quantum yields and stability upon binding due to their high affinity for the nucleic acids. We have recently synthesized a series of homodimeric dyes including 6-chloro-YOYO-1 and 6-chloro-TOTO-1 that are analogs of the commercial dyes YOYO-1 and TOTO-1. They differ from the commercial products in that they have chloro-substituents at the six-position of the benzoxazole or benzothiazole rings respectively. Each dye has also been prepared with different anionic counterions imparting improved water solubility. In addition, their excitation and emission spectra upon DNA binding (EX 450 and 520 nm; EM 510 - 540 nm, quantum, yield 0.5) overlaps well with common fluorescence detection systems.
These new analogs have been found to have an approximately two-fold increased sensitivity in gel electrophoresis detection of dsDNA prestained with these new dyes over their parent analogs. The new dyes have also found application in a number of cell biology assays. Staining of fixed chromatin of human breast cancer cells (MDA-MB-231) exhibited bright green signal, providing an alternate fluorescence detection of chromatin/DNA over DAPI. The new dyes have also found use for post-staining amplification products in Loop Mediated Isothermal Amplification (LAMP) assays. In this assay, we utilized 4 primer sequences which recognize 6 distinct regions of bacteriophage or lacZ DNA and a strand disrupting polymerase for amplication under isothermic conditions. In addition, upon application at 1 uM concentration, the new dyes have been found to be suitable for staining flash frozen, ethanol fixed brain tissue

sections. Since these new dyes are cell-membrane impermeant, they have been found to be suitable for distinguishing dead cells when used at a 0.5 uM concnetration and therefore act as a vital reagent in a Live:Dead format assay. In addition, they are less hazardous than other DNA stains and can thus be utilized in live cell analysis formats. In conclusion, our new 6-chloro-YOYO-1 and 6-chloro-TOTO-1 dyes have increased sensitivity in detection and can be a suitable reagent for a number of important biological assays. This work is funded in part by grant number NSF-IIP0923953 from the National Science Foundation-USA.

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Toward Unbiased Direct DNA and RNA Labeling Systems.

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Problems with current genomic microarray analysis techniques include hybridization perturbation caused by base labeling and enzyme-introduced sequence bias for labeling. To solve these problems, we have developed a series of new ultrasensitive labeling reagents for directly labeling DNA or RNA samples isolated from live cells or tissues or prepared by PCR. From their unique diazo reactive group as well as 31P-NMR studies, these labeling reagents have been found to directly modify terminal and backbone phosphate groups of DNA or RNA samples. We developed these new reagents for use in fluorescent (TAMRA, Cy3, Coumarin) as well as hapten (biotin) labeling protocols. The new labeling reagents have been shown to cause less hybridization perturbation.

The effects of the direct labeling method on hybridization efficiency as well as in comparison with existing techniques were examined. Biotin labeling of miRNA isolated from NIH3T3 murine fibroblasts was hybridized to Affymetrix GeneChip miRNA (v1) arrays and compared to poly(A) polymerase enzyme-based labeling which is the adopted labeling method used in most miRNA microarray protocols. We observed a 0.68 Pearson Correlation Coefficient between these two labeling methods, with the new direct labeling reagent detecting 24.4% more sequences. A 13.3% bias in labeling was found for sequences ending with a 3'-uracil for the enzymatic labeling method versus a 1.7% bias for our chemical labeling method. Certain hairpin sequences also exhibited biased labeling. Reports from multiple labs regarding enzyme-based bias in labeling are consistent with these observations. To confirm the differential labeling is due to the enzymatic sequence bias, we also compared biotin labeled miRNA with a chloroethylamino reactive biotin direct-labeling reagent which directly modifies the bases of nucleotides. We observed a 0.97 Pearson Correlation Coefficient with 17.8% higher mean intensity using our new direct labeling over the chloroethylamino direct labeling biotin system. In addition, all four miRNA probes miR-{467a,328,206,214}, which lack G,A,C and U respectively, were labeled and exhibited minimal sequence dependence compared with a 10% hybridization bias for the reactive chloroethylamino biotin labeling of miR-328 and 214 over miR-467a and 206.

We also isolated miRNA from both etoposide-treated and non-treated NIH3T3 cells, and labeled them using our direct biotin labeling reagent and analyzed the miRNA patterns using the miRNA Affymetrix (v1) arrays. The differential expression miRNA patterns obtained upon etoposide treatment were consistent with the literature as well as confirmed by northern blot and qPCR. Uses of these methods to determine the pattern of gene expression upon therapeutic treatment as well as for pharmacokinetic analysis of DNA or RNA based drug therapies have been developed. This work is supported by NSF grant IIP-0923953.

Voltage-Gated Channels and Neurotransmission

1158

Molecular dissection of epilepsy-related neuronal secreted protein, LGI1.

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LGI1 is a neuronal secreted protein, and its mutations cause an inherited form of human epilepsy, autosomal dominant partial epilepsy with auditory features (ADPEAF). Also, autoantibodies against LGI1 is involved in limbic encephalitis with seizures and memory impairment. We have recently reported that 1) LGI1 functions as a ligand for epilepsy-related transmembrane proteins, ADAM22 and ADAM23, and enhances AMPA receptor (AMPAR)-mediated synaptic transmission and that 2) loss of LGI1 in mice reduces AMPAR-mediated synaptic transmission and causes lethal epilepsy. However, the molecular function of LGI1 still remains unclear. Here, we focused on human epilepsy-related LGI1 mutations, as characterization of these mutations helps understand LGI1 patho-physiological functions. We isolated LGI1 mutants identified in ADPEAF pedigrees. Most of LGI1 mutants were not secreted as previously reported (secretion negative mutation, Sec-). Notably, we found that some of reported point mutations did not affect LGI1 secretion process (secretion positive mutation, Sec+). When expressed in the LGI1-knockout mice, both LGI1 mutants could not rescue the epileptic phenotype, under the conditions wild-type LGI1 could rescue it. We found that LGI1(Sec-) did not bind to ADAMs in the brain, and instead that it robustly interacts with proteins involved in biosynthetic secretion pathway. Consistently, this mutant protein was mislocalized in the hippocampus. In contrast, LGI1(Sec+) showed reduced binding to ADAM receptors, and its protein stability was robustly hampered. Thus, secretion and binding to ADAM22/23 are essential for anti-epileptic function of LGI1.

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Antibodies and Autoimmunity: a role in neurodegeneration.

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Objective: To demonstrate a link between autoimmunity to heterogenous ribonucleoprotein A1 (hnRNPA1) and neurodegeneration.
Methods: We utilized microarray analysis, protein and mRNA immunoprecipitation, proximity ligation assay (PLA) and immunohistochemistry.
Results: Multiple sclerosis (MS) is an autoimmune disease that is increasingly recognized as a neurodegenerative disorder. To further investigate this concept, we explored the impact of autoantibodies on neuronal cells. hnRNPA1 autoantibodies are present in biological fluids of MS patients. Whether these molecules penetrate neuronal cells is unknown. One potential mechanism of antibody entry into neurons is endocytosis. To test if endosomes contributed to antibody penetration, we labeled early endosomes with GFP using baculovirus expressing mammalian promoter (BacMam, Invitrogen) and Rab5a targeting sequence. Antibodies to hnRNPA1 and human IgGs were labeled with Atto 550 ester dye for colocalization analysis. The results of these experiments indicate that fluorescently tagged antibodies penetrate neuronal cells by endocytosis. In addition to neuronal penetration, antibodies to hnRNPA1 appeared to alter neuronal phenotype. Next, we hypothesized that antibodies to hnRNPA1 present in the neuronal cytoplasm might interfere with normal protein function and alter RNA levels. Following transfection of anti-hnRNPA1 antibodies into neurons, microarray analysis showed alterations in

spastin (SPG4) RNA levels. Mutations in SPG4 result in hereditary spastic paraplegia; a genetic disease clinically indistinguishable from progressive forms of MS. Further, spastin contains an MIT (microtubule interacting and trafficking protein) site and has been shown to play a role in microtubule stability, synaptic growth and transmission in neurons. Due to this potential link between an autoimmune reaction and a neurodegenerative disease, we evaluated protein-protein interactions between hnRNPA1 and SPG4. Through protein and mRNA immunoprecipitation and proximity ligation assays (PLA) we show that molecular interactions occur between hnRNPA1 and SPG4.
Conclusion: These data suggest a strong connection between autoimmunity and neurodegeneration in MS patients. Autoantibodies penetrate neuronal cells by endocytosis and have a negative effect on the cellular viability. In addition, we have also shown that an interaction exists between hnRNPA1 and SPG4 at the protein and mRNA levels. Further studies are currently taking place to map the site of protein and mRNA interaction between hnRNPA1 and SPG4.

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Ca^{2+} Binding by Synaptotagmin's C2A Domain Essential for Synchronous Synaptic Transmission.
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Synaptotagmin is the major calcium sensor for fast synaptic transmission which requires synchronous fusion of multiple synaptic vesicles. Synaptotagmin contains two calcium binding domains: C2A and C2B. Ca^{2+} binding to C2A has been thought to be extraneous for triggering synchronous fusion because mutations that remove the negatively-charged residues required for Ca^{2+} binding support efficient synchronous fusion. Yet C2A Ca^{2+}-dependent effector interactions *are required* for efficient synchronous fusion. How can the effector interactions be more significant for function than Ca^{2+} binding itself? Based on a structural analysis, we generated a novel mutation of a single Ca^{2+}-binding residue in C2A that blocked Ca^{2+} binding, but *maintained* the negative charge of the pocket. Electrophysiological analyses demonstrated that this C2A mutation resulted in ~80% decrease in synchronous transmitter release and a decrease in the apparent Ca^{2+} affinity of release. Our findings now reveal that previous aspartate to asparagine mutations in C2A inadvertently mimicked Ca^{2+} binding. We show the function of Ca^{2+} binding to C2A is to neutralize the negative charge of the pocket, thereby unleashing the fusigenic activity of synaptotagmin. Our results now demonstrate that Ca^{2+} binding by C2A is a critical component of the electrostatic switch that triggers synchronous vesicle fusion during synaptic transmission.

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Role of the Na+/K+ ATPase in auditory mechanosensation in Drosophila melanogaster.
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Plasma membrane localized Na+/K+ ATPase is important for maintaining ionic homoeostasis in most biological systems. The alpha subunit is the main catalytic pump, which depends on a beta subunit for its transport to the plasma membrane and also for regulating its activity. In adults,

both alpha subunit (ATPα) and Nrv3 (one of the 3 known beta subunit proteins) are expressed in brain, eye and neurons of fly auditory organ (Johnstons Organ or JO) located in the second antennal segment. In addition, ATPα has high expression in the ablumenal plasma membrane of scolopale cells, a support cell type in the auditory organ. The goal of our study is to investigate the functional role of Na+/K+ ATPase in hearing and we hypothesize that it plays a critical role in establishing and maintaining the ionic content of the scolopale space, an extracellular receptor lymph space surrounding the sensory dendrites and enclosed by the scolopale cell of the JO sensory organs. We knocked down expression of ATPα and each of the 3 beta subunits in the JO with UAS/Gal4 mediated RNA-interference (RNAi). Knocking down the ATPα in neurons and scolopale cells using specific drivers resulted in complete loss of hearing as was tested by our auditory electrophysiology assay. Immunohistochemistry shows that these animals lack ATPα expression in the scolopale cells and Electron Microscopy (EM) shows abnormal accumulation of electron dense material in the scolopale space. Knocking down nrv3 in neurons results in hearing loss but knockdown in the scolopale cell does not. RNAi mediated knockdown of nrv2 but not nrv1 (genes encoding the other two beta subunits) in the scolopale cell resulted in almost complete loss of hearing, suggesting its scolopale cell specific role in audition. In conclusion, the Na+/K+ ATPase is likely to be an important molecular player in auditory mechanotransduction by virtue of its role in maintaining the ionic homeostasis in a specific support cell type of the hearing organ.

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All -Trans-Retinoid Acid Induces the Differentiation of Encapsulated Mouse Embryonic Stem Cells into GABAergic Neurons.

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Embryonic stem (ES) cells are pluripotent cells that can differentiate into all three main germ layers: endoderm, mesoderm, and ectoderm. ES cells can be differentiated into several specific cell types depending on the growth factors used to induce their differentiation. A number of methods have been developed to differentiate ES cells into neuronal phenotypes such as sensory neurons, motor neurons, and interneurons. However, differentiation of ES cells into GABAergic interneurons has progressively been a challenge. The main output of inhibitory GABAergic interneurons is the gamma-aminobutyric-acid (GABA). A reduction in GABA signaling can cause neuronal disorders such as epilepsy, autism, seizure, and insomnia. In this study, we generated GABAergic neurons by encapsulating mouse ES cells in alginate-based hydrogels followed by treatment with 5μM all-trans-retinoid acid (RA). Encapsulated ES cells were cultured for eight days and RA was applied at days 4 and 6. On day 8, cells were removed from the hydrogels followed by plating on poly-D-lysin and laminin-coated dishes. The final maturation step was accomplished by culture in supplemented Neurobasal medium for an additional period of 6 days. Our encapsulation-based protocol resulted in a 2-fold increase in neuronal differentiation as compare to a standard non-encapsulation encapsulation protocol. Furthermore, our results indicate that encapsulation of mouse ES cells in alginate-based hydrogels generate GABA-positive neurons (~87-93%) after RA treatment and without the addition of expensive growth factors. The results obtained from these studies may result in more efficient and scalable differentiation strategies for the generation in culture of GABAergic interneurons that are suitable for future therapeutic applications. Thus, the relevance of this technology extends beyond the enrichment of research to the fields of cell replacement therapy and functional CNS treatment modalities.

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Calpain and calcineurin in low-frequency depression at phasic synapses of neuromuscular junction.

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Low-frequency depression (LFD) at crayfish leg extensor phasic synapse of neuromuscular junction (NMJ) is regulated by presynaptic calcineurin. Since calcium buffering by slow chelator EGTA-AM does not block LFD, but the fast chelator BAPTA-AM inhibits LFD, depression is not caused by residual free calcium, but rather the Ca2+ sensor for LFD may be close to a Ca2+ source at active zone. LFD may require activation of presynaptic calcineurin by calpain. Both proteins are present at nerve terminals as shown by immunostaining and Western blot analysis. Calpain inhibition with calpain inhibitors (calpain inhibitor I, MDL-28170, and PD 150606, but not by control PD 145305) inhibits LFD both in intact and in dissected preparations probably acting presynaptically as suggested by miniEPSP analysis. Calpain activity was detected in crayfish preparations using a fluorimetric assay and was modulated by calcium and calpain inhibitors. LFD inhibition by calpain inhibition causes rearrangement of tubulin cytoskeleton at phasic terminals. Another form of synaptic depression, high frequency depression (HFD) does not involve protein phosphorylation- or calpain-dependent mechanisms. In conclusion LFD involves a specific pathway in which local Ca2+ signaling activates calpain and calcineurin at active zones and causes changes of tubulin cytoskeleton during LFD.

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Up-regulation of TREK-2 Potassium Channels in Astrocytes Requires de novo Protein Synthesis: Relevance to Neuroprotection.

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Background: Excitotoxicity due to glutamate receptor over-activation is one of the key mediators of neuronal death after an ischemic insult. A major function of astrocytes is to maintain low extracellular levels of glutamate. Glutamate transport by astrocytes requires a hyperpolarized membrane potential conferred by the presence of potassium channels in the astrocytic membrane. In normal conditions, Kir 4.1 potassium channels are predominantly responsible for maintaining the hyperpolarized membrane potential of astrocytes, but these channels are inhibited during ischemia when ATP is depleted, therefore, an alternative pathway is necessary to maintain glutamate homeostasis during ischemia. We have previously shown that ischemia functionally up-regulates TREK-2 potassium channels in astrocytes.

Objective: The purpose of the present study was to determine the mechanism leading to TREK-2 up-regulation and to assess the contribution of TREK-2 potassium channels to glutamate clearance during ischemia.

Methods: Rat cortical astrocytes were subjected to simulated ischemia (anoxia and reduced glucose) and compared to astrocytes grown in under normoxic conditions with normal glucose. Electrophysiological measurements were used to determine functionality of the TREK-2 channels, whereas real time RT-PCR and Western Blot were performed to determine mRNA and protein levels, respectively. Glutamate clearance was assessed using a colorimetric assay.

Results: Ischemia increased TREK-2 protein levels by 65% and this effect was reversed by the protein synthesis inhibitors emetine (60nM) or cyclohexmide (1μg/ml). In contrast, TREK-2 mRNA levels were not increased in response to ischemia. Whole cell voltage-clamp studies revealed that astrocytes exposed to ischemic conditions had increased temperature sensitive outward currents as compared with control suggesting greater numbers of TREK-2 functional

channels in the astrocytic membrane. Finally, we found that the contribution of TREK-2 channels to overall astrocytic glutamate clearance was greatly increased after ischemia.
Conclusions: Taken together, these data suggest that TREK-2 channels in astrocytes are up-regulated during ischemia through a mechanism requiring de novo protein synthesis. This up-regulation may help to maintain lower extracellular glutamate concentrations and contribute to neuronal rescue.

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A Novel Fluorescent Protein and Biotin Tagged Nav1.6 Channel Allows Analysis of Voltage-gated Sodium Channel Dynamics in Hippocampal Neurons.

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Voltage-gated sodium channels (Nav) are essential for most neuronal excitability and are concentrated at the axon initial segment (AIS) where they are thought to be responsible for action potential initiation. While much is understood about channel structure and function, there is limited information on how cells process sodium channel proteins in real time. How sodium channels are trafficked and localized to the AIS remains unknown. In addition, it is unclear what percentage of AIS channels are actually sodium conducting. Since the large sodium channel cDNAs are difficult to manipulate, the most elegant trafficking work to date has utilized chimeric proteins containing the sodium channel ankyrin-binding motif fused to other membrane proteins. While these approaches address aspects of sodium channel localization, they cannot address sodium channel function and there is always the question of whether these chimeras faithfully reproduce wild-type sodium channel behavior. Appropriately tagged full-length and functional sodium channels are required if sodium channel cell biology is to advance. In the present study, Nav1.6 was tagged with GFP or Dendra2 fluorescent proteins and an extracellular biotin acceptor domain (BAD) in order to allow for visualization and single particle tracking of functional sodium channels on the surface of living cells. This Nav1.6 construct demonstrated wild-type Nav1.6 activity when expressed in hippocampal neurons. Confocal microscopy indicated the tagged channel trafficked efficiently to the cell surface and had a dense accumulation at the AIS. Alexafluor 594-conjugated streptavidin binding indicated the surface density of channels at the AIS was approximately 60 times greater than on the soma. This density compares with that for the endogenous Nav1.6 channel. Fluorescence recovery after photobleaching (FRAP) and single particle tracking showed that channels at the AIS had recovery time constants of greater than 2 hours and were confined to 60nm +/- 20nm compartments. In summary, our study demonstrates the creation of a functional sodium channel construct with fluorescent protein and extracellular biotin reporters that has both wild-type trafficking and biophysical properties. This construct will be utilized to examine channel turnover, trafficking, diffusion and location-dependent function in neuronal cells.

Anterograde and Retrograde Axonal Transport

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Axonal transport of neurofilaments in the mouse optic nerve: a single population of neurofilaments moving intermittently at a broad range of average rates.

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The axonal transport of neurofilaments in mouse optic nerve has been controversial for more than two decades. The central issue is whether or not neurofilaments are deposited into a persistently stationary cytoskeletal network during their transport along this nerve. We have used computational modeling to address this controversy, taking advantage of the wealth of published radio-isotopic pulse-labeling and morphometric data available for neurofilaments in the mouse visual system. We show that the published data can be explained fully by a “stop and go” model of neurofilament transport, in which axons contain a single population of neurofilaments that move in a rapid, intermittent and bidirectional manner. Importantly, we find that a single set of parameters in our model is sufficient to fully explain three different aspects of the data: the shape and location of the transport waves at multiple time points, the decay kinetics from a nerve window, and the observed gradient in neurofilament content along these axons. In addition, we find that the transport kinetics are not consistent with deposition of neurofilaments into a persistently stationary phase, and that a simple deposition model cannot account for the observed gradient in neurofilament content. Finally, we show that the original published evidence upon which the deposition model was based is most likely an experimental artifact due to contamination of the neurofilament transport kinetics with cytosolic proteins that move at faster rates. While neurofilaments alternate between moving and pausing states on a time scale of minutes or hours, our data indicate that on a time scale of days or weeks they behave as a single population. All the neurofilaments in these axons move, and they do so with a single broad and continuous distribution of average rates that is dictated by the intermittent and stochastic nature of their transport behavior.

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Local acceleration of neurofilament transport at nodes of Ranvier.

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Neurofilaments are abundant structural components of the axonal cytoskeleton and are actively transported along axons in a rapid, intermittent and bidirectional manner. In myelinated axons, the axonal thoroughfare is dramatically constricted at regular intervals by nodes of Ranvier, where the number of neurofilaments can decrease locally by tenfold or more. This local constriction creates a potential bottleneck for axonal neurofilament traffic. To avert neurofilament traffic jams and accumulations, we hypothesize that neurofilament transport must be more efficient in nodes than in the flanking internodes, much as water flows more rapidly at a narrowing of the banks of a river. To test this hypothesis, we developed a novel Thy1-PAGFP-NFM transgenic mouse, which expresses a photoactivatable neurofilament protein (photoactivatable green fluorescent protein linked to neurofilament protein M) in neurons. Tibial nerves were removed from adult mice and perfused with oxygenated saline at 37°C during imaging. Axon health was monitored by observation of organelle movement using bright field illumination, and neurofilament transport was monitored by epifluorescence microscopy using the fluorescence photoactivation pulse-escape technique. We activated the neurofilament fluorescence in contiguous nodal and internodal segments along single myelinated axons and

then imaged the activated regions at intervals for thirty minutes to analyze the kinetics of neurofilament departure. On average, the axonal neurofilament content and cross-sectional area in the nodes was 14±7% and 8±3% of the flanking internodes, respectively (n=11). In all cases, the fluorescent neurofilaments departed the nodes faster than the internodes, with 65±15% remaining in internodal regions and 37± 12% in the nodal regions after thirty minutes, and this difference was statistically significant ($p<0.0001$, n=14; paired t-test). No fluorescence decay was observed in the presence of metabolic inhibitor, confirming that the loss of fluorescence was due to active transport. These data demonstrate that the velocity of neurofilament transport is regulated at nodes of Ranvier to ensure a stable neurofilament distribution and axonal morphology. We speculate that the neurofilament accumulations and swollen axons seen in many neurodegenerative diseases could arise to a disruption of these regulatory mechanisms.

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Local regulation of neurofilament transport by myelinating cells.

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Two basic mechanisms by which animals increase axonal conduction velocity are to increase axonal diameter and to insulate axons by myelination. Intriguingly, the growth of axon caliber during vertebrate development is triggered by myelination; most unmyelinated axons do not exceed ≈1μm in diameter, whereas most myelinated axons grow much larger. This radial growth is caused principally by an accumulation of neurofilaments, which are space-filling polymers that function to increase axonal cross-sectional area. Neurofilaments are transported along axons in a rapid, bidirectional and intermittent manner and their motile behavior is dominated by prolonged pauses. We propose that the balance of movements and pauses determines the average velocity, and that this in turn regulates axonal neurofilament content. Thus a slowing of neurofilament transport could contribute to the radial expansion of myelinated axons. To test this hypothesis, we established long-term myelinating co-cultures from rat dorsal root ganglia. After 4-6 weeks in culture, we co-transfected the neurons with photoactivatable neurofilament fusion protein (PAGFP-NFM) and mCherry, a diffusible red fluorescent protein, and analyzed the kinetics of neurofilament transport using the pulse-escape fluorescence photoactivation technique. To determine the effect of myelination on neurofilament transport, we took advantage of the discontinuous myelination in these cultures, which results in contiguous myelinated and unmyelinated regions along many axons. On average we observed a 40% accumulation of neurofilaments in myelinated regions compared to contiguous unmyelinated regions along the same axons, and a 47% expansion of axonal cross-sectional area (n=27, $p<0.05$ and $p<0.001$, respectively; paired t-test). The kinetics of neurofilament departure from both the myelinated and unmyelinated activated regions were biphasic, indicative of distinct long- and short-term pausing states, as we have described previously. However, the filaments departed the myelinated regions more slowly. On average, 34% of the neurofilaments remained in the unmyelinated activated regions after 2 hours compared to 47% in the myelinated activated regions, and this difference was statistically significant (n=27, $p<0.001$; paired t-test). Computational modeling revealed that this change in the decay kinetics can be explained by a 40% decrease in the neurofilament transport velocity, which would be sufficient to explain the observed neurofilament accumulation. Thus the local neurofilament accumulation in myelinated axonal segments can be explained by a local slowing of neurofilament transport. These data indicate that myelinating cells locally regulate axonal morphology by modulating the kinetics of neurofilament transport.

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Neurofilaments are transported along axons in an unfolded and fully extended conformation.

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Live-cell imaging studies in cultured nerve cells have demonstrated that neurofilament polymers move intermittently along microtubule tracks in both anterograde and retrograde directions, powered by kinesin and dynein motors. The instantaneous velocity is fast but the average velocity is slow because the movements are interrupted by long pauses. We have reported previously that the moving neurofilaments typically average about 5-10μm in length, with some as long as 40μm or more, and that they appear to be very flexible. We do not know how motors interact with neurofilaments, but the length and flexibility of these polymers has interesting implications for the site of this interaction. For example, if a motor binds to one end of a filament, it will pull the filament behind it in a fully extended conformation. However, if a motor binds away from the ends, the filament will be pulled from the somewhere in the middle, causing it to fold back on itself like an uneven hairpin with both ends trailing. To investigate the conformation of neurofilaments when they are moving, we transfected primary cultures of cortical neurons from newborn mice with GFP-tagged neurofilament protein M and then imaged the neurofilaments, which appear diffraction-limited in width, by time-lapse fluorescence microscopy. Our observations of 90 filaments revealed that the filaments are indeed very flexible and that they frequently exhibit complex and dynamic folding and unfolding behaviors, including hairpin loops, while they are pausing. Remarkably, however, when the neurofilaments move they almost always appear to be in a fully extended conformation. To test this quantitatively, we performed axial fluorescence intensity profiles along these filaments. Folding events were associated with a 2-3 fold increase in the fluorescence intensity along the folded portion of the filament, accompanied by a corresponding decrease in the filament length. To determine whether moving filaments ever show evidence of such folds, we analyzed 50 moving filaments, some anterograde and some retrograde. In all cases, the fluorescence was uniform along the filaments with no evidence of folding, even at the leading end. A statistical comparison of the fluorescence intensities in the leading, middle and trailing portions of the filaments revealed no significant difference. Thus we suggest that motors must bind to the ends of moving neurofilaments, regardless of their direction of motion, and this has intriguing implications for the mechanism by which motors interact with these cytoskeletal polymers.

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Interaction of neurofilaments with kinesin-1A and dynein/dynactin.

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Neurofilaments are synthesized in the nerve cell body and transported bidirectionally along axons by slow axonal transport. Direct observations of neurofilament transport in cultured neurons indicate that the slow rate is due to rapid movements interrupted by prolonged pauses. Previous studies suggest that the neurofilament motors include dynein/dynactin and kinesin-1A (KIF5A). However, while there is evidence for an interaction between neurofilaments and dynein/dynactin, there is no such evidence for kinesin-1A. Therefore, we examined the interaction of kinesin-1A and dynein/dynactin (p150 subunit) with neurofilaments using immunoprecipitation with magnetic beads. About 0.1% of the total kinesin-1A (average=0.08±0.05%; n=5) and about 0.5% of the total p150 (average=0.5±0.4%; n=6) immunoprecipitated with neurofilaments from mouse brain homogenate. In addition, about 0.1% of the neurofilament protein immunoprecipitated with kinesin-1A (average=0.13±0.1%; n=7), and about 0.7% with p150 (average=0.7±0.6%; n=5). These small percentages are expected

because (1) dynein and kinesin motors have multiple cargoes, (2) only a small proportion of neurofilaments are moving at any time, and (3) it is likely that a few motors are sufficient to move a single neurofilament. To test the specificity of the interaction, we designed a sequential immunoprecipitation approach, in which we first precipitated all the neurofilaments, and then we performed a second immunoprecipitation with the same antibody using the immuno-depleted supernatant from the first immunoprecipitation. We found that neurofilament antibody failed to precipitate kinesin-1A and p150 from the neurofilament-depleted supernatant in the second immunoprecipitation, even though both kinesin-1A and p150 were present in abundance. The reciprocal sequential immunoprecipitation experiment, using kinesin-1A or p150 antibody, yielded similar results. Finally, we found that kinesin-1A and p150 also co-purified and co-immunoprecipitated with neurofilaments from mouse and rat spinal cords. Together, these data suggest a specific interaction between neurofilaments and kinesin-1A and confirm the previously reported interaction between neurofilaments and dynein/dynactin.

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Presenilin and gamma-secretase control kinesin-1 and dynein motor activity in axonal transport.

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Neurons and other cells require intracellular transport of essential components for viability and function. Previous work suggested that the amyloid precursor protein (APP) can function as a kinesin-1 receptor during transport. However, how APP vesicle transport is regulated remains poorly understood. In this context we use genetic analysis and in vivo imaging in *Drosophila* to test the role of normal presenilin (PS) in APP transport. Our results show that reduction of PS suppresses axonal transport defects and blockages induced by excess human APP (hAPP). However, simultaneous reduction of PS and Drosophila APP-like protein (APPL) enhances axonal blockages induced by APPL loss. This PS-dependent enhancement is specific to APPL. No effect is seen with reduction of PS in the context of sunday driver (*syd*) mutants, which disrupt transport of a different class of vesicles. Further, increased velocities are observed for anterograde and retrograde APP vesicles in PS reduced axons. Strikingly, increased retrograde velocities can also be induced by pharmacological inhibition of gamma-secretase activity. These increased velocities require functional kinesin-1 and dynein motors. Our findings suggest that PS and gamma-secretase activity regulate APP intracellular transport by repressing kinesin -1 and dynein motor activity. Thus axonal transport defects induced by excess APP or loss of PS-mediated regulatory effects on APP vesicles could be a major cause of the neuronal defects observed in AD pathogenesis.

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Vesicle Transport without Vesicle Motility: Firmly Attached Vesicles Take a Ride on Moving Microtubules.

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Intracellular transport of vesicles over long distances – a process that occurs in all cells, but is particularly active in neurons - is powered by molecular motors, kinesins (plus-end movement) and cytoplasmic dynein (minus-end movement) that are recruited to, and move, individual vesicles along stationary microtubules. Yet, a fraction of intracellular vesicle trafficking may operate in a different, unexpected way. Earlier observations made by us while studying vesicle transport in squid axons and mammalian neurons suggest that some vesicles, rather than

recruiting molecular motors, appear steadily attached to short microtubules that glide over stationary cytoskeletal tracks. In this way, the vesicles take the ride by piggybacking on moving microtubule segments. Here, we present data in support of this novel form of vesicle transport. First, in proof of principle experiments, we found that in vitro generated vesicle-microtubule complexes move robustly over surfaces coated with active microtubule motors, kinesin-1, as well as cytoplasmic dynein. Second, video-enhanced DIC microscopy with extruded squid axoplasm often showed moving trains of vesicles, maintaining their relative interspacing, as if they were piggybacking on sliding microtubules; that this was the case was confirmed by observing the concerted movement of the clearly identifiable microtubule ends, and of the attached vesicles. Third, in cultured neuronal cells, we identified a specific set of vesicles that are likely transported in association with short, moving microtubules. These vesicles carry sAPP, a secreted polypeptide generated by proteolytic cleavage of the transmembrane protein, amyloid-β precursor protein (APP). We found that axonal transport of sAPP requires kinesin-1. Within neurites, sAPP – but not several other kinesin-1 cargo proteins - localizes exclusively to linear structures, with discontinuous distribution, which we identified as short, acetylated microtubules. With biochemical approaches, we found that, unlike the ATP-dependent binding of kinesin-1 to microtubules, the association of sAPP with microtubules is insensitive to ATP; this indicates that sAPP binding to microtubules does not involve motor proteins. The finding that, in axoplasmic spreads, anti-kinesin-1 antibodies heavily decorate microtubule segments, and not the attached vesicles, is consistent with our nonconventional model of vesicle transport by association with gliding microtubules. Overall, our data expand the current view on vesicle transport; we propose that, in addition to the long-accepted paradigm of individual vesicles moving independently along microtubules, molecular motors could transport en bloc trains of vesicles firmly attached to moving microtubules. Supported by NIH awards GM068596 (V.M., Z.M.) and AG039668 (Z.M.).

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GSK-3 negatively regulates bidirectional transport of APP and other cargos by altering the number of active molecular motors.

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Alzheimer's disease is characterized by abnormal deposits of amyloid plaques which block important processes in neurons, leading to their deterioration. Deficient transport of the Amyloid Precursor Protein (APP) by kinesin-1 molecular motors has been linked to the development of these plaques. Uncovering the regulatory factors that enhance or inhibit axonal transport can lead to new targets for pharmaceutical treatments of neurodegenerative diseases. Glycogen Synthase Kinase 3 (GSK-3) , which has been linked to many aspects of the development of Alzheimer's disease, has also been proposed as a regulator of axonal kinesin-1. We use a combination of biophysical, biochemical and genetic techniques and analyses to examine the role GSK-3 plays in neuronal transport. Using *Drosophila* embryos and larvae with altered GSK-3 expression as our model system, we characterize motor transport of various cargos including APP using DIC microscopy, high-resolution video tracking, fluorescence, and force measurements made with optical tweezers. Comparing the forces, velocities, run lengths, and global distributions of cargos in environments with normal or deficient levels of GSK-3, we find that GSK-3 is a required negative regulator of *in vivo* transport by both kinesin-1 and cytoplasmic dynein, and that GSK-3 directly affects the motion of APP in larval axons and of lipid droplets in embryos. Stall force measurements indicate that GSK-3's regulatory role is not accomplished by weakening the motors, but by altering the number of motors that are actively

pulling at a given time. We find that motor-cargo binding is not affected by GSK-3, leading us to propose that its regulation occurs via a mechanism of changing motor-microtubule interactions.

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Cargo-Specific and Region-Dependent Axonal Transport Defects Induced by Tau.

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Tau-mediated neurodegeneration has been identified as an important pathway for several human neurodegenerative diseases including Alzheimer's disease. It is thought that a possible mechanism of disease development is through tau induced axonal transport defects, which cause neuronal cell death. However, whether, and if so how, tau directly mediates transport defects *in vivo* remains controversial. In this study, we imaged and analyzed with high spatiotemporal resolution axonal transport *in vivo* in two tau transgenic *Drosophila* fly lines that express wild type human tau ($hTau^{WT}$) and a mutant human tau ($hTau^{R406W}$), respectively. To examine the effect of tau on cargoes carried by different motor proteins, transport of two types of vesicles were analyzed. Vesicles containing amyloid precursor protein (APP) tagged by yellow fluorescent protein (YFP) have been shown to be driven by kinesin-1, whereas vesicles containing sytaptotagmin (Synt) tagged with GFP have been shown to be driven by kinesin-3. Using the UAS/Gal4 system with a single neuron driver line, APP-YFP or Synt-GFP was expressed in several neurons in the ventral ganglion of Drosophila 3rd instar larvae. We collected and analyzed time-lapse movies of four equally spaced regions along the proximal-distal axis of axons within the segmental nerves. For APP-YFP vesicles in both tau transgenic animals, there was a significant increase in the fraction of stationary vesicles as well as significant decrease in anterograde velocity, whereas the retrograde transport was not affected. In contrast, transport of Synt-GFP vesicles was not significantly impaired in terms of their velocity, run length and pause frequency in either anterograde or retrograde direction. We then checked for any regional differences of axonal transport defects and found stronger impairment closer to the distal end. Surprisingly, both vesicles paused less frequently under the background of either type of tau in almost all regions along the axon. Together, our data revealed that tau induced cargo-specific and region-dependent axonal transport defects.

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The Schizophrenia-Related Protein, DISC1, Controls Production of Amyloid-β (Aβ), a Pathogenic Peptide Implicated in Alzheimer's Disease (AD), by Regulating Trafficking of Aβ Precursor Protein (APP) along Secretory, Endocytic, and Degradative Routes.

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APP, a type-I transmembrane protein is proteolytically processed into N-terminal (NTFs) and C-terminal fragments (CTFs), and the 38-43-residue Aβ peptides. Overaccumulation of Aβ fragments in neurons is a risk factor for AD. The mechanisms that control the levels of Aβ in neurons are still elusive. We discovered that DISC1, the product of a susceptibility gene for schizophrenia, positively regulates the neuronal load of Aβ: decreased expression of DISC1 renders intracellular Aβ undetectable, whereas the neurons that express high levels of DISC1 accumulate high amounts of Aβ, in culture and in the AD brain. Since DISC1 binds the microtubule motors kinesin-1 and cytoplasmic dynein, we examined whether the transport function of DISC1 is required for accumulation of Aβ in neurons. We found that Aβ is generated through endocytic processes that begin in the soma, and continue during transport into the neurites. We also found that CTFs and Aβ, but not NTFs accumulate in the Rab11-positive, endosomal recycling compartment (ERC) in the soma, indicating that the proteolytic processing

of APP, and sorting of generated fragments begins in the early endocytic route. Within neurites, Aβ is detected within the Rab7-positive, late endosomes, with size and frequency increasing towards the terminals. This suggests that Aβ is generated from CTFs within the anterograde-moving endosomes. DISC1 precisely colocalizes with the CTFs and Aβ at ERC, and with Aβ at the Rab7-positive endosomes, indicating that it is implicated in the transport of endosomal vesicles, where Aβ is generated from CTFs. The inactivation of kinesin-1, by silencing expression of the light chains with siRNA, decreases accumulation of Aβ in neurites to 25%, suggesting that transport of the Aβ- and DISC1-containing late endosomes requires kinesin-1. Silencing DISC1, or impeding dynein function with Lis1 siRNA, decreases the level of Aβ in ERC, indicating that these treatments block APP processing upstream of ERC. These results suggest a model were generation of Aβ begins in early endosomes that progress into ERC by DISC1-, and dynein-mediated transport, and continues in late endosomes, transported by kinesin-1 into neurites. DISC1 facilitates both transport events by recruiting dynein to the APP-containing endocytic vesicles moving toward ERC, and kinesin-1 to ERC-derived endosomal vesicles, carrying Aβ and CTFs, which gradually mature into late endosomes as they advance into neurites. Thus, DISC1 could modulate neuronal levels of Aβ by participating in generation and clearance of Aβ, as part of the molecular complex regulating the transit of APP species through the compartments where Aβ is generated and degraded. These data implicate DISC1 – a schizophrenia-related protein – in the pathogenic mechanism of AD. Supported by NIH awards AG039668 (Z.M.), GM068596 (V.M., Z.M.).

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BDNF mediates competitive interactions between individual cortical neurons.

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Reduced expression of Brain Derived Neurotrophic Factor (BDNF) is observed in many neurological disorders, including Alzheimer's, Huntington's, and Parkinson's diseases and may contribute to neurodegeneration. We previously reported that the loss of forebrain BDNF in conditional knockout mice recapitulates aspects of these diseases including shrinkage of associated brain regions, smaller neuron soma size, decreased dendritic arbor complexity, and reduced dendritic spine density. A variety of evidence indicates that BDNF can be expressed and secreted both pre- and post-synaptically by cortical neurons. The relevance of the associated anterograde and retrograde signaling pathways to features of cellular neurodegeneration has not been described at the single cell level. To determine if BDNF is cell autonomously required for the formation and maintenance of dendritic spines, the structural site at which most excitatory synapses in the mammalian brain occur, we injected visual cortex of mice that were heterozygous or homozygous for a floxed BDNF transgene with Adeno-associated virus with cre (AAV-Cre). AAV-Cre infects isolated cortical neurons, and analysis of the resulting isolated mutant layer 2/3 neurons demonstrated a cell-autonomous requirement for BDNF. Dendritic spine density was reduced in isolated mutant neurons, when these isolated neurons were surrounded by cells with higher levels of BDNF. In contrast mutant neurons surrounded by cells with lower BDNF levels did not show the same reduction of spine density. This comparison amongst genotypes provides strong evidence for an essential requirement for BDNF in mediating competitive interactions that determine dendritic spine density. Furthermore, these data demonstrate that isolated BDNF mutant neurons cannot be rescued by their wild-type neighbors, indicating that BDNF signals at the level of individual neurons in the cortex. These data support a model in which the formation and/or stabilization of some cortical excitatory synapses relies upon competition for a post-synaptic supply of BDNF.

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Amyloid-beta oligomers induce tau-independent disruption of fast axonal transport via calcineurin in cultured hippocampal neurons.

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Emerging evidence suggests that fast axonal transport (FAT) defects are early pathological manifestations of Alzheimer's disease (AD). We have previously shown that soluble amyloid beta oligomers (AβOs), a causative agent of AD, impede FAT of dense core vesicles (DCVs) in cultured hippocampal neurons. AβOs induce hyperphosphorylation of the axonal microtubule-associated protein, tau; however, it remains unclear how this modification disrupts transport. To determine whether tau is required for AβO-induced transport defects, we treated hippocampal neurons from wildtype (WT) and tau knockout (KO) mice with nanomolar concentrations of AβOs. We subsequently assessed DCV transport by live cell imaging of fluorescently-tagged brain-derived neurotrophic factor (BDNF). AβOs reduced DCV bidirectional transport similarly in WT and tau KO neurons, suggesting that tau is not initially involved in AβO-induced DCV transport defects. To investigate whether tau-independent FAT disruption results from changes in microtubule modifications and stability, we assessed tubulin acetylation, tyrosination and polymerization by immunocytochemistry and immunoblotting. In WT and tau KO neurons, AβOs did not induce significant changes in these characteristics. Thus, transport defects may result from dysregulation of intracellular signaling cascades that are implicated in AD pathogenesis. We hypothesized that AβO-disrupted transport results from tau-independent dysregulation of intracellular signaling cascades that activate calcineurin (CaN) and protein phosphatase 1 (PP1). PP1 is inhibited by protein phosphatase inhibitor-2 (I-2) and activated by CaN, a calcium/calmodulin-dependent phosphatase that is upregulated in the presence of AβOs. CaN inhibition using FK506 in cultured neurons from WT and tau KO mice not only rescued, but reversed AβO-induced FAT defects. Moreover, overexpression of I-2 prevented AβO-induced transport defects in hippocampal neurons from WT and tau KO mice. Our results indicate that AβO-induced FAT disruption is independent of tau and microtubule destabilization, and that this dysregulation is mediated by CaN. Work is ongoing to investigate novel roles for aberrant calcium signaling in motor protein inhibition and AD pathogenesis.

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Autophagosome biogenesis in primary neurons occurs distally followed by maturation during transport to the cell soma.

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Autophagy is an essential lysosomal degradation pathway in neurons. To determine the spatial and temporal dynamics of autophagosome biogenesis in primary neurons, we isolated dorsal root ganglion neurons from transgenic mice expressing the autophagosome marker GFP-LC3. Live-cell imaging of GFP-LC3 revealed the robust, constitutive initiation of autophagosomes in the distal end of the neurite. GFP-LC3 is incorporated into ring-like structures an average of 813 ± 23 (± SEM) nm in diameter. Multiple rings accumulate within a single bulbous region at the neurite tip. We used FRAP to analyze the origin of these autophagosomes and observed *de novo* formation of rings, while pre-existing rings did not recover their GFP-LC3 fluorescence indicating stable incorporation of LC3. While the distal population of GFP-LC3 rings exhibited bidirectional motility, autophagosomes that escaped from this compartment moved in a robust retrograde manner over hundreds of microns to the cell soma. 82 ± 2 (± SEM) percent of autophagosomes were minus end-directed, moving along microtubules at an average velocity of

0.45 ± 0.01 (± SEM) µm/sec with few pauses or reversals in direction. This motility is driven by cytoplasmic dynein and was blocked by expression of the dynein inhibitor CC1. To observe the maturation of autophagosomes along the axon, we expressed the dual reporter construct mCherry-GFP-LC3; the GFP is preferentially quenched in acidic environments. Distal organelles were positive for both markers, but we observed an increasing gradient of red-only organelles along the axon. Proximal to the cell body 69 ± 5 (± SEM) percent of LC3-puncta were positive for mCherry only. These results suggest that autophagosomes mature as they move distally to proximally along the axon. This maturation was accompanied by a shift in motility from robust retrograde movement toward more bidirectional motility characteristic of lysosomes. Bidirectional autophagosomes proximal to the cell body were positive for the lysosomal marker LAMP-1, consistent with maturation into autolysosomes. Taken together, these results support a model in which autophagosomes originate distally and mature into autolysosomes during transport toward the cell soma in primary neurons.

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A kinesin-II tail guides the diffusive flow of choline acetyltransferase in *Drosophila* axons.

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More than 200 soluble proteins are transported at about 2-10 mm/day in the axon, which is far slower than the transports of membrane-bound cargoes. The underlying mechanisms are mostly unknown. For instance, choline acetyltransferase (ChAT), a soluble enzyme that catalyzes acetylcholine synthesis, is produced in the neuronal cell bodies and transported to the presynaptic compartments at 1.25 mm/day, which is perceived to be diffusion based. Previous studies showed that the process requires heterotrimeric kinesin-II family motor comprising KLP64D, KLP68D and DmKAP in *Drosophila*. Soluble ChAT also coprecipitates with kinesin-II subunits. Here, we show that both endogenous ChAT and Green Fluorescent Protein (GFP)-tagged recombinant ChAT (GFP:ChAT) associate with kinesin-II through a specific and direct binding to KLP64D tail, which is essential for its transport in the axons. In addition, the apparently diffusive propagation of GFP:ChAT has an anterograde bias in the axons for a limited period. Altogether, these evidences suggest that direct interactions of soluble proteins with motors could actively regulate diffusive movements in cytoplasm.

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Mutation of a Zinc Finger Polyadenosine RNA Binding Protein Causes Autosomal Recessive Intellectual Disability in Humans and Neuronal Dysfunction in a Drosophila Model: A Critical Role for Control of Poly(A) Tail Length.

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We have identified the first gene encoding a polyadenosine RNA binding protein, ZC3H14, which is mutated in inherited nonsyndromic autosomal recessive intellectual disability. ZC3H14 is a member of a novel class of tandem zinc finger polyadenosine RNA binding proteins. The polyadenosine RNA binding module in these proteins consists of tandem CCCH zinc fingers

such as those found in the essential Saccharomyces cerevisiae Nuclear poly(A)-Binding protein 2 (Nab2) and human ZC3H14. Studies of ZC3H14 in higher eukaryotes are extremely limited. Most information about this class of proteins thus far comes from studies of S. cerevisiae Nab2. Although Nab2 mutants have defects in poly(A) RNA export from the nucleus and control of poly(A) tail length, neither the precise molecular function of Nab2 nor the function of this class of proteins in higher eukaryotes is known. We present here the first studies aimed at understanding the function of ZC3H14. We show that ZC3H13 is located in nuclear speckles and also targeted to axons of cultured primary neurons supporting a role in post-transcriptional regulation of gene expression. Behavioral studies in a dNab2 mutant fly model confirm a critical role for dNab2 in neurons consistent with the nonsyndromic form of intellectual disability seen in patients. Taken together, results from these studies show that this class of zinc finger polyadenosine RNA binding proteins is required for proper development and brain function.

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Transport of mRNA in the Projections of Maturing Hippocampal Neurons.

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Introduction: Translation of mRNA in axons and dendrites enables a rapid supply of proteins to specific sites of localization within the neuron. Distinct populations of mRNA-containing cargoes, including granules and mitochondrial mRNA, are transported with neuronal projections. The distributions of these cargoes appear to change during neuronal development, but details on the dynamics of mRNA transport during these transitions remain to be elucidated. The goal of this project is to characterize transport of mitochondrial and non-mitochondrial mRNA in neuronal projections during the development of hippocampal neurons.

Materials and Methods: Hippocampal neurons of one day old rat neonates were cultured on poly-lysine-coated glass cover slips. The neurons were co-labeled with dyes (Invitrogen) marking mRNA and mitochondria, to distinguish mRNA from mitochondrial mRNA. Live fluorescence imaging were performed on three different days, corresponding to different stages of development: **day 4,** when neurons have differentiated, but do not contact other neurons, **day 7**, when they form immature synaptic contacts, and **day 12** when they form mature synapses. Maturity of the neurons was determined via immunolabeling with PSD95, a post-synaptic marker. Immunolabeling with SMI-31, an axon-specific marker, was performed to differentiate axon and dendrites. Parameters of mRNA trafficking were quantified via kymograph (graph of spatial position over time). Statistical analysis was performed by Kolmogorov-Smirnov (K-S) test and ANOVA : Tukey.

Results and Discussion: The results suggest differences in the transport pattern of mitochondrial and non-mitochondrial mRNA, and also indicate significant differences in transport parameters at different time points. Higher mRNA velocity in growing neurons compared to mature neurons was observed. In addition, the mRNA flux increases during growth as it forms mature synapse, presumably because local protein synthesis is essential for long term potential.

Conclusions: The results suggest significance of local protein synthesis during growth of the neurons. To better understand the logic underlying altered mRNA transport, we are currently exploring transport mechanism of mRNA, and its importance in regeneration. This work has important implications for the regulation of neuronal plasticity during neuronal development and in response to neuronal injury.

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The effects of oxygen concentration on mitochondrial function and morphology in SH-SY5Y cells.

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Neurodegeneration is a high-priority matter facing the aging population. Recent research has identified mitochondrial function as critical in the development of neurodegenerative disorders. As such, mitochondria have been extensively studied with loss of mitochondrial integrity being linked to disease progression. While oxygen levels are tightly regulated in vivo, neurons grown in vitro are commonly exposed to atmospheric oxygen levels which is up to 10-fold higher than in tissues. On this basis, we hypothesized that culturing SH-SY5Y cells, a neuroblastoma cell line, at atmospheric oxygen levels detrimentally impacts mitochondrial homeostasis when compared to culturing at physiological oxygen levels. To test this hypothesis, quantitative imaging techniques were utilized. Through the use of a combination of mitochondrial dyes including JC-1, dihydrorhodamine 123, and Mitotracker Red, the contribution of oxygen level to mitochondrial polarization, reactive oxygen species production, and mitochondrial morphology was identified. Furthermore, the impact of oxygen level on mitochondrial markers such as Mfn1, Mfn2, the oxidation-phosphorylation complexes, DRP1, SOD2, Pink1, and GAPDH was evaluated through immunoblotting. Our results show that SH-SY5Y cells cultured at 5% oxygen, an oxygen level similar to the level found in the brain, exhibited a higher mitochondrial potential, a lower production rate of reactive oxygen species, a larger cytosolic fraction of mitochondria, and a more extensive mitochondrial network than neurons cultured at 2% or 21% (atmospheric) oxygen. Furthermore, the expression of several subunits of complexes in the oxidation-phosphorylation cycle were altered in SH-SY5Y cells cultured at 5% oxygen compared to those cultured in higher or lower oxygen, suggesting a correlation between mitochondrial protein expression and mitochondrial structure/function under optimal oxygen levels. Further tests of the effects of these changes in expression on mitochondrial polarization and cellular ATP production were conducted using inhibitors of the various mitochondrial complexes. These data indicate that neuronal cultures in vitro require close regulation of oxygen levels to properly mimic in vivo systems.

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Impaired Mitochondrial Function, Axonal Transport and Morphology in the Neurons of *PINK1* Mutants of *Drosophila.*

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Parkinson’s disease is a common neurodegenerative disease that can be caused by loss-of-function mutations in the mitochondrial serine/threonine kinase *PINK1* and the E3 ubiquitin ligase *Parkin*. Studies in cultured cells suggest that these proteins play roles in the mitochondrial quality control system that targets depolarized mitochondria for autophagic degradation. To explore the role of PINK1 in the intact nervous system, we used live imaging of the intact larval nervous system of *Drosophila PINK1* mutants to examine mitochondrial function, behavior and morphology. We observed substantially diminished mitochondrial membrane potential in the neurons of *PINK1*^{B9}mutants compared to wild type. Analysis of mitochondrial transport parameters in motor neurons of segmental nerves (SNs) of *PINK1*^{B9} mutants showed that mitochondrial velocities were decreased in both anterograde and retrograde directions and mitochondrial duty cycles were also reduced, with a higher percentage of time being stationary. In addition, we observed an increase in the lengths of the stationary pool of mitochondria in motor axons of SNs of *PINK1*^{B9}mutants compared to wild type. These

results suggest that the cellular neuropathology of *PINK1* mutants involves not just mitochondrial turnover, but also defects in mitochondrial function, transport and morphology.

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Expression of Mitochondrial Molecular Motors in Substantia Nigra Before Neurodegeneration-Related Protein Aggregation. A Study in Primary Cell Cultures and Aged Rats.

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Neurodegeneration is often accompanied by intra- and extracellular protein aggregation found throughout the central nervous system. However whether the aggregation is the cause or consequence for cell physiology impairment remains to be determined. The better understanding of the relationship between mitochondria trafficking and protein aggregation is an important step to clarify many aspects of neurodegeneration. In view of this the objective of the present study is to analyze the expression of synthaphilin, kinesins 1 and 5, dynein C1H1 and dynactin which are related to anchoring, anterograde and retrograde traffic of mitochondria. This study was performed before protein aggregation promoted by low doses of rotenone. METHODS: Primary cell cultures from substantia nigra (SN) of newborn Lewis rats were exposed to 0.1, 0.3 or 0.5nM of rotenone during 48h. Twelve-month old Lewis rats received continuous subcutaneous infusion of rotenone (1mg/kg/day) through minipumps during 4 weeks. Cells and age-matched rats were exposed to DMSO as control. Extracted protein from SN (cultures and rats) was analyzed through Western Blot. Experiments were performed in triplicates and evaluated by Student's t-test or one-way ANOVA. RESULTS: Rotenone decreased the expression of syntaphilin in SN cultured cells in a dose-dependent manner, and increased it by 200% in SN of aged rats exposed to 1mg/kg/day of rotenone. Kinesin 1 was 150% up-regulated in vitro after 0.5nM of rotenone, while it was observed a decrease in its expression in aged rats. Rotenone decreased (30%) the expression of kinesin 5 at the concentration of 0.1nM; and increased it (50%) after 0.5nM in cultured cells. Aged rats did not show any change in kinesin 5 expression after 1mg/kg/day of rotenone. In vitro dynein expression slightly decreased after 0.3nM and increased 30% after 0.5nM of rotenone. Aged rats presented decreased expression of dynein C1H1 in SN after rotenone. Dynactin expression was 50% decreased after 0.1nM and increased 30% after 0.3nM of rotenone in cultured cells. Aged rotenone-exposed rats showed a 2-fold increase in dynactin expression. CONCLUSION: Overall the results suggest that mitochondrial traffic may be altered before protein aggregation in SN, and the in vivo results may be comparable to cultured cells exposed to 0.3nM of rotenone. The present findings add to the understanding of the pathophysiology underlying neurodegenerative diseases.

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Analysis of competition between cargo motor receptors during active transport in the squid giant axon: Evidence for a hierarchy of motor-affinities.

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Transport of membrane bound organelles requires microtubule tracks and molecular motors such as the kinesins and dynein to carry cargo. To attach vesicular cargo to specific motors requires binding sites on the cargo which we have identified as peptide zipcodes. We first described a 15 amino acid sequence within the carboxyl-terminus of APP uniquely sufficient to mediate transport of 100 nm plastic beads in the squid giant axon, and now report a 14 amino acid sequence from the JIP-1 MAPK scaffolding protein as another cargo-motor receptor. This adds to two other already known cargo motor receptors, negative charge and the lipid, phosphatidyl insotide. While JIP-1, APP-C and negative charge all display similar velocities consistent with fast axonal transport, they display markedly different biophysical properties such as run length, frequency of pauses, and pause durations. JIP-1 is significantly more efficient in these properties than APP-C and negative charge. All of these cargo motor receptors are competitive with each other for transport machinery. As analyzed by non-parametric statistical algorithms such as cumulative probabilities and Kolmogorov-Smirnov null hypothesis, these differences are highly significant ($P<0.004$). We are applying multiscale mathematical modeling using partial differential equations combined with individual motor-cargo dynamics informed by vector-based analysis of peptide-bead trajectories to predict and characterize behaviors of these cargo motor receptors. Three-dimensional immunofluorescence analysis of the squid axonal cytoskeleton is used to produce dimensions for inter-microtubule distances necessary to predict transit time between microtubule tracks. This powerful combination of engineered cargo dynamics, biophysical measurements and mathematical modeling will enable us to better understand how cargo motor receptors recruit and compete for transport machinery. Supported by NS062184; NS046810 and P5OGM08273 (ELB), DGE-0549500 (P.E.S.) and 1U54CA143837 (V.C.)

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Mechanistic logic behind intracellular trafficking of cytosolic cargoes - a new trafficking paradigm.

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Proteins vital to axonal and presynaptic function are synthesized in the neuronal perikarya and delivered into synapses via two modes of axonal transport. While membrane anchoring proteins are conveyed in fast axonal transport via motor-driven vesicles, cytosolic proteins travel in slow axonal transport via mechanisms that are poorly understood. We found that in cultured axons, populations of cytosolic proteins tagged to photoactivatable GFP (PAGFP) move with a peculiar slow motor-dependent anterograde bias distinct from both vesicular trafficking and diffusion of untagged PAGFP (1, see fig.). The overall bias is likely generated by an intricate particle kinetics involving transient assembly and short-range vectorial spurts; qualitatively and quantitatively distinct from classical modes of axonal transport. In vivo biochemical studies reveal that cytosolic proteins are organized into higher order structures within axon-enriched fractions that are largely segregated from vesicles. Data-driven biophysical modeling best predicts a scenario where soluble molecules dynamically assemble into mobile supramolecular

structures. We propose a model where cytosolic proteins are transported by dynamically assembling into multiprotein complexes that are directly/indirectly conveyed by motors. As cytosolic protein mobility is seen in all cells, this is likely a conserved mechanism for such transport, and we will show some evidence supporting that. We will also present MudPIT-MS data identifying the members of cytosolic protein complexes.

1. Mechanistic logic underlying the axonal transport of cytosolic proteins. Scott D, Das U, Tang Y and Roy S. **Neuron** May **2011** 12;70(3):441-54.
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Jip3-Jnk interaction mediates retrograde axonal transport and axon health.

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Neural circuit assembly and maintenance requires active, axonal transport of proteins and organelles away from (anterograde) and towards (retrograde) the neuronal cell body. This transport is driven by conserved, microtubule-based motors of the Kinesin and Dynein families. Abnormalities in axon transport have been implicated in numerous neurodegenerative and neurodevelopmental diseases but, to date, a vertebrate system in which to analyze this process *in vivo* during development and maintenance of a neural circuit had not been developed. We have developed the posterior lateral line system of the zebrafish for this purpose. The posterior lateral line nerve is comprised of a small number of long, superficial, and planar axons whose stereotyped development has been thoroughly characterized. Additionally, as larval zebrafish are optically transparent, we can visualize transport of fluorescently tagged cargo of interest, expressed under the control of neuron-specific promoters, in these superficial axons without the use of invasive techniques. We used this new approach in conjunction with an ENU-based forward mutagenesis screen to identify zebrafish mutants with abnormalities in axon transport. One of these mutants, *rogue*^{nl7}, carries a loss-of-function mutation in *jnk-interacting protein 3* (*jip3*). Jip3 has been shown to interact with both the light and heavy chains of the Kinesin-1 molecular motor, components of the Dynein complex, and the Jnk signaling module. *rogue*^{nl7}mutant axons displayed large swellings at axonal terminals and, surprisingly, accumulation of activated Jnk (pJnk). *In vivo* analysis of Jnk transport utilizing our newly developed technique revealed a specific decrease in the frequency of retrograde transport of Jnk though distance and velocity traveled by motile puncta were normal. Similarly, general membrane-bound cargo also displayed decreased frequency of retrograde movement and accumulation in the terminal swellings. Immunolabeling experiments indicated that endogenous dynein motor components, Dynein Heavy Chain and p150^{glued}, were distributed normally at *rogue*^{nl7}axon terminals, indicating that retrograde transport was not globally disrupted in the absence of Jip3. Importantly, exogenous expression of Jip3 lacking the Jnk binding domain rescued neither retrograde transport deficits nor axonal swellings. This implicates Jip3-Jnk interaction specifically in retrograde Jnk transport and the generation of axonal swellings. In summary, our results reveal a novel role for Jip3 in the retrograde transport of an active kinase, which in turn affects retrograde transport of other cargo and subsequent axon health.

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The vesicular SNARE Synaptobrevin is required for Semaphorin 3A axonal repulsion.

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Attractive and repulsive molecules such as Semaphorins (Sema) trigger rapid responses which control the navigation of axonal growth cones. The role of vesicular traffic in axonal guidance is still largely unknown. We set out to address the potential role in axonal guidance of exocytic vesicular SNARE Synaptobrevin 2 (Syb2). Here, we took advantage of clostridial neurotoxins and genetic invalidation to demonstrate that Syb2 is required for Sema3A-dependent repulsion but not Sema3C-dependent attraction in cultured neurons and in the mouse brain. Further, more we have shown that Syb2 associates with Neuropilin 1 and Plexin A1, two essential components of the Sema3A receptor, via its juxta-transmembrane domain and Sema3A receptor and Syb2 colocalize in endosomal membranes. Moreover, upon Sema3A treatment, Syb2 deficient neurons fail to collapse and transport PlexinA1 to cell bodies. Reconstitution of Sema3A receptor in non-neuronal cells revealed that Sema3A further inhibits the exocytosis of Syb2. Therefore, Sema3A-mediated signalling and axonal repulsion require Syb2-dependent vesicular traffic.

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Possible Protein Interaction between Maspardin and ALDH18A1 in Mast Syndrome.

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Hereditary Spastic Paraplegias (HSPs) are a family of neurological disorders characterized by weakness and spasticity of the lower limbs and sensory impairment. Mast syndrome is a complicated, autosomal recessive form of HSP exhibiting symptoms of dementia, thinning of the corpus callosum, white matter abnormalities, and spastic paraperesis. Patients with Mast syndrome carry a mutation along their SPG21 gene producing a frame shift and premature stop codon. Thus, Mast syndrome is likely due to loss of functional maspardin protein. Previous studies demonstrate maspardin interacts with ALDH16A1 and following maspardin deletion aldehyde dehydrogenase ALDH18A1, another member of the ALDH superfamily, was upregulation 1.66 fold suggesting an interaction between ALDH18A1 and maspardin may occur. Previous studies indicate patients with a missense mutation, R84Q, along the ALDH18A1 gene have reduced activity of both isoforms thus causing deficiencies of proline, arginine, and ornithine. Since other enzymes are dependent on the correct production of these amino acids, homeostasis throughout the body is also disturbed. Patients with this mutation exhibit progressive neurodegeneration, joint laxity, and bilateral subcapsular cataracts suggesting a link between ALDH18A1 and other neurodegeneration diseases. Interactions between ALDH18A1 and maspardin are being studied using numerous molecular, genetic, and cellular techniques. Correct ALDH18A1 gene and Myc tag vector have been identified and a PCR reaction performed amplifying ALDH18A1 insert. Dephosphorylation of vector, purification of vector and insert, ligation, and transformation of vector and insert into E. coli cells have also been executed. Successful cloning of insert and orientation has been confirmed. In vitro and in vivo immunoprecipitation analyses suggest an interaction occurs, whether this is interaction is direct or indirect will be determined as well as the interaction domains.

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Possible Contribution of Kinesin-1 to Mast syndrome.

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The hereditary spastic paraplegias (HSPs) are a group of neurological disorders characterized by progressive lower-limb spasticity and weakness. One autosomal recessive form of HSP, Mast syndrome (SPG21; MIM 248900), is caused by mutation in the SPG21 gene, which encodes a protein, maspardin, of unknown function. Studies suggest that normal functions of maspardin include protein sorting in the late endosomal/lysosomal pathway and mediating changes in intracellular protein targeting. We will investigate this function by studying the interaction of maspardin and a family of kinesin-1 motor proteins. The kinesin-1 proteins are microtubule motors involved in transport of membranous organelles down the axon and include Kif5a, Kif5b, and Kif5c. Another form of HSP, SPG10, is caused by mutations in Kif5a. In addition, it was revealed through gene chip analysis that Kif5b was upregulated 2.70 fold in the absence of maspardin, which suggests a possible interaction exists. Immunoprecipitation confirmed an interaction between maspardin and kinesin-1 protein, however there was no distinction between the Kif5 isoforms. The various Kif5 isoforms have been cloned into a pGW1-myc-tagged mammalian expression vector, and overexpressed in cell culture. Following SDS-PAGE, isoforms are detected using anti-myc monolclonal antibodies via western analysis. Direct interaction is supported by GST-fusion pull down. We hypothesize that a Kif5 isoform interacts with maspardin and disruption of this interaction contributes to neuropathogenesis. Therefore investigation into subcellular localization and changes following protein deletion between maspardin and Kif5a, Kif5b, and/or Kif5c have commenced.

Host Pathogen/Host-Commensal Interactions II

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Mechanisms of endothelial cell infection by *Listeria monocytogenes.*

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The ubiquitous food-borne bacterium *Listeria monocytogenes*, though ordinarily an agent of gastroenteritis, causes sepsis in pregnant women and meningitis in immune-compromised hosts. The interaction of *L. monocytogenes* with the mammalian gut epithelium has been extensively studied, but less is understood about how *L. monocytogenes* spreads from the digestive tract to infect distal sites, such as the placenta or the central nervous system. Generally, upon entering a host cell, the bacterium harnesses that cell's actin machinery to move within individual cells and from one cell to another, without exposure to the extracellular space. In particular, the bacterium expresses the protein ActA, which activates Arp2/3 to polymerize actin at the bacterial surface; polarized actin polymerization propels the bacterium through the cytoplasm.

We are examining how *L. monocytogenes* is able to subvert cellular barriers during systemic infection; we are primarily focused on its subversion of the vascular endothelium, a single layer of cells that lines the blood vessels and represents a significant barrier to bacterial spread to organs distant from the gut. The most likely mechanisms of bypassing the barrier properties of the endothelium include: direct infection of endothelial cells, infection of endothelial cells via cell-to-cell spread from infected circulating immune system cells, and transmigration of infected

immune system cells across an uninfected endothelium. We have demonstrated that *L. monocytogenes* in infected macrophages can robustly infect monolayers of HUVEC via direct heterotypic cell-to-cell spread, in an ActA-dependent manner. Additionally, we have used fluorescence microscopy to confirm that *L. monocytogenes* can also directly invade a monolayer of human umbilical vein endothelial cells (HUVEC). In the latter case, experiments with an *ΔactA* strain have revealed that while a small fraction of cells (<5%) are directly invaded by *L. monocytogenes*, most infected cells in a sheet are infected by homotypic cell-to-cell spread of bacteria from previously infected HUVEC. Furthermore, we have determined that infected HUVEC divide normally; this process likely contributes to bacterial spread in a sheet. Using time-lapse microscopy, we have observed the *L. monocytogenes* life cycle in infected HUVEC and determined that intra-cytoplasmic bacteria form long filaments in HUVEC that we did not see with epithelial or endothelial cell lines. We are currently exploring how the specific cytoplasmic milieu of endothelial cells influences the bacterial life cycle.

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Defensins Enable Macrophages to Inhibit the Intracellular Proliferation of *Listeria monocytogenes.*

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Listeria monocytogenes is an intracellular pathogen that infects a wide range of host cells including macrophages. To avoid the phagosome microbicidal environment, *L. monocytogenes* secretes the pore-forming toxin listeriolysin O (LLO) that disrupts the phagosomal membrane allowing for bacterial proliferation in the cytoplasm. In this work, we hypothesized that the defensin HNP-1, which is an antimicrobial peptide abundantly found in infected tissues, could protect macrophages from infection by *L. monocytogenes*. We found that *L. monocytogenes* did not grow in murine and human macrophages (RAW 264.7 cells, bone marrow-derived macrophages, and PMA differentiated THP-1 cells) incubated with HNP-1 before or during infection. Using fluorescence microscopy based invasion assays, we observed that HNP-1 did not alter the efficiency of phagocytosis, but markedly enhanced *L. monocytogenes* entrapment in LAMP-1 positive phagosomes and inhibited intracellular proliferation. Concentrations of HNP-1 that did not affect bacterial viability efficiently inhibited the release of LLO as determined by western blotting analysis of proteins precipitated from bacterial culture supernatants. In addition, HNP-1 inhibited LLO perforation of macrophage membranes as evidenced by measuring the incorporation of a small fluorescent nuclear dye into macrophages. These anti-LLO activities likely account for the inhibition of *L. monocytogenes* escape from the phagosome. We tested a second defensin, retrocyclin-1 (RC-1) that is a proposed therapeutic tool to treat bacterial and viral infections. Similar to HNP-1, RC-1 prevented *L. monocytogenes* intracellular growth and exerted anti-toxin activities. Also, RC-1 was a more potent anti-listerial peptide than HNP-1. In conclusion, the cooperation between antimicrobial peptides, such as HNP-1, and macrophages at the site of infection likely plays a critical role in the innate immune defense against *L. monocytogenes*. This is a property that could be exploited in novel therapeutic treatments against intracellular pathogens.

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The listeria P60 protein coopts the inflammasone in mature DCs to stimulate NK cell activation.

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The intracellular pathogen Listeria monocytogenes (Lm) activates the inflammasome in host cells, leading to downstream innate immune responses, including IFNγ; production by activated natural killer (NK) cells. Activated NK cells enhance Lm growth at early times after systemic infection. We have shown that NK cell IFNγ; production requires IL-18 produced by infected dendritic cells (DC), yet the roles of specific bacterial factors in DC/NK cell activation are poorly defined. We previously reported the NK response is weakened in mice infected with an Lm strain lacking expression of the secreted virulence protein p60. In this study, we hypothesized that the released p60 protein interacts with DCs to enhance NK cell stimulation.
We employed a co-culture system in which bone marrow-derived DCs (BDCs) were infected with Lm prior to the addition of naïve NK-enriched splenocytes. We found that wildtype Lm-infected BDCs produced robust levels of the inflammasome-processed cytokines IL-18 and IL-1β;. However, production of these cytokines was significantly reduced in BDCs infected with Δp60 mutant Lm strains. The reduced IL-18 correlated with decreased amounts of IFNγ; secretion from NK cells co-cultured with the infected BDCs. Highly purified, endotoxin-free p60 protein stimulated processing of IL-18 and IL-1β in treated mature BDCs. The addition of p60 to BDC/NK co-cultures was also sufficient to induce IFNγ; from NK cells. Using DCs from *NLRP3*$^{-/-}$ and *IL-18*$^{-/-}$ mice, we demonstrated that p60 elicits processing of IL-18 by the NLRP3 inflammasome, and that such IL-18 production was essential for NK activation in response to p60. Inflammasome and NK cell activation by p60 were independent of its putative endopeptidase catalytic activity and mapped to an N-terminal LysM-domain containing region of the protein, termed L1S. L1S bound to the surface of DCs and was sufficient to stimulate NK cell activation both in co-culture and when administered to naïve animals. These data support the model that the N-terminal region of the secreted p60 protein promotes Lm pathogenicity by binding DCs to stimulate pro-bacterial NK cell activation. The mechanism for such immune subversion involves processing of IL-18 by the NLRP3 inflammasome in infected, or perhaps bystander, DCs. Our data reveal a mechanism by which an Lm virulence factor appears to intentionally elicit activation of the NLRP3 inflammasome, arguing against the assumption that inflammasome activation is universally protective for the host during infections. Furthermore, our findings suggest that p60 or L1S may prove useful for stimulation of innate immune responses in experimental and possibly therapeutic contexts.
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The Pore-Forming Toxin Listeriolysin O Mediates a Novel Entry Pathway of L. monocytogenes into Host Cells.

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Despite the diversity of bacterial virulence factors promoting host cell invasion, only two mechanisms of entry have been observed. First, bacterial invasins bind to host cell receptors to activate signaling cascades that orchestrate the internalization of the bacterium. Second, some pathogens bypass the requirement for a host receptor by utilizing a secretion system that injects effectors into the host cell to activate bacterial uptake. Using Listeria monocytogenes as a

model intracellular pathogen, we have discovered a novel mechanism of entry that involves host cell attack by a pore-forming toxin. Several virulence factors are known to play critical roles in L. monocytogenes intracellular lifecycle. Two surface invasins -InlA and InlB- induce L. monocytogenes internalization into nonphagocytic cells and the pore-forming toxin listeriolysin O (LLO) facilitates bacterial escape from the internalization vesicle. We demonstrate that in addition to InlA and InlB, LLO is a critical invasion factor required for efficient internalization of L. monocytogenes into human hepatocytes (HepG2). Surprisingly, we found that LLO is sufficient to induce the internalization of noninvasive Listeria innocua or polystyrene beads into host cells in a concentration-dependent fashion including the concentrations produced by L. monocytogenes. As shown by electron scanning and fluorescence microscopy, LLO-coated beads stimulate the formation of plasma membrane extensions that ingest the beads into an early endosomal compartment. This pathway requires the activity of host tyrosine kinase(s), the Rho GTPase Rac1, and leads to F-actin polymerization at the entry site. To decipher how LLO could induce these events we constructed and characterized novel LLO variants that bind to the host cell membrane but are unable to form oligomers or that oligomerize but are unable to form pores. Our data demonstrate that membrane perforation is a key event required for F-actin polymerization and bead entry. In conclusion, this is the first demonstration that host cell attack by a pore-forming protein can be used by a bacterial pathogen as an invasive strategy.

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Invasion of Lung Epithelial Cells By Filamentous Legionella pneumophila.

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The term Legionellosis is used to define a group of respiratory illnesses that vary in severity from mild flu like symptoms to fatal pneumonia. It is estimated that Legionellosis is responsible for 8,000 to 18,000 hospitalizations annually in the United States. The severe form of the disease known as Legionnaires' disease has a high mortality rate of upto 80% in immuno-suppressed patients.
Legionella is the etiological agent responsible for Legionellosis. It is an opportunistic pathogen that is found ubiquitously in association with protozoan hosts in natural and engineered water systems as well as in multispecies biofilms. Human infection is established upon inhalation of aerosolized water contaminated with the bacteria. Once internalized the bacteria can evade phagosomal clearance and establish a replicative niche inside macrophages.
Legionella exhibits pleomorphism and while the filamentous forms of the bacteria are present in sputum, bronchoalveolar lavage and alveolar tissue from infected patients and in biofilms, their role in the infectious process in not well studied. Our work is aimed at investigating the mechanisms used by these filaments to infect host epithelial cells.

Our results show that L.pneumophila filaments as long as 80 μm can invade epithelial cells and survive intracellularly. A membrane rich zipper structure forms around attached bacteria causing their internalization. Using various microscopy and biochemical approaches we have elucidated the cellular processes that mediate the internalization of bacterial filaments into cells. We have determined that L.pneumophila exploits both E-cadherin and β1 Integrin receptors on host cells to attach to them. Engagement of these receptors activates intracellular signaling that leads to bacterial internalization through a process that depends on
actin polymerization and the activity of actin motor myosin II. Additionally, we have demonstrated a role for Rab11 GTPase dependent receptor recycling in the progression of the zippers and consequently in bacterial internalization.

The invasion of macrophages by Legionella has been extensively studied, however 95% of the human lung surface consists of epithelial cells. As a result, these cells provide an important niche for bacterial infection. The ability of the filamentous form of the L.pneumophila to invade lung epithelial cells presents additional challenges for the host that have not been addressed in literature so far. Our work examines two important and previously under-studied factors that potentially play a significant role in Legionella pathogenesis.

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Invasion of Polarized Epithelial Cells by Filamentous *Legionella Pneumophila.*

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Legionella pneumophila (Lp.) is an emerging respiratory pathogen that inhabits natural as well as engineered aquatic environments. *Lp.* causes two distinct illnesses; mild flu like Pontiac fever and a severe pneumonia called Legionnaires' disease. *Lp.* is a pleomorphic bacteria and although filamentous forms have been documented in biofilms and tissue/sputum from infected patients, they are not well studied. The ability of bacteria to invade and survive inside mammalian cells is crucial for *Lp.* infection. This process has been very well studied in macrophages, where it is considered to resemble the life cycle of Legionella in its natural host, amoeba. However, the invasion of lung epithelial cells by *Lp.* has been less studied and consequently is poorly understood.
Preliminary data from our laboratory indicate that filamentous *Lp.* attaches to and invades non-polarized cultured lung epithelial cells. This invasion process is mediated by cell surface receptors E-cadherin and ß1-integrin. While these receptors are available at the apical surface in non-polarized epithelia, in polarized epithelial cells tight junctions keep them segregated to the basolateral membrane. The objectives of our research are to investigate the invasion of polarized epithelial cells by filamentous *Lp.*
In order to study *Lp.* infections in polarized epithelial cells, we have utilized two well-established cell models, 16HBE14o- and MDCK cells. 16HBE14o- cells are polarized lung epithelial cells while MDCK cells are widely used as models of highly polarized epithelial cells. Following 7-10 days of differentiation after seeding, polarized cells are infected with late exponential phase *Lp.* and analyzed by fluorescent microscopy. Our results show that Lp. attachment to epithelial cells depends on the level of cell polarity with highly polarized cells being less susceptible to *Lp.* infection. We have also shown that in highly polarized MDCK and 16HBE14o- cells E-cadherin and ß1-integrin receptors are not exposed for *Lp.* attachment. However, wounding the cells can expose more receptors, leading to *Lp.* attachment and invasion.
These results suggested that intact tight junctions in polarized epithelial cells obstruct *Lp.* attachment and inhibit their internalization. However, *Lp.* can take advantage of exposed receptors in non-polarized or wounded epithelia in order to attach or invade epithelial cells. This may also be the case in patients with underling disease or injuries, which are reported to be more susceptible to Legionellosis. These data indicate that tight junctions may play an important role in regulating *Lp.* infection and generate knowledge on the poorly studied but important basic cellular microbiological aspects of *Lp.* pathogenesis.

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The Role of Protein Kinase C in Coxiella burnetii infection.

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Coxiella burnetii, the agent of human Q fever, is an environmentally stable, Gram-negative bacterium that infects humans via contaminated aerosols. The acute form of Q fever includes flu like symptoms and less prevalent chronic disease typically presents as endocarditis. *C. burnetii* targets alveolar macrophages *in vivo* where the organism generates a phagolysosome-like parasitophorous vacuole (PV) for replication. The lengthy growth cycle of *C. burnetii* makes PV formation and maintenance critical for survival. Using a panel of pharmacologic compounds, we previously found that inhibition of 11 kinases and 2 phosphatases prevented proper PV formation. Five inhibitors from this panel targeted protein kinase C (PKC), suggesting a critical role for this protein during *C. burnetii* intracellular growth. In the present study, we further examined the role of classical, novel, and atypical PKC isoforms for their role in PV generation. In cells treated with isoform-specific PKC inhibitors, individual bacteria were harbored in tight-fitting phagolysosomes, as confirmed by CD63 (LAMP-3) and cathepsin D labeling, indicating *C. burnetii* replication was halted. Phosphorylation levels of myristoylated alanine-rich C-kinase substrate (MARCKS), a specific downstream substrate of PKC, remained elevated through 120 hpi, indicating continual stimulation of PKC signaling by *C. burnetii*. Phosphorylation of the classical isoform PKC-α and the novel isoform PKC-δ was observed from 24-96 hpi, indicating activation of both isoforms during infection. Furthermore, Immunofluorescence microscopy demonstrated differential trafficking of PKC-α to the plasma membrane at 48-72 hpi similar to artificial activation by phorbol myristoyl acetate treatment. Together, our current results suggest PKC signaling is critical for proper PV formation and *C. burnetii* replication. Elucidation of isoform-specific consequences will further our understanding of this unique host-pathogen interaction.

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Host PKA Activity is Required for Coxiella burnetii Parasitophorous Vacuole Formation and Replication.

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Coxiella burnetii is a highly infectious intracellular bacterial pathogen that causes the zoonotic disease Q fever. *In vivo*, the organism replicates in a unique phagolysosome-like parasitophorous vacuole (PV) within human alveolar macrophages. Establishment of this intracellular niche is required for replication and survival and necessitates regulation of numerous host cell signaling pathways, presumably via the activity of a specialized Dot/Icm type IV secretion system. We recently showed that a commonly used inhibitor of cAMP-dependent protein kinase (PKA) reversibly impairs typical PV formation, suggesting *C. burnetii* regulates this versatile cascade throughout infection. In the current study, we further probed the role of PKA during infection using more specific inhibitors and downstream readouts of kinase activity. Using a cAMP analog, we found that specific pharmacologic inhibition of PKA activation antagonized PV formation and replication. PKA phosphorylation, indicative of activation, increased through 96 hours post-infection. Downstream PKA substrates were differentially phosphorylated during infection, and inhibition of bacterial protein synthesis abrogated these changes. Interestingly, the prototypical PKA substrate cAMP response element-binding protein was not differentially phosphorylated during infection. However, increased phosphorylation of Bad and p105 was observed, suggesting *C. burnetii* regulates specific PKA substrates related to

apoptosis and the host immune response. Additionally, we investigated cAMP-dependent changes in host transcription and found expression of several downstream PKA targets was altered at three days post-infection. Importantly, PKA was also activated during virulent *C. burnetii* infection of primary human alveolar macrophages, indicating the importance of this signaling cascade in natural infection. We are also currently investigating the role of Dot/Icm in mediating these events. Our results collectively suggest PKA activity is required for *C. burnetii* infection, and the pathogen triggers activation of distinct downstream targets with important implications for host cell responses.

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Chlamydia trachomatis infection induces anchorage independence in a 3T3 soft agar assay.

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Chlamydiae are gram negative, obligate intracellular bacteria, and *Chlamydia trachomatis* is the cause of the most commonly reported sexually transmitted disease in the United States. *Chlamydiae* undergo a unique life cycle that takes place inside a vacuole termed an inclusion. Chlamydial infections have been epidemiologically linked to increased rates in cervical cancer in patients simultaneously infected with human papillomavirus (HPV). Our lab has previously shown that a chlamydial infection induces supernumerary centrosomes and multipolar spindles inhibiting accurate chromosome segregation during mitosis. Many studies indicate that centrosome abnormalities, spindle defects, and chromosome segregation errors can lead to tumors. The soft agar assay is a commonly used technique to detect *in vitro* cellular transformation by measuring anchorage independence. We hypothesize that a chlamydial infection may lead to transformation of 3T3 cells by induction of centrosome abnormalities, spindle defects, and chromosome segregation errors. Here we demonstrate that *Chlamydia trachomatis* is, in fact, able to transform 3T3 cells in soft agar resulting in increased colony formation compared to uninfected 3T3 cells. These results contribute to a growing body of research implicating a role for *Chlamydia* in cervical cancer development.

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Salmonella enterica Typhimurium associated with enterocyte extrusion in an in vivo bovine enteric infection model.

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Salmonella enterica Typhimurium is a major food-borne zoonotic agent with a broad host range causing high morbidity enteritis and low to moderate mortality. The Gram-negative bacterium invades and replicates within a membrane bound niche in several cell host cell lineages, including enterocytes, macrophages and neutrophils. A recent report observed *S.* Typhimurium hyper-replicating in the cytosol of colonic epithelial cells *in vitro* and murine gall bladder epithelial cells *in vivo*. Interestingly, the cytosolic bacteria were expressing the SPI-1 invasion-associated gene profile and induced inflammatory cell death and extrusion. The objective of this study was to further analyze and compare the previous observations in an *in vivo S.* Typhimurium bovine ileal loop model. *S.* Typhimurium harboring conditional expression of destabilized GFP- driven by either the *invF* promoter, a SPI-1 associated gene, or the *ssaG* promoter, a SPI-2 associated gene, were injected into ligated bovine ileum. Following incubation for 2 or 8 hours, ileal sections were excised and biopsies collected for morphological and

molecular analyses. Histological, confocal and transmission electron microscopy confirmed the presence of extruding bovine enterocytes containing multiple *S.* Typhimurium positive for P*invF*-GFP[LVA] fluorescence. Laser-capture microdissection and subsequent molecular analysis of infected extruding cells confirmed expression of a SPI-1 invasion-associated gene profile and host cell inflammatory cell death. These *in vivo* data confirm in the gut the recently described novel survival and dissemination strategies of *S.* Typhimurium.

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The Giant Phosphoprotein AHNAK is Required for Salmonella Invasion of Non-Phagocytic Cells.

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During invasion of non-phagocytic cells, the Gram-negative bacterium Salmonella enterica uses a type III secretion system (T3SS) to deliver bacterial effector proteins into the host cell cytosol. Once inside the cytosol, these effectors drive actin polymerization at the invasion site. Rapid rearrangement of the actin cytoskeleton leads to the formation of prominent ruffles on the surface of the host cell, macropinosome formation, and internalization of the bacteria. While several components of this process are well understood, particularly actin remodeling, the role of membrane trafficking in ruffle formation has not been well studied. Using a proteomics approach we identified the giant phosphoprotein AHNAK (700 kDa) as a host cell protein that is localized to the plasma membrane in response to Salmonella invasion. AHNAK is a membrane-associated protein that has been implicated in vesicle-mediated membrane repair processes, can interact with actin, and act as a scaffolding protein during membrane signalling events. Using fluorescence microscopy we found that AHNAK is recruited to the Salmonella invasion site in HeLa cells. siRNA mediated knockdown of AHNAK results in a significant decrease in Salmonella invasion. To confirm this data we also examined Salmonella invasion in wild-type and AHNAK-null mouse embryonic fibroblasts and determined that AHNAK is required for efficient Salmonella invasion. AHNAK is known to complex with Annexin A2/p11 at the plasma membrane and this interaction has been implicated in cortical actin organization. siRNA mediated knockdown of Annexin A2 and p11 also result in a significant decrease in Salmonella invasion. Together these results suggest that the AHNAK/Annexin A2/p11 complex is required for efficient Salmonella invasion.

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Selective targeting of Rab29 by an effector protein distinguishes the intracellular compartment of human-adapted and broad-host Salmonella.

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Unlike other *Salmonella enterica* serovars (e.g., *Salmonella* Typhimurium), *Salmonella* Typhi can only infect humans, where it causes typhoid fever, a life-threatening disease. The objective of this work is to identify the molecular and cellular bases for different pathogenic properties of human-adapted and broad-host-range *Salmonella* serovars. Here through an RNAi screen to identify Rab GTPases involved in the transport of Typhoid toxin, a unique virulence factor of *Salmonella* Typhi, we found that Rab29 (Rab7L1) is recruited to the *Salmonella* Typhi-containing vacuole but not to the vacuole containing the broad-host serovar *Salmonella* Typhimurium. We observed that in *Salmonella* Typhimurium-infected cells Rab29 is specifically cleaved by the proteolytic activity of GtgE, a novel type III secreted bacterial effector that is absent from *Salmonella* Typhi. We also found that a *Salmonella* Typhi strain engineered to express GtgE and therefore able to cleave Rab29 exhibited increased intracellular replication in human macrophages. These findings indicate fundamental differences in the intracellular biology of human adapted and broad host range *Salmonella enterica* serovars and have

implications for the understanding of *Salmonella* Typhi's ability to cause systemic infection. These results also show how subtle differences in the assortment of type III secreted effector proteins encoded by highly related pathogens can have a major impact in their biology.

1203

Visualizing Salmonella effectors in host cells and characterizing the intracellular niche.

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Salmonella species are gram-negative enteropathogenic bacteria that cause diseases in humans ranging from enteritis to typhoid fever. In order to successfully invade the host, *Salmonella* use two type three secretion systems (T3SSs 1 and 2) to secrete bacterial effector proteins into the host cell. Effector proteins secreted from T3SS1 and T3SS2 are called SPI-1 and SPI-2 effectors, respectively. These effector proteins are responsible for hijacking the host cell machinery to allow for bacterial engulfment and uptake into the host cells (SPI-1 effectors) as well as promoting bacterial replication and furthering *Salmonella* infection (SPI-2 effectors). Our lab has developed a split GFP system to image the localization and dynamics of SPI-2 effectors in real time in live cells. Using this methodology, we have labeled a number of different effector proteins and tracked their localization between 4 hours and 24 hours post invasion. Although numerous effectors localize to the *Salmonella* containing vacuole and tubules that emanate from the vacuole, we observe distinct populations of tubules that colocalize with different pools of host cell markers. We also observe significant perturbation of host cell organelles such as the Golgi apparatus. We are determining which host cell organelles are involved in the formation of the tubules in order to characterize the biochemical composition of these tubules and their role in the overall infection cycle.

1204

Alternative Mating Type Configurations (a/α versus a/a or α/α) of *Candida albicans* Result in Alternative Biofilms, One Pathogenic and the Other Sexual, That Are Regulated By Different Pathways.

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Similar multicellular structures can evolve within the same organism that may have different evolutionary histories, be controlled by different regulatory pathways and play similar but non-identical roles. In the human fungal pathogen *Candida albicans*, a quite extraordinary example of this has occurred. Depending upon the configuration of the mating type locus (**a**/α versus **a/a** or α/α), *C. albicans* forms alternative biofilms that appear similar morphologically, but exhibit dramatically different characteristics and are regulated by distinctly different signal transduction pathways. Biofilms formed by **a**/α cells have all of the characteristics expected of a pathogenic biofilm. They are impermeable to molecules in the size range of 300 Da to 140 kDa, are poorly penetrated by human polymorphonuclear leukocytes (PMNs) and are resistant to antifungals. In contrast, **a/a** or α/α biofilms are permeable to molecules in this size range, are readily penetrated by PMNs and are susceptible to antifungals. Moreover, they facilitate mating between mating competent opaque cells, suggesting that they represent sexual biofilms. By mutational analyses, pathogenic **a**/α biofilms are demonstrated to be regulated by the Ras1/cAMP pathway that includes Ras1→Cdc35→cAMP(Pde2—)→Tpk2(Tpk1)→Efg1→Tec1→Bcr1, and sexual **a/a** biofilms by the MAP kinase pathway that includes Mfá→ Ste2→Ste4, Ste18, Cag1)→Ste11→Hst7→Cek2(Cek1)→Tec1. The permeable, sexual biofilm can be made impermeable by overexpression of the transcription factor Bcr1. These observations suggest the hypothesis that while the upstream portion of the newly evolved

pathway regulating sexual **a/a** and α/α cell biofilms was derived intact from the upstream portion of the conserved pheromone-regulated pathway for mating, the downstream portion was derived through modification of the downstream portion of the conserved pathogenic pathway for **a**/α biofilm formation. *C. albicans* therefore forms two alternative biofilms, one pathogenic and one sexual, depending upon the configuration of the mating type locus.

1205

Investigation of Candida albicans adhesion to endothelial cell monolayers under controlled shear flow.

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Candida albicans is an important pathogen in several biological niches which are constantly irrigated by biological fluids such as saliva or blood. One important step to dissemination of organisms during a septic infection is adhesion to endothelial cells. To approximate the vascular niche more closely, we developed an in vitro model of endothelial cell culture under flow in microfluidic channels. We used these model monolayers to examine adhesion rate and strength of C. albicans yeast and hyphal forms to the monolayers under variable flow. We then tested adhesion rate and strength in monolayers stimulated with TNF-alpha. Under conditions of maximal adhesion, we tested adhesion inhibition with anti-PECAM, anti-VCAM, anti-vitronectin and anti-ICAM. We found maximum rapid adhesion with yeast forms at lower flow rates. However, we found that filamentous forms adhered under the strongest shear forces(in excess of 20dyn/cm2) . Inhibition of adhesion by filamentous forms on unstimulated endothelial cells was noted using anti-PECAM, anti-VCAM-1, anti-ICAM-1, and anti-vitronectin. This model is further extensible to the study of downstream events related to C. albicans pathogenesis such as transmigration.

1206

Host factor screening cleaved by hepatitis C virus protease using wheat cell-free system.

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Hepatitis C virus (HCV) causes acute and chronic hepatitis and leads hepatocellular carcinoma. After infection with HCV, it is not easy to be cured although there are effective anti-viral medicines such as PEG-interferon and NS3/4A protease inhibitor. Thus, new drug targets are required to eliminate HCV. In general, a virus exploits proteins in the host cells for replication, and HCV is also known to use some host factors. Because drugs targeting viral proteins largely produce resistances, recent studies consider possibility that the host proteins are good candidates as drug target. We have an idea that HCV protease may provide an appropriate environment for viral growth by cleavage of host proteins. In this study, we challenged to identify new host factors that are cleaved by HCV protease.

We constructed N- and C-terminally tagged protein libraries consisting of protein kinases and single transmembrane proteins by the wheat cell-free translation system, and monitored the cleavage of these proteins by a HCV protease, NS3/4A, by AlphaScreen system. As a result, we identified 12 human proteins that were cleaved by HCV protease in vitro, and their cleavage sites. Now, we are investigating whether these candidate proteins are cleaved by HCV protease in the infected cells and the cleavage effects on HCV growth.

1207

The effects of hepatitis C virus NS3-4A protein on stress-induced apoptosis.

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Hepatitis C virus (HCV) NS3 functions as a viral protease that together with the NS4A cofactor promotes the viral polyprotein processing. We have previously demonstrated an internal NS3 cleavage activity and its association with an enhanced transforming activity of NS3. In this study, an inducible cell line HepG22-NS3-4A was established. Expression of the NS3-4A polyprotein upon induction and its further processed into individual NS3 and NS4 protein in the cell line were evident. Immunostaining assay demonstrated distribution of the NS3/4A complex to the mitochondria. In addition, expression of the NS3-4A protein alleviated a reduced mitochondria membrane potential caused by deoxycholic acid (DCA). Furthermore, DCA-induced releasing of mitochondria cytochrome C into cytoplasm can be alleviated by overexpression of the NS3-4A polyprotein. Reduced sub-G1 population in DCA treated, NS3-4A expressing 293 cells suggested a role of the NS3-4A polyprotein in protecting cells from DCA-induced apoptosis. This study implies the anti-apoptotic effect of NS3-4A protein involving in HCV tumorigenesis.

1208

Isolation of a hepatitis C virus mutant adapted to mouse CD81 in hepatic cell culture system.

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Infection of hepatitis C virus (HCV) is a rapidly increasing global public health problem, with approximately 170 million people infected worldwide. HCV infects only humans and chimpanzees naturally, which has restricted development of animal models for HCV. Although molecular determinants responsible for the narrow host range of HCV are not well-defined, cell surface CD81 as well as occludin at least contributes to the species tropism at the entry step. From human hepatic Huh7.5.1 cell clones resistant to HCV infection, we isolated a CD81-defective cell clone, designated as 751r. The 751r cells stably expressing human CD81 or mouse CD81 (named 751r/hCD81 and 751r/mCD81, respectively) were also established. In consistent with previous observations, 751r/hCD81cells were permissive to the HCV-JFH1 strain, but both 751r and 751r/mCD81 cells were not. Interestingly, the HCV-JFH1 variant strain with two adaptive mutations (K74T/I414T), which we reported as a highly infectious HCV mutant at the last meeting, however, significantly infected 751r/mCD81 cells. Then we tried to isolate HCV mutants more adapted to mouse CD81. By repeated infections of naïve 751r/mCD81 cells (and Huh7.5.1 cells or 751r/hCD81 cells) with the HCV-JFH1 (K74T/I414T) variant, we finally obtained a HCV-JFH1 mutant showing similar infectivity toward 751r/mCD81 and 751r/hCD81 cells. Sequencing analysis of the mutant virus genome revealed that there are five additional mutations in the structural protein region: F172C in the core protein; N234D, V293A, and T331S in the E1 protein; and V402A in the E2 protein. Since its infectivity and all these mutations were preserved after further repeated infections, the phenotype of the mutant was very stable and the

mutation sites would be important for adaptation to mouse CD81. These results suggest that the adapted mutations in the structural proteins contribute to overcome the CD81-mediaed species-specific restriction of HCV entry into cells. In the future this study should lead to the development of a mouse model permissive to HCV.

1209

Dynamics of ESCRT protein recruitment during retroviral assembly.

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The assembly of retroviruses is the dependent upon the interaction of various components encoded both by the virus and the host cell. The separation of retroviral particles from host membranes is promoted by the cellular ESCRT (Endosomal Sorting Complex Required for Transport) complexes and associated proteins. The ESCRT complexes and associated proteins mediate other membrane scission reactions, such as multi-vesicular body formation and the terminal stages of cytokinesis. These proteins are believed to be sequentially recruited to the site of membrane scission, and then complexes are disassembled by the ATPase Vps4A. However these events have never been observed in living cells and their dynamics are unknown. Characterizing the relative time course of these molecules is complicated in cells where each virion is in a different state of assembly. To characterize the dynamics of these molecules, we studied the assembly of individual virions. By quantifying the recruitment of several ESCRT and associated proteins during the assembly of two retroviruses, EIAV and HIV-1, we show that Alix progressively accumulated at viral assembly sites, coincident with the accumulation of the major viral structural protein, Gag, and was not recycled after assembly. In contrast, ESCRT-III and Vps4A were only transiently recruited when the accumulation of Gag was complete. These data suggest that the rapid and transient recruitment of proteins that act late in the ESCRT pathway and carry out membrane fission is triggered by prior and progressive accumulation of proteins that bridge viral proteins and the late-acting ESCRT proteins.

1210

Sub-localization of Murine Norovirus proteins and its genome RNA.

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Human norovirus (HuNoV), a member of the family Caliciviridae, is the dominant agent of acute gastroenteritis. HuNoV replication is difficult to study because there is no cell culture system or small animal model. However murine norovirus (MNV) was recently found to replicate in RAW264.7 cells. To understand MNV life cycle in cells, we used immunofluorescence deconvolution microscopy to visualize MNV proteins (VPg, RNA dependent RNA polymerase: RdRp, NTPase, and structural proteins VP1 and VP2), double stranded viral RNA (dsRNA), and newly synthesized viral RNA (nsRNA) during replication in RAW264.7 cells. At 12 hours post infection, the dsRNA was observed at the perinuclear region. The small amount of nsRNA was also localized with the dsRNA at the perinuclear region, however most of the nsRNA were spread diffusely throughout the cytoplasm. The RdRp, NTPase and VPg proteins were localized at the perinuclear region, indicating that MNV replicates at perinuclear region, and the replication complex is composed of dsRNA, RdRp, NTPase, VPg and a small amount of nsRNA. The structural protein, VP1, was detected in the cytoplasm and appeared to co-localize with nsRNA, suggesting that virion assembly, which involved nsRNA and VP1, was at a different site to the replication complex. The other structural protein, VP2 was detected in the cytoplasm

in particle forms, but interestingly it was not co-localized with any viral proteins (RdRp, NTPase, and VPg) and with any genome RNA (nsRNA and dsRNA).

Defining Therapeutic Targets and New Therapeutics

1211

Transduced Tat-Frataxin protein ameliorates MPTP-induced Parkinson′s disease mouse model.

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Frataxin is an essential mitochondrial protein which deficiency causes increased reactive oxygen species (ROS), and deficit of iron-sulphur cluster, and mitochondrial dysfunctions. Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by selective loss of dopaminergic neurons in the substantia nigra pars compacta and it is associated with ROS. Thus, we investigated the protective effects of Tat-Frataxin protein on neuronal cell death in vitro and in vivo. Tat-Frataxin protein showed a protective effect on 1-methyl-4-phenylpyridinium ion (MPP+)-induced SH-SY5Y neuronal cell death by reduction of ROS and caspase-3 activation. Furthermore, a histological analysis indicated that transduced Tat-Frataxin protein markedly protects against dopaminergic neuronal cell death in 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP)-induced PD mouse models. These results strongly suggest that Tat-Frataxin may provide a useful therapeutic strategy for the prevention of progressive neurodegenerative disorders such as PD.

Keywords: Antioxidant, MPP+, Parkinson disease, Protein transduction, Reactive oxygen species (ROS), Tat-Frataxin protein.

1212

PEP-1-heat shock protein 27 protects from neuronal damage in cells and in a Parkinson′s disease mouse model.

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Heat shock proteins (HSPs) are induced in response to a variety of cellular responses including reactive oxygen species (ROS). HSP27 is a chaperone protein with the ability to increase cell survival in response to oxidative stress. Parkinson's disease (PD) is a neurodegenerative disorder characterized by loss of dopaminergic neurons. Although the mechanism of PD remains unclear, oxidative stress is known to be important in the pathogenesis of PD. This study investigated the protective effects of PEP-1-HSP27 on neuronal damage induced by 1-methyl-4-phenyl pyridinium (MPP+) in SH-SY5Y cells and a 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP)-induced PD mouse model. PEP-1-HSP27 rapidly entered the cells and protected them against MPP+-induced toxicity by inhibiting the ROS levels and DNA fragmentation.

Furthermore, transduced PEP-1-HSP27 prevented dopaminergic neuronal cell death in the substantia nigra of MPTP-induced PD mouse models. These results demonstrate that PEP-1-HSP27 protects against neuronal cell death in vitro and in vivo, and suggest that PEP-1-HSP27 may have therapeutic potential in preventing or delaying progression of PD, and utility as a therapeutic agent for various human diseases related to oxidative stress, including PD.
Keywords – PEP-1-HSP27; ROS; Protein transduction; Parkinson's disease; Protein therapy.

1213

Tat-proline rich Akt substrate 40 (Tat-PRAS40) attenuates MPP+-induced dopaminergic neuronal cell death in a mouse model of Parkinson′s disease.

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Parkinson's disease (PD) is caused mainly due to loss of dopaminergic neurons in the substantia nigra (SN), leading to accumulation of Lewy bodies inside nerve cells. Primary characteristic symptoms include tremors, bradykinesia, rigidity and postural instability. Phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin signal pathways play crucial roles in controlling cell growth, survival and apoptosis. Proline-rich Akt substrate (PRAS40) is implicated in negative regulation of mTORC1 activity through binding to the raptor-mTORC1 complex. In this study, we examined whether Tat-PRAS40 fusion protein could protect dopaminergic neuronal cells from 1-methyl-4-phenylpyridinium (MPP^+)-induced oxidative stress, *in vitro* and *in vivo*, thereby increasing survival of dopaminergic neuronal cells. Transduced Tat-PRAS40 effectively suppressed MPP^+-induced ROS production and attenuated apoptosis to a considerable extent in SH-SY5Y cells. Also, in a MPTP-induced mouse model of PD, cresyl violet staining and tyrosine hydroxylase immunostaining demonstrated that Tat-PRAS40 could readily and effectively cross the blood-brain barrier and protect dopaminergic neuron cells in the SN. The collective results suggested that Tat-PRAS40 could be utilized as a potent therapeutic agent against oxidative stress-induced PD.
Keywords: Apoptosis, Mammalian target of rapamycin, Oxidative stress, Parkinson's disease, Proline-rich Akt substrate 40, Protein transduction domain.

1214

Chronic central administration of valproic acid increased pro-survival proteins and growth cone signaling without altering rat behavior.

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Valproic acid (VPA) is the most widely prescribed antiepileptic drug due to its ability to treat a broad spectrum of seizure types. Direct delivery of VPA intracerebroventricularly (ICV) could circumvent the toxic effects normally seen with the oral route of administration. ICV use would maximize VPA brain concentration which may ameliorate neuron and astrocyte death associated with seizures. After ICV administration of VPA in the Sprague Dawley rat, near-infrared fluorescence was used to image coronal brain tissue slices and analyze brain lysates. Immunopositive complexes of VPA were localized to the periventricular zone in tissue slices and pro-survival phospho-proteins pAkt-Ser473, pGSK3β-Ser9 and growth cone protein 2G13p were

also increased in this area of the brain slice (compared to chronic saline injection; significance determined by ANOVA and Scheffe a posteriori). In regional tissue lysates, there were increases in pro-survival pAkt-Ser473, pAkt-Thr308, pGSK3β-Ser9, pErk1/2-Thr202/Tyr204 and growth cone associated protein GAP43 (Western blotting). However, ICV administration of VPA did not change the total protein amount of doublecortin, NeuN, synaptotagmin, and synaptophysin. This lack of change could be due to dilution during tissue extraction or stalled growth cone formation. Elevated VPA concentration (3 mM) may have induced hydrocephaly through higher drug viscosity which would decrease ciliary flow of the CSF. VPA did not alter animal anxiety or drugged behavior as analyzed by open field (no change in percent time against the wall of the field, or distance traveled in region), and elevated plus mazes (no change in percent time in the elevated open arms). Analysis of brain slices from the cortical region showed high levels of VPA that had traveled from the ventricle along an incipient space formed by the insertion of the cannula. Future studies will examine whether VPA conjugated to high molecular forms of poly(ethylene glycol) (PEG) will significantly decrease diffusion and concentrate the drug to minimize leak from the CSF into the peripheral blood.

1215

Gene Expression Profiling of Oxidative Stress on Vascular Tissue in Diabetic Patients.

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Diabetes Mellitus is a worldwide epidemic and the main risk for coronary artery disease. Diabetes is the third leading cause of death in Puerto Rico and it contributes to the major cause of mortality, coronary artery disease (CAD). Diabetic patients with advanced, symptomatic CAD are usually referred for coronary artery bypass grafting (CABG). High blood sugar levels causes generation of free radicals that leads to oxidative stress in several tissues. We hypothesize that Puerto Rican diabetic patients undergoing coronary artery bypass grafting comprise molecular differences compared to normal patients who experienced identical surgery. The aim of this study is to evaluate the molecular differences in the left internal thoracic artery and saphenous vein conduits in diabetic Puerto Rican patients undergoing coronary artery bypass grafting. Mammary artery (MA) and saphenous vein (SV) tissues from 10 patients undergoing CABG surgery were provided. RNA was extracted and the gene expression levels were compared between diabetic and non-diabetic patients using the super array for 84 genes of oxidative stress. Our results show that the expression level of the Glutathione peroxidase 1 (GPX1) gene is significantly higher in conduits of diabetic patients with respect to non-diabetic patients. This suggest that oxidative stress modulates certain antioxidant defense in type 2 Diabetes, improving our understanding of the molecular pathways that are modulated in the conduits of diabetic patients undergoing CABG. Moreover, this information will help to develop new treatments to improve the long-term prognosis of diabetic patients undergoing CABG.

1216

Amelioration of ischemic neuronal cell damage by transduced Tat- glyoxalase fusion protein.

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Methylglyoxal (MG), a metabolite of glucose, is the major precursor of protein glycation and induces apoptosis. MG is associated with neurodegeneration including oxidative stress and

impaired glucose metabolism, and is efficiently metabolized to S-D-lactoylglutathione by glyoxalase (GLO). Although GLO has been implicated as being crucial in various diseases including ischemia, its detailed functions remain to be elucidated. To this end, we investigated the protective effect of GLO in neuronal cells and an animal model of ischemia using cell-permeable Tat-GLO fusion proteins. Purified Tat-GLO was efficiently transduced into neuronal cells and protected cells against MG-induced cell death, DNA fragmentation, and activation of caspase-3 and mitogen-activated protein kinase. Gerbils injected intraperitoneally with Tat-GLO displayed delayed neuronal cell death in the CA1 region of the hippocampus compared with control cells. Those results demonstrate that transduced Tat-GLO protects neuronal cells by inhibition MG-mediated toxicity. Therefore, Tat-GLO could be useful as a therapeutic agent for various human diseases related to MG including oxidative stress.
Keywords - Methylglyoxal (MG), Glyoxalase (GLO), Oxidative stress, Protein therapy, Ischemic damage.

1217

Tat-DJ-1 protein protects against oxidative stress and ischemic injury in gerbils.

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Reactive oxygen species (ROS) have been implicated in the pathogenesis of various human diseases including ischemic brain injury. Although the exact mechanism remains elucidated, oxidative stress plays critical roles in the pathogenesis of ischemic insult. DJ-1 is widely distributed and is highly expressed in the brain. DJ-1 has an antioxidant and chaperone. However, the protective effect of DJ-1 in brain ischemic injury is unclear. Therefore, we investigated the protective effects of transduced Tat-DJ-1 protein against cell death and ischemic insult. Tat-DJ-1 protein efficiently transduced into astrocytes and protected them against oxidative stress-induced neuronal cell death by reduced intracellular ROS and apoptosis. In addition, we found that Tat-DJ-1 protein prevented neuronal cell death in the CA1 region of the hippocampus when Tat-DJ-1 protein intraperitoneally injected into gerbils.
These results demonstrate that transduced Tat-DJ-1 protein is a tool for the treatment of ischemic insult and it can be used as protein therapy for various disorders related to ROS, including stroke.

Key words: Antioxidant; Cell viability; Ischemic damage; Oxidative stress; Protein therapy; Tat-DJ-1

1218

Anti-inflammatory effect of transduced PEP-1-Cyclophilin A in Raw 264.7 cells and 12-O-tetradecanoylphorbol-13-acetate-induced mice.

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Cyclophilin A (CypA) is an immunophilin that acts as a receptor for the immunosuppressant drug cyclosporine A (CsA). CypA has emerged as a potential drug target for several inflammatory diseases, although the details of its mechanism are not unclear. To elucidate the protective effects of CypA on inflammation in Raw 264.7 cells and animal models, a human CypA gene was fused with a protein transduction domain, PEP-1 peptide, to construct a cell permeable PEP-1-CypA fusion protein. Transduced PEP-1-CypA protein markedly inhibited lipopolysaccharide- and 12-O-tetradecanoyl phorbol-13-acetate-induced expression levels of cyclooxygenase-2 as well as pro-inflammatory cytokine levels *in vitro* and *in vivo*. Furthermore, transduced PEP-1-CypA protein resulted in a significant reduction in the activation of nuclear factor-kappa B (NF-*k*B) and mitogen-activated protein kinase (MAPK). The results indicate that PEP-1-CypA inhibits inflammatory response cytokines and enzymes by blocking NF-*k*B and MAPK activation upon stimulation of inflammation *in vitro* and *in vivo*. PEP-1-CypA protein may potentially be used as a therapeutic agent against skin diseases-related inflammation.
Keywords: Cyclophilin A, Inflammation, NF-*k*B, Mitogen-activated protein kinase, Protein therapy.

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Investigation of Superparamagnetic Iron Oxide Nanoparticles across Scales in the Inner Ear.

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Today, there is enormous potential for therapeutic innovations for organs which are difficult to access by systemic drug application such as the inner ear. One challenge is to identify suitable multifunctional nanoparticles for drug delivery. This includes the demand for better visible and traceable nanoparticles.
Imaging properties of nanoparticles should enable visualization and analysis across scales down to sub-cellular localization within the target cells.

Objective of this work was to evaluate superparamagnetic iron oxide nanoparticles (SPIONs) within a novel thermo-sensitive copolymer for future pharmacological treatment of hearing loss and drug delivery to the inner ear.

We reported successful application of this ferrogel. SPIONs were tested in two model systems of the inner ear. Results from organotypic explant culture from mouse and human temporal bones are presented.
Distribution and localization of SPIONs in the tissue was visualized by light microscopy and energy filtered transmission electron microscopy (EF-TEM).

We conclude that iron oxide is a powerful imaging agent in multifunctional nanoparticles which can be used for multiscale evaluations especially in inner ear studies. Novel SPIONs have a great potential for traceable targeted drug delivery into inner ear.

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Protective effects of p53 in a mouse model of progressive hereditary kidney disease Alport syndrome.

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Alport syndrome (AS) is one of the hereditary, progressive kidney diseases caused by mutations in the genes encoding type IV collagens such as *COL4A3, 4* or *5*. These collagens compose the glomerular basement membrane (GBM), a key component of the filtering unit in the kidney. Mutation in *COL4* genes causes aberrant GBM structure that results in podocyte detachment from GBM and proteinuria leakage, which are responsible for the symptoms such as chronic inflammatory and fibrotic phenotypes in the kidney of AS. To ameliorate the symptoms and phenotypes of AS, identification of molecules other than COL4 proteins that modulate disease progression could provide novel therapeutic approach for AS. Here, we identify p53, a stress responsive transcriptional factor known to regulate various genes expression, as an inhibitory molecule of the progression of renal symptoms in a mouse model of AS (*Col4a5* mutation). A p53 heterozygosity in AS mice ($p53^{+/-}$ AS mice) caused rapid progression of proteinuria increasing rate compared with $p53^{+/+}$ AS mice. Furthermore, expression of inflammatory cytokine genes in the kidney of $p53^{+/-}$ AS mice was significantly increased, and glomerular injury score and fibrotic phenotype were more exaggerated in $p53^{+/-}$ AS mice compared with $p53^{+/+}$ AS mice. Moreover, survival rate of $p53^{+/-}$ AS mice was significantly lower than that of $p53^{+/+}$ AS mice, suggesting that p53 has a protective role against renal symptoms in AS. Finally, expression analysis of p53 in the kidney of $p53^{+/+}$ AS mice proved that renal expression of p53 is decreased during progression of AS, supporting the idea that induction and maintenance of p53 expression in the kidney may contribute to its protective effects against AS. Collectively, our data demonstrate the critical role of p53 in a mouse model of AS.

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Cleavage of the Polycystin-1 cytoplasmic tail: pathogenic and proper modulation of STAT3.

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Autosomal-dominant polycystic kidney disease (ADPKD) is a common genetic disorder that frequently results in renal failure by midlife. Functional nephrons are progressively replaced by non-filtering cysts and fibrous tissue. Mutations in *polycystin-1* (PC1) underlie ~95% of ADPKD cases, but the function of PC1 has remained poorly understood. PC1 is a multi-pass integral membrane protein, which has been implicated in the regulation of multiple signaling pathways

including the JAK/STAT pathway. PC1 protein levels are high during development and acute renal injury but drop with maturity and the resolution of injury. Unexpectedly, PC1 levels are also high in ADPKD patients.

Similar to cancer, the “loss of heterozygosity” sets the stage for disease. However, it is an environmental “third hit” that induces cystogenesis, leading to the growth of clonal cysts, which differ in their geneotypes due to unique mutations in each PC1 allele. We report here two mechanisms by which PC1 can regulate STAT3 activity. The first is characteristic of wild type PC1 and critical for development and acute injury response, the second characteristic of ADPKD and executed by a pathogenically cleaved PC1.

We show that, as in a normal kidney, membrane-anchored PC1 tyrosine-phosphorylates STAT3 in a JAK2-dependent manner, leading to its nuclear translocation and activating transcriptional activity. We show that the cleaved PC1 tail accumulates in ADPKD kidneys, including a unique ~15-kDa fragment (P15). Cleavage of the P15 fragment from the PC1 cytoplasmic tail abolishes the ability of PC1 to activate STAT3. Additionally, patient mutations in the PC1 tail affect its ability to activate STAT3.

Also, a ~30kDa cleavage product (P30) is enriched in ADPKD kidneys. The cleaved P30 PC1 product readily undergoes nuclear translocation. The P30 fragment co-activates STAT3 by a mechanism requiring phosphorylated STATs, which are activated by auto- or paracrine secretion of cytokines/growth factors. This leads to an exaggerated cytokine/growth factor response and amplified levels of STAT3 signaling.

STAT3 is strongly activated in cyst-lining epithelial cells in human ADPKD, and orthologous and nonorthologous polycystic mouse models compared to normal kidneys in which it is barely detectable. STAT3 is also activated in developing, postnatal kidneys but inactivated in adult kidneys.

Altogether, these results indicate that PC1 can differentially regulate STAT3 signaling depending on the state of PC1 cleavage, and on the local cytokine environment. Normally, PC1 may integrate mechanical and chemical signals to direct the appropriate cellular response of renal epithelial cells. In disease, the cleaved PC1 tail hypersensitizes cells to STAT3-dependent cytokine signaling. We believe that STAT3 signaling is a driving factor for renal epithelial proliferation during normal renal development and during cyst growth.

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The Structural Investigation of Protective Role of Antiepileptic Vigabatrin in Epilepsy Treatment by FTIR Spectroscopy and Neural Networks.

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Epilepsy is a well-known neurological disorder affecting 1-2 % of the population of the world, however; epileptic seizures remain uncontrollable in at least 30% of all the cases. Clinical studies have been carried out to improve new antiepileptic drugs. Vigabatrin (γ-vinyl-GABA) is a newer well-characterized antiepileptic, which differs from old drugs due to high rate of metabolic transformation. In the current study, the therapeutic role of VGB on PTZ-induced epileptic rat brain cell membrane was investigated at molecular level by using Fourier Transform Infrared Spectroscopy (FTIR) together with Neural Network (NN) analysis.

Animals were divided as control (n=6), PTZ (n=5) and PTZ-VGB (n=6). Whilst PTZ group was administrated by 60 mg/kg pentylenetetrazol, PTZ-VGB group received first 100 mg/kg VGB and 4 hours later 60 mg/kg pentylenetetrazol injection. The isolated rat brain plasma membrane samples were studied in FTIR spectroscopy in 4000-1000 cm-1 frequency range with 2 cm-1 resolution. The variations in the secondary structure of membrane proteins upon VGB treatment were determined by NN and second derivative analysis of Amid I band. Based on spectral variations cluster analysis was applied to different spectral regions which provided successful differentiation of control, epileptic and VGB-treated groups.
In epileptic group, there are significant ($p<0.05^*$) changes in the spectral parameters of lipid bands e.g. the bandwidth of the CH2 asymmetric and symmetric, the C=O band frequency, olefinic band area and also the lipid to protein ratio (the ratio of the total areas under CH2 asymmetric and symmetric stretching bands to the total areas under Amid I and II bands). With the application of VGB to PTZ group, these alterations approached to control group values, however; the C=O, the PO2 asymmetric and symmetric band areas related to phospholipid molecules and the secondary structure of membrane proteins were significantly ($p<0.05^*$) different from control group.
The results revealed that VGB protects some of the epilepsy-induced alterations such as the decrease in membrane fluidity, membrane disorganization, protein degradation, lipid to protein ratio and lipid peroxidation. However, it was observed that VGB had no positive effects to prevent phospholipid degradation and changes in the secondary structure of proteins.

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Light and Electron Microscopic Examination of The Effects of Long Term-Low Dose CyclosporinA (CsA) and Its Combination with Prednisolone on Hematopoietic Cells.

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Objective:
A very potent immunosuppressive agent, CsA, is a common drug after transplantations. The side effects of CsA at high doses after short term applications have been examined in previous studies. However; many immunosuppressive treatment protocols contain long term-low dose CsA administration. There are concerns about the side effects of the vehicle of CsA, Cremophore-EL. Some protocols also include low dose Prednisolone, which might cause additional side effects.
The current study focuses on the effects of long term immunosuppressive therapy on hematopoietic cells. It was aimed to analyse the morphologic effects of CsA, Cremophor-EL and Prednisolone on bone marrow both at light and electron microscope level.
Methods:
The study included four experimental groups: 1st group was control, 2nd group had oral form of CsA(4mg/kg/day) for 10 weeks. 3rd group had i.v. form of CsA(4mg/kg/day) for 10 weeks. This form contains Cremophor-EL. Finally, 4th group had i.v. form of CsA(4mg/kg/day) and prednisolone(1 mg/kg/day) for 10 weeks. 24 female Sprague Dawley rats had been used for the study: Bone marrow specimens from these animals were embedded in Araldite. Semi-thin sections were stained with toluidine blue and examined under Axioscope. Thin sections were stained with uranyl acetate and lead citrate. They were examined under Jeol 1200 FX.
Results:
Bone marrow specimens from control group included myelomonocytic cells, erythroid cells, and megakaryocytic cells. There were also lymphocytes, plasma cells, connective tissue cells and stromal cells. Myeloid to erythroid ratio (ME ratio) was about 3:1.. After long term application of either oral or intravenous form of CsA; cellularity was slightly decreased. Distribution of

precursor cells was different from control group. Additional therapy with Prednisolone caused significant increase at the number of adipocytes.

Conclusions:

These morphologic results suggest that long term-low dose CsA therapy has impact on hematopoiesis. Long term effects of the drug differs from the results after short term-high dose applicatons. Cremophore EL does not cause any additional microscopic changes on bone marrow cells. The most drammatic changes are observed after the application of CsA with Prednisolone.

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Novel therapeutic strategy for the inhibition of pseudorabies virus (PRV) infection in C57BL/6 mouse by recombinant nuclease catalytic antibody.

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3D8 scFv is a recombinant monoclonal antibody with nuclease activity originally isolated from autoimmune-prone MRL mice. Previously, we have demonstrated that HeLa cell lines expressing 3D8 scFv conferred resistance to HSV-1 and PRV by nuclease activity of 3D8 scFv. In this study, we show that 3D8 scFv can be delivered to the target tissues and cells by intraperitoneal injection and have a therapeutic effect against PRV. Intraperitoneal injection of 5 μg and 10 μg 3D8 scFv did not develop any noticeable toxicity. C57BL/6 mouse showed 9% survival rate after $10LD_{50}$ PRV intramuscular infection. By contrast, 3D8 scFv injected C57BL/6 mouse exhibited 57% (5 μg; group E) and 47% (10 μg; group F) survival rates. This therapeutic effect of 3D8 scFv against PRV was also supported by qRT-PCR, southern hybridization, and immunohistochemistry. Comparative mRNA expression data using several chemokines (iNOS and CXCL10) revealed that the antiviral mechanism on C57BL/6 mice was due to the nuclease activity of 3D8 scFv. Therefore, our results indicated that the nuclease activity of 3D8 scFv can be utilized as an effective antiviral agent on several animal models.

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Binding Affinity of a Phosphopeptide Analogue of Heat Shock Protein β6 to Various Isoforms of 14-3-3.

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AZX100, a phosphopeptide mimetic of Heat Shock Protein β6 (HSPβ6 or HSP20) containing a protein transduction domain, has been previously shown to bind to 14-3-3γ. In cells expressing 14-3-3γ, AZX100 binding results in the displacement and dephosphorylation of cofilin. Upon dephosphorylation, cofilin dismantles actin, leading to reduced contractility and migration of fibroblasts. AZX100 has also been shown to reduce filamentous actin and change cell shape in both 3T3 fibroblasts and keloid fibroblasts (KF).

Since the binding motif for phosphoserine peptides is conserved across all 14-3-3 isoforms, the ability of AZX100 to bind to 14-3-3β, –ε, and –σ was investigated via Surface Plasmon Resonance (SPR) measurements conducted with the Biacore® 2000. Peptides were coated on a CM5 chip and various concentrations of each 14-3-3 isoform were flowed over the chip surface. Binding affinities were calculated from the measured binding curves. AZX100 bound to all isoforms of 14-3-3; however, the binding affinity was highest to 14-3-3γ. AZX100 binding affinity to 14-3-3γ was 157 ± 24.9 nM, whereas AZX100 binding affinity to 14-3-3β, -ε, and –σ was 1702 ± 135, 607.9 ± 66.3, 2192 ± 60, respectively. Small variations in the binding pocket of

the 14-3-3 isoforms for phosphoserine peptides could account for the differences in binding affinity.

Additional experiments with peptide variants of AZX100 were measured with SPR to determine important residues associated with binding. Variants W12A, L13A, and L23A did not significantly reduce binding to 14-3-3γ (215 ± 33.5, 177 ± 16.5, and 218 ± 29.4 nM, respectively). These variants also bound to 14-3-3β, -ε, and –σ with similar binding affinity as AZX100. However, a single amino acid substitution at P19A abolished all binding to 14-3-3γ, -β, and -ε. Interestingly, P19A bound to 14-3-3σ with a binding affinity of 1832 ± 480 nM. Therefore, further optimization can be conducted at the 12, 13, and 23 positions in AZX100 that may lead to improved binding and specificity.

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Development of a novel antiviral mouse model harboring a nuclease antibody against pseudorabies virus (PRV) infection.

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In the previous experiments, recombinant 3D8 scFv, isolated from an autoimmune-prone MRL mice, showed antiviral effects. HeLa cell lines expressing 3D8 scFv conferred the resistance to HSV-1 and PRV by nuclease activity of 3D8 scFv and then 3D8 scFv can be utilized as an effective antiviral agent on C57BL/6 mice. In this Paper, we identified preventive effect on 3D8 scFv transgenic C57BL/6 mice against PRV. STG90 mouse (3D8 scFv transgenic mouse line 90) exhibited high mRNA expression of 3D8 scFv by qRT-PCR in muscle and brain. Wild type C57BL/6 mouse showed 0% survival rate after PRV challenging while STG90 have a 56% survival rates after PRV intramuscular infection. Preventive effect against PRV on 3D8 scFv TG mouse was verified by qRT-PCR, southern hybridization, and immunohistochemistry. These results demonstrated that 3D8 scFv transgenic C57BL/6 mice showed preventive effect by nuclease activity and will exhibit antiviral effects against DNA and RNA virus.

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Calretinin Interacts with Mutant Huntingtin and Suppresses Its Cytoxicity in Cell Culture.

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Huntington's disease (HD) is a fatal neurodegenerative disorder and is caused by an abnormal expansion of polyglutamine repeats in the Huntingtin (Htt) protein. Abnormal mutant Htt-protein interactions have been implicated in the pathogenesis of HD. To identify novel proteins that interact with mutant Htt, we employed the approach of tandem affinity purification and discovered that Calretinin (Cr), a calcium-binding protein, interacts with mutant Htt. This was confirmed by co-immunoprecipitation. Overexpression of Cr reduced mutant Htt-induced cytotoxicity in both non-neuronal and neuronal cell models of HD, whereas knockdown of Cr expression in the cells facilitates mutant Htt-caused cell death. These data taken together suggest that Cr might be a novel therapeutic target for treating HD and other polyglutamine disorders.

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Mechanistic studies of fibronectin peptide P12: a co-factor of PDGF-BB.

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Objective: P12 is a small cationic peptide derived from the first type III repeat of human fibronectin based on its growth factor binding activity. P12 has shown promise limiting injury progression in both rat and porcine burn injury models. The overall goal is to investigate the mechanism of P12 to promote cell survival by in vitro models created to mimic the ischemic condition in the peri-burn tissue. This is a logical extension of previous biochemical experiments demonstrating P12 has synergistic effects with platelet derived growth factor-BB (PDGF-BB) to prolong cell survival. The hypothesis is that P12 functions through augmenting PDGF-BB survival signals as a PDGF-BB co-factor. Results: P12 in the presence of PDGF-BB promoted adult human dermal fibroblast (AHDF) cell metabolism and survival under nutrient deprivation stress as measured by XTT and TUNEL assay. P12 modulated PDGF-BB signaling in AHDF under nutrient deprivation stress on both serum coated and collagen coated surfaces. Compared to PDGF alone or PDGF with scrambled P12, authentic P12 prolonged Akt phosphorylation at Ser473 for 2hs on both surfaces. It also reduced JNK and ERK1/2 phosphorylation to control level on serum coated surfaces. It did not change p38 phosphorylation and eIF4e protein level. P12 showed efficient binding to PDGF-BB and penetrated AHDF as a protein transduction domain. Briefly, P12 labeled by Alexa Fluor 488 entered human dermal fibroblast in a temperature and energy dependent manner as measured by confocal microscopy and FACS. P12 entry is also partially blocked by removing cell surface glycosaminoglycans. Conclusion: P12 promotes cell survival by augmenting survival signals from PDGF-BB and possibly by affecting PDGF-BB/PDGFR internalization/trafficking.

Signaling from the PM/Cytoplasm to the Nucleus

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IFN-γ and TNF-α induced GBP-1 inhibits epithelial cell proliferation through suppression of β-catenin/TCF signaling.

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Proinflammatory cytokines induce Guanylate Binding Protein 1 (GBP-1) protein expression in intestinal epithelial tissues. GBP-1 has been described as influencing a number of cellular processes important for epithelial homeostasis, including cell proliferation. However, the role of GBP-1 in intestinal mucosa remains incompletely understood. We therefore sought to investigate the function of proinflammatory cytokine induced GBP-1 during intestinal epithelial cell proliferation. Through the use of complementary GBP-1 overexpression and siRNA-mediated knockdown studies, we now show that GBP-1 acts to inhibit pro-mitogenic β-catenin/ T cell factor (TCF) signaling. Interestingly, proinflammatory cytokine induced GBP-1 was found to be a potent suppressor of β-catenin protein levels and β-catenin serine 552 phosphorylation. Neither GSK3-β nor proteasomal inhibition alleviated GBP-1-mediated suppression of cell proliferation or β-catenin/TCF signaling, indicating a non-canonical mechanism of β-catenin inhibition. Together, these data show that cytokine-induced GBP-1 retards cell proliferation by forming a negative feedback loop that suppresses β-catenin/TCF signaling.

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The mechanism of IL-33-induced Th2 cytokine production in natural helper cells.

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We have previously identified novel IL-2R$^+$IL-7R$^+$IL-25R$^+$IL-33R$^+$ lymphocytes named 'natural helper (NH) cells' in mouse, rat and human adipose tissues. NH cells produce large amounts of Th2 cytokine such as IL-5, IL-6 and IL-13 by IL-33 stimulation. The ability of NH cells to produce Th2 cytokine is much higher than mast cells and basophils. IL-33 is a member of IL-1 family cytokine, which is known to localize in the nucleus of epithelium and endothelium cells. During helminth infection, allergy and other diseases, IL-33 is released by necrotic cells in injured tissues. In this study, we aimed to clarify why NH cells can produce such large amounts of Th2 cytokines compared to other cells by focusing on IL-33 signaling pathways. NH cells and bone marrow derived mast cells (BMMC) were stimulated with IL-33, and examined the kinetics of Th2 cytokine production at protein levels by ELISA. In NH cell, production of IL-5, IL-6 and IL-13 was significantly accelerated 48 hrs after IL-33 stimulation, leading to the production of more than 10 ng of these cytokines by 5,000 NH cells. In contrast, BMMC produced IL-6 from 3 hrs and peaked at 24 hrs after IL-33 stimulation but total amounts were less than 3% of NH cells. Only small amounts of IL-5 and IL-13 (~200 pg) were produced by BMMC. Western blot analysis revealed that IL-33 stimulation of NH cells activated NFκB and MAPK pathways more strongly than in BMMC. The activation of NFκB and MAPK signaling pathways were sustained even 48 hrs after IL-33 stimulation. To clarify what signaling molecules are important for IL-33-induced Th2 cytokine production in NH cells, we examined the effect of several inhibitors of signaling pathways. Intriguingly, a p38 inhibitor, SB203580 markedly inhibited IL-33-induced IL-5, IL-6 and IL-13 production in NH cells. Taken together, these results suggest that NH cells produce high amounts of IL-5, IL-6 and IL-13 through p38 mediated signal transduction pathways downstream of IL-33 receptor. Now, we are analyzing more detailed mechanisms of p38 mediated Th2 cytokine production in NH cells.

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In Situ Calcium Imaging During Mechanical Shear Applied to Articular Cartilage.

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Osteoarthritis is a degenerative disease of articular cartilage in the joint that affects over 27 million people in the US alone (Lawrence, 2008). Cartilage cells (chondrocytes) are influenced by mechanical loading which in turn regulates a variety of biochemical responses, including Ca2+ concentrations. Recent studies have shown how intracellular Ca^{+2} concentrations in chondrocytes change in response to different mechanical loading such as shear flow. Many studies of this type involve isolated cells in monolayer cultures, but the role of the surrounding three-dimensional extracellular matrix in delivering the mechanical stimuli has been widely discarded from such studies. When shear stress is applied to cartilage, deformation of the extracellular matrix and consequently of the pericellular matrix occurs, changing the environment that surrounds the cell and initiating signal transduction pathways. To study how the application of shear stress affects the intracellular concentrations of calcium, we carried out calcium imaging using the ratiometric dye Fura-2 AM in cells in their native environment, i.e. in explants (plugs) obtained from bovine articular stifle joints. A dual piezoelectric device was used to deform the cartilage explants every 30 seconds during imaging. In one of the experiments ionomycin was added after 10 minutes and EGTA was added after 16 minutes while applying

compression. Results show that 340/380 nm ratio intensity increases steadily in calcium-free media while applying compression to the explants. These results indicate that biochemical responses to mechanical loading can be measured *in situ* in chondrocytes, and they further enable the study of potentially altered cell responses to disease states in articular cartilage.

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Increased expression of Aquaporin 9 in inflammatory cells as a response to LPS.

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The aquaporins (AQPs) are a family of water channels involved in volume regulation through water transport in and out of the cell and are thereby of importance in a number of cellular events. The aquaglyceroporin, AQP9 has been suggested to play a pivotal role in inflammatory cell migration, differentiation and metabolism. Thus, we wanted to investigate the role and regulation of AQP9 in human primary inflammatory cells in response to inflammatory stimuli and inhibitors especially the effects of lipopolysaccharide- (LPS) and TNF-alpha-stimulation as analyzed with molecular and imaging techniques,.
qPCR analyses of human primary macrophages obtained from healthy blood donors showed an increase of the mRNA expression of AQP9 upon LPS stimulation. Protein analyses with Western blot further confirmed this effect at the protein level. Moreover, preliminary data from human primary neutrophils indicated similar responses.
These results implicate a role of AQP9 in inflammatory cell responses to LPS and are in line with a suggested role of AQP9 in inflammatory cell motility. AQP9 could thus have crucial effects on cell functions at sites of infection and bacterial antigen deposition, like their volume regulation, motility and metabolism. Here we present a hypothetical model for AQP9 upregulation that is dependent on bacterial agents and inflammatory mediators.

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Regulation of p21 in urothelial carcinoma cells.

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Background
The PTEN/Phosphatidylinositol 3’-kinase (PI3-kinase) growth factor signaling pathway plays a critical role in epithelial tumor development in a multitude of tissue types. Deletion of the Pten tumor suppressor gene in murine urothelial cells in vivo results in upregulation of cyclin-dependent kinase inhibitor p21. We have previously shown in mice that p21 expression blocks an increase in urothelial cell proliferation due to Pten deletion. In this study, we utilized human urothelial carcinoma cells UMUC-3 and UMUC-14 to identify the signaling pathways downstream of PI3-kinase that regulate p21.
Methods
Cells were treated with a combination of PI3-kinase stimulating growth factors and kinase inhibitors, or transfected with exogenous genes in order to identify the signaling events that are necessary for p21 induction. Mice with conditional deletion of Pten in bladder urothelium were also examined for evidence of PI3-kinase pathway signaling events that affect p21 expression.
Results
When cells were treated with PI3-kinase activating growth factors EGF or PDGF, we found that p21 levels increased, in a manner similar to that observed in mice. We used the inhibitors LY294002, Akti-1/2, and rapamycin, to show that p21 induction is dependent upon PI3-kinase and AKT activity, and partially dependent on mTOR. We treated the cells with proteasome inhibitor MG-132 and found that p21 may be degraded in the proteasome to regulate protein

levels. Importantly, our findings show that GSK-3β plays a role in diminishing p21 levels in cells. Treatment of cells with the GSK-3β inhibitor SB-216763 increased p21 levels, while exogenous expression of GSK-3β caused a decrease in p21, indicating that GSK-3β actively reduces p21 levels. We found that a combined treatment of LY294002 and SB-216763 improved the cytotoxic effect against UMUC-3 and UMUC-14 carcinoma cells over LY294002 alone, suggesting potential therapeutic uses for GSK-3β inhibitors. Immunohistochemical staining in bladders from wild-type and Pten-deleted mice indicated that GSK-3β inhibitory phosphorylation increases when Pten is deleted.
Conclusion
PI3-kinase and AKT cause an upregulation of p21 by suppressing GSK-3β activity and activating mTOR in both cultured human urothelial carcinoma cells and mouse urothelial cells in vivo.

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Regulation of β-distroglycan nuclear localization by ezrin.
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Dystroglycan is a protein member of the Dystrophyn-Associated Protein Complex (DAPC). DAPC is composed of extracellular, transmembrane and cytoplasmic proteins, and has central role in muscular integrity. Dystroglycan subcomplex comprises the extracellular subunit α–dystroglycan and the transmembranal protein β-dystroglycan (β-DG); the latter links to α-dystroglycan by its N-terminus domain, and to different intracellular proteins (dystrophin, actin, ERK, MAPK, Grb2, and ezrin) through its C-terminus domain. Nuclear presence of β-dystroglycan has been recently demonstrated in different cell lines, and a functional nuclear localization sequence (NLS) that is recognized by importins α and β was identified within the cytoplasmic domain. It is worth to note that the NLS of β-DG also serves as an ezrin-binding site, making possible that ezrin and importins compete each other for binding to the same motif on β-DG. Therefore, the aim of this study was to analyze the influence of ezrin on the nuclear localization of β-DG, we showed that ezrin over-expression increases rather than decreases the nuclear levels of endogenous β-DG. Furthermore, we revealed that over-expression of an ezrin variant that mimics its phosphorylated state (ezrinT567D) favors even more the nuclear accumulation of β-DG. On contrary, over-expression of a nonphosphorylable variant of ezrin (ezrinT567A) causes a marked decrease in the nuclear levels of β-DG. In consistency, induction of ezrin phosphorylation by LPA treatment increased the nuclear levels of β-DG, while inhibition of ezrin phosphorylation by C3-transferase exotoxin treatment reduces the nuclear accumulation of β-DG. Altogether, our results suggest that phosphorylation of ezrin favors the nuclear import of β-DG, likely by ezrin-mediated cytoskeleton remodelation.

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Pathway Selection by Nod1 and Nod2.
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Defects in both Nod1 and Nod2 signaling pathways are associated with human disease. Perhaps most notably, polymorphisms in Nod2 and ATG16L are strongly associated with susceptibility to Crohn’s Disease. The two main effectors for Nod1 and Nod2 are Receptor-interacting serine/threonine-protein kinase 2 (Ripk2) and Autophagy-related protein 16 (ATG16L). Upon recruitment to the Nod1 or Nod2 signaling complex, the proinflammatory

effector Ripk2 is autophosphorylated and stimulates the canonical NF-κB pathway by activating the IKK complex. How ATG16L activates autophagy remains poorly understood, but recent evidence suggests that its recruitment by Nod1 or Nod2 signaling complexes to sites of intracellular bacterial invasion serves to enhance autophagocytosis of those bacteria.
NMR spectroscopy and affinity matrix binding assays were used to map protein:protein interactions between Nod1, Nod2, ubiquitin, Ripk2 and ATG16L. We found distinct but overlapping binding interfaces for Ripk2 and ubiquitin exist on Nod1. Accordingly, we show that ubiquitin binding is competitive with Ripk2 binding to both Nod1 and Nod2. Similarly to how Ripk2 binds Nod1 and Nod2, ATG16L binds the Caspase Activation and Recruitment Domains (CARDs) on Nod1 and Nod2.
The finding that ubiquitin competes with the interactions between Ripk2 and Nod1 and Nod2 suggests that ubiquitin plays a regulatory role in Nod1 and Nod2 signaling. In addition to our findings, recently reported results on the regulation of another innate immune receptor by poly-ubiquitin chains support a general role for ubiquitin:CARD interactions in modulating activity of innate immune receptors. Ongoing functional studies of ubiquitin- and Ripk2-specific binding mutants of Nod1 and Nod2 will determine the functional relevance of these interactions. As well, investigations into the role of ubiquitin in the interaction between ATG16L and Nod1 and Nod2 are underway. These studies will not only advance our understanding of the Nod1 and Nod2 pathways, but will also contribute to the broader field of cell biology and immunology.

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Muscle Plasticity Is Regulated by a Process Involving ATP Release and Inositol Trisphosphate Production in Skeletal Myofibers.

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Introduction: In skeletal muscle, myofibers can be classified as slow- and fast-twitch depending on its metabolic and contractile properties. Transcription of specific gene programs is regulated by different electrical stimulation patterns, process known as muscle plasticity. We have previously described the presence of post-tetanic, inositol trisphosphate (IP_3)-dependent calcium signals that are frequency dependent in adult muscle fibers. We reported that extracellular ATP can act as an autocrine signal in primary myotubes, inducing intracellular calcium movements and regulating gene expression.
Objective: How myofibers can discriminate among different frequencies and activate specific gene programs? In the present work we propose a pathway for muscle plasticity is modulated by IP_3-dependent calcium transients, with ATP release as a key regulator.
Methods: We used adult myofibers obtained from both FDB and soleus muscles. Myofibers were stimulated at 20 and 90 Hz (270 pulses, 0.3 ms each). We analyzed gene expression (qPCR), ATP release (luciferase) and IP_3 production (ELISA).
Results: We found that ATP release from myofibers is different depending on stimulation frequency, with an important, biphasic release at 20 Hz with a peak 3-5 min after stimulation; this release is dependent on pannexin channels and dihydropyridine receptors because it is inhibited by fiber pre-incubation with 25 μM nifedipine. With this stimulation pattern we found two IP_3 increments, one immediately after stimulation another one 5 min after stimulation. We observed an increase in slow troponin I (TnIs) mRNA levels and a decrease in fast troponin I (TnIf) mRNA. These changes are inhibited by ATP release inhibitors (apyrase, carbenoxolone) and IP3R blockers (xestospongin B). We see an increase of citrate synthase mRNA and a decrease in those of enolase mRNA after 20 Hz stimulation, which are not inhibited by xestospongin B. At 90 Hz there was no significant ATP release and IP_3 production was much less evident. In these conditions there is an increase in TnIf mRNA levels and a decrease in TnIs, that was IP3R-independent.

Conclusions: We propose that both ATP release and IP_3 production are part of a frequency-dependent integrated process that activates the slow type muscle fiber program.
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Hypertrophy Induced by Testosterone in Skeletal Muscle Cells: Not Only an Androgen Receptor Mediated Process.

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Introduction: Since description of testosterone as the major male anabolic hormone in humans, questions about its action mechanism in skeletal muscle have arisen. Reports about rapid non genomic effects of this hormone raised the question about their impact in hypertrophy elicited by testosterone. We studied whether the classic androgen receptor was necessary to elicit hypertrophy in skeletal muscle myotubes. Concomitantly activation of classic hypertrophic pathways by testosterone and the potential relationship between the androgen receptor and these hypertrophic pathways was also studied.
Materials and Methods: Five to seven day old rat myotubes, obtained from neonatal rat hind limbs, were used. 100nM testosterone, a dose that produces oscillatory calcium transients and induces activation of ERK1/2, was utilized. Hypertrophy was measured by microscopy, western blot and qPCR. Activation of the putative pathways was studied by western blot and inhibitors. Androgen Receptor action was studied by western blot and small interference RNA (siRNA).
Results: Testosterone induced an increase in myotube cross sectional area (control=134,5μm^2±10,21; testosterone=181,9μm^2±14,41) and in α-actin protein levels at 12 hours. Morphological development of myotube sarcomerisation was more clearly established in testosterone stimulated myotubes at this time. Earlier times of testosterone stimulation showed increased transcription of α-actin gene, concomitantly with no changes in atrogenes (MAFfbx and MuRF-1) activity at 6 hours. Classic hypertrophy pathways were studied at short times: ERK1/2 and Akt showed an increase in phosphorylation status after testosterone at 5 and 15 minutes, respectively. p70S6K was phosphorilated at 60 minutes. This response was inhibited with LY294002, Akt-inhibitor-VIII and Rapamycin but not by PD98059. Similarly, the hypertrophic response at 12 hours was abolished with these inhibitors as well as partly by a siRNA against androgen receptor but not by PD98059.
Discussion: In this work we showed that testosterone activates the Akt/mTOR pathway at short times. Two selected parameters of hypertrophy showed an increase at 12 hours. Inhibition of Akt/mTOR pathway at 12 hours, demonstrated a direct relationship between the early hypertrophy response and the activation of mTOR pathway. siRNA to the androgen receptor also unveiled an important role of this receptor in the early hypertrophy response.
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JNK1 inhibits GluR1 expression and GluR1-mediated calcium influx through phosphorylation and stabilization of Hes-1.

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The GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor plays an important role in excitatory synaptic transmission and synaptic plasticity in the brain, but the regulation mechanism for GluR1 expression is largely unknown. *Hairy* and *Enhancer of split 1* (Hes-1) is a mammalian transcription repressor that regulates neuronal

differentiation and development, but the role of Hes-1 in differentiated neurons is also less known. Here we examined the molecular mechanism in regulation of GluR1 expression in cultured cortical neurons from rat. We found that Hes-1 suppressed GluR1 promoter activity and decreased GluR1 expression through direct binding to the N box and through preventing Mash1/E47 from binding to the E box of GluR1 promoter. We also found that Hes-1 could be regulated by c-Jun N-terminal kinase (JNK1). JNK1 directly phosphorylates Hes-1 at Ser-263, and JNK1 phosphorylation of Hes-1 stabilized the Hes-1 protein and enhanced its suppressing effect on GluR1 expression. Moreover, this JNK1-mediated signaling pathway was found to inhibit AMPA-evoked calcium influx in cortical neurons and this regulation mechanism is Notch-independent. Further, JNK1 regulation of GluR1 expression is independent of GluR1 phosphorylation. Here we provided the first evidence that Hes-1 plays an important role in synaptic function in differentiated neurons. We also identified a novel JNK1-Hes-1 signaling pathway that regulates GluR1 expression involved in synaptic function in rat cortical neurons.
Keywords: JNK1, Hes-1, GluR1, phosphorylation, stabilization, calcium influx

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The N-terminal Projection Domain of The Microtubule Associated Protein Tau Inhibits TNFa Signaling.

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Tumor Necrosis Factor-alpha (TNFα) is a multifunctional pro-inflammatory cytokine implicated in inducing both tumor promotion and tumor inhibition. This paradoxical effect can be partly explained by the fact that TNFα acts as a regulator of both proliferation and apoptosis; however the mechanisms and factors associated with how or whether a tumor cell responds to a microenvironment rich in TNFα have not been fully resolved. Here we provide evidence that the microtubule associate protein Tau inhibits TNFα signaling through its N-terminal domain and implicate it as a novel regulator of the cellular response to this key cytokine. Our findings indicate that over-expression of Tau in the human breast cancer cell lines MCF7, SkBr3 and in the pheochromocytoma cell line PC12 suppresses TNFα-induced apoptosis evident in the control cells. TNFα-induced cell apoptosis in control cells is triggered as early as 6 hrs post-treatment with hallmark features of cell shrinkage, membrane blebbing, disruption of tubulin architecture and caspase-3 activation. While over-expression of wild type full length Tau isoform in these cell lines, completely abrogates all TNFα-associated apoptotic phenotypes. Aside from inhibiting TNFα-induced apoptosis, Tau over-expression also interferes with TNFα-induced NFKB activation. This is evident by a significant decrease in NFKB nuclear translocation in Tau over-expressed cells compared to controls. Suppression of TNFα-signaling by Tau is not associated with its tubulin interaction since a truncated N-terminal projection domain lacking all microtubule binding domains still provides resistance to TNFα-induced apoptosis and NFKB activation. These findings lead us to conclude that the functional domain of Tau that regulates TNFα response lies within its N-terminal region and current studies investigating regions within this domain

are ongoing. Our data provide direct evidence that the N-terminal domain of the microtubule associated protein Tau can inhibit TNFα signaling in tumor cells. These findings indicate a novel function for Tau and add to our knowledge of how a tumor cell responds to TNFα-signaling.

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Substratum topography modulates Nesprin1,2 but not FAK or ROCK1 in corneal epithelial cells.

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Human corneal epithelial cells *in vivo* attach to the underlying stroma through a basement membrane that possesses a rich 3 dimensional topography. The impact of submicron and nanoscale topographic cues on fundamental cell behaviors including adhesion, shape, size, proliferation, and migration has been widely reported. However, the processes that allow the cells to identify and react to topographic features are still poorly understood. To study the impact of topographic cues on various cell types, our lab is using topographically patterned substrates with anisotropic features of parallel ridges and grooves in the biomimetic micron- and nanoscale range. Topographic cues appear to be transmitted from the outside of the cell to the nucleus resulting in changes in the cells' gene expression patterns. We therefore set out to investigate the effects of micron- through nanoscale topographic features on signaling events both proximal/near the extracellular matrix interface and distal/near the nucleus. One of the signaling molecules near the cell membrane is focal adhesion kinase (FAK), which has been proposed to serve as a mechanosensor. Perinuclear proteins, such as Nesprins, link the cytoskeleton to the nucleus via the LINC complex and can transfer forces to the nucleus hence influencing nuclear shape and signaling. To investigate the relevance of FAK as a signaling molecule, we depleted FAK in immortalized human corneal epithelial cells by siRNA transfection and studied their response to topographic cues. Our results indicate that FAK knockdown has no impact on the cells' ability to align to topographic cues. FAK depleted cells remain able to migrate along the ridges and grooves. Also, topography alone did not modulate the inherent expression of FAK or ROCK, a signaling molecule downstream of FAK, in corneal epithelial cells. However, qPCR analyses revealed that the expression levels of Nesprin 1 and 2 are altered by topographic cues. Interestingly, FAK depletion in corneal epithelial cells leads to an increased Nesprin 2 expression accompanied with decreased Nesprin 1 expression.

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Diarylheptanoid mediates GSK-3beta-dependent activation of Wnt signaling pathway.

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Estrogen promotes growth in many tissues by activating Wnt/β-catenin signaling pathway. We recently isolated diarylheptanoids from *C.comosa* (compound-049) which contains phytoestrogens, is widely used as an alternative supplement for treatment of unpleasant symptoms in menopausal women. However, it is not clear whether these phytoestrogens act in a similar manner to that of estradiol. In the present study, we aim to investigate the mechanistic effect of compound-049, which exhibits the highest estrogen-like activity, on Wnt/β-catenin signaling pathway. By using a TOPFlash luciferase assay in HEK 293T cells, compound-049

rapidly activated β-catenin activity and induced a significant increase in β-catenin protein levels similar to those of estradiol. Moreover, immunofluoresence in CHO cells demonstrated that β-catenin was predominantly localized in the nucleus after treatment with E2 and compound-049. Treatment with LiCl, the specific inhibitor of GSK-3β, abolished the effect of compound-049 and E2 on the activation of the TOPFlash luciferase reporter assay. In addition, Wnt/β-catenin signaling stimulated by the constitutively active β-catenin mutant, S33Y, which is insensitive to GSK-3β, was not susceptible to activation induced by compound-049 and E2. Treatment of compound-049 and E2 induced the phosphorylation at serine 473 of Akt and serine 9 of GSK-3β, which in turn inhibited GSK-3β activity. Collectively, these results suggest that compound-049 and E2 activate the canonical Wnt/β-catenin signaling through a mechanism that is dependent of Akt and GSK-3β. Therefore, our results provide an important information required for further development of this natural compound as supplement for post-menopausal women.

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Grb7-mediated transcriptional regulation of EphA4 gene expression in cancer progression.

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Signaling through Grb7, a multi-domain adaptor, has been shown in capable of transmitting EGF receptor family kinases- and/or integrin cell adhesion- dependent events. Conceivably, Grb7 involves in EGFR- and integrin-mediated tumor progression, such as invasion and metastasis. In this study, we found that Grb7 mediated STAT3 activation gives rise to the elevation of EphA4 gene expression in EGF dependent manner in both SK-Br3 breast cancer and A549 lung cancer cell lines. Moreover, the Grb7-mediated *EphA4* upregulation enhanced cancer invasion/metastasis in lung adenocarcinoma cells. Furthermore, EphA4 could involve in EGF-induced cytoskeleton reorganization as well as cell proliferation, migration and anchorage-independent growth. Our data suggest that a novel mechanism by which Grb7 regulates tumorigenesis through the formation of a novel EGF-triggered EGFR-Grb7-STAT3-EphA4-ERK1/2 signaling pathway, thereby highlighting the potential strategy of targeting Grb7 as an anti-lung cancer therapy.

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Insulin regulated expression of adiponectin receptors in muscle vs. fat cells.

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Adiponectin, an adipocyte secreted hormone, exerts its effect via its specific receptors, AdipoR1 and AdipoR2, in insulin sensitive cells in muscle, liver, and adipose tissues and plays an important role in lipid and glucose metabolism. The purpose of the current study was to investigate if the effect of insulin is also modulated through adiponectin receptor expression in muscle and fat cells. Differentiated fat (3T3-L1), skeletal muscle (L6), and vascular smooth muscle (PAC1) cells were serum starved and exposed to 100 nM insulin for 1-24 hours. AdipoR1 and AdipoR2 mRNAs and AdipoR1 protein expression were respectively monitored by real time PCR and Western blotting. The results demonstrate that insulin down regulates both AdipoR1 and AdipoR2 mRNAs levels with little or no changes at the protein level in L6 and PAC1 cells. On the other hand, insulin has little or no effect in the regulation of AdipoR1 expression (both mRNA and protein levels) in 3T3-L1 cells, while significantly up regulating AdipoR2 mRNA level in a biphasic manner. These data suggest that insulin differentially regulates expression of adiponectin receptors R1 and R2 in muscle (L6 and PAC1) vs. fat (3T3-L1) cells; and perhaps insulin regulated expression of the receptors is also dependent on the availability of the endogenous ligand such as adiponectin for AdipoR1 and R2 in fat cells.

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The LINC-anchored actin cap connects the extracellular milieu to the nucleus for ultrafast mechanotransduction.

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Cells continuously sense and respond to external mechanical forces through their cytoskeleton. Here we show that only a small subset of actin fibers, those forming the perinuclear actin cap that wraps around the nucleus, form in response to low physiological mechanical stresses. While conventional basal stress fibers form only past a threshold shear stress of 0.5 dyn/cm^2, actin-cap fibers are formed at shear stresses 50 times lower and within 30 s, orders-of-magnitude faster than biochemical stimulation with serum. This fast differential response is uniquely mediated by focal adhesion protein zyxin at low shear stress and by talin at high shear stress, actomyosin fibers of the actin cap, lamin A/C of the nuclear lamina, and linkers of nucleus to cytoskeleton (LINC) molecules Nesprin2giant and Nesprin3, which anchor actin cap fibers to the nucleus. These results map an interconnected physical pathway for mechanotransduction, from the extracellular milieu to the nucleus.

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Cell Adhesion and Spreading Regulate a Map Kinase/Ternary Complex Factor Transcriptional Switch to Control Proliferation.

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Much of our understanding of adhesion-regulated proliferation is based on studies comparing suspended vs. adherent cells. However, these conditions do not recapitulate the changes in adhesion and spreading that occur *in vivo*. Using microcontact printing to control the degree of spreading and adhesion, we uncovered a previously undescribed mechanism by which these more subtle adhesive changes modulate cell proliferation through regulation of the map kinases (MAPKs) and the Serum Response Factor (SRF) cofactors, the ternary complex factors (TCFs). Increasing adhesion preferentially enhanced JNK activity while suppressing p38 activity. This switch in MAPK activity led to the exchange of the Sap-1 TCF for the Net TCF at the promoters of the immediate early genes *egr1* and *fos*. Modulation of MAPK signaling and knockdown of TCFs demonstrated that MAPK/TCF switching controlled both immediate early gene expression and proliferation. Proliferative regulation by the TCFs was conserved in an *ex ovo* model of angiogenesis, validating the physiological significance of this MAPK/TCF switch. These data highlight the pivotal role that cell spreading and adhesion play in the transcriptional regulation of proliferation and points to a novel mechanism by which this occurs.

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Oxygen signaling via the regulation of protein localization.

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Oxygen provides a crucial energy source in eukaryotic cells. Hence, eukaryotes ranging from yeast to humans have developed sophisticated mechanisms to respond to changes in oxygen

levels. Regulation of protein localization, like protein modifications, can be an effective mechanism to control protein function and activity. We examine how hypoxia affects protein distribution on a genome-wide scale in the model eukaryote, the yeast *Saccharomyces cerevisiae*. We demonstrated, by live cell imaging, that hypoxia alters the cellular distribution of 203 proteins in yeast. These hypoxia-redistributed proteins include an array of proteins with important functions in various organelles. Many of them are nuclear and are components of key regulatory complexes, such as transcriptional regulatory and chromatin remodeling complexes. Under hypoxia, these proteins are synthesized and retained in the cytosol. Upon reoxygenation, they relocalize effectively to their normal cellular compartments, such as the nucleus, mitochondria, ER and cell periphery. The resumption of the normal cellular locations of many proteins can occur even when protein synthesis is inhibited. Further, we show that the changes in protein distribution induced by hypoxia follow a slower trajectory than those induced by reoxygenation. These results show that the regulation of protein localization is a common and potentially dominant mechanism underlying oxygen signaling and regulation. Experiments are underway to examine the time course characteristics of the relocalization of certain regulatory proteins and to determine their direct contributions to oxygen regulation.

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Probing the functional integration of mechanosignals to chromatin organization in living cells.

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Cells sense physical and chemical cues from their microenvironment and selectively transmit these cues to the nucleus – resulting in altered gene expression. However the underlying mechanisms are still unclear. In this work, we provide evidence for transduction of physical forces between the plasma membrane and the chromatin organization through the actin cytoskeleton. For this controlled forces are applied using an electromagnet on cell membranes adhered with magnetic beads. The effect of force and its transduction to the nucleus is visualized using high–resolution fluorescence anisotropy imaging of chromatin assembly within single living cells. Our studies evidence physical links anchoring cytoskeleton and the nucleus. Force induced disruption of these links relaxes the prestressed organization of the cell nucleus in a reversible manner. In addition cellular networks are shown to be correlated with 3D organization of chromosomes and their gene expression patterns. Taken together our results evidence a strong architectural coupling between physico-chemical networks and spatial organization of the chromosomes within the nucleus facilitating mechanotransduction.

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Post-Translational Modifications in Signaling

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Stimulus-dependent ubiquitination of a G protein β subunit is required for proper cell polarization.

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Ste4 is the β subunit of a heterotrimeric G protein that mediates mating responses in Saccharomyces cerevisiae. Here we show that Ste4 undergoes mono-ubiquitination in response to pheromone stimulation. Ubiquitination of Ste4 is dependent on the E3 ligase Rsp5. Disrupting the activity of Rsp5 abolishes ubiquitination of Ste4 in vivo, and recombinant Rsp5 is capable of ubiquitinating Ste4 in vitro. We find also that Lys340 is a major ubiquitination site on Ste4, as pheromone-induced ubiquitination of the protein is prevented when this residue is mutated to an arginine. Functionally, ubiquitination does not appear to regulate the stability of Ste4, as blocking ubiquitination has no apparent effect on either the abundance or the half-life of the protein. However, when presented with a concentration gradient of pheromone, $Ste4^{K340R}$ mutant cells polarize significantly faster than wild type, indicating that ubiquitination limits pheromone-directed polarized growth. Together, these findings reveal a novel stimulus-dependent post-translational modification of a Gβ subunit, establish Ste4 as a new substrate of the E3 ligase Rsp5, and demonstrate a role for G protein ubiquitination in cell polarization.

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Hypoxia-Induced Estrogen Receptor Alpha Activation Is Mediated by Both the MAPK and PI3K Phosphorylation Pathways.

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The estrogen receptor (ER) plays an important role in breast cancer development and progression. Hypoxia was shown to modulate the level of ERα expression and induce ligand-independent transcriptional activation of ERα, which may be intimately associated with the biology of breast carcinomas. Given that phosphorylation affects the transcriptional activity and stabilization of ERα, we examined the changes in phosphorylation of ERα under hypoxic conditions. Hypoxia induced phosphorylation of ERα at serine residues 118 and 167 in the absence of estrogen. Phosphorylation-defective ERα mutants with serine-to-alanine replacements at residues 118 and 167 had impaired hypoxia-induced ERα activation, showing that serine residues 118 and 167 are involved in hypoxia-induced ERα activation. The hypoxia-induced ERα-mediated transcriptional response was dependent on both the ERK1/2 MAPK and PI3K pathways, but not the p38 pathway, as assessed using chemical inhibitors. These data show that ERα phosphorylation via both the MAPK and PI3K pathways is one mechanism leading to ERα activation under hypoxia.

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Novel components of the SRR (Snf3/Rgt2/Rgt1) glucose sensing pathway of S. cerevisiae.

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Novel components of the SRR (Snf3/Rgt2/Rgt1) glucose sensing pathway of *S. cerevisiae*
Kobi (Jamie) Simpson-Lavy and Mark Johnston

Introduction: *S. cerevisiae* cells undergo many transcriptional, proteomic and metabolic changes when glucose becomes available. Glucose is sensed at the cell surface by glucose receptors that activate Casein Kinase 1 (CK1) to cause degradation of Mth1 and Std1, (which collaborate with the Rgt1 DNA-binding protein to represses expression of *HXT* genes), leading to expression of glucose transporters. The AMP-activated protein kinase orthologue Snf1 opposes this process by an unknown mechanism.
Results: We discovered that SUMO is involved in this glucose-sensing pathway. The SUMO E3 ligase activity of Mms21 is required for degradation of Mth1 and induction of *HXT3* expression; overexpression of the Ulp1 deSUMOylase prevents these events. Mutations in *mms21* are suppressed by deletion of *STD1*, raising the possibility that Std1 is a (direct or indirect) target of SUMO. *STD1* dosage modulates both induction of *HXT* gene expression and Snf1 activity, and its deletion suppresses defects in glucose sensing caused by Snf1 hyperactivity.
Conclusions: SUMO E3 ligase activity of Mms21 is crucial for glucose sensing and expression of glucose transporter genes. This is intriguing because Mms21 provides a minor portion of total SUMO E3 ligase activity in *S. cerevisiae*, and all Mms21 substrates found to date are involved in genome maintenance and repair. Std1 is a key player in regulating glucose transporter expression, and may serve as a link between Snf1 and its regulation by glucose, SUMO, and glucose sensing though the glucose sensors and CK1.

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TNF-alpha alters microRNA expression profile in macrophages.
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The NF-κB signaling pathway is a pivotal mediator of the chronic inflammatory process that drives atherosclerotic plaque development, and has been proposed as a therapeutic target for clinical management. Additionally, microRNA species are rapidly being uncovered as equally prominent components of atherosclerosis, but little is known about the interplay between NF-κB signaling and microRNA in this context. We aim to define the interaction between NF-κB and microRNA in the macrophage, since it is an abundant inflammatory cell type in plaques. Tumor Necrosis Factor alpha (TNF-α) is a known stimulator of NF-κB signaling. Therefore, as the first step of this investigation, TNF-α was applied to induce alterations in microRNA expression, which were quantified by real time RT-PCR. RAW264.7 cells were treated with 10 ng/ml TNF-alpha for 15 min, 30min, 1hr or 2hrs, respectively. We found that the nuclear tranaslocation of NF-kB protein, p65, peaked at the 30 min treatment time. Therefore, we then treated RAW264.7 with 10 ng/ml TNF-alpha for 30 minutes and evaluated expression of 88 inflammation-related microRNAs. The microRNA expression profiles were dramatically altered, such that 14 out of 88 microRNAs had altered expression by more than 7 fold change, indicating a potent effect of TNF-α potentially operating both NF-κB signaling axis and microRNA system in macrophages. By far, we validated and optimized initial experimental conditions to study interplay between NF-κB and microRNA in macrophage.

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PIAS1 SUMO ligase regulates apoptosis in response to UV damage.
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Cells respond to UV damage by activating damage repair responses and/or initiating apoptosis. The choice between survival and death is influenced by a number of factors, including post-translational modifications. Post-translational modification of proteins by SUMO has been known to alter both the localization and the stability of DNA repair factors and influence the activity of transcription factors. In addition, SUMO E3 ligases PIAS1 and PIASy have been implicated in

the DNA repair process by modifying double stranded DNA break foci with SUMO2/3 and SUMO1 respectively. However, the specific role for each member of the PIAS family of SUMO ligases in DNA damage response, specifically damage caused by UV irradiation, has not been elucidated. In order to understand the role of PIAS family of SUMO ligases in UV-induced DNA damage response, we ectopically expressed mCherry fused PIAS1, PIASXa, PIAS3 and PIASy in HeLa cells. Cells expressing PIAS1 show increased sensitivity to UV irradiation. Each of the PIASes shows distinct localization and SUMOylation profile, indicating PIAS1 modified substrates may have a specific role in UV-induced apoptosis. We identified Daxx as a possible downstream protein in PIAS1 mediated apoptosis based on its co-localization at PIAS1 foci. Daxx is a pro-apoptotic protein that localizes to heterchromatic regions and within PML bodies in the nucleus. It has two SUMO interacting motifs that enable it to bind SUMOylated proteins. Daxx is known to act as a co-repressor of a number of SUMO-modified transcription factors, including those required for production of anti-apoptotic proteins. Daxx is proposed to interact with only a subset of SUMOylated proteins; however the basis for this specificity is not clear. Using siRNA against Daxx, we show that PIAS1 induced cell death is mediated by Daxx. We hypothesize that Daxx interacts with PIAS1 specific substrates and suppresses production of anti-apoptotic factors. This may be the molecular mechanism by which ectopic PAIS1 expression sensitizes cells to genomic insults such as UV irradiation.

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Girdin/GIV's partner Rabenosyn-5 controls turnover of Akt.

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Background: Girdin/GIV is a substrate of Akt. Akt-mediated phosphorylation of Girdin is essential for cancer cell migration, postnatal angiogenesis and neuron development. The regulation between Girdin and Akt is reciprocal; Akt phosphorylates Girdin at serine-1416, whereas overexpression of Girdin enhances phosphorylation of Akt at threonine-308 and serine-473, which is partly due to Girdin's GEF function for Gαi3. However, the detailed mechanism underlying this regulation is still unclear.
Objective: To investigate the mechanism of Girdin-mediated enhancement of Akt signaling.
Methods: Mass spectrometry was used to find novel Girdin-interacting proteins. Immunoprecipitation and GST-pull down assays were applied to confirm the interaction and identify interacting domains between Girdin and Rabenosyn-5. Furthermore, overexpression and siRNA-mediated knockdown of Rabenosyn-5 were performed to check their effects on Akt signaling.
Results: We identified Rab5 effector Rabenosyn-5 as a Girdin partner by mass spectrometry. Endogenous interaction between Girdin and Rabenosyn-5 was confirmed in HeLa cells by immunoprecipitation. In COS7 cells, GST-fused Girdin fragments was transfected combining with myc-Rabenosyn-5. Girdin CT domain was found interacting with Rabenosyn-5. Furthermore, immunoprecipitation assays using lysates of COS7 cells transfected with T7-tagged Rabenosyn-5 fragments and Girdin CT domain revealed that C3 domain of Rabenosyn-5 is responsible for the interaction with Girdin. In addition, we found that knockdown of Rabenosyn-5 by siRNA decreases the expression level of total Akt. Interestingly, overexpression of Rabenosyn-5 increases the expression level of total Akt, which indicates the role of Rabenosyn-5 in Akt turnover or degradation. Further experiment is required to check the effect of Girdin-Rabenosyn-5 complex on Akt expression level and signaling.
Conclusion: Girdin's partner Rabenosyn5 may have a role in regulating expression level of Akt.

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SOX9 induces irreversible chondrogenic phenotypic change in diabetic nephropathy.

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Study's objective: Diabetic nephropathy (DN) is a most important chronic kidney disease. We have previously reported that SOX9, a master gene of chondrogensis, is ectopically induced and promotes glomerulosclerosis in mouse experimental nephritis and recently reported that BMP4-Smad1signaling pathway regulates the expression of extracellular matrix proteins in DN. Phenotypic changes of mesangial cells(MCs)are key pathologic findings which underlies the increased glomerular sclerosis seen in DN. Furthermore a recent report revealed that HIF-1α also could be important factors in DN. The aim of this study is to investigate that activation of HIF-1α,BMP4 and SOX9 is responsible for chondrogenic phenotypic change, a new potential of phenotypic switch in DN.
Methods and Results: In vitro cell cultures, when MCs were cultured under micromass culture method, sulfated proteoglycans and Col2 were confirmed. Activation of SOX9 and Col2 in response to BMP4 induction were confirmed. Chondrogenic potential of MCs, accompanied by activation of HIF1 α, BMP4, SOX9 and Col2, could also be induced under diabetic or hypoxic condition. Furthermore forced expression of SOX9 in MCs also induced ectopic expression of proteoglycans and Col2 in MCs.BMP4-Smad1 signaling pathway played a key role in phenotypic change in MCs.
In streptozotocin-induced diabetic model mice, the induction of SOX9 and Col2 in glomeruli were confirmed. In another diabetic model, transgenic mice expressing NOS2 constitutively in pancreatic βcells exhibited pathological change resembling an advanced stage of human DN. In this model, the induction of HIF1α, BMP4,SOX9 and Col2 were observed. Positivity of these proteins were detected in sclerotic lesions in the glomeruli and a major part of the positive staining area of SOX9 was coincided with the area of HIF1αand BMP4.
We have developed transgenic mice with inducible expression of BMP4 (BMP4 Tgm) by using thetamoxifen-regulated Cre/LoxP system. BMP4 Tgm exhibited an expanded mesangial area and a thickened glomerular basement membrane that remarkably resembles human DN. BMP4 Tgm also showed significant induction of glomerular expression of SOX9 and Col2 compared to the control mice. Furthermore, the heterozygous BMP4 knockout mice did not show the induction of SOX9.
Conclusions: Our present data provide a new aspects in the progression of glomerular injury, MCs have a chondrogenic potential. As chondrocytes are unique cells that locate at avascular and hypoxic condition, it makes sense that MCs acquire the chondrogenic phenotype mediated by ectopic BMP4 and SOX9 to operate a cellular protective mechanism for chronic pathological hypoxic stress in the kidney.

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Cytosolic CD38 Protein Forms Intact Disulfides and Is Active in Elevating Intracellular Cyclic ADP-ribose.

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Cyclic ADP-ribose (cADPR) is a Ca^{2+} messenger molecule responsible for regulating a wide range of physiological functions from social behavior in mice to abscisic acid-dependent signaling in sponges and plants. Currently, no method is available for manipulating the cellular levels of cADPR, greatly impeding the mechanistic elucidation of its signaling function. We have now modified the native CD38 and engineered a soluble form for transfection into cells. The soluble CD38 efficiently used the cytosolic NAD as substrate and produced cADPR intracellularly. The activity of the engineered CD38 can be tuned downward by mutating the

catalytic residue, Glu226, and upward by a double mutation, E146A and T221F, which increased its cADPR synthesis activity by more than 11 folds. Remarkably, the engineered CD38 exhibited the ability to form the critical disulfide linkages required for its enzymatic activity. This was verified by using a monoclonal antibody generated against a critical disulfide, C254-C275. The specificity of the antibody was established by X-ray crystallography and site-directed mutagenesis. The engineered CD38 is thus a novel example challenging the general belief that cytosolic proteins do not possess disulfide. Functionally, the intracellular calcium was increased in HEK 293 cells by co-overexpressing this engineered CD38 (double mutant) and ryanodine receptor (RYR), which is the potential target of cADPR. This study has set the stage for *in vivo* manipulation of cADPR signaling pathway.

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Sumoylation-promoted enterovirus 71 3C degradation correlates with a reduction in viral replication and cell apoptosis.

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Enterovirus (EV) 71, a member of the Picornaviridae family, may cause serious clinical manifestations associated with the central nervous system. Enterovirus 3C protease is required for virus replication and can trigger host cell apoptosis via cleaving viral polyprotein precursor and cellular proteins, respectively. While the role of the 3C protease in processing viral and cellular proteins has been established, very little is known about the modulation of EV71 3C function by host cellular factors. Here, we show that sumoylation promotes EV71 3C protein ubiquitination for degradation, correlating with a decrease of EV71 in virus replication and cell apoptosis. SUMO E2 conjugating enzyme Ubc9 was identified as an EV71 3C-interacting protein. Further studies revealed that EV71 3C can be SUMO modified at residue K52. Sumoylation downregulated 3C protease activity in vitro and also 3C protein stability in cells, in agreement with data suggesting 3C K52R protein induced greater substrate cleavage and apoptosis in cells. More importantly, the recombinant EV71 3C K52R virus infection conferred more apoptotic phenotype and increased virus levels in culture cells, which also correlated with a mouse model showing increased levels of viral VP1 protein in intestine and neuron loss in the spinal cord with EV71 3C K52R recombinant viral infection. Finally, we show that EV71 3C amino acid residues 45-52 involved in Ubc9 interaction determined the extent of 3C sumoylation and protein stability. In addition, sumoylation can further regulate 3C subcellular localization. We also demonstrate that 3C protein degradation is through ERAD (ER-associated degradation) pathway, and identify HRD1 is the 3C ubiquitin E3 ligase. Furthermore, USP14 is the 3C-deubiquitinating enzyme. More importantly, USP14 inhibitor IU1 treatment significantly reduced the protein half-life of EV71 3C and inhibited EV71 virus replication. Collectively, our results uncover a previously undescribed cellular regulatory event against EV71 virus replication and host cell apoptosis by sumoylation and ubiquitination at 3C protease.

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AOD2 and AOD5 localize to the nucleus and synergistically induce alternative oxidase in *Neurospora crassa.*

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Alternative oxidase (AOX) provides an alternative respiratory pathway in plants, some other eukaryotes, and some prokaryotes. It functions as a terminal oxidase in the electron transport chain as it directly delivers electrons from ubiquinol to molecular oxygen and produce water as a final product. Production of the enzyme responds to various signals in different organisms including inhibition of the standard electron transport chain, increased production of reactive

oxygen species, temperature changes, and pathogen attacks. AOX serves as an excellent model to study mitochondrial retrograde signaling because it is encoded by a nuclear gene and can be induced by effects on mitochondrial function. So far, the mechanism(s) by which AOX is induced is poor elucidated. In *Neurospora crassa*, the induction of AOX requires two zinc binuclear cluster transcription factors, AOD2 and AOD5. Here, we report that both AOD2 and AOD5 are located in the nucleus under either normal conditions or conditions that result in induction of AOX. Furthermore, pulldown experiments suggest that they interact with each other *in vivo*, again under both normal and inducing conditions. Finally, both AOD2 and AOD5 are required for growth on poor carbon courses, suggesting a role in gluconeogenesis. Future studies are aimed at examining the two proteins under normal and inducing conditions for changes in protein modifications.

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Coordinated Regulation of Human GCM1 Activity by Phosphorylation and Desumoylation in Placental Cell Differentiation.

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cAMP signaling and the placental transcription factor GCM1 regulate expression of syncytin-1 and -2 fusogenic proteins, which are critical for syncytiotrophoblast formation by trophoblast fusion. We recently revealed a cAMP/PKA/CBP signaling pathway that activates GCM1 by coordinating GCM1 phosphorylation and acetylation. In contrast, GCM1 activity is downregulated by sumoylation of Lys156. How GCM1 sumoylation is regulated was unknown. Here we identify a novel PKA-independent cAMP signaling pathway as the critical regulator of GCM1 sumoylation. We show that Epac1 and Rap1, in response to cAMP, activate CaMKI to phosphorylate Ser47 in GCM1. This phosphorylation facilitates the interaction between GCM1 and the desumoylating enzyme SENP1 and thereby leads to GCM1 desumoylation and activation. Using RNAi, we further demonstrate that 8-CPT-AM, an Epac activator, stimulates syncytin-1 and -2 gene expression and cell fusion of placental BeWo cells in a GCM1-dependent manner. Importantly, the cell fusion defect in GCM1-knockdown BeWo cells can be reversed and enhanced by the RNAi-resistant phosphomimetic GCM1S47D mutant. Our study has identified a novel cAMP/Epac1/CaMKI/GCM1 signaling cascade that stimulates trophoblast fusion through promoting GCM1 phosphorylation and desumoylation.

Computational, Systems, and Synthetic Biology

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Modeling and Simulation Analysis of Insulin Secretory Functions of Pancreatic β-cells.

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BACKGROUND: Pancreatic β-cells secrete insulin in response to elevated plasma glucose levels, and the dysfunction of these cells is a major contributor to the development of diabetes. Diabetes is classified roughly into two types, type 1 diabetes (T1D) and type 2 diabetes (T2D). Usually, T1D onset is associated with an autoimmune reaction against pancreatic β-cell constituents, and T2D onset is associated with insulin resistance and insulin secretory dysfunction. Furthermore, genetic and environmental factors also play important roles in diabetes onset. The insulin secretory capacity of Asians including Japanese is relatively lower than that of western populations; thus, insulin secretory dysfunction, rather than insulin resistance, may play a more critical role in the development of T2D in Asians. Therefore, an

integrated understanding of the insulin secretory function of pancreatic β-cells is important for the study and therapy of diabetes. In systems biology, modeling and simulation analyses of biological pathways, such as metabolic pathways, gene regulatory networks, and cell signaling networks, are available using a computational approach. Cell Illustrator (CI), which is a graphic platform, implements the Hybrid Functional Petri net with an extension (HFPNe) theory suitable for the modeling and simulation of biological pathways and enables realistic modeling and simulation of biological pathways in silico. We developed a biological pathway model of insulin-secretion-related molecules in pancreatic β-cells, and the insulin secretory function, which is induced by glucose, incretin, and antidiabetic drugs, was simulated using CI. METHODS: To develop a computer simulation model of the insulin secretory function of pancreatic β-cells, we compiled and interpreted measurement data from the literature on insulin secretion of pancreatic β-cells using CI. RESULTS: A biological pathway model of the insulin secretory function was developed and was visually presented similarly to a pathway map. Glucose-induced time-dependent changes in β-cell secretory function parameters such as ATP/ADP ratio, membrane depolarization, Ca^{2+} oscillation, and biphasic insulin secretion were simulated successfully in the graph data form. CONCLUSION: This simulation model may be useful for providing a visually easy-to-understand explanation of the pathophysiology and treatments of diabetes to medical professionals and patients. Moreover, this model may contribute to the development of novel therapies and drugs for T2D.

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Modeling the Interplay of Plasmin and Thrombospondin-1 in TGF-β1 Activation : A Bistable Switch *in Silico* and *in Vitro.*

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Background: Transforming growth factor-β1 (TGF-β1) is a potent and influential cytokine that accumulates in the extra-cellular matrix as a latent, inactive complex. The bioavailabilty of TGF-β1 for cell signaling is controlled by its extracellular activation, for example by conformational change upon binding Thrombospondin-1 (TSP), by plasmin proteolysis, or by other activating factors. Although plasmin is a direct molecular activator of TGF-β1, animal experiments have paradoxically shown the opposite effect of the plasmin pathway toward TGF-β1.
Methods: We built a systems-level computational model using ordinary differential equations to represent the kinetics of TGF-β1 activation, mutual antagonism between plasmin and TSP, and gene expression feedback from TGF-β1. Model-based predictions were tested with hepatic stellate cells by adding plasminogen, the stable precursor of plasmin, to cell culture media. TGF-β1 was measured with an ELISA specific for the active form.
Results: Mathematical simulations predict that plasmin will decrease TGF-β1 activation in the presence of TSP-mediated activation, and increase TGF-β1 otherwise. Furthermore, simulations predict that the TGF-β1 activation system can exhibit cooperative, bistable switching between two distinct modes of TGF-β1 activation: a TSP-dependent mode with high activation of TGF-β1, and a plasmin-dependent mode with TSP suppressed and lower activation of TGF-β1.
In vitro experiments showed that increasing plasmin caused increased cleavage of TSP and decreased concentration of active TGF-β1, with a sigmoidal (cooperative) dose-response curve. In the presence of a specific inhibitor to block TSP-mediated TGF-β1 activation, plasmin was instead able to increase TGF-β1 activation. Bistability was demonstrated experimentally in the form of hysteresis.

Conclusion: Antagonistic interplay between plasmin and TSP is an important feature of our model, and is confirmed by our experiments. These results suggest novel mechanisms and hidden pitfalls for addressing TGFβ-related diseases.

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A new method for measuring intracellular dynamics reveals a negative feedback linking cell growth to proliferation in mammalian cells.

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Intracellular dynamics are studied from either time course measurements on live cells (e.g. time lapse microscopy) or time course measurements on synchronized populations. Limitations of these two different techniques range from the unknown affects of synchronizing agents to the difficulty of probing live cells for both post-transcriptional modifications and bulk properties such as total cell mass. In this study we develop ergodic rate analysis (ERA) as a third independent path to investigate intracellular dynamics. ERA is based on the fact that in non-synchronized populations, rapid intracellular events are represented by fewer cells than slower events. By mathematically harnessing this point, ERA provides an interpretive framework transforming measurements made on large ensembles of fixed cells into information about cellular dynamics. Applying ERA to investigate cell growth dynamics reveals, at the G1/S transition, a negative feedback linking growth rate to cell size. We show that this feedback functions by cell-size dependent protein degradation and is regulated by the mTOR pathway. By affectively decreasing the cell-to-cell size variation, this feedback partly explains the relative homogeneity in cell-size in proliferating populations and, perhaps, in tissues.

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Novel graphical approaches to analyze phosphoproteomic data reveal signaling pathways activated in lung cancer and neuroblastoma.

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Phosphoproteomics, which identifies a large number of phosphorylated proteins from tumors and cell lines, has shown that individual cancerous tumors express different combinations of active tyrosine kinases, including multiple receptor tyrosine kinases. A major challenge is to sort out relationships between individual signaling pathways for targeted therapy. Hierarchical clustering dendrograms, heat maps, and network graphs have been employed to attempt to visualize patterns that may indicate functional relationships among different groups within data, but graphical visualization of large data sets rapidly becomes unwieldy and too often conveys little meaning. We developed new methods that combine statistical analyses (using R) and novel graphic approaches (using RCytoscape, Cytoscape, and PyMOL) to visualize the statistical structure of data. We applied these methods to phosphoproteomic data from lung cancer tumors and cell lines and neuroblastoma cell lines. Multidimensional scaling was used to translate Pearson and Euclidian distance into Cartesian coordinates in three dimensions, into a data structure that we call a Pearson Euclidian Distance (PED) graph. Proteins that are close to one another on this graph have similar phosphorylation patterns as measured by two independent statistical criteria. Groups selected from the data structure were plotted as networks using evidence for protein-protein interactions sought from String, GeneMANIA, and the kinase-substrate data from PhosphoSitePlus. The analysis supports the hypothesis that activation of one or more different receptor tyrosine kinases (RTKs) and/or SRC-family kinases (SFKs) occurs in different individual cancerous tumors. Remarkably, many regions of the data

structure appear to contain at least one RTK and/or SFK and known effector proteins thereof. The data-driven clusters suggest potential links between several different cancer driver pathways and proteins that have not previously been characterized. These clusters suggest strategies for more effective cancer treatments involving combinations of drugs tailored to individual tumors.

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EGFR Regulation of Epidermal Barrier Function.

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The epidermal growth factor (EGF) receptor (EGFR) plays an important role in keratinocyte terminal differentiation, a process that ultimately forms the epidermal barrier. Defective epidermal differentiation and disrupted barrier are primary features of many human skin diseases, including psoriasis and atopic dermatitis. Abnormalities in expression of the EGFR and its ligands are common features found in these diseases. Here, we explored the genome-wide effects of EGF, a ligand of the EGFR, on normal human epidermal keratinocytes undergoing density-induced differentiation. We identified 2,676 density-dependent EGF-regulated genes. Contrary to density effects, EGF inhibited expression of 91% of the density-induced genes and promoted that of 96% of the density-suppressed genes, suggesting that EGF plays a critical role in inhibiting epidermal differentiation. Specifically, EGF significantly reduced free fatty acid synthesis to half by suppressing expression of PTPLB and TECR, enzymes that form stearic and oleic acids, two major fatty acid components in the stratum corneum. EGF also inhibited mRNA levels of genes encoding enzymes in the *de novo* and salvage ceramide pathways, causing a decrease in ceramide 1, 3, 6, 7, 8, and acyl-glucosylceramides. Our expression and protein results strongly suggest that activation of EGFR signaling inhibits cornified envelope formation by altering levels of enzymes and structural proteins essential for the synthesis of this differentiated structure. Further, we showed that EGF caused a significant reduction in levels of tight junction proteins such as CLDN1 and TJP1, leading to an increase in paracellular permeability and disrupting tight junction barrier function. EGF impaired the epidermal barrier integrity as a whole by increasing the transepidermal water loss in organotypic culture. Finally, bioinformatics and statistical analyses revealed that genes associated with skin diseases were enriched in the set of EGF-regulated genes. Our work advances the current understanding of EGFR signaling in regulating epidermal barrier function. We identified many new EGFR-regulated genes and, more importantly, connected the function of these genes to major processes involved in epidermal differentiation. These findings provide a reference for subsequent studies of EGFR in the regulation of epidermal cell fate and homeostasis, and may lead to novel therapeutic approaches for the treatment of dermatological diseases.

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Differential Expression of Transcripts Linked to Wound Healing, Epithelial-Mesenchymal Transition (EMT), and Inflammation during Primary Explant Culture of Zebrafish Keratocyte.

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Fish keratocytes derived from 1 – 3 day old explant cultures are routinely used to study mechanisms of cell motility. Significant cellular changes have been reported in a number of other systems during these initial stages of primary culture but 1) a systematic characterization of these changes is lacking and 2) it is uncertain if these effects are seen in the keratocyte system. Data presented here suggest that keratocytes undergo significant changes in gene expression patterns during primary explant culture including genes controlling processes involved in wound healing, epithelial-mesenchymal transition (EMT), and inflammation. Changes in gene expression begin as early as 12-hours after establishment of explant cultures. Additionally, our data show that 17.5% of the genome is differentially expressed between 1 and 7 days of explant culture. Cell motility and morphology appear to undergo little change within the initial 72-hours of explant culture, when changes in gene expression are significant and increasing, but are dramatically altered in 7-day cultures. The variability in the expression profile within a well-controlled, genetically-defined explant culture system provides an important contextual framework for interpretation of data from keratocyte primary explant cultures and other primary culture systems.

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Animating Cell Biology: New Tools for Research and Communication.

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In recent years, there has been a rapid growth in the use of animation as a means to communicate complex biological processes to a wide range of audiences. Using 3D animation software from the entertainment industry, it is possible to synthesize data from diverse sources to create a coherent and contextualized view of how molecular and cellular systems operate. These visualizations have served not only to make molecular concepts more accessible to students and the public at large, but have also proven to be extremely useful for researchers seeking to build and refine their hypotheses. In an effort to make animation tools more readily available to researchers, we have embarked on a project to create a novel molecular biology-centric 3d animation application created specifically for cell and molecular biologists.

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The Open Microscopy Environment (OME): Informatics for Biological Imaging.

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OME is a multi-site collaborative effort across academic laboratories and commercial entities that produces open tools to support data visualization, management, and analysis for biological microscopy and high content screening (HCS). All OME specifications and software are free, and all source code is available under GNU public "copyleft" licenses. With a strong foundation for biological light microscopy in place, OME has begun extending its coverage to HCS, electron microscopy, scanning probe microscopy, other emerging modes of biological imaging.

OME develops and releases:
-- The OME Data Model (http://ome-xml.org) provides a specification for saving and exchanging metadata in biological imaging.
-- The OME-TIFF file format (http://ome-xml.org/wiki/OmeTiff) and the Bio-Formats file format library (http://openmicroscopy.org/site/products/bio-formats) provide an easy-to-use set of tools for converting data from proprietary file formats into an open, accessible format.
-- OMERO (http://openmicroscopy.org/site/products/omero) is a Java-based server and client application suite that combines an image metadata database, a binary image data repository and high performance visualization and analysis. OMERO works as an image data management system for a laboratory, department or institution. For computational analysis of microscopy or HCS images, OMERO's standardised interface and Bio-Formats provide a single mechanism for accessing image data and metadata regardless of original file format. OMERO includes interfaces for Java, C++ and Python to support standard image processing tools like ImageJ, Matlab, and CellProfiler, a scripting facility for Python-based data processing, and custom clients that enable remote image viewing, processing and analysis. Processed images, calculated regions-of-interest, and quantitative measurements can be stored using OMERO's internal databases or in an HDF5-based tabular data store. OMERO is used in a number of commercial products, and runs the JCB DataViewer (http://jcb-dataviewer.rupress.org), the first publication system for original image data in the life sciences.
More information is available at http://openmicroscopy.org.

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A synthetic molecular tool for post-transcriptional control of gene expression in E. coli.

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We here present a synthetic, modular, molecular device for the post-transcriptional control of the expression of any gene of interest in E. coli. To this aim, a CIS- and a TRANS-acting non-coding sequences were cloned into the same high copy number plasmid: the CIS element contains a non-coding region and a Ribosome Binding Site (RBS) upstream of a GFP reporter gene; the TRANS element (T4) is complementary to the CIS non-coding region and to the initial 4 nucleotides of the RBS, to be able to inhibit translation of the gene placed downstream of the CIS. T4 is regulated by the Lac repressor to tune, via IPTG, the number of available interfering molecules. To this aim a lac operator is placed upstream of the TRANS sequence. The Lac repressor is produced by a co-transformed low copy number plasmid, built up in two configurations, with either O1 or Os lac operator upstream of the lacI sequence. All parts used were in the BioBrick format. A mathematical model was also developed to describe/predict the behaviour of the entire molecular device upon characterization [see also Ceroni F., 2010] of its elementary parts (except of CIS/TRANS binding affinity).
Experimental measurements were performed in cells grown up to 14 hours in 5 ml M9 medium. 200 ul samples were taken every hour and fluorescence and OD measured with a Tecan® multiplate reader. Cells transformed only with the CIS-reporter yielded a GFP output of 10307±1561 arbitrary units (a.u.). When both CIS and TRANS sequences were present, fluorescent signal decreased down to 776±387 a.u., as the result of their interaction. Indeed, when the TRANS sequence was absent, fluorescence was restored to 9974±1154 au. The presence of O1- lacI or Os- lacI yielded respectively 11598±2768 a.u. and 11308±2152 au, in absence of IPTG inducer, proving that the LacI repressor can efficiently inhibit TRANS production. When up to 2 mM IPTG was added to the cells fluorescence did not change when O1- lacI was used. This suggests that the increase in the transcription of TRANS induced by IPTG, in this case was not sufficient to block GFP translation via CIS-TRANS annealing. On the

other hand, IPTG (0.1, 0.25, 0.5, 1 and 2 mM) added when Os- lac operator was upstream of the lacI sequence, produced respectively 8455±911, 8093±1524, 6814±1170, 5908±727 and 5679±1398 a.u. in fluorescence, showing that the device can tune gene expression. The behaviour of the genetic circuit was well described by the mathematical model, and model fitting allowed the identification of CIS-TRANS binding affinity (2.3*10-3 min-1*molecule-1). These results show that our synthetic, modular, molecular device allows post-transcriptional regulation of gene expression in E. coli, useful for multiple application in synthetic biology as well as a tool to study RNA interfering process in living cells.

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Characterization of a Fast Version of SNAP-tag for In Vitro and Live Cell Protein Labeling.

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Protein synthesis, transport, localization, and degradation are highly dynamic and are modulated during a variety of cellular processes. However, the tools to study these events in living cells are limited. Site-specific labeling of cellular proteins with chemical probes allows for imaging and analyzing protein dynamics in live cells and small animals. One of the labeling systems is achieved through the reaction of O6-benzylguanine (BG) derivatives with SNAP-tag based on human O6-alkylguanine-DNA alkyltransferase (hAGT). The efficiency of the labeling depends mostly on the reactivity of SNAP-tag. In order to study fast dynamic cellular events, such as receptor endocytosis and protein turnover, we have recently reported a fast-labeling variant of SNAP-tag, termed SNAPf (Sun, X. et al., ChemBioChem, 2011, in press). SNAPf carries 19 amino acid substitutions and a C-terminal deletion compared to wild-type AGT, and 10 extra mutations compared to SNAP26m, a previously described hAGT variant. In this study, SNAPf has been further characterized and utilized in conjunction with a wide variety of SNAP-tag substrates, including fluorogenic probes and near-infrared dyes, to label proteins in vitro and in vivo. In vitro kinetics studies show that SNAPf has up to tenfold increased activity towards several fluorophore BG conjugates compared to SNAP26m. Live cell labeling assays using mammalian cell lines expressing different SNAP-tag fusion proteins confirm that SNAPf exhibits faster labeling kinetics than SNAP26m. Fluorescence microscopy and image analysis of labeled SNAP-tag fusion proteins in live mammalian cells demonstrates that SNAPf allows for shorter labeling times and improved signal-to-noise ratios. The increased reactivity of the SNAPf variant shows promise for analysis of proteins with rapid trafficking or fast turnover rates.

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Yeast-based Biosensors for Environmental Monitoring.

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Water contamination is a major concern in both the developed and developing worlds, and current technologies to monitor water quality and detect contamination are inadequate. To address gaps in our water quality monitoring, we are developing yeast that respond to water contamination and testing sensor platforms to utilize these biosensors in diverse situations. Some of these yeast sensor strains are based on the genetic memory system developed by the lab of Dr. Pam Silver (Ajo-Franklin *et. al.* 2007). This system allows the yeast to respond to water contaminants at a threshold concentration and continue to signal exposure if contaminant levels drop. We will use these and other genetically engineered yeast as the core of devices that detect water contamination in various circumstances. For developed world applications, we are designing a device for continuous, long-term, in line water monitoring. This device will have two monitoring capabilities. Each strain will have a specific contaminant response, indicating

when that toxin is detected above an acceptable threshold, using a fluorescent signal. In addition, all strains will serve as monitors of catastrophic toxicity leading to death or halted growth through monitoring of yeast concentration. This can provide an early warning for changes in water quality that could lead to catastrophic disruption of water treatment plants. For developing world and field testing applications, we are developing paper analytical devices (PADs) incorporating yeast which will respond to water contamination by producing a color change. These devices will serve as an inexpensive, low technology method (from the use standpoint) to determine whether water sources are safe to drink.

1270

Computational Modeling of Cyclooxygenase in Drosophila.

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Eicosanoids are important mediators that are involved in reproduction, immune system, ion transport and etc. Eicosanoids are oxygenated from C20 polyunsaturated fatty acids. Mammalian eicosanoid biosynthesis consists of three pathways, COX (cyclooxygenase) pathway, LOX (lipoxygenase) pathway, and epoxygenase pathway. Cyclooxygenase (COX) catalyzes the committed step of biosynthesis of prostaglandins, thromboxanes and prostacyclins. There are two isoforms of COX: COX-1 and COX-2, both of which are membrane bound proteins. COX exists in many organisms including vertebrates and invertebrates, but has mostly been studied in mammalian systems. In this study, we used computational methods to identify several putative COX homologues in Drosophila. We have built three-dimensional models of all the putative COX enzymes in Drosophila using computational biology methods and compared them both at the sequence and structural levels. Our results are a preliminary attempt at uncovering the functional role of these putative COX proteins in the metabolic pathways of Drosophila with an emphasis on their role in immune regulation.

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Genome-wide Analysis for Enrichment of Seed Sequences Identifies Prominent Off-targeted Genes in RNAi Screens.

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Off-target effects can hamper the ability to interpret the results of genome-wide RNAi screens, but there are no general methods available to rapidly identify potential off-targeted genes prior to performing validation studies. We developed a new method, Genome-wide Enrichment of Seed Sequences (GESS), which identifies candidate off-targeted genes from direct analysis of primary screening data. By comparing siRNAs with and without phenotype, GESS identifies genes that are enriched for miRNA-like binding sites among the group of siRNAs with phenotype. GESS identified MAD2 as frequently off-targeted in a screen for components of the spindle assembly checkpoint, which we confirmed experimentally. We applied GESS to several other published RNAi screens, and identified known and novel genes sensitive to seed-match based off-target effects. We provide GESS as standalone package and recommend it be implemented as a standard step in screen validation to identify genes sensitive to off-target effects.

1272

The proteome of a thermophilic Eukaryote.

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Chateomium thermophilum, a thermophilic filamentous fungus, is on its way to becoming an established model organism. With an optimal growth temperature of 55°C, its proteins are extraordinarily stable and very amenable for structural studies [1]. We have used extensive peptide fractionation and tandem mass spectrometry to measure its proteome to saturation. We identified 3977 proteins and annotated almost 60% of the predicted ORFs using Gene Ontologies [1]. To investigate which functional protein categories in the cell adapt to thermophily, we systematically compared absolute protein abundances measured in *C. thermophilum* to *Saccaromyces cerevisiae* and *Chaetomium globosum*, the closest mesophilic relative. The preliminary results show that proteins responsible for cell wall and integral to plasma membrane, display a significantly higher abundance in *C. thermophilum.*

[1] Stefan Amlacher, Philip Sarges, Dirk Flemming & Vera van Noort. et al. Insight into Structure and Assembly of the Nuclear Pore Complex by Utlizing the Genome of a Eukaryotic Thermophile. *Cell*, 146, 277-289, July 2011.

1273

Validation of automated subcellular tracking tool for kinetic image analysis.

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Background: Timelapse imaging of biological sample at microscopic level generates a wealth of novel, dynamic phenotypes that, if quantitatively analyzed, could provide new insights into biological processes and disease formation. Automated tracking of the subcellular objects is critical to the unbiased and complete quantification of the dynamic phenotypes. A large amount of work is being done in the scientific community to develop particle tracking and characterization tools for data sets composed of massive images. However, these tools are generally difficult to adopt in broad lab settings and are often application specific and limiting. We have reported in last meeting about development of a robust and flexible tracking method called "soft tracking" that can be taught to handle challenging tracking applications.
Objectives: Here we validate the automated subcellular tracking performance of our soft tracking tool by comparing with multiple conventional benchmark software including Image J (PTA Plugin), Matlab, Imaris and Metamorph.
Study Data: As a comprehensive set of benchmark movies, we used (A) 6 public movies: 3 synthetic movies containing different kinetic scenarios (exit and no enter; enter and exit; allowed to exit), a rotating golf ball (~180 points entering and exiting the scene), Birds (at different altitudes with frequent occlusions), Fishes (a flock of 150+ fishes in the sea); (B) 4 movies of COS7 cell expressing GFP in endosome with systematically reduced temporal sampling rates; (C) a movie containing microtubule (MT) tips transition through "growth", "pause" and "shrink" phases. It is challenging to track as the MT-tips lose contrast or even disappear during pause phase.
Results: In terms of tracking sensitivity and the error rate (sensitivity, error), our soft tracking tool had superior performance overall (0.718±0.033, 0.052±0.003), followed by Imaris (0.475±0.037, 0.154±0.006), and ImageJ PTA (0.398±0.037, 0.171±0.006).
Conclusion: the results indicate that our soft tracking could support subcellular tracking for comprehensive quantification of dynamic phenotypes.

Chaperones, Protein Folding, and Quality Control

1274

Adaptation to hydrogen sulfide protects against hypoxia in *C. elegans.*

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Oxygen is an essential environmental nutrient for animals. Physiological responses to decreased oxygen availability (hypoxia) play important roles in development, metabolism and disease. We have found that, in *C. elegans*, exposure to specific hypoxic conditions cause disruptions in cellular protein homeostasis, as monitored by aggregation and toxicity of polyglutamine-containing proteins. This response to hypoxia is mediated by genetic factors, showing that it is an active response to changes in oxygen availability. Our data further indicate that the response to hypoxia has long-lasting effects on cellular physiology that persist even after return to room air. Recent evidence suggests that exogenous hydrogen sulfide can protect against the effects of ischemia-reperfusion injury in mammals. We have shown previously that adaptation to hydrogen sulfide increases lifespan and thermotolerance in *C. elegans*. Here we show that adaptation to hydrogen sulfide also protects against, and even reverse, the effects of hypoxia on protein homeostasis. Thus, as in mammals, hydrogen sulfide can protect against the damaging effects of decreased oxygen. We are currently exploring the molecular genetic mechanisms that mediate effects of adaptation to hydrogen sulfide on protein homeostasis. This work will provide unique insight into the mechanisms by which adaptation to hydrogen sulfide is integrated with the response to hypoxia at the cellular and organismal level.

1275

Peripheral neuropathy-linked mutations cause mislocalization and aggregation of ESCRT-I-interacting protein SIMPLE.

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Missense mutations in SIMPLE cause an autosomal dominant, demyelinating form of peripheral neuropathy known as Charcot-Marie-Tooth disease type 1C (CMT1C), but the pathogenic mechanisms of these mutations remain unknown. SIMPLE contains a PSAP motif that is known to bind the UEV domain of TSG101, a component of the ESCRT-I (endosomal sorting complex required for transport-I) complex involved in sorting ubiquitinated cargo proteins to the lysosomal pathway for degradation; however, the cellular function and subcellular localization of SIMPLE remain poorly characterized. Here, we report that SIMPLE is highly expressed in the peripheral nerves and Schwann cells. We found that SIMPLE is an early endosomal membrane protein that markedly co-localizes with TSG101 and have identified a transmembrane domain (TMD) for anchoring SIMPLE to the membrane. We find that CMT1C-associated mutations are clustered within or around the TMD of SIMPLE and that these mutations cause mislocalization of SIMPLE from the early endosome membrane to the cytosol. The CMT1C-associated SIMPLE mutant proteins are unstable and prone to aggregation, and they are selectively degraded by both the proteasome and aggresome-autophagy pathways. Our findings suggest that SIMPLE mutations cause CMT1C peripheral neuropathy via a combination of loss-of-function and toxic gain-of-function mechanisms and highlight the importance of both the proteasome and autophagy pathways in the clearance of CMT1C-associated SIMPLE mutant proteins.

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Global molecular mechanisms underlying hypoxia response and tolerance.

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Hypoxia is a widely occurring condition experienced by diverse organisms under numerous physiological and disease conditions. To probe the molecular mechanisms underlying hypoxia responses and tolerance, we previously performed a genome-wide screen to identify mutants with enhanced hypoxia tolerance in the model eukaryote, the yeast *Saccharomyces cerevisiae*. We identified five genes whose deletion significantly enhanced hypoxia tolerance. They are *RAI1, NSR1, BUD21, RPL20A, and RSM22*, all of which encode functions involved in ribosome biogenesis. Further analysis of the deletion mutants showed that they minimized hypoxia-induced changes in polyribosome profiles and protein synthesis. Strikingly, proteomic analysis using the iTRAQ profiling technology showed that hypoxia induced changes in a substantially fewer number of proteins in the deletion mutants. Computational analysis of the iTRAQ data indicated that the activities of a group of regulators were regulated by hypoxia in the wild type parent cells, but such regulation appeared to be diminished in deletion strains. Analysis of previous microarray gene expression profiling data also shows that a group of regulators, particularly certain components of Swi/Snf complexes, play important roles in oxygen regulation of gene expression. Experiments are currently underway to investigate the molecular mechanisms by which these regulators control gene expression in response to hypoxia or reoxygenation.

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Impairing PERK ameliorates neuropathy in Charcot Marie Tooth 1B mouse.

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Charcot-Marie-Tooth 1B (CMT1B) neuropathy is characterized by demyelination in peripheral nerves resulting in slowed nerve conduction velocity, muscular atrophy, and postural abnormalities. It is caused by mutations in Myelin Protein Zero (*MPZ*, P0), abundantly expressed by myelin-forming Schwann cells. When expressed in transgenic mice, P0S63del produces a demyelinating neuropathy that mimics the corresponding human CMT1B disease. P0S63del is retained in the endoplasmic reticulum of Schwann cells where it elicits an unfolded protein response (UPR) characterized by phosphorylation of eIF2alpha, ATF6 cleavage, IRE-1 induced Xbp-1 splicing and persistent CHOP synthesis. Genetic ablation of *Chop* restores motor capacity and ameliorates demyelination in S63del mice, suggesting that the UPR is maladaptive and pathogenic. Since CHOP is downstream of the PERK/eIF2alpha pathway, we studied the effect of *Perk* ablation in models of normal and S63del myelination. Myelinating DRG explant cultures prepared from S63del//*Perk -/-* mice show more and longer myelin internodes as compared to S63del. Ablation of *Perk* specifically in Schwann cells (S63del/P0Cre/*Perkf/f* mice) ameliorates myelination and nerve conduction velocities. Surprisingly, despite reduced levels of eIF2alpha phosphorylation in *Perk*-deficient S63del nerves, CHOP levels remain elevated. Interestingly, preliminary data suggest that IRE1 signaling could be more active in these nerves as compared to S63del. From these data we suggest that impairing PERK signaling ameliorates myelination in S63del/CMT1B neuropathy mice, perhaps by altering the balance between IRE-1 and PERK signaling in Schwann cells.

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Purinosome Formation is Altered in Cells from Patients with Defect in De Novo Purine Synthesis.

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De novo purine synthesis (DNPS) requires ten enzymatic steps to generate IMP. This process is in eukaryotes catalyzed by six enzymes, which are organized into a multimeric protein structure: the purinosome. The purinosome is a transient structure which appears to be dynamically regulated by actual need and availability of purines. Dysfunction of DNPS is represented by two genetic diseases: AICA-ribosiduria and adenylosuccinate lyase deficiency (dADSL) both presenting mostly with devastating neurologic involvement. We hypothesized that some of the mutations may affect selectively formation or stability of the purinosome in vivo and lead thus to more severe phenotypes. In this work we therefore studied purinosome formation in skin fibroblasts from 10 patients with ADSL deficiency and from single case with AICA-ribosiduria.
We performed immunofluorescence labeling and subsequent analysis of confocal microscopy data of DNPS proteins produced by cells cultured in purine depleted conditions. We colocalized combinations of PPAT and ATIC, ADSL and ATIC, ADSL and PPAT enzymes in patient's skin fibroblasts.
PPAT protein formed clusters at reduced rate and ATIC remained diffuse in the patients` fibroblasts. When we focused on ADSL and ATIC proteins, we observed diffuse distribution of both proteins in patients cells, suggesting no purinosome formation. When compared to the control fibroblasts, the amount of the produced enzymes was reduced especially in AICA-ribosiduria and in more severe forms of dADSL. Colocalization of PPAT and ADSL proteins showed strong reduction of purinosome formation in patients with more severe dADSL, while reduction was only slight in AICA-ribosiduria and in moderate forms of dADSL. Control fibroblasts formed the complex very strongly in all tested enzyme combinations.
We observed correlation between purinosome formation and severity of the disease: no creation of the purinosome in neonatal and severe type of dADSL and strongly reduced formation in moderate type of dADSL and AICA-ribosiduria. These results indicate that genetic defects of DNPS affect not only activity and structure of the individual enzymes, but according to disease severity, also the functionality of the DNPS pathway by purinosome complex destabilization.
Study was granted by Ministry of Education of the Czech Republic MSM0021620806 and 1M6837805002 and LH11031

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Roles for nucleotide binding and multiple chaperones in *de novo* septin protein folding and higher-order assembly.

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Members of the septin family of GTP-binding proteins are found in nearly every eukaryote, typically as complexes composed of multiple septins. Filamentous septin arrays control cytokinesis in dividing cells, and morphogenesis in non-dividing cell types. As is true of other cytoskeletal polymers like actin and tubulin, binding and hydrolysis of nucleotide appear to regulate the structural integrity of septin filaments, presumably by stabilizing inter-subunit interfaces. However, unlike actin and tubulin, which traverse complex folding pathways and are substrates of multiple chaperone systems that prevent non-native interactions (e.g.,

aggregation), very little is known about nucleotide-dependent septin folding in vivo and roles for chaperones therein. In budding yeast, where septin filament formation is essential, mutations that abrogate septin nucleotide binding render cells temperature-sensitive, and destabilize oligomeric assemblies. At permissive temperatures such mutants display a striking defect in their ability to incorporate into higher-order structures when expressed *de novo* in a wild-type cell. Here, we show that this "competition" between wild-type and nucleotide-binding-mutant septins is mediated by two co-chaperones, prefoldin/GimC and Ydj1, a DnaJ-family Hsp40. As was known to be the case for multiple artificially-misfolded proteins, nascent Cdc10 polypeptides are apparently subject to Ydj1-dependent quality-control destruction. However, a large pool of each nucleotide-binding-defective septin that cannot "compete" with the wild-type is immune to degradation, and remains diffusely localized throughout the cell. Intriguingly, the CCT/TRiC chaperonin – which was known to bind septins and to help fold actin and tubulin – maintains the solubility of these misfolded septins, because they aggregate upon its inhibition. Our findings have important implications for the understanding of septin aggregation in human pathologies, including Parkinson's and Alzheimer's diseases.

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Chaperonin-Assisted Protein Folding at Low Temperature: Co-Evolution of Antarctic Fish CCT and Its Substrates.

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Marine ectotherms of the cold Southern Ocean (-2 to +1.5°C) surrounding Antarctica face energetic challenges to protein folding assisted by the chaperonin CCT. We hypothesize that CCT and its client proteins (CPs) from these organisms have co-evolved compensatory molecular alterations that facilitate CP-CCT binding and the ATP-driven folding cycle at low temperature. To evaluate this hypothesis, we compared CP/CCT systems purified from testis tissues of an Antarctic fish, *Gobionotothen gibberifrons*, and of the cow, *Bos taurus* (body T = 37°C). Purification of folding-competent *G. gibberifrons* CCT required substantial modifications to protocols used to isolate testis CCT from mammals. After establishing the fish CCT protocol, we tested the binding of denatured CPs (β-actin, β-tubulin) by the CCTs, both in homologous and in heterologous combinations and at temperatures of 4 and 20°C. Samples were processed for negative-stain EM at the incubation temperature, micrographs were recorded and digitized, and 1,000-2,000 particles from each reaction were scored as apo- or holo-CCT using maximum-likelihood classification procedures. In homologous combination, *G. gibberifrons* CCT possessed a higher affinity for β-actin or β-tubulin at 4 °C compared to 20 °C, whereas the converse was true for bovine CCT and its actin/tubulin CPs. When tested in heterologous combination, the binding affinities of the CCTs for the CPs were low at both Ts. The temperature dependence of the binding affinities of homologous CCT/CP pairs was analyzed in triplicate experiments at four Ts between -4 and +20°C. Irrespective of CP, the percentage of client bound by *G. gibberifrons* CCT declined linearly with increasing T, whereas the % of client bound by bovine CCT increased linearly with increasing T. The kinetics of the folding and release of native actin by *G. gibberifrons* CCT at 2°C were slow, reaching a plateau at 48 h. Together, our results suggest that CCT and CPs from Antarctic fishes have evolved interaction surfaces that place a greater reliance on polar and electrostatic bonds, which increase in strength as T decreases, at the expense of hydrophobic interactions, which weaken with decreasing T. Supported by NSF grants ANT-0635470 and ANT-0944517 (HWD) and by

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Chaperones in the Early-Diverged Eukaryote *Porphyra* (Rhodophyta): Revealing Hsp70 and Hsp40 Activities in the Cytoplasm of Thallus Cells under Control and Stress Conditions.

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Heat shock proteins are intensively studied in a number of organisms, but still very little is known about these stress proteins in early-diverged free-living photosynthetic eukaryotes, particularly in Rhodophyta. In algae, most attention is traditionally paid to sequencing genes that encode Hsp70 from different cell compartments, mainly chloroplast Hsp70, which is related to the cyanobacterial dnaK gene. Though cytoplasmic Hsp70 bears the main responsibility for heat shock response in the cell, there are no data on correlation between cytoplasmic hsp70 gene expression and the level of Hsp70 content in the cells of multicellular red algae. In this study, we investigated stress proteins of the thallus cells of *Porphyra* from the Kattegat area of the Baltic Sea (10 °C, salinity 28 psu) which were cultivated under control and stress conditions by Western blotting and ECL method using anti-Hsp70 antibodies. We revealed a faint band representing a 70 kDa protein and additionally a band with 40 kDa polypeptide. After heat shock at 28 °C during 1 hr we observed significant decrease of Hsp70 while Hsp40 was induced. The content of Hsp40 was 1.5 times higher in 4 hrs, and 10 times higher in 24 hrs after heat shock, if compare with control samples. To check the applicability of Remane's species-minimum concept to red algae, the experiments have been designed to study chaperone activities in *Porphyra* cells in the salinity gradient and compare the effects of different stresses (salinity and temperature) on Hsp expression in these early-diverged eukaryotes. Supported by IB/BMBF (RUS-09/038) and RFBR (10-04-00943).

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S. cerevisiae genome-wide analysis identifies cellular processes affecting aggregation of Alzheimer's amyloid beta 42-EGFP fusion reporter.

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Alzheimer's disease (AD) is the leading cause of dementia in individuals over 65 years of age; and the risk of developing Alzheimer's disease increases steadily with age. Amyloid-beta (Aβ) plaques are a major neuropathological feature of AD. These plaques are primarily composed of aggregates of Aβ peptides generated via the amyloidogenic processing of the amyloid precursor protein. The two major isoforms of Aβ peptide are Aβ40 and Aβ42, of which the latter is highly prone to aggregation and shown to support a central role in AD disease progression. The increased presence and aggregation of intracellular Aβ42 peptides has been shown to be an early event in the disease progression of AD. Improved understanding of cellular processes involved in Aβ42 aggregation may have implications for our understanding of AD disease progression and possible development of therapeutic strategies.

In this study, Aβ42 fused to GFP (Aβ42GFP) was expressed in each mutant of the Saccharomyces cerevisiae genome-wide deletion library, to identify on a genome-wide scale the proteins and cellular processes that affect intracellular Aβ42 aggregation by assessing the fluorescence associated with the Aβ42GFP reporter. The genome-wide screening identified 98 mutants exhibiting intense Aβ42GFP-associated fluorescence. These deletion mutants were categorised according to broad biological functional groups/cellular processes based on the established or putative cellular roles of each of the respective 98 gene products. This approach identified four major cellular processes that were over-represented in the data set, of which mutants that were categorised into the lipid homeostasis functional group is discussed. In addition to the identification of cellular processes, distinct localisation of Aβ42GFP in the these lipid mutants were observed.
To identify the specific lipid macromolecule(s) that may potentially interact with amyloid-beta and hence abate its aggregation, a shotgun lipidomics approach was undertaken, to allow quantitative measurements of all cellular lipids. These data provide the first genome-wide evidence of cellular processes and macro-molecules that affect intracellular Aβ42GFP aggregation and may have important implications for our understanding of cellular mechanisms that affect intracellular Aβ42 aggregation and ultimately disease progression of AD.

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Expression of clusterin decreases with aging and oxidative stress in human normal epidermal keratinocytes and dermal fibroblasts and is stimulated by phospholipids.

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Clusterin is a glycoprotein widely distributed in mammalian tissues and biological fluids and is implicated in a variety of physiological and pathological processes. Mature form of clusterine is secreted in extracellular spaces as an heterodimeric glycoprotein of 80 kDa. It acts as a molecular chaperone to stabilize proteins in a folding competent state and has a role in scavenging extracellular misfolded proteins produced following stress-induced injury. It has also a role in the clearance of cellular debris and phagocytosis promotion. Clusterin is associated with altered elastic fibers in aged human skin, and inhibits UV-induced aggregation of elastin. In epithelial cells, clusterin protects from exogenous H_2O_2 stress and over expression protects human skin fibroblasts against premature senescence
Objectives: to examine a possible alteration of clusterin expression on human skin cells in vitro during ageing and after oxidative stress.
Methods: Normal human dermal fibroblasts (NHDF) were obtained from donors from 25, 40 and 69 yo and epidermal keratinocytes (NHEK) from donors (n=18) from 49 to 66 years old. Expression of clusterin was followed by western blot and RT-QPCR with and without 20 min treatment of cells with H202 (100 to 500µM) or phospholipids vesicles are composed of a mixture of phosphatidylcholine and phosphatidylserine 70% and 30% respectively in weight
Results: NHDF and NHEK highly express clusterin at the mRNA and protein levels. This expression significantly decreases in NHDF from 25 year old (yo) to 40 yo (1.5 times less) and 69 yo (2,5 times less) donors. More over fibroblasts from the 69 yo donor are more sensitive to H_2O_2 particularly at the lowest 100µM dose. Expression of clusterin mRNA is also significantly decreased in NHEK from 49-66 yo donors (-54%) compared to cells from the younger group. Clusterin expression is also reduced after H202 exposure with a maximal effect observed at 200 µM. Vesicles of phospholipids are able to stimulate at 600µmol/L the expression of clusterin mRNA (2 times at 24h) and the extracellular protein secretion (1.8 times at 72h) in NHEK. More over a 24h preventive treatment of NHEK with these phospholipids, protects clusterin expression from H_2O_2 exposure.

Conclusion: These results demonstrate that aging and oxidative stress decrease clusterin expression in dermal and epidermal skin cells and that phospholipids could be used to stimulate its expression, and to protect it from oxidative stress.

Regulation of Aging

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Susceptibility of epidermal stem cells to 4-HNE stress: Oxidized protein measurement and aging studies.

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Accumulation of oxidized proteins is a hallmark of cellular ageing and is believed to be one of the main contributors to the aged phenotype. This process is dependent on an increased occurrence of oxidative damage to proteins and also on a decreased elimination of oxidative modified protein.
Stem cells have an extraordinary ability to multiply and consequently allow cell renewal and help maintain the quality of the skin. When no stress occurs, they remain dormant in the skin but multiply several times in case of damage. Subsequently, the cells differentiate, lose their ability to divide and migrate towards the surface of the skin, where they are eliminated via exfoliation.
Objective
The aim of the study is to set up a method of visualization and quantification of oxidized proteins. It has been done on young (mean 22 years old) and aged (mean 55 years old) Normal Human Keratinocytes (NHK) in culture. Furthermore, this technique has been performed on Colony Forming Unit in order to better characterized stem cells from skin epidermis. Oxidized proteins have been quantified with 4-HNE induction, a lipidic peroxidation product which can crosslink with proteins. We tried to counteract the effect of 4-HNE with a complex of anti-oxidants compounds.
Methods
The Oxidized proteins staining was performed with a monoclonal mouse anti-DNP antibody, and alexafluor 546 Goat anti-mouse secondary antibody. This staining has been performed on NHK and CFU cultivated on irradiated fibroblasts feeder-layer.
The OE complex is composed of 0,1% Phaeodactylum tricornutum extract + 0,1% Aframomum angustifolium extract and 0,002% dimethyl methoxychromanol. 4-HNE is used at 10 µM.
Results
In comparison with young donor keratinocytes, aged donors cells showed a very significant increase (+858 %) of oxidized proteins content. We can see a significant increase in the amount of oxidized proteins induced by HNE (+179 %) compared to the untreated control. The OE complex is able to significantly reduce the amount of oxidized proteins produced during HNE stress (-55.8 %). OE complex showed a significant decrease (-61,8 %) of oxidized proteins on CFU from epidermal stem cells.
We can see a 61% decrease of big colonies (from stem cells) with 4-HNE stress. A treatment with the complex counteracts the effect of HNE stress with 88 % increase of stem cells number. We observed that CFU from old keratinocytes donors are more sensitive to HNE stress compared to young donors cells (-69,1% for old cells and –35% for young cells). OE complex applied with HNE increased the number of big clones, and this particularly true for old cells: +302% compared to +84,7% for young cells.

Conclusions

We observed that epidermal stem cells from old donors are more sensitive to HNE stress compared to young cells maybe because old NHK (which contains fewer stem cells than young donor cells) have already a higher level of oxidized proteins. This damage can be significantly reduced by a unique anti-oxydant complex which protects old donor stem cells from HNE stress.

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SIRT2 is downregulated in insulin-resistant HepG2 cells: a possible role of oxidative stress.

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Sirtuin 2 (SIRT2), a member of class III histone deacetylases, has been reported to protect cells against oxidative and other types of stress. Moreover, increasing evidence suggests the existence of a link among increases in oxidative stress, activation of stress-sensitive pathways, and the development of insulin resistance. We therefore hypothesized that the antioxidant effect of SIRT2 could lead to improved insulin sensitivity. In this study, we established an in vitro model of insulin resistance by treatment of the human hepatocarcinoma cell line HepG2 with glucosamine (GlcN). We found that in GlcN-treated cells insulin-stimulated glycogen synthesis was impaired and this was associated with an elevation in H_2O_2 production. Moreover, insulin-resistant HepG2 cells showed an increase in basal ERK1/2 phosphorylation and failed to respond to acute insulin stimulation. We further demonstrated that GlcN induced SIRT2 downregulation in a time-dependent manner. The antioxidant N-acetylcysteine decreased H_2O_2 levels and partially improved insulin sensitivity, suggesting that insulin resistance was mediated to a significant extent via increased oxidative stress. Taken together, our results indicate that hepatic insulin resistance is associated with reduced expression of SIRT2. Further studies will address whether the modulation of SIRT2 levels and/or activity is able to improve insulin sensitivity through a ROS-mediated mechanism.

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Transcriptional profiling of hypoxia and/or UVB modulated genes in human epidermal keratinocytes reveals novel targets for reducing skin aging-induced effects.

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There is increasing evidence for hypoxic conditions (1.5 to 5% oxygen) in human epidermis. Because of the skin changes associated with skin aging, in particular with chronic photodamage, hypoxia appears to increase with aging of the skin. However, the effects of chronic hypoxia on skin growth and function, and on the UVB-mediated skin damage, are currently unknown.

The major goals of this project were to characterize the effects of chronic mild hypoxia, alone or in combination with UVB-induced skin damage, on the transcriptional profile and the functional behaviour of human epidermal keratinocytes in vitro.

HaCaT immortalized human epidermal keratinoctes were cultured under normal or hypoxic conditions (≈ 2% O2) for 24 hours or for 7 days and/or were irradiated with UVB light (≈ 20 mJ/cm2). Total RNA was extracted, and gene microarray profiling of the transcriptional profiles of the treated cells was performed in triplicates, using the Applied Biosystems gene array platform, followed by qRT-PCR confirmation of lead candidates.

We found that hypoxia and UVB irradiation induced distinct, partially overlapping gene expression patterns of human epidermal keratinocytes. Typical hypoxia-response pathways were activated by hypoxia, including glycolysis and HIF activation pathways, and typical hypoxia-response genes such as lysyl oxidase-like 2 and angiopoietin-like 4 were upregulated. After UVB irradiation, several genes including placental growth factor and filaggrin were induced or reduced. The modulation of candidate genes was confirmed by qRT-PCR. Current studies investigate the functional effects of these genes on the growth, differentiation and colony forming ability of normal human epidermal keratinocytes. Overall, we have identified a number of potential targets to modify the skin response to hypoxia and UVB damage.

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Slimming potential of a salicornia herbacea extract via its effect on adiponectin protection against oxidative stress.

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OBJECTIVE: Ageing alters mitochondrial functions and biogenesis, increases the production of free radicals by mitochondria with dramatic consequences for epidermal and dermal cells but also for hypodermal cells (mainly adipocytes). The problem concerning ageing of adipocytes is how to protect them against oxidative stress in order to restore the lipolytic functions. Ageing alters the production of adipose-specific cytokines (adipokines) involved in the control of lipid synthesis, and in particular adiponectin. The aim of this work was to present a new potential ingredient to fight against adipocyte ageing and some of its effects, the decrease of adiponectin synthesis, and the decrease of lipolysis by human adipocytes.
METHODS: The cells used were adipocytes isolated from human adipose tissue or adipocytes obtained after differentiation of human adult mesenchymal stem cells. These cells were exposed to oxidative stress induced by UV rays (UV) or by glucose oxidase (GO). The effects of these treatment on lipolysis activity were evaluated by the measure of non esterified fatty acids (NEFA) released by adipocytes treated or not with the classical lipolytic agent caffeine (10mM). Adiponectin level was measured by ELISA method. An aqueous extract of the halophyte plant *Salicornia herbacea* was evaluated on fat cells.
RESULTS: Treatments with UVB (50 mJ/cm²) reduced the lipolytic response of isolated adipocytes to caffeine (increase of NEFA by +53% with UV versus +166% without UV) and reduced by 20% the adiponectin production by adipocytes. Treatment with GO (2 mU/ml) also limited caffeine –induced lipolysis and reduced by 50% the adiponectin production.
The extract of *Salicornia herbacea* has a high antioxidant activity evaluated by ORAC test (659 ± 14 mg ET/L ; ET = Equivalent Trolox). It protected adipocytes against reduction of the lipolytic response induced by UVB (50 mJ/cm²) or by GO (2 mU/ml). The adiponectin level was also protected against GO-induced stress: protection by 50% and 92% with *Salicornia herbacea* extract tested at 0.2% and 0.4%, respectively.
CONCLUSION: By protecting adiponectin against its degradation by free radicals, the anti-oxidant plant extract protects adipocyte metabolism.

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Role of Notch signaling in stem cell behavior in dystrophic muscle.

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Duchenne muscular dystrophy (DMD) is a progressive disease that features chronic inflammation and continuous myofiber regeneration. The dystrophin gene was found to be defective in DMD patients; however, it has always been a puzzle why mdx mice, a model of

DMD being defective in the dystrophin gene, only develops very mild skeletal muscle defects and exhibits a very potent muscle regenerative capacity. It would therefore be of interest to find out the potential molecular mechanism that determines the differential behavior of muscle stem cells derived from the skeletal muscle of mdx mice and DMD patients. Notch signaling has been indicated to play an important role in the development and regeneration of skeletal muscle, and it can maintain muscle stem cells in a quiescent or undifferentiated state, which suggests a role for Notch signaling in reserving the stem cell pool in skeletal muscle. Moreover, Notch signaling was shown to potently repress myogenesis by affecting the function of the myogenic factor MyoD. However, the investigation of the role of Notch signaling in dystrophic muscle is lacking. Interestingly, it was recently reported that TNF-α can actually inhibit Notch1 activation in *in vitro* cultured muscle cells of mouse, and a potential similar mechanism in dystrophic muscle was suggested. Our current study was conducted to determine whether Notch signaling could be a critical contributor to the differential stem cell behavior and muscle regeneration potential of mdx and DMD muscle. Using semi-quantitative PCR, our results showed that, the expression of Notch signaling-related genes (*Notch1, 3, Stat3* and *Hes1*) were all greatly down-regulated in muscle cells from the dystrophic muscle of mdx mice, compared to those from wild type normal mice; however, Notch signaling in the muscle cells of DMD patients was found to be strongly activated. Meanwhile, the inactivation of Notch signaling in muscle cells isolated from DMD patients, using a Notch inhibitor, reduced the expression of inflammation marker genes and improved the myogenesis potential of the DMD cells. Our results indicate that the Notch signaling pathway could be the potential molecular switch which determines the differential behavior observed between muscle stem cells and the muscle phenotype of mdx verses DMD muscle. Therefore, a beneficial effect of muscle regeneration could be expected by the inactivation of Notch signaling in the dystrophic muscle of DMD patients, and the development of a potential new therapeutic regime for treatment of the disease.

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KLOTHO Regulates Retinal Pigment Epithelia functions through TRPV5 Ca2+ channels; Implication in Age-Related Macular Degeneration.

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Purpose: Age-related macular degeneration (AMD) is the leading cause of blindness in people over 55-years old in developed countries. AMD starts with the deposit of drusen between Retinal Pigment Epithelia (RPE) and Bruch's membrane and progresses with the death of RPE, photoreceptor degeneration, and eventually loss of central vision. RPE comprise a monolayer of pigmented cells and play many crucial roles in the retina. Recent discovery of KLOTHO (KL) gene that encodes for a transmembrane protein secreted primarily by the kidney, known for its senescence-suppression function in the mouse, has opened new avenues for studying of the age-related diseases. In this study we investigate the role of KL in the regulation of RPE functions that could be directly implicated in retinal degeneration and AMD.

Methods: Real-time PCR, Western blot, Ca2+ imaging, immunostaining, EIISA, knockdown assays.

Results: Here we show for the first time that human RPE express KL, and KL expression is significantly reduced in the eye during aging and due to oxidative stress. Using Ca2+ imaging we demonstrated that KL protein regulates the activity of Transient Receptor Potential cation channel subfamily V member 5 (TRPV5) in human RPE and increases the Ca2+ entry across

the apical membrane. We further demonstrate that reduction of KL expression by oxidative stress can increase the vascular endothelial growth factor (VEGF) expression in RPE, that is known to be implicated in neovascularization inducing visual loss in AMD.

Conclusion: Since Ca+2 is involved in many RPE functions (i.e., phagocytosis, VEGF secretion, light absorption, migration), its tight regulation is crucial for RPE functions and survival. Our results show for the first time that KL regulates RPE functions by increasing Ca+2 entry through TRPV5 channels, therefore, a decrease in KL expression during aging might be directly implicated in AMD. This study if completed can open new avenues for understanding the mechanisms of AMD and development of new treatments for the disease.

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Examining the Role of TPP-I in the Degradation of Fibrillar Aβ by Microglia.

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The aggregated fibrils of β-amyloid (fAβ) associated with Alzheimer's disease are not completely degraded by microglia. These monocyte derived phagocytic cells of the CNS internalize fAβ and deliver it to the lysosomes, but the lysosomal pH of microglia is higher than that of macrophages and degradation of fAβ is incomplete. The lysosomal enzymes of microglia that we have examined are identical to those present in macrophages, but the activity of these enzymes is decreased by the higher pH. We hypothesize that the enzyme tripeptidyl-peptidase I (TPP1), which has a low optimal pH and has been shown to have an endopeptidase activity, is the key enzyme for initiating degradation of fAβ to allow further degradation by other lysosomal enzymes. This enzyme is present in all of the cell types that we have examined that degrade fAβ. We have previously shown that microglia treated with cytokines such as interleukin-6 (IL-6), lipopolysaccharide (LPS), or macrophage colony stimulating factor (MCSF) degrade more fAβ than untreated microglia and have a lower lysosomal pH. We have found that the level of TPP1 is increased in microglia treated with MCSF. We examined the role of TPP1 in the degradation of fAβ through enzyme addback experiments with microglia, chemical inhibitor additions to macrophages, and RNAi knockdown of the enzyme in osteosarcoma cells expressing Scavenger Receptor A (SRA), another cell line able to degrade fAβ. We are currently examining fAβ degradation by TPP1 knockout microglia treated with MCSF and wildtype and TPP1 knockout murine bone marrow macrophages. We have also begun to examine the levels of plaque deposition in an Alzheimer's disease mouse model that has limiting levels of the TPP1 enzyme. Our results to date show that although the lysosomal pH in microglia is more alkaline than that of macrophages, addition of either pooled lysosomal enzymes or purified TPP1 to microglia increases the degradation of fAβ by these cells. Without TPP1, MCSF-treated microglia do not degrade fAβ as well as wildtype MCSF treated cells. The addition of specific TPP1 inhibitors to macrophage and osteosarcoma cells, as well as RNAi knockdown of TPP1, blocks fAβ degradation. These results indicate that TPP1 plays an important initial role in the degradation of fAβ by microglia.

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Molecular Mechanisms distinguishing Reproductive Aging from Somatic Aging.

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Female reproductive cessation is the earliest aging phenotype humans experience, occurring midway through life, and is governed by declining oocyte quality. We have shown previously

that C. elegans reproduction, which also persists for only half the worms' lifetime, is similarly limited by oocyte quality decline (Luo, et al. Cell 2010). Moreover, the transcriptional changes that worm oocytes undergo with age are similar to those in aging mammalian oocytes, and these changes are reversed in mutants with extended reproductive spans.
Both the TGF-b Sma/Mab and Insulin/IGF-1 signaling pathways regulate reproductive aging (Luo, et al. PLOS Genetics 2009), but IIS extends life span, as well (Kenyon, et al. 1999). We had previously identified the downstream DAF-16 targets of IIS that are responsible for long life span (Murphy, et al. Nature 2003). We wondered to what degree the mechanisms that extend reproductive span and life span are shared. In fact, very few of the terms overlap, suggesting that post-mitotic, non-proliferating tissues utilize one specific set of genes, while mitotically proliferating and cell-cycle-arrested cells utilize completely different mechanisms to keep cells youthful and functioning.

Establishment and Maintenance of Polarity II

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CPEB-mediated ZO-1 mRNA Localization Stimulates Mammary Epithelial Tight Junction Assembly and Cell Polarity.

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CPEB is a sequence-specific RNA binding protein that regulates cytoplasmic polyadenylation-induced translation; in so doing, it controls several biological processes including vertebrate germ cell development. CPEB knockout female mice do not produce mature oocytes and thus are sterile; their vestigial ovaries do not secrete sufficient levels of hormones to promote mammary gland development. CPEB heterozygous mice, however, are fertile but their mammary epithelial cells are disorganized and ZO-1 and claudin-3, apical tight junction proteins, are mis-localized. CPEB depletion from cultured mammary epithelial cells disrupts ZO-1 apical localization and tight junction distribution; conversely, ectopic expression of CPEB enhances apical ZO-1 localization. CPEB and ZO-1 mRNA are co-localized apically and ZO-1 3′UTR binding sites for CPEB promote the RNA localization. In a 3-dimensional culture system that models lumen-containing mammary ducts, depletion of CPEB or ZO-1 impairs central cavity formation, indicating a loss of tight junction formation and cell polarity. Cavity formation in ZO-1 depleted cells is substantially rescued when they are transduced with ZO-1 mRNA containing, but not lacking, the CPEB binding sites. These and other data indicate that CPEB promotes tight junction assembly and mammary epithelial cell polarity, and that one mRNA important for this process encodes ZO-1.

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Two B-type cyclins regulate anterior PAR protein localization in the early *C. elegans* embryo.

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We are using *C. elegans* as a model to study asymmetric cell division, an essential process to generate cell diversity during development. Establishment and maintenance of polarity are fundamental processes for a normal asymmetric division in all eukaryotes.
In the *C. elegans* embryo, the establishment of an anterior-posterior axis of polarity takes place after fertilization and depends on the asymmetric localization of the conserved PAR proteins in

two mutually exclusive groups: the anterior complex, with PAR-3, PAR-6, PKC-3 and the posterior group with PAR-2 and PAR-1. Depletion of any PAR protein causes a loss of polarity and embryonic lethality. A genome-wide RNAi screen identified two B-type cyclins, *cyb-2.1* and *cyb-2.2*, (together referred to as *cyb-2*) as suppressors of *par-2(it5ts)* lethality. The goal of this project is to determine the role of these cyclins in cell polarity.
Using double and triple mutants for *par-2* and each cyclin, we showed that the loss of *cyb-2.1* or *cyb-2.2* suppressed lethality and polarity defects in *par-2(it5ts)* mutants and that loss of both cyclins promoted the anterior displacement of the PAR-6 cortical domain. Suppression of *par-2(RNAi)* lethality was also observed in a thermosensitive mutant for the cyclin-dependant kinase *cdk-1*, suggesting that CYB-2 acts with its CDK-1 partner in this pathway. *cyb-2* mutants did not show defects in cell cycle progression, timing of polarity establishment or velocity of polarizing myosin flows, indicating that CYB-2 acts in polarity independently of these processes. Interestingly, western blot analyses revealed that PAR-6 levels are decreased in *cyb-2* mutants. Another suppressor of *par-2* lethality, *nos-3*, was previously shown to regulate PAR-6 levels through the activity of a CUL-2-based E3 ubiquitin ligase complex. Using epistatic analysis, we found that CYB-2 requires CUL-2 to suppress *par-2*(RNAi) lethality, suggesting that it also functions through this E3 ubiquitin ligase. Furthermore, mutations in *cyb-2* and *nos-3* were found to be additive, suggesting that these genes act independently on CUL-2 to regulate PAR-6 levels.
These results support a model in which a CYB-2/CDK-1 complex acts on CUL-2 to modulate PAR-6 levels independently of cell cycle progression during *C. elegans* embryonic polarization and suggest that the control of PAR-6 levels is a regulated step important for cell polarity. We are currently using biochemical and proteomic approaches to further define the molecular mechanism by which CYB-2/CDK-1 acts in this pathway.

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PAR-3 oligomerization gives rise to a bistable switch mechanism that may help maintain Par protein domains in the early *C. elegans* embryo.

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Shortly after fertilization, embryos of the nematode worm *Caenorhabditis elegans* polarize by segregating Par proteins to spatially distinct domains at the anterior and posterior poles. Recent experimental work demonstrated that during the maintenance phase, these domains are held with minimal drift in the position of the boundary between the domains, even in the absence of cortical actomyosin (Goehring et al, 2011). In this talk, we will discuss a mathematical model of Par protein interactions that incorporates mutual cross-inhibition of Par proteins and PAR-3 oligomerization. This model exhibits bistability and is able to maintain distinct Par protein domains independently of asymmetries or dynamics of cortical actomyosin. The model dynamics predict a sudden loss of polarization as the anterior Par protein PAR-6 is depleted, and we confirm the prediction experimentally using RNA interference.

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Crumbs prevents photoreceptor cell death by repressing Rac1/NADPH oxidase-dependent superoxide production.

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Drosophila Crumbs is crucial for establishment and maintenance of epithelial cell polarity. In addition to its role in apicobasal polarity, Crumbs is essential to prevent light-induced retinal degeneration. Similarly, mutations in CRB1, which encodes a human ortholog of Crumbs, cause

retinal dystrophies in humans. Recent findings have highlighted that Crumbs controls signaling pathways regulating growth and apoptosis in epithelial tissues. However, the molecular mechanisms by which Crumbs maintains photoreceptor cell viability remain largely unknown.

We have previously shown that Crumbs promotes epithelial tissue integrity by inhibiting Rac1 and PI3K in Drosophila embryos. Here we show that this function is conserved in photoreceptor cells, as loss of Crumbs increased active GTP-bound Rac1 levels and PI3K activity in retinas. This was associated with an increase in NADPH oxidase activity, which produces superoxide in a Rac1-dependent manner. Strikingly, inhibition of Rac1 and NADPH oxidase as well as antioxidant rescued the crumbs mutant phenotype. Similarly, knockdown of Rac1 and NOX (catalytic subunit of NADPH oxidase) prevented photoreceptor cell death.

Together, our data show that Crumbs represses Rac1/NADPH oxidase-dependent superoxide production, thereby preventing photoreceptor cell death. As Crumbs functions are evolutionarily conserved, it is likely that human CRB1 maintains retinal integrity through a similar mechanism.

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Identification of Par1b substrates required for regulating epithelial polarity.

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Simple epithelial cells share the same basic morphological structure. They have distinct luminal (apical) surfaces separated by tight junctions from the intercellular and blood barrier-interacting (baso-lateral) surface. The maintenance of these highly polarized cells is crucial for proper functioning of tissues such as kidney tubules, liver, and gastric epithelia. Par1b is a serine/threonine kinase which is well known for its role in maintaining polarity of epithelial cells. In MDCK cells, knockdown of Par1b causes cells to lose their apico-basal polarity. Conversely, overexpression promotes the establishment of a hepatic-type polarity, where the apical surface forms intercellularly. How Par1b acts as a molecular switch to regulate polarity is unknown. However, overexpression of a kinase-dead Par1b does not lead to the development of hepatic-type polarity, suggesting that the polarity regulation is controlled by Par1b's kinase activity. Par1b has few known substrates, none of which can single-handedly account for maintaining polarity. It is the goal of this project to 1) identify novel substrates of Par1b and 2) characterize those substrates for their role in the establishment and maintenance of epithelial cell polarity.

The identification strategy is based on a chemical genetics approach in which the ATP-binding pocket of a kinase is altered to accommodate bulky ATP-analogues that when offered as phosphate donor in complex mixtures can only be utilized by the engineered kinase to selectively phosphorylate its substrates. The ATP-analogues contain a chemical affinity tag that is transferred onto the substrates and allows their isolation along with the identification of the phosphorylation sites by LC-MS/MS analysis. We have generated an analogue-sensitive Par1b (Par1b-ATP*), and utilizing a radioactive ATP analogue, have successfully labeled substrates from Par1b immuno-precipitated complexes, Tx100 soluble cell lysates, and whole digitonin permeabilized cells. From the immuno-isolated Par1b complexes, we have identified 4 known substrates and 76 putative substrates. Thus far we have validated 10 of the candidates as *in vivo* substrates. We will expand our candidate list by identifying and validating novel substrates from MDCK cell lysates and digitonin permeabilized cells. Furthermore, we will examine how Par1b regulation of the substrate regulates epithelial polarity.

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RAB25 regulates polarity in intestinal epithelial cells.

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Objective: Epithelial cells establish apical and basolateral membrane domains during polarization. This conserved function of polarized epithelial cells requires efficient and organized vesicular trafficking to the correct apical or basolateral membrane domains. Rab25 is an epithelial-specific small GTPase. Rab25 regulates transcytosis in MDCK cells and recent studies indicate that it acts as a tumor suppressor in the intestine and colon. Here we have investigated Rab25's role during polarization of the intestinal epithelial cells using CaCo2–BBE cells. These cells form polarized monolayers in culture when grown on permeable supports. **Methods**: The CaCo2-BBE cells either stably expressing a scrambled shRNA or shRNA directed against Rab25 were plated on transwell filters for 3, 8 and 15 days, the cells were analyzed for apical and junctional proteins by western blotting, qPCR and immunofluorescence. The apical membrane was analyzed by scanning EM. **Results:** Rab25, but not Rab11a, expression increased from 1.5 to 2.5 fold at the both in protein and mRNA level as the cells polarized. Knockdown of Rab25 expression altered the expression both at the transcriptional and translational level of the microvillar proteins such as villin-1 and sucrase isomaltase (SI). Rab25 loss also caused improper assembly of microvilli/brush-border in the apical domain. Interestingly, knockdown of Rab25 caused a loss of β1, α1 and α5 integrins and altered expression of the tight junctional proteins like claudin-1,2,3,4 and 7. These tight junctional alterations decreased the transepithelial resistance in Rab25knockdown cells. The anchorage independent growth assay showed 2-fold increase in the number of colony formed and increased in diameter of the colony in the Rab25 knockdown cells when compared to control. All these changes were restored when knock-down cells were rescued with expression of rabbit Rab25. **Conclusions:** Taken together, our studies demonstrate that Rab25 is important for the proper assembly of microvilli/ brush-border in polarized intestinal epithelial cells.

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Phosphorylation of Rab11-FIP2 regulates polarity in MDCK cells.

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The Rab11 effector, Rab11-FIP2 regulates transcytosis through its interactions with Rab11a and Myosin Vb. Previous studies have implicated Rab11-FIP2 in the establishment of polarity in MDCK cells through phosphorylation of serine 227 by MARK2. Here we have examined the dynamic role of Rab11-FIP2 phosphorylation on MDCK cell polarity. Endogenous Rab11-FIP2 phosphorylated on serine 227 coalesced on vesicular plaques during the re-establishment of polarity following either monolayer wounding or calcium switch. While expression of the non-phosphorylatable Rab11-FIP2(S227A) elicited a loss in lumen formation in MDCK cell cysts grown in matrigel, the putative pseudophosphorylated Rab11-FIP2(S227E) mutant induced the formation of cysts with multiple lumens. On permeable filters, Rab11-FIP2(S227E)-expressing cells exhibited alterations in the compositions of both the adherens and tight junctions. At the

adherens junction, p120 catenin and K-cadherin were retained, while the majority of the E-cadherin was lost. While ZO-1 was retained at the tight junction, occludin was lost and the claudin composition was altered. Interestingly, the effects of Rab11-FIP2 on cellular polarity did not involve Myosin Vb or Rab11a. These results indicate that serine 227 phosphorylation of Rab11-FIP2 regulates the composition of both adherens and tight junctions and is intimately involved in the regulation of polarity in epithelial cells.

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The Role of FIP5 and Kinesin-2 in the Regulation of Epithelial Morphogenesis.

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Epithelia outlining the organs possess multiple functions, including protection, secretion and selective absorption. To accomplish these functions, epithelia are structurally and functionally polarized. The plasma membrane (PM) of these polarized epithelial cells is divided into the apical and basolateral domains, each with distinct protein compositions. Appropriate protein transport via endocytic recycling is of great importance in establishing and maintaining epithelial polarity. The protein transport toward the apical membrane, apical recycling, has recently been shown to mediate lumen initiation and expansion during organogenesis. Recent work suggests that the formation, transport and docking (to PM) of the apical cargo-containing endocytic vesicles play a key role during the initiation of the apical lumen. However, the molecular mechanisms regulating apical lumen formation remain to be fully understood. In this study we demonstrated that the Rab11-binding protein, FIP5, mediates apical recycling via sequential binding of the Rab11/FIP5 complex to various endocytic transport proteins, including sorting nexin 18 (SNX18) and Kinesin-2. We further investigated the interactions between SNX18, Kinesin-2 and FIP5 by in vitro binding assays and have shown that SNX18 and Kinesin-2 forms mutually exclusive complexes with FIP5. We also used a combination of shRNA knockdown technique and 3D tissue culture assays to study how FIP5 and Kinesin-2 regulate lumen formation. We visualized the stepwise process of lumen initiation by live cell imaging. Thus, we propose that the Rab11/FIP5 complex acts as a scaffold for the sequential recruiting of the effector proteins to mediate the delivery of the apical proteins during the initiation of the apical lumen.

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Ankyrin-G Mediates a Conserved yet Versatile Pathway for Assembly of Specialized Membrane Domains in Epithelial Cells and Neurons.

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Epithelial lateral membranes and axon initial segments (AIS) represent physiologically important sites that host a number of structurally distinct proteins. It has long been hypothesized that a conserved core polarity pathway is shared between epithelial cells and neurons. While an epithelial-specific AP1B pathway has been identified, a common molecular mechanism remains elusive. Here we examine how ankyrin-G (AnkG) controls the polarized localization of E-cadherin and neurofascin to the epithelial lateral membrane and AIS, respectively. AnkG controls polarity in these very different cell types through binding to independently-evolved unstructured peptides in the cytoplasmic domains of its membrane partners. Interestingly, although the membrane-binding domain of AnkG is responsible for these interactions, there is a large range of binding affinities, with a direct high affinity binding to neurofascin and a tenfold lower binding affinity for E-cadherin. We show that the AnkG-binding-deficient mutants of E-cadherin and neurofascin are mislocalized to the apical membrane in epithelial cells, while mutant neurofascin is misdirected to both the axon and dendrite in hippocampal neurons. Stowe

and colleagues proposed that a dileucine motif within E-cadherin is responsible for interactions with the clathrin pathway. Interestingly, this dileucine motif is located in the putative AnkG binding site suggesting that epithelial cells have evolved a pathway by which these unstructured peptides sites can "moonlight" to further enhance polarity. Expression of the AnkG binding-deficient E-cadherin in clathrin shRNA MDCK cells demonstrates a worsened apical localization, showing that AnkG and clathrin cooperate in epithelial cells. In contrast, silencing of clathrin heavy chain in neurons has no effect on neurofascin localization to the AIS, demonstrating the epithelial specificity of the clathrin component of the polarity pathway. Finally, we show that the mechanism by which AnkG controls polarity is at least in part due to retention within the membrane and tethering to the spectrin-based membrane skeleton. This is based on increased E-cadherin mobility within the membrane upon expression of an inducible dominant-negative consisting of the AnkG binding site from beta 2 spectrin. These studies show that AnkG is the central component of a conserved yet versatile core pathway for the formation of specialized membrane domains in neurons and epithelial cells, and perhaps other polarized cells.

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Role of Uroplakins in Kidney Development.

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Mammalian Uroplakin3a (UPK3a) is a transmembrane protein expressed on the apical plasma membrane of umbrella cells lining the uroepithelium of kidney and bladder. Genetic defects in UPK3a cause altered kidney development and function, but the mechanism(s) involved remain elusive. To understand the role of UPK3a in these events, we analyzed its expression and function in the zebrafish kidney (pronephros), whose organogenesis mimics that of the early mammalian kidney. Earlier studies showed that the zebrafish genome encodes UPK3l, an ortholog of the mammalian UPK3a. We confirmed the expression of UPK3l mRNA by RT-PCR and western blotting. Further, *in situ* hybridization (ISH) showed its expression in pronephric tubules (PT) and ducts and Immunocytochemical analysis revealed its localization at the apical surface of PT epithelial cells. UPK3l-knockdown using an UPK3l-morpholino (MO) led to the development of pericardial edema in zebrafish embryos 2.5-day post fertilization (dpf), which was rescued upon expression of a MO-resistant variant. The morphants expressed Pax2.1, an early developmental marker, and formed tubules positive for the adherens junction-associated protein Cdh-17, indicating that early pronephros development is not obviously altered. Also, ISH analysis of relevant markers showed that glomerular development and PT segmentation is not altered significantly. However, morphants were unable to effectively clear injected dextran, indicating a defect in pronephric clearance. Moreover, morphants showed apical mislocalization of Na^+/K^+-ATPase suggestive of an altered polarity of the PT epithelial cells. Immunofluorecence and electron microscopic analysis also revealed an alteration in the structure of cilia and microvilli lining the PT epithelial cells in morphants. Our studies indicate that UPK3l is a key regulator of PT structural and functional integrity. Further, we identified that the cytosolic domain of UPK3l harbors the key functional residues suggesting that it may regulate signaling events crucial for its function.

1303

Mitochondrial fusion regulates hepatocyte polarization.

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We previously demonstrated that activation of AMPK, the cells' metabolic sensor which increases ATP production, is required for polarization of rat hepatocytes in collagen sandwich cultures (Fu D. et al, J Cell Sci 2010, PNAS 2011). Mitra et al, (PNAS, 2009) proposed that mitochondrial fusion enhances ATP production and metabolic efficiency. Therefore, we used similar cell biologic and immunologic methods to investigate the relation between mitochondrial fusion and function, and hepatocyte polarization.

Sequential live cells imaging of mitochondria (mitotracker green) and immunofluorescent staining of mitochondrial Tom20 revealed that day 1 hepatocytes were not polarized, and almost all mitochondria were small and not fused. Polarization and mitochondrial fusion progressively developed in parallel (days 2-6). By day 4-5, most mitochondria were fused and formed tubular structures. In Western blots sequentially performed during polarization, mitochondrial fusion proteins, Mfn1 and 2, selectively increased; fission-facilitating protein Drp-1 was not specifically activated, and structural proteins, Tom20, Hsp60 and Cox IV, were unchanged. Mitochondrial potential and ATP levels progressively increased (2 and 3 fold, respectively) during polarization. FCCP inhibited polarization, and decreased ATP production and mitochondrial potential by ~50%. Mitochondrial fission inhibitor, MDIVI1, did not affect mitochondrial morphology or hepatocyte polarization.

This study reveals that mitochondrial fusion is associated with AMPK activation, increased mitochondrial ATP production and membrane potential during hepatocyte polarization as manifested by bile canalicular network formation. Our results suggest that mitochondrial fusion may be an important determinant of energy production and polarization. Mitochondria may thus be a potential therapeutic target in inheritable and acquired diseases affecting hepatocyte polarization.

1304

Nematode Sperm Secrete Serpin to Coordinate both Sperm Motility and Sperm Competition.

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Sperm competition has been widely recognized as one of most potent driving forces in the evolution, studies on sperm competition mechanisms have been focused on some physical traits of sperm and on seminal fluid produced by several accessory glands in the male body. Completion of secretory events in sperm and subsequent release of sperm components are required for reproductive success in most animal species, however, whether these released components are involved in sperm competition remains unknown. Here we identified a Serpin family protein (As_SRP-1) that is secreted from spermatids during nematode Ascaris suum spermiogenesis (or sperm activation) and showed that As_SRP-1 coordinates both spermatozoon motility and sperm competition. First, As_SRP-1 functions in cis to support Major Sperm Protein (MSP)-based cytoskeletal assembly in the spermatid that releases it, thereby facilitating sperm motility and enhancing the competitiveness of the resulting spermatozoon. Second, As_SRP-1 released from an activated sperm inhibits, in trans, the activation of surrounding spermatids by blocking vas deferens secreted As_TRY-5, a trypsin-like serine protease necessary for sperm activation. These findings suggest that sperm play an active role

in sperm competition and that sperm activation and sperm competition are mechanistically connected in nematodes. Given the fact that the exocytosis of essential components in sperm vesicle(s) is necessary to create fertilization-competent sperm in many animals, sperm-secreted components might mediate sperm competition more widely among animals than was previously appreciated.

1305

Functional Genomics of Cell Regeneration in the Giant Ciliate *Stentor coeruleus*.

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The mechanisms that specify cell shape and organization are not currently understood. Ciliates provide ideal model systems to tackle problems of cell morphology due to their complex cell organization and unique patterning. With current advances in technology along with work done in *Paramecium tetraurelia* and *Tetrahymena thermophila*, it is now possible to apply these tools to studying other organisms. *Stentor coeruleus* is a large, ~1mm long, single cell with a highly patterned cell cortex and the ability to regenerate and reorganize after surgical or chemical manipulations. The ease of surgical manipulations gives *Stentor* significant advantages over other ciliate models. Using the surgical techniques unique to *Stentor* as well as modern RNA interference (RNAi) methods, visualization techniques, and genomic sequencing I will revive *Stentor* as a model for studying cell polarity and organization. With the current state of Next Generation Sequencing it has become feasible for a lab to sequence the genome of a Eukaryotic organism. We have begun our own sequencing effort for *Stentor*'s macronuclear genome in order to facilitate the development of a better experimental toolbox. We have been able to repeat many of the surgical experiments performed by Vance Tartar, Noël de Terra and others. Using data obtained from preliminary sequences I constructed RNAi vectors that target endogenous *Stentor* genes and here I provide evidence that methodology developed for other ciliates can function in *Stentor* as well. Results for RNAi of Alpha-Tubulin and Mob1 result in dramatic changes in cell polarity and organization of the cortex and provide strong evidence that studies in *Stentor* can yield exciting and useful results. Knocking down Alpha-Tubulin, a key structural component in the cortex, results in clear cortical defects and problems with cell regeneration. This is very different from the Mob1 knockdown, which results in the drastic elongation of cells and other cortical aberrations. Using RNAi in conjunction with the unique microsurgical methods available in *Stentor*, it should be possible to restore this classical system to its previous status as a central model for addressing many important questions, including centriole structure, cell polarity, biological pattern formation, and cellular regeneration.

1306

Dynamic compartmentalization of SCAR/WAVE signaling scaffolds and active pools of ARP2/3 during polarized growth.

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In plants, the epidermis is a biomechanical shell, the growth of which can affect the behavior of underlying cell layers and the overall architectural properties of the leaf. Understanding the genetic and molecular basis of its morphogenesis is an important problem in basic research and applied plant science. Arabidopsis pavement cell and trichome morphology mutants continue to identify new genes and biochemical pathways that affect the growth process; however, it is unclear how these complicated networks of proteins interact with the endomembrane and cytoskeletal systems to initiate and maintain polarized growth. The Rac/ROP small GTPase

exchange factor SPIKE1 and an actin based growth control system defined by the "distorted" mutants is a perfect example. Genetic and biochemical analyses provide a logic model for information flow from the formation of a Rac/ROP activation scaffold through a series of heteromeric protein complexes (WAVE/SCAR and ARP2/3) that generate an actin filament nucleation response. The cellular deployment of the pathway is unknown, but commonly believed to occur at specialized cortical domains. Contrary to this notion, our most recent results suggest that abundant subdomains of the ER termed ER exit sites (ERES) serve as a distributed network of SPK1-ROP activation sites. We describe recent live cell imaging, genetic, and biochemical analyses indicating that SPIKE1 signals diverge from ERES to control distinct trafficking and cytoskeletal activities. We will discuss how signal generation and cytoskeletal responses are compartmentalized and how this unexpected cellular deployment might regulate the cytoskeleton and morphogenesis at the spatial scale of a cell.

1307

Polarization and traffic of cell wall building enzymes in the filamentous fungus Neurospora crassa.

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Apical growth in filamentous fungi is supported by the constitutive exocytosis of secretory vesicles, which maintain the normal complement of plasma membrane proteins and lipids through "full fusion" and provide the necessary enzymes and building-blocks for cell wall synthesis. Our main interest is to identify the cargo of the different populations of vesicles presumably reaching the cell tip and to analyze their traffic during polarized growth in the filamentous fungus Neurospora crassa. Using chromosomal fluorescent tagging of candidate proteins, we found that vesicles containing cell-wall building enzymes accumulate temporarily in an apical body, the Spitzenkorper (Spk), in a stratified manner. Four out of the seven chitin synthases present in N. crassa, localized at the core of the Spk, in microvesicles. In contrast, a glucan synthase related protein localized in macrovesicles in the Spk periphery. These vesicles are distributed from the Spk outwards until reaching the plasma membrane, where they are tethered presumably by an exocyst-mediated process. The exocyst components SEC-8, Exo-70 and EXO-84 accumulated in N. crassa surrounding the frontal part of the Spk, whereas SEC-3, -5, -6, and -15 were found in a delimited region of the apical plasma membrane, which correlates with the zone of maximum exocytosis predicted earlier by the Vesicle Supply Center model for hyphal morphogenesis.

1308

Quantitative analysis of organelle distribution and dynamics in Physcomitrella patens protonemal cells: new insights in tip growth.

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In the last decade, the moss Physcomitrella patens has emerged as a powerful plant model system, amenable for genetic manipulations not possible in any other plant. This moss is particularly well suited for plant polarized cell growth studies, as in its protonemal phase, expansion is restricted to the tip of its cells. Based on pollen tube and root hair studies, it is well known that tip growth requires active secretion and high polarization of the cellular components. However such information is still missing in Physcomitrella patens. To gain insight into the mechanisms underlying the participation of organelle organization in tip growth, it is essential to determine the distribution and the dynamics of the organelles in moss cells. Here, we used fluorescent protein fusions to visualize and track Golgi stacks, mitochondria, and peroxisomes in

live protonemal cells. We also visualized and tracked chloroplasts based on chlorophyll auto-fluorescence. We showed that all four organelles are gradually distributed in protonemata from the tip of the apical cell to the base of the sub-apical cell. For example, the number of Golgi dictyosomes is 4.7 and 3.4 times higher at the tip than at the base in caulonemata and chloronemata respectively. While Golgi stacks are concentrated at the extreme tip of the caulonemal cells, chloroplasts and peroxisomes are totally excluded. However, such compartmentalization was not observed in chloronemata. Interestingly, caulonemal cells, which grow faster than chloronemal cells, also contain significantly more Golgi stacks and less chloroplasts than chloronemal cells. Moreover, the motility analysis revealed that organelles in protonemata move with low persistency and instantaneous velocities ranging from 29 to 75 nm/sec, which are at least three orders of magnitude slower than those of pollen tube or root hair organelles. This study reports the first quantitative analysis of organelles in Physcomitrella patens and by comparing the distribution and dynamics of organelles from different tip growing plant cells, may help better understand the mechanisms of plant polarized cell growth.

1309

Structurally and Functionally Mimicked Three-dimensional Hepatic Structure in vitro.

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Introduction

Hepatocyte-based tissue engineering has been considered as an attractive possibility for treatmenting metabolic disorder and liver injury. However, hepatocytes are known to lose their functions within several days under monolayer culture conditions. Effective hepatocytes culture systems, which allow hepatocytes to preserve their morphologies and functions for a long-term, need to be established. The present study created a triple-layered structure mimicking hepatic cord in vivo structurally by stratifying monolayer hepatocyte sheets and other types cell sheets (3T3 cell and endothelial cell) using a cell-sheet technology for preserving hepatic functions and morphologies for a long-term. Functional and morphological analyses of the triple-layered co-culture were performed, and the results were compared with those of mono- and double-layered homotypic hepatocyte sheets.

Materials and Methods

Hepatocytes were isolated from the livers of six-week-old rats. Isolated hepatocytes were cultured on 35-mm temperature responsive culture dishes (TRCDs) for forming monolayer hepatocyte cell sheets. After 4 days culturing, a monolithic hepatocyte sheet (Hep) was harvested from TRCD at 20? and sandwiched with two endothelial cell sheets (EC, bovine endothelial cell) or 3T3 cell sheets (3T3, mouse embryonic fibroblast cell line) on a culture insert by a fibrin gel-coated manipulator.

Results

Triple-layered hepatic structures (EC-Hep-EC and 3T3-Hep-3T3) were successfully created, and their albumin secreting functions and cytochrome P450 (CYP) activities related with drug metabolism were investigated. Albumin secretion levels of homotypic co-cultured mono- and double-layered hepatocyte sheets showed a steep decline. In contrast, triple-layered heterotypic hepatic structures (EC-Hep-EC and 3T3-Hep-3T3) were found to preserve albumin secretion levels until 20 days. Especially, the albumin secretion level of 3T3-Hep-3T3 was higher than those of EC-Hep-EC and monolayer hepatocytes (Hep). Furthermore, CYP3A induction assay by pregnenolone-16a-carbonitrile known as CYP3A inducer showed that CYP activities of 3T3-Hep-3T3 and EC-Hep-EC were 3.3- and 2-fold increases compared with that of monolayer hepatocyte, respectively.

Conclusions

This study succeeded in creating a triple-layered structure that structurally resembles the microstructure of the liver by stratifying hepatocyte and other type cell sheets (ECs and 3T3 cells). Hepatic functions such as albumin secretion and CYP activities were highly preserved for a long-term in the present triple-layered co-culture system. These results demonstrate the cell sheet-based 3-D culture system was a potentially valuable method to create useful assessment tools for drug screening and a valuable tissue source for liver tissue engineering.

Embryogenesis

1310

Formation of early axon tracts in the embryonic vertebrate brain.

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Initial nerve connections in the vertebrate brain form an array of longitudinal tracts, transversal tracts and commissures, from clusters of neurones. These tracts will act as a scaffold for later, follower axons that allow more complex connections to be set up in the brain. The early axon scaffold has been identified in a number of species, and many of the tracts appear remarkably conserved between all vertebrates analysed. However, a direct comparison of early tracts between different species is lacking, and many of the tracts are poorly characterised. By using a range of pan-neural antibodies the early axon scaffold was mapped in cat shark, *Xenopus*, chick and mouse embryos. The aim was to provide a comparative description of early neurones and their tracts in the embryonic vertebrate brain. This has provided insight into the evolution of the embryonic brain architecture and is critical for the interpretation of early neuronal phenotypes in gain- and loss-of-function studies. The longitudinal tracts, the MLF and TPOC and transversal tract TPC are highly conserved in all these vertebrates. The MLF forms first in the cat shark, *Xenopus* and chick brains, however the DTmesV forms first in mouse brain. The genes involved in specification of neurones to an MLF fate are unknown; microarray analysis was used to identify candidate genes with a possible role in MLF neurone specification in the embryonic chick brain. Once the neurones have differentiated, the axons then need to project along the correct path. The role of Netrin1 and Netrin2 in the guidance of axon tracts within the early axon scaffold have been investigated using expression constructs in gain-of-function experiments. This led to a reduction or loss of the TPC in the embryonic chick brain.

1311

TULP3 is an effector of the IFT-A complex in regulating neural tube morphogenesis.

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Tissues are built through an orchestrated process of morphogenesis coordinated by morphogen gradients, which prime distinct zones of cellular differentiation; for example, sonic hedgehog (Hh) gradients regulate patterning of the neural tube. Primary cilia and intraflagellar transport (IFT), an ancient conserved trafficking mechanism within the cilia, are fundamentally important in vertebrate Hh-mediated signaling. The tubby-like proteins (Tulps) comprise of a family of poorly understood proteins with roles in neural development and function. Mutants in tubby-like protein 3 (Tulp3) and IFT-A complex (implicated in retrograde IFT within the cilia) show over-activation of the Hh pathway in the neural tube. However, mice carrying mutations in the retrograde IFT motor dynein 2 show reduced Hh signaling in the neural tube. This suggests that IFT-A may be modulating other processes in addition to retrograde IFT during neural tube

differentiation. While generating the tubby family interactome using tandem affinity purification and high-confidence mass spectrometry-based proteomics, we were surprised to discover that specific tubby family proteins, notably TULP3, bind to the IFT-A complex. Furthermore, systematic depletion of the IFT-A subunits using RNA interference suggested that a "core" IFT-A sub-complex comprising of WDR19, IFT140, and IFT122, associates with TULP3. Surprisingly, in addition to its known role in retrograde IFT, the "core" IFT-A sub-complex also functions in providing ciliary access to TULP3. What does TULP3 and IFT-A do in this second role? We find that TULP3 and IFT-A promote ciliary trafficking of a subset of rhodopsin-family G protein-coupled receptors (GPCRs) in a wide variety of cells. Both IFT-A and membrane phosphoinositide binding properties of TULP3 are required for ciliary GPCR localization. Notably, Tulp3 does not affect the ciliary trafficking of the frizzled-family GPCR Smoothened (Smo) raising the possibility that an additional GPCR regulates neural tube patterning. We are characterizing knockouts of candidate GPCRs, and have generated a detailed IFT-A-Tulp3 proteomic network in order to discover key players in neural tube differentiation. To conclude, the Tulp3-IFT-A protein interaction network is a major regulator of neural tube patterning, and provides us with important insights into its morphogenesis.

1312

The expression and characterization of cysteine sulfinic acid decarboxylase during zebrafish embryogenesis.

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Cysteine sulfinic acid decarboxylase (CSAD) is the rate-limiting enzyme in the biosynthesis of taurine. There are numbers of physiological roles of taurine such as bile salt synthesis, osmoregulation, lipid metabolism and oxidative stress inhibition. To investigate the roles of de novo synthesis of taurine during embryonic development, zebrafish CSAD was cloned and the cDNA encodes for a protein of 482 amino acids with its sequence highly homologous to mammalian CSAD and conserved throughout evolution. Semi-quantitative RT-PCR detected CSAD mRNA as early as 0 hours post fertilization (hpf) indicating the existence of maternal CSAD message. Whole-mount in situ hybridization demonstrated that CSAD was expressed in yolk syncytial layer and various mesoderm tissues such as pronephric duct, notochord and cardiogenic field during early embryogenesis. Knockdown of CSAD by morpholino oligos (MOs) reduced taurine level in embryos dramatically. The CSAD morphants showed increased early mortality, elevated cell death in cardiogenic region and tail, pericardial edema and malformation of tail. mRNA coinjection and taurine supplementation rescued the mortality and cardiac phenotypes in CSAD morphants suggesting that the heart malformation in CSAD morphants was due to taurine deficiency via de novo synthesis pathway. Our findings indicated that de novo synthesis pathway via CSAD plays critical role in taurine acquisition in zebrafish early embryos and taurine plays a role in zebrafish cardiac development.

1313

Akt1 regulates Hox gene expression in mouse embryonic fibroblast.

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In mammals, precise spatiotemporal expressions of Hox genes control body pattern and provide positional information along the body axis during embryogenesis. However, the mechanism by

which Hox genes are regulated is poorly understood. To search for novel regulators of Hox genes, we analyzed the archived gene expression profiles from the Gene Expression Omnibus (GEO) database. In a particular dataset, clustered Hox gene expressions were largely altered in Akt1-/- mouse embryonic fibroblasts (MEFs) compared to the wild type, suggesting a hypothesis that Akt1 is required for proper expression of the Hox genes during mouse embryonic development. We therefore examined all 39 Hox gene expressions using quantitative RT-PCR and found that the transcripts of 5¡Ç Hoxc genes, Hoxc10, 11, 12 and 13 including a noncoding RNA, were upregulated in Akt1 null MEFs. Particularly, the upregulation of Hoxc11 was further confirmed in the Akt1 null embryonic limbs using quantitative RT-PCR and in situ hybridization. Finally, epigenetic modifications correlated with active transcription, such as DNA hypomethylation at promoter regions and increased acetylation of histone H3K9, underlied the upregulation of Hoxc11 and 12 in Akt1 null MEFs. These results suggest that Akt1 is necessary for epigenetic regulation of a group of Hox genes during embryogenesis.

1314

Robo-Slit Pathway: Molecular Gatekeeper for Corneal Nerves during Embryonic Eye Development.

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PURPOSE: During avian cornea development, trigeminal nerves reach the cornea margin at embryonic day (E)5 where they are initially repelled for days, instead encircling the cornea's periphery in a nerve ring. The molecular events coordinating nerve guidance during cornea development are poorly understood. Here we evaluated a potential role for the Robo-Slit nerve guidance family.

METHODS: mRNA expression for *Slit 1-3* and their cognate *Robo1* receptor was examined in E5-10 corneas, lens and trigeminal ganglions (TG) by RT-PCR or *in situ* hybridization. TG explants (a source of neurons) were co-cultured adjacent to lens vesicles or corneas which secrete nerve guidance molecules, in the presence or absence of inhibitory Robo1-Fc proteins, and neurite guidance assessed.

RESULTS: *Slit 1-3* expression in the cornea and lens persisted during all stages of cornea innervation examined. *Robo1* expression was developmentally regulated in TG cell bodies, expressed robustly during nerve ring formation then later declining concurrent with projection of growth cones into the cornea. In organotypic co-cultures, lens and cornea tissues strongly repelled E7 TG neurites, except in the presence of inhibitory Robo1-Fc protein. Nerve guidance of older, E10, neurons was not impacted by Robo1-Fc protein and repulsion by cornea diminished.

CONCLUSIONS: This study suggests that nerve repulsion from the cornea during nerve ring formation is mediated by lens- and cornea-derived Slit molecules and Robo receptors on trigeminal nerves. Later, a shift in nerve guidance behavior occurs, in part due to molecular changes in trigeminal neurons including *Robo1* downregulation, allowing nerves to find the *Slit*-expressing cornea permissive of growth cones.

1315

Telescoping: characterizing a new cellular movement in gastrulation.

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Gastrulation is a complex orchestration of movements by cells that are specified early in development. Live imaging and experimental techniques allow examination of morphogenesis at the cellular level in the optically clear sea urchin embryo. Using time-lapse microscopy, our lab

has discovered a new phenomenon that we have termed “telescoping” which contributes significantly to gut elongation. During gastrulation, the cells of the endoderm lineage slide anteriorly alongside one another away from the vegetal pole. These cells move in a fashion similar to opening a telescope to elongate the gut. Until now, it was thought that lateral rearrangement of endoderm cells by convergent extension was the main contributor to gut elongation. This project characterizes the telescoping phenomenon and attempt to distinguish it from possible convergent extension movements that are coincident, or follow the initial invagination.

The objective of this project is to analyze the movements of telescoping cells and their molecular control. The sea urchin is a strong model system because of its well-studied endomesoderm gene regulatory network (GRN) that describes the cell fate specification of the future larval gut. However, the GRN does not describe specific cell biological events driving morphogenesis. Still, several molecules known to be involved in aspects of morphogenesis such as adhesion, signaling, polarity, and cytoskeletal remodeling rely on the circuitry described in the GRN for their contribution to morphogenesis. Rac1, a small RhoGTPase, is localized to the adherens junctions prior to gastrulation where it co-localizes with the adhesion molecule β-catenin. Both cytoskeletal regulators are removed from adherens junctions of the forming gut. This suggests that the absence of these molecules at cellular membranes destabilizes adherens junctions of endoderm cells not only enabling the sliding mechanism of telescoping, but also allowing the intercalation events of convergent extension while maintaining stability of the forming larval gut. Connecting the endomesoderm GRN to the morphogenetic and adhesion events of telescoping will provide a framework for characterizing this remarkable sequence of cell movements in the simplest of deuterostome models.

1316

A Sub-circuit of the Ectoderm GRN Integrates Positional Information and Patterns the Sea Urchin Embryonic Skeleton.

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The Gene Regulatory Network (GRN) driving the first 30 hours of development in the sea urchin is at an advanced state of understanding. It effectively explains how most tissues in the embryo are specified, and it has been used to identify a variety of evolutionarily conserved regulatory modules. Yet the current GRN is not sufficient to explain how the complex morphological patterns present in the developing embryo arise. In particular, the pattering of the embryonic skeleton is not explained, even though the specification of the mesenchyme cells which produce it is one of the best understood GRNs to date. We know that signals including VEGF, coming from a few ectodermal cells bordering the endoderm, communicate this patterning information to the mesenchymal cells that will produce the calcium based skeleton. Yet these signals are a final messenger, the end result of a process which identifies the site of skeleton formation from a thin band of ectodermal cells neighboring the endoderm. We call this region the border ectoderm. To understand this process we identified a group of transcription factors expressed in the border ectoderm. These include IrxA, Nk1, FoxJ1, Msx, and Lim1. Perturbations showed however that while the D-V axis is needed to restrict the expression of these border ectoderm genes, the activation of these genes must originate elsewhere. This led us to hypothesize that the border ectoderm is activated by a signal from neighboring cells, most likely in the endoderm. Several lines of evidence support this hypothesis. Finally, perturbations of genes in the border ectoderm cause defects in skeleton formation, consistent with our hypothesis that these genes partition the border ectoderm and are necessary to establish the signaling centers for skeletogenesis. We conclude that cells in the border ectoderm execute a GRN capable of

integrating positional information from the two major embryonic axes in order to establish the pattern of the embryonic skeleton.

1317

Gap junctional transport through endoderm cells is required for LR asymmetry in mouse embryos.

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The development of left-right (LR) asymmetry is an integral part of the organization of the body plan. In developing mouse embryos at day 8 post fertilization, the initial asymmetric signal is generated by the leftward flow of extracellular fluid called Nodal flow, over the midline located node cells. This LR signal is transferred to the left lateral plate mesoderm (LPM) which then expresses Nodal and downstream targets Lefty2 and Pitx2, conferring left side characteristics to the LPM. An important unresolved question is the mechanism of this asymmetric signal transfer from the node at the cellular as well as molecular level. In this study, we have focused on the role of endoderm cells in the signal transfer process, which form a single epithelial layer tightly apposed to the node along the outer surface of the embryo and show elevated levels of free intracellular calcium ions after the onset of Nodal flow in the node. To specifically target the endoderm cells for study, we studied the Sox17 mouse mutants, which show defects specifically in the definitive endoderm population arising from the primitive streak. We found that the asymmetric gene expression of Nodal, Lefty2 and Pitx2 in the left LPM was absent in a majority of mutant embryos, moreover the mutants also showed randomized heart looping orientation. Functional analysis of the mutant nodes revealed that the initial asymmetric is determined and the LR signal is generated in the left side, suggesting that the LR defect of the Sox17 mutants is most likely due to absent or reduced signal transfer from the node. To uncover the mechanisms by which endoderm cells may relay the asymmetric signal from the node, we analysed the properties in endoderm differentiation in the Sox17 mutants. Most significantly, we found that the mutant endoderm cells failed to localize Connexin43 to the cell membrane and showed highly reduced gap-junction permeability assayed by dye iontophoresis. Although the endoderm cells labelled by a Sox17-GFP knock-in construct formed a surface epithelial sheet as evidenced by ZO1 immunohistochemistry, they were also found interspersed in the underlying mesoderm cells, suggesting delamination due to loss of epithelial polarity. Disorganized adherens junctions, abnormal microtubule assembly and actin polarization suggest possible causes for defective gap-junction assembly in the mutants. These studies have led to novel insights into the LR development process and will be useful to identify the molecules that pass through gap–junctions to relay the asymmetric LR signal to the left LPM.

Cell Fate Determination

1318

Wnt signaling through Lef1 regulates mechanosensory organ progenitor cell identity in the zebrafish.

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The formation of the zebrafish posterior lateral line (pLL) provides an excellent model in which to study the role of progenitor cell behavior during organogesis. The pLL is a mechanosensory system comprised of discrete sensory organs called neuromasts (NMs) that are stereotypically arrayed along the trunk of the fish. The pLL NMs are derived from a pLL primordium (pLLp), a group of ~100 migratory cells that are organized into 2-3 polarized rosettes called proto-NMs. During migration, NMs are formed in a cyclical fashion: after a proto-NM is deposited from the trailing region of the primordium and a new proto-NM forms from proliferating progenitor cells in the leading region. Wnt signaling is active in the leading zone of the pLLp and global Wnt inactivation leads to a failure of the pLLp patterning, migration and NM deposition. However, the exact cellular events that are regulated by the Wnt pathway are not known. We identified a mutant strain, *lef1^{nl2}*, which contains a lesion in the canonical Wnt effector gene *lef1*. *lef1^{nl2}*mutants invariably lack terminal NMs and live imaging revealed that proto-NM renewal fails during later stages of pLLp migration. The overall pLLp patterning, as assayed by the expression of various markers, was not altered in *lef1^{nl2}* mutants. Lineage tracing and mosaic analyses revealed that the presumptive progenitors move out of the pLLp and are incorporated into NMs; this results in a decrease in the number of proliferating progenitor cells and an eventual pLLp disorganization. We concluded that Lef1 function is not required for the initial pLLp organization or migration, but is necessary for regulating the behavior of progenitor cells that contribute to proto-NM renewal during later stages of pLL formation. These findings revealed novel a role for canonical Wnt signaling during the formation of the pLL in the zebrafish and may shed light on mechanism for progenitor cell regulation in other organ systems.

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Transcriptome Transfer Generates tCardiomyocyte a Model for Understanding the Phenotype of Cardiomyocytes.

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Cardiomyocytes are among the most sought after cells in regenerative medicine because they may help to repair an injured heart by replacing lost tissue. Functional cardiomyocyte-like cells have been induced from embryonic stem cells (ESCs), induced from pluripotent stem cells (iPSCs) and generated from direct conversion of fibroblast using defined transcription factor transduction but the induced cardiomyocytes often develop undesired phenotypes such as carcinogenesis or early senescence. In order to generate a model for understanding the phenotype of cardiomyocytes, we used TIPeR (Transcriptome Transfer Induced Phenotype Remodeling) methodology that we have developed in this lab. Our methodological approache was to transfect primary mouse embryonic fibroblast with poly-A+ RNA isolated from wild type adult mouse ventricular myocytes, assess the expression of cardiac antigenic markers, analyze expression profiles of single cells and perform patch-clamp recordings on putative

tCardiomyocytes using whole-cell patch recordings. We also transfected primary mouse cortical astrocyte with poly-A+ RNA from ventricular myocytes to test the dependency of transdifferentiation on initial cell type. Transcriptome-effected cardiomyocytes (tCardiomyocytes) from fibroblasts and astrocytes display morphologies, immunocytochemical properties and expression profiles of postnatal cardiomyocytes. Those global phenotypic changes occur in a time-dependent manner and confer electro-excitability to the tCardiomyocytes. These data highlight the dominant role of the gene expression profile in developing and maintaining cellular phenotype. The Transcriptome Induced Phenotype Remodeling (TIPeR) generated tCardiomyocyte has significant implications for understanding and modulating cardiac disease development.

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Investigating the role of CACN-1 in the Canonical Wnt Signaling Pathway.

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Development of the *C. elegans* gonad is an excellent eukaryotic system in which to study cell specification, differentiation, and migration *in vivo.* CACN-1, the novel and highly conserved *C. elegans* homolog of Cactin, is expressed in a number of tissues, including the somatic gonad. Our project aims to characterize the role of CACN-1 in the canonical Wnt signaling pathway, one of the signaling cascades responsible for setting up proper cell identities in the somatic gonad. Using an integrated strain of *C. elegans* containing POPTOP, a TOPFLASH-like reporter, we can measure changes in the activity of the downstream Wnt signaling component, TCF/LEF, *in vivo*. Knockdown of *cacn-1* by RNA interference in POPTOP-containing worms causes a dramatic increase in POPTOP expression levels, and this increase is dependent on POP-1, the single worm TCF/LEF homolog. Other TCF/LEF-related processes including determination of embryonic cell polarity, endoderm specification and seam cell specification also require CACN-1 for proper execution. Our data provides support for the hypothesis that CACN-1 acts in the canonical Wnt/beta catenin pathway as a TCF/LEF repressor. Further experiments will explore the effect of CACN-1 on other components of the canonical Wnt signaling pathway and may help reveal its role in eukaryotic development.

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Regulation of endothelial cell differentiation by Prx1 during embryonic development.

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Homeobox genes encode highly conserved transcription factors that control tissue patterning and morphogenesis throughout development, yet relatively little is known about their roles in lung vascular development. Previously, we have shown that paired related homeobox gene transcription factor Prx1 is expressed in and around the developing lung vessels and plays a key role in vessel maturation. However, whether Prx1 plays a role on endothelial cell specification (i.e. arterial, venous and lymphatic endothelial cell) during lung vessel functional maturation is unknown. In this study, we examined blood vessel, undifferentiated and lymphatic vessel endothelial cell marker expression (CD31, Lyve1 and Prox1 respectively) in embryo lungs from Prx1-null and wild type mice. At E14.5d, lung immunostainings showed that the number of Lyve1 positive endothelial cells was higher in Prx1-null lungs while the number of CD31 positive endothelial cells was lower as well as highly co-localized with Lyve1 positive undifferentiated endothelial cells. In contrast, wild type lungs expressed higher CD31 positive endothelial cells than Lyve1 positive cells. In parallel to immunostaining results, real time PCR

showed that mRNA expression of Prox1, which is essential for lymphatic vessel differentiation, was two-fold higher in Prx1-null lungs than wild type. Downstream of Prox1, VEGFR3 and Lyve1 mRNA expressions were also up-regulated in Prx1-null lungs compare to wild type. These results indicate that Prx1-null lungs have more uncommitted endothelial cells in developing vessels compare to wild type. Furthermore, gain-of-function studies at the cellular level demonstrated that overexpression of Prx1 gene down-regulated the mRNA expression of Prox1 and VEGFR3 in human umbilical vein endothelial cells and lymphatic endothelial cells. We suggest that Prx1 may interact with Prox1 and regulate blood vessel endothelial cell specification and functional vessel development in the embryonic lung.

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AuroraA regulates the endocytic activity of Numb.

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During asymmetric cell division of Drosophila Sensory Organ Precursor (SOP), the cell fate determinant Numb localizes at the anterior cortex and is unequally inherited by the anterior daughter cell. In this cell, Numb negatively regulates Notch signalling at least in part by promoting the internalisation of the four pass transmembrane protein Sanpodo (Spdo), a Notch-binding partner essential for Notch activation in asymmetrically dividing cell. The anterior cell adopts the pIIb cell identity. Notch is activated in the posterior cell that adopts the pIIa identity. The Ser/Thr kinase AurA was shown to phosphorylate PAR complex to regulate Numb asymmetric localization (Wirtz-Peitz F et al., 2008). We here report the identification of Numb in a 2-Hybrid screen using AurA as a bait. AurA phosphorylates Numb on the evolutionarily conserved Ser304 residue that falls into the AurA consensus phosphorylation site, suggesting a direct regulation of Numb localization and/or activity by AurA. Using three distinct mutant alleles of AurA that exhibits strong to complete loss of kinase activity in vitro, we confirmed that AurA is a negative regulator of Notch signaling. Using time-lapse confocal microscopy of aurA MARCM clones, we observed that mitosis lasts four-time longer than in wild-type cells and that the asymmetric localization of Numb is delayed compared to control cells. In contrast to previous reports (Berdnik and Knoblich, 2002), live imaging revealed that in prometaphase, Numb is asymmetrically localized and unequally inherited. Despite the unequal segregation of Numb, the anterior SOP daughter cell systematically adopts the pIIa fate. Thus, in the absence of AurA kinase activity, asymmetrically localized and inherited Numb fails to negatively regulate Notch activation in the anterior cell. These observations raised the possibility that AurA could control Numb endocytic activity and prompted us to analyse the localisation of Spdo. In control cells, Spdo localized in intracellular dotted compartments in cells expressing or inheriting Numb, namely during interphase and mitosis of SOP and in the anterior SOP daughter cell. In contrast, loss of AurA activity causes Spdo to localize at the plasma membrane during SOP division and in the anterior daughter cell, strongly suggesting that Numb does not longer promote Spdo endocytosis in aurA mutant cells. Numb was previously shown to bind to the clathrin endocytic adaptor AP-2 and Spdo. Coimmunoprecipitation experiments indicate that the ability of Numb to interact with Spdo and AP-2 relies on the phosphorylation of Ser304. We thus favour a model in which AurA regulates asymmetric cell division at two distinct steps. First, AurA phosphorylates PAR to control the kinetics of Numb asymmetric localization at mitosis. Second, AurA phosphorylates Numb and directly regulates Numb endocytic activity of Spdo, that ultimately regulate Notch signalling.

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Latent Process Genes for Cell Differentiation are Common Decoders of Neurite Extension Length.

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A latent process involving signal transduction and gene expression is needed as a preparation step for differentiation. We previously found that nerve growth factor (NGF)-induced cell differentiation has a latent process, which is dependent on ERK and gene expression and required for subsequent neurite extension. A latent process can be considered as a preparation step by decoding extracelluar stimulus information into cellular functions. However, molecular mechanisms of the latent process remain unknown. We examined the time course change of gene expression and neurite extension in response to NGF. Using the siRNA-mediated knockdown experiments, we identified *Metrnl*, *Dclk1*, and *Serpinb1a* as the latent process (LP) genes, which were induced during the latent process and required for subsequent neurite extension in PC12 cells. The LP genes showed distinct ERK dependency and were also induced during the latent process of pituitary adenylate cyclase-activating polypeptide (PACAP)- and forskolin-induced cell differentiation. Regardless of neurotrophic factors, expression levels of the LP genes during the latent process always correlated with subsequent neurite extension length. Although ERK activity was required for the expression of the LP genes and neurite extension, phosphorylation level of ERK was not correlated with neurite extension length. Considering the fact that expression of the LP genes occurred during the latent period (0-12 h) and neurite extension was induced during the extension period (12-24 h), information of neurite extension length contained in ERK is likely to be decoded into the expression levels of the LP genes before the extension process starts. Co-overexpression of all LP genes, but not that of each LP gene alone, enhanced NGF-induced neurite extension. This result suggests that all LP genes can cooperatively enhance neurite extension. Our findings provide a molecular insight and physiological meaning of the latent process as the preparation step by decoding information of future phenotypic change.

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PCL Nanopillars Vs Nanofibers: A Stark Contrast in Progenitor Cell Morphology, Proliferation, and Fate Determination.

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Background and Objective: Plastic-adherent, fibroblast-like populations of bone marrow cells, termed multipotent mesenchymal stromal cells (MSCs), have garnered increasing attention in the tissue engineering community resulting from their ease of acquisition, ex vivo expandability, and exciting potential for tissue regeneration. Previous work with human MSC populations have shown that they are capable of differentiation in response to a variety of stimuli, including soluble cues, surface chemistry, matrix elasticity, seeding density, and cell geometry. Recently, MSCs have been demonstrated to differentiate into osteoblast-like cells without the use of bone differentiation media, by modifying the substrate nanotopography to contain nonordered nanopits or nanotubes. In this work, the role of nanotopography on the long-term response of progenitor cells is explored using polycaprolactone (PCL) nanopillar and nanofiber surfaces seeded with plastic-adherent rat MSCs.

Study Design/Materials and Methods: Electrospun PCL nanofibers and gravimetrically extruded PCL nanopillars serve as experimental surfaces to investigate progenitor cell response to nanotopography. Smooth PCL surfaces act as negative topographical controls, while the combination of standard tissue culture polystyrene with bone differentiation media serves as positive controls for osteogenesis. MSCs extracted from the bone marrow of Wistar and Sprague-Dawley rats were seeded on all four surface types at equal densities. Following two, three, and four weeks in culture, MSC morphology was assessed via immunofluorescence imaging of the actin cytoskeleton. On the fourth week, surface induced osteogenesis was investigated by staining for bone marker proteins.
Results: After four weeks in culture under normal expansion media conditions, MSCs cultured on nanofibers exhibit better adherence, increased proliferation, and maintain increasingly dense fibroblast-like morphologies. In contrast, MSCs seeded on nanopillar surfaces display lowered adherence, reduced proliferation, and adopt highly elongated cellular morphologies. Immunofluorescent staining of MSCs on PCL nanopillars reveals the presence of two bone marker proteins, osteopontin and osteocalcin, providing evidence for surface induced stretching and subsequent differentiation into osteoblast-like cells. Unlike the nanopillar topography, MSCs cultured on nanofiber and smooth PCL surfaces did not appear to undergo osteogenesis.
Conclusions: Observed differences in cellular response to the PCL nanotopographies offer strategies to direct progenitor cell populations solely based upon submicron surface modifications. This study provides a foundation for additional work exploring variations in PCL nanopillar topography with the goal of optimizing adherence and osteogenic response of MSCs.

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Differentiation potential of a line of human hematopoietic stem cells.

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A line of commercially available human hematopoietic stem cells was characterized for its potential to differentiate into osteocytes and transdifferentiate into neuron-like cells. This cell line expresses the cell surface marker CD 34, and was found to have a perinuclear arrangement of mitochondria, a marker that is becoming more apparent as representing the undifferentiated state of mesenchymal adult stem cells. Upon stimulation of this cell line to osteocytes, mitochondria dispersed throughout the cytoplasm, an apparent marker for the initial differentiation, followed by the synthesis of a calcium-based extracellular matrix. This cell line was also able to undergo transdifferentiaiton into neuron-like cells with a concomitant dispersion of mitochondria away from the nuclear region, loss of CD34 expression, and the formation of polymerized neurofilments. Expression of unpolymerized neurofilament protein is not a marker of neurons since the undifferentiated stem cells also express this protein. The transdifferentiation is rapid, starting within 12 hours after exposure to neural induction medium, with approximately 30% of the cells assuming a neuron-like morphology after 48 hr. The number of neuron-like cells does not increase with subsequent exposure to induction medium. The transdifferentiation process is not stable in the absence of the neural-induction medium with cells either reverting back to the undifferentiated state or becoming necrotic.

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Multispectral cell tracking and dynamic analysis in intact tissues using confocal and multiphoton microscopy of five fluorescent proteins.

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The use of multicolor fluorescent proteins (FPs) to label cells has emerged as a powerful tool for cell tracking and lineage tracing during morphogenesis or regenerative processes. However many FP variants have similar excitation and emission properties, making unambiguous separation of signals from each reporter difficult. When multiple FPs are combined in the same experiment, high resolution multidimensional imaging could provide new insights into complex biological processes. We approached this challenge using lentiviral vectors encoding five FPs: Cerulean, eGFP, Venus, tdTomato, and mCherry, respectively to stably mark cells. Co-transduction with these vectors generates a large palette of combinatorial colors and gives the ability to track individual cells and their progeny. We optimized conditions of transduction and detection of different FPs using NIH3T3 cells. Confocal spectral imaging was used to record the five individual FP fingerprints, and software-aided linear un-mixing was performed to separate them. Although spectral imaging can be performed in 3D, the lengthy time for data collection prohibits imaging of large volumes or dynamic tracing of cells. We overcame these obstacles using the individual FP spectra and intensities to set sequential imaging channels for accurate separation of the 5FPs. We subsequently used these vectors to mark murine hematopoietic stem and progenitor cells (HPSCs) and followed engraftment and regeneration of hematopoiesis in the bone marrow (BM). In these studies we found that spectrally-coded HPSC-derived cells can be detected non-invasively in various intact tissues, including the BM, for extensive periods of time following transplantation. Moreover, we demonstrate that confocal imaging can be combined with multiphoton microscopy, revealing complementary information from autofluorescent and second-harmonic-generating structures. The unprecedented high resolution images from optical sections were used to computationally reconstruct 3D-patterns of great complexity to depths of 150-300μm. These revealed multiple expanding clones derived from individual transduced HPSCs in the BM, clearly demarcated by color-marking, in the bone proximity. HPSC-derived individual cells could be resolved in the thymus, lymph nodes, spleen, liver, lung, heart, skin, skeletal muscle, adipose tissue, and kidney as well. The 3D-images can be assessed qualitatively and quantitatively to appreciate the distribution of cells with minimal perturbations of the tissues. Finally, we illustrated the feasibility of live dynamic studies in 4D by combining resonant scanning multiphoton and confocal time-lapse imaging. This methodology enables non-invasive high resolution, multidimensional cell-fate tracing of spectrally marked cells populations in their intact 3D architecture, providing a powerful tool in the study of tissue regeneration and pathology.

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The Mouse Mammary Microenvironment Redirects Cells from Mesoderm-derived Bone Marrow Cells to a Mammary Epithelial Progenitor Cell Fate.

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Mammary stem cells reside in protected tissue locales (niches), where their reproductive potency remains essentially unchanged through life. Disruption of the tissue leads to a reduced capacity of dispersed epithelial cells to recapitulate complete functional mammary structures. Previous studies demonstrate that during the reformation of mammary stem cell niches by dispersed epithelial cells in the mammary stroma, non-mammary cells of ectodermal germ origin may be sequestered and reprogrammed to perform mammary epithelial cell functions including

those ascribed to mammary stem/progenitor cells. To test whether tissue cells from organs derived from different germ layers could respond to mammary epithelial-specific signals, we utilized FACS-purified Lin- and Lin-/cKit+ adult male bone marrow cells to mix with mammary epithelial cells. We mixed wild type mammary epithelial cells with bona fide bone marrow cells isolated from the femurs of WAP-Cre/R26R males at a 1:1 ratio and inoculated them into the cleared inguinal fat pads of immune-compromised Nu/Nu female hosts The host mice were bred 6-8 weeks later and examined 20-30 days post involution. This approach allowed for growth of mammary tissue, transient activation of the WAP-Cre gene, recombination and constitutive expression of LacZ from the Rosa 26 promoter. PCR analysis of the DNA isolated from the chimeric outgrowths demonstrated the presence of both transgenes and sequences specific to the Y chromosome, verifying the presence of mesodermal-derived male cell DNA. Similar results were obtained when secondary outgrowths from BMC/MEC outgrowths were sectioned and analyzed by in situ fluorescent hybridization for male Y-chromosome associated DNA Our evidence shows that the signals provided by the mammary microenvironment are capable of redirecting mesoderm-derived adult progenitor cells to produce functional mammary epithelial cell progeny.

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Reprogramming of the osteoclast lineage using murine macrophages.

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Macrophages are cells produced by the differentiation of monocytes in tissues. They function in innate, as well as adaptive, immunity. In addition to their roles as phagocytes, macrophages can be differentiated, by the addition of RANKL and M-CSF, into osteoclasts (large, multinucleated cells that are responsible for bone resorption). In this work, we set out to characterize whether the addition of various stimuli to RANKL pre-treated murine macrophages (RAW264.7), can alter the type of multinucleated cell obtained. Following a four-day differentiation protocol, along with LPS/IFNgamma as one stimulus, and IL-4 as the other, three types of multinucleated cells were generated. The cells were then characterized using various microscopy techniques (light, epifluorescent and scanning electron) and western blotting for osteoclast markers. We found that, as expected, RANKL treatment alone resulted in osteoclasts whereas the addition of LPS/IFNgamma to RANKL pre-treated macrophages generated Langhan's Giant Cells-type cells while IL-4 led to giant cells resembling those found in Giant Cell Tumors of the Bone. While osteoclasts had irregular morphologies, with nuclei located throughout the cell, LPS/IFN-treated cells were epitheliod in shape with peripherally located nuclei. In contrast, IL-4-treated cells were "fried-egg" shaped in appearance with centrally located nuclei. Western blotting for two osteoclast markers, tartarate-resistant acid phosphatase (TRAP) and cathepsin K, showed all three cell types being positive for TRAP and varying degrees of cathepsin K expression. Current research is focused on how the nuclei within these cell types are organized and what protein motors are involved in their movement.

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Genetic mapping and characterization of a mutation that affects male-specific neural development in *C. elegans*.

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Objective: The *C. elegans* male and hermaphrodite nervous systems display sexually dimorphic development characterized, in part, by the presence of 8 hermaphrodite-specific neurons and 89 male-specific neurons. We are interested in identifying the genes and molecular mechanisms that govern sex-specific neural development in *C. elegans*. **Methods:** Through a

mutagenesis screen using a *pkd-2::GFP* reporter to label male-specific neurons, we recovered several mutants that display defects in sex-specific neural development. **Results:** Males carrying the *sm129* mutation lack *pkd-2::GFP* expression in the male-specific CEM neurons that are involved in mate finding. Since CEMs undergo programmed cell death in hermaphrodite embryos, we investigated whether CEMs inappropriately undergo cell death in *sm129* males or whether these cells adopt an alternative cell fate. Blocking programmed cell death in *sm129* mutants, using a mutation in the caspase gene *ced-3*, does not result in the appearance of *pkd-2::GFP*-marked CEMs. This suggests that CEMs in *sm129* mutant males have adopted an alternative cell fate or are improperly specified. *sm129* mutant males also have reduced *pkd-2::GFP* expression in neurons in the male tail and have a very poor mating efficiency suggesting that male-specific neurons associated with the male tail are not formed or are not properly specified. The *sm129* mutant phenotype appears to be male-specific; hermaphrodites homozygous for the *sm129* mutation are not affected. The recessive *sm129* mutation is not a null allele; the phenotype of *sm129/Deficiency* trans-heterozygotes is more severe than *sm129/sm129* homozygotes. Three-factor mapping and deficiency mapping place *sm129* within a small region on linkage group II between 1.84cM and 2.83cM that spans roughly 100 genes. *mab-3* is a gene located in this region that affects sexually dimorphic development, but *sm129* complements a null allele of *mab-3* suggesting that *sm129* affects a different locus. There are no other genes in this region that are known to affect sex-specific development. **Conclusions:** We present evidence for a previously uncharacterized gene that regulates male-specific neural development in *C. elegans*.

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Derivation of skeletal muscle progenitor cells from human embryonic stem cells.

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Muscular dystrophy comprises a group of genetic diseases that cause progressive weakness and degeneration of skeletal muscle resulting from defective proteins critical to muscle integrity. This leads to premature exhaustion of the muscle stem cell pool that maintains muscle integrity during normal use and exercise. Stem cell therapy holds promise as a treatment for muscular dystrophies, since clinical trials with human skeletal myoblasts demonstrated that these fuse with diseased fibers, providing a source of normal muscle proteins. Embryonic stem cells (ESCs) harbor infinite proliferation capacity, and can differentiate into cell types comprising most body tissues. To date, consistent approaches to the directed differentiation of ESCs into skeletal muscle precursors have been elusive and laborious. The development of efficient methods for directing skeletal muscle differentiation of ESCs would provide a model system for elucidating the control and regulation of skeletal myogenesis, and lead to the derivation of muscle progenitor cells for therapy. To address this, we tested several conditions to direct the differentiation of H9 human ESCs into skeletal muscle. Embryoid bodies (EBs) were formed in the presence or absence of the transforming growth factor-beta inhibitor, SB431542, in defined medium containing 10% fetal bovine serum (FBS) or a cocktail of insulin, transferrin and selenium. By quantitative RT-PCR we observed dramatically increased expression of muscle transcription factors, *Pax3* and *Pax7*, and increased expression of *hepatocyte growth factor receptor* (*cMET)*, a marker of satellite cells, in the presence of 10% FBS and the inhibitor after 3 days of differentiation. We also detected an increase in the percentage of cells expressing neural cell adhesion molecule, a marker of myogenic precursors, by flow cytometry. When plated on Matrigel in 2% horse serum, adherent EBs showed outgrowth of desmin-expressing myoblasts, as detected by immunocytochemistry, after 2 weeks. These studies demonstrate a validated approach to derive skeletal muscle progenitor cells from human ESCs for the study of

human myogenesis, and the development of cell therapy for the treatment of muscular dystrophies.

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Potential Role for Liver X Receptor in Osteoblast Function.

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The liver X receptors (LXRα and LXRβ) are nuclear receptors that function in cholesterol metabolism. Despite their name, the oxysterol-activated LXRs may also have an active role in bone and joint health. While LXRα is expressed mostly in the liver, kidney and intestines, LXRβ is expressed ubiquitously. Based on microarray and other data, a potential role for oxysterols in osteoblast (OB) differentiation has been proposed. This study investigates whether activation of LXR with a synthetic ligand would enhance OB differentiation. A human fetal pre-osteoblastic cell line (hFOB) was treated with and without 10μM synthetic LXR-agonist (N,N-dimethyl-3beta-hydroxy-cholenamide, DMHCA) in culture for 3, 7 and 14 days. At these time points, gene expression by each culture was measured using quantitative real-time PCR (RT-qPCR). Two known downstream targets of LXR, ATP-binding cassette A1 (ABCA1) and ABCG1; a marker of OB differentiation and function, osteocalcin (OCN); and β-actin for a housekeeping gene were measured. ABCA1 and ABCG1 were significantly increased with DMHCA treatment at days 7 and 14 ($P<0.05$). OCN significantly increased with treatment on day 7 ($P<0.05$). These data indicate that OBs have functional LXR receptors that can be activated with DMHCA and further that LXR activation may promote OB differentiation and/or function.

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Canonical Wnt signaling regulates the renewal of differentiated taste cells of adult mice.

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In adult mice, cells within taste buds are continually renewed. Signaling pathways including Sonic Hedgehog, BMP, and Notch are thought to control this turnover, but specific molecular mechanisms have not been elucidated. Moreover, while Wnt/β-catenin signaling controls embryonic taste bud development (Liu et al., 2007, Nat Genet 39(1):106-12), its involvement in adult taste cell turnover is unexplored. We used conditional doxycycline-inducible Cre recombination of either exons 2-6 or exon 3 of the β-catenin gene to induce β-catenin loss- (LOF) or gain- (GOF) of function, respectively, in the taste epithelium of adult mice. After two weeks of doxycycline, β-catenin LOF resulted in a significant reduction in PLCβ2-IR (type II) cells in taste buds in the circumvallate papilla (CVP). Moreover, we found that proliferation of perigemmal basal cells in the CVP was reduced after 4 days of drug treatment, as determined by the proliferation marker Ki67. In the β-catenin GOF mice, we found that PLCβ2-IR cells appear taller and more numerous after 4 days on doxycycline, yet paradoxically, these cells were greatly reduced after 2 weeks of doxycycline. Interestingly, proliferation was virtually abolished at day 4 of β-catenin GOF, and then only somewhat recovered by day 7. Although both GOF and LOF of β-catenin both result in reduced proliferation and type II taste receptor cells, we suspect that the underlying mechanisms are quite different. Reduced proliferation following β-catenin LOF is likely because β-catenin is required for proliferation of perigemmal

basal cells, which are the progenitor population for taste buds; thus in the absence of β-catenin, new taste cells are not generated. Beta-catenin GOF also results in an early reduction in proliferation, but we hypothesize that this is because elevated β-catenin forces progenitor cells to exit the cell cycle and/or differentiate into mature taste bud cells, depleting the proliferative progenitor population.

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Distinct protein domains regulate stability and patterning of MEX-3 in the *C. elegans* embryo.

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PAL-1, the *C. elegans* caudal homolog, is a homeodomain protein required to specify posterior cell fates. While *pal-1* mRNA is present throughout the embryo, translation is restricted to the posterior in a process that requires the 3' untranslated region (3'UTR) of the *pal-1* mRNA, and the RNA-binding protein MEX-3. MEX-3 protein is present uniformly throughout the newly fertilized embryo, but becomes depleted in the posterior by the four-cell stage. Normal MEX-3 patterning requires the CCCH Zn-finger protein MEX-5, the RNA Recognition Motif protein SPN-4, and the serine/threonine kinase PAR-4. Genetic and biochemical evidence suggests the following model: MEX-5 binds to MEX-3 in the anterior and protects it from degradation, allowing MEX-3 to bind the *pal-1* 3'UTR and repress translation. MEX-3 that is not bound to MEX-5 becomes inactivated in a *par-4* dependent fashion, possibly through phosphorylation, then targeted for degradation through binding to SPN-4.

To determine which regions of the MEX-3 protein are required for stability and which for degradation, different regions of the protein were fused to GFP and introduced into the C. elegans embryo. Full-length MEX-3 expressed in this manner can functionally replace endogenous MEX-3. Results indicate that the N-terminus of MEX-3, which contains two KH domain RNA binding motifs, confers unusual stability to GFP. In contrast, the C-terminus of MEX-3, which contains potential phosphorylation sites, causes GFP to be degraded over the same timeframe as endogenous MEX-3. Furthermore, a region in the C-terminus containing a glutamine-rich region and just a few potential phosphorylation sites is required for timely degradation. Research on neurodegenerative diseases resulting from expansion of polyglutamine tracts, including Huntington's Disease, indicates that polyglutamine regions can serve as sites for protein cleavage and that polyglutamine proteins can be ubiquitinated and targeted to the proteosome. This raises the possibility that MEX-3 is inactivated by phosphorylation, then ubiquitinated and targeted to the proteosome for degradation. Intriguingly, the four human Mex-3 homologs and the apparent ascidian homolog all contain a RING finger motif, which can serve as an E3 ubiquitin-protein ligase. By creating additional fusion proteins that are missing potential ubiquitination and/or phosphorylation sites, we are determining which residues are required for inactivation and degradation.

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Differentiation Analysis of Alveolar Bone Derived Mesenchymal Stromal Cells (Al-MSCs) and Bone Marrow Mesenchymal Stem Cells (BMSCs): A comparison.

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Tissue engineering is a promising approach for regenerative procedures in oral and maxillofacial surgery. Here we investigated the suitability of oral skeletal tissue as an applicable source of human mesenchymal stromal cells and an alternative to the iliac crest bone marrow. The aim was to compare the multilineage differentiation potential of alveolar bone derived mesenchymal stromal cells (Al-MSCs) and bone marrow mesenchymal stromal cells (BMSCs). Al-MSCs were isolated using explant cultures of intra-orally harvested bone chips during routine oral surgery. BMSCs were obtained from iliac crest bone marrow aspirates and used as positive control for multilineage differentiation analysis. Cells were immunocytochemically characterized. Differentiation capacities into the osteogenic, adipogenic and chondrogenic lineages were investigated using cytochemical tests (alkaline phosphatase activity, Oil Red O and Alcian blue staining) and RT-PCR analysis. Al-MSCs showed characteristics of BMSCs like plastic adherence and fibroblast like shape. Immunocytochemical analysis revealed a specific surface antigen expression pattern considered typical for multipotent mesenchymal stromal cells. Al-MSCs showed consistently high expression of CD73, CD90, CD105 and lack of expression of CD14, CD34, CD45, CD79α, HLA-DR surface molecules. Their differentiation capacity into the osteogenic, adipogenic and chondrogenic lineages was comparable to that of BMSCs. These findings suggest that Al-MSCs have a similar differentiation potential to BMSCs' in vitro. Oral skeletal tissue may be considered as a suitable source of cells for tissue engineering therapies in regenerative dentistry.

1336

Characterization of Stem Cell Differentiation and Dedifferentiation using Gene Expression Signatures.

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Stem cell-based therapies are promising for the treatment of currently intractable medical conditions. These therapies rely on transplantation of differentiated stem cells that are functionally analogous to the differentiated tissues they replace. A major bottleneck in efforts to efficiently differentiate stem cells is derived from the inability to quickly and accurately monitor the effectiveness of a protocol and thereby determine if a functional cell type has been obtained. Ultimately the final product may require functional testing to satisfy this requirement, but this is impractical when differentiation protocols are under development and evolving quickly. Therefore, a fast and accurate method to monitor the efficacy of differentiation protocols is needed.

Here we describe the use of a series of qPCR panels specifically designed to identify molecular profiles that accurately monitor functional hepatocyte differentiation. We demonstrate the use of a small set of lineage-specific markers to effectively track the differentiation of human embryonic stem cells into mature hepatocytes. We show that these cells express the appropriate immunocytochemical markers and possess functional activities of mature hepatocytes, such as glycogen storage, albumin production and indocyanine green dye uptake. In addition, key genes in the cytochrome P450 superfamily are highly upregulated. These CYPs include CYP3A4, CYP2C9 and CYP2E1 which are all critical for proper hepatocyte function in drug metabolism.

Levels of other markers associated with mature hepatocytes such as Albumin and Alpha-1 antitrypsin are also found to closely follow that of their respective proteins. By validating our qPCR panels with functional testing, we show that gene expression signatures can be a useful method for monitoring differentiation protocols for functional relevance.

1337

Highly-reproducible transfer of cyclic mechanical stretch to stem cells: a novel bioreactor.

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Dynamic culture of cells seeded on biocompatible 3D-poymeric matrix is a suitable condition to create a engineered pseudo-tissue useful for the regenerative medicine. Several studies has demonstrated that mechanical forces influence the growth and the shape of virtually every tissue. The aim of this work was the development of a novel bioreactor (WIPO WO2011013067 entitled “Bio reactor for stem cell stimulation”) able to transfer controlled and standardized mechanical stimuli to Mesenchymal Stem Cells (MSCs) for their commitment towards the muscle phenotype.
Bone marrow MSCs were isolated from wild type adult male Wistar rats and were seeded onto selected scaffold (3x2 cm hyaluronan-based woven mesh) at the concentration of 10^6 cells per cm^2. Control cells were cultured on scaffold in conventional static condition during 2 weeks, whereas dynamic experimental test was carried out maintained the cells in standard condition during 1 week and transferring them in the bioreactor during the following week. Dynamic cultured conditions were: frequency = 600 ms (1.66 Hz; 100 bpm), mean load over the cycle = 10 g (0.1 N) obtained with 2.5 mm amplitude of stretching (< 10% of scaffold length available for cell growth = 2.5 cm). At the end of experiment the cells were examined in light microscopy (LM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and protein extracts were submitted by SDS-PAGE and Western blot.
After 7 days of “training” in dynamic condition, we observed a significant difference between MSCs cultured in dynamic condition compared with the cells in static control culture. Two main differential features were scored by LM: the large number and the multilayer organization of cells in the dynamic setting. SEM and TEM showed structural differences and cellular alignment along with stretching direction. In addition, the mechanical stretching stimulation increased the expression of typical muscle markers (smooth muscle actin and α-sarcomeric actin) witness of an ongoing differentiation process.
The dynamic bioreactor presented in this work aims to support maintenance of constant parameters during mechanical stimulations, in order to obtain a suitable, standard method for dynamic cell culture where strain is required. Therefore, this bioreactor may represent a new basic research tool in the field of tissue engineering and in the study of stem cells differentiation towards specialized phenotypes.

Oncogenes and Tumor Suppressors II

1338

Inhibition of Myosin Light Chain Kinase by Aurora B Kinase Leads to Cytokinesis Failure and Tetraploidization in Cancer Cells.

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Cancer cells can fail in cytokinesis leading to tetraploidy and chromosomal instability, a common phenotype of malignant cells. A key regulator of the process of cytokinesis is the enzyme Aurora B kinase. Dys-regulation of Aurora B is known to cause cytokinesis failure and tetraploidization. However, the mechanism of Aurora B mediated tetraploidization is not yet known. An essential step in cytokinesis is phosphorylation of myosin regulatory light chain (MLC), required for actin–myosin interaction and formation of the cleavage furrow. Previous studies have shown that MLC phosphorylation is deficient in cancer cells and that MLC phosphorylation deficiency is the cause of cytokinesis failure in the tested cancer cell lines. Myosin light chain kinase (MLCK) is a key enzyme that phosphorylates MLC during cytokinesis and is inhibited in cancer cells. As Aurora B kinase is known to phosphorylate MLCK, we hypothesize that Aurora B is an inhibitor of MLCK and the source of MLCK deficiency in cancer cells. Consistent with our hypothesis, we show that Aurora B is an inhibitor of MLCK *in vitro* and also in cultured mammalian cells. Our data also suggests that chromosome segregation errors can be a cause of Aurora B over-expression and MLCK inhibition in cancer cells. Thus, we have identified what we believe is the molecular explanation for Aurora B-induced cytokinesis failure in tumor cells. These results contribute to understanding of the consequences of Aurora B over-expression and the causes of cytokinesis failure in malignant cells.

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Hepatitis B Virus X (HBx) induces chromosomal instability through reduction of aurora B kinase activity.

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Correction of mal-attached microtubules is required to prevent chromosome mis-segregation and subsequent chromosomal instability and tumorigenesis. Although Hepatitis B virus X (HBx), an onco-protein encoded by HBV genome, is involved in the development of HBV-mediated liver cancer, its precise mechanism of action remains unclear. Here we show that HBx reduces Aurora B activity, which induces chromosomal segregation defects. Expression of HBx induces severe anaphase lagging and micronuclei formation after monastrol release, indicating insufficient correction of mal-attachment leading to segregation defects. In addition, HBx causes chromosome congression defects at metaphase. Interestingly, HBx interacts with Aurora B in the presence of HBxAP, an associating protein with HBx, which leads to a significant decrease of the kinase activity of Aurora B, but not the expression of Aurora B based on western blot analysis using phospho-specific antibodies. Likewise, HBx colocalizes with Aurora B at the centromere. Together, our data strongly suggest that the decrease of Aurora B kinase activity through binding with HBx protein is involved in HBx-induced chromosomal instability. This provides a novel mechanism for mitotic aberrations by a viral pathogen, which contributes to the accumulation of genomic instability during hepatocarcinogenesis.

1340

Depletion of cellular poly (A) binding protein prevents protein synthesis and results in p53 mediated apoptosis in HeLa cells.

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The cytoplasmic poly (A) binding protein (PABP) is an important activator of mRNA translation and also regulates mRNA stability. It interacts with eIF4G at the 5'cap which leads to the circularization of translating mRNA and enhances mRNA translation. In yeast, PABP gene is essential and its conditional mutation leads to inhibition of mRNA translation. However, it is uncertain, whether mammalian PABP is essential for mRNA translation. PABP depletion from a cell free extract prevents mRNA translation whereas according to a recent report ablation of PABP expression by siRNA did not prevent mRNA translation in HeLa cells in culture. We re-examined the effect of PABP depletion on mRNA metabolism in both HeLa and HEK 293 cells by using small interfering RNA. We found that depletion of PABP reduced protein synthesis by approximately 50-75%, and consequently leads to cell death in both cell lines. Significant reduction of cellular PABP level resulted in the nuclear translocation of the glycolytic enzyme GAPDH. Presumably the nuclear GAPDH activated an apoptotic pathway by enhancing acetylation and serine 46 phosphorylation of p53.We have shown that p53 translocates to the mitochondria to initiate Bax mediated apoptosis in PPABP depleted cells. It is likely that p53 induce apoptosis by a transcription independent mechanism in PABP depleted cells We have shown that p53 binds to the pro survival protein Bcl2. Bcl2 normally binds to pro apoptotic protein Bax and prevents apoptosis by inhibiting its oligomerization and mitochondrial translocation. Therefore, perhaps in PABP depleted cells binding of p53 to Bcl2 released Bax from Bcl2 inhibition and allowed Bax to oligomerize and triggered apoptosis.

1341

Cytoplastic sequestration of p53 by the heat shock protein 70 family member, mortalin, in human colorectal carcinoma cell lines.

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Colorectal carcinomas are among the leading cancer deaths in Western countries and typically arise due to mutations in tumor suppressors and oncogenes, as well as functional inactivation of tumor suppressor proteins. The examination of 16 colorectal adenocarcinoma cell lines to determine expression levels for p53 and a heat shock protein family member mortalin was conducted. Inactivation of p53 through cytoplasmic sequestration has been suggested to promote carcinogenesis by tethering of the p53 protein by mortalin thereby rendering it non-functional. All cell lines displayed punctate p53 and mortalin localization in the cell cytoplasm with the exception of cytoplasmic p53 protein in HCT-8 which was never detected, possibly because p53 levels are below detectable limits. Nuclear and therefore actively functioning p53 was only observed in HCT-116 40-16, LS123, and HT-29 cell lines. Co-immunoprecipitation revealed that p53 and mortalin were bound and co-localized in five of eight cell lines examined thus far. HCT-116 40-16 and 386, HT-29, LS123 and LoVo, were preventing p53 from being shuttled into the nucleus and therefore causing its inactivation. Elevated mortalin gene expression levels were observed in all p53-containing cell lines and most cell lines showing cytoplasmic p53 sequestration resulting in elevated p53 gene levels. Current experiments include the disruption of p53-mortalin interaction using either MKT-077, a cationic rhodacyanine dye analogue or siRNA targeted to mortalin. Initial results for the HCT-116 cell lines show a return of p53 to its functional site, the nucleus. Our data reveal the first evidence of cytoplasmic sequestration of p53 by the heat shock protein mortalin in colorectal adenocarcinoma cell lines,

which had previously been suggested but not yet proven as it has been the case for other unrelated cancers.

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Annexin A2 facilitates cell cycle by sustaining c-Jun N-terminal kinase-inhibited p53 expression in lung adenocarcinoma.

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Overexpression of annexin A2 is required for cancer cell proliferation and invasion; however, the molecular mechanism underlying cell cycle is still unknown. Annexin A2 richly expressed in lung adenocarcinoma and positively correlated to poor prognosis. Non-small cell lung cancer cell A549 lacking annexin A2 exhibited defects on tumor growth *in vivo* and cell proliferation *in vitro* without cytotoxicity. Knockdown of annexin A2 induced cell cycle arrest in G_2 phase of interphase. Unexpectedly, silencing annexin A2 increased expression of p53 and its downstream genes followed by p53-mediated G_2 arrest, at least in part. Aberrant c-Jun N-terminal kinase (JNK) inactivation in annexin A2 deficient cells caused cell proliferation inhibition following G_2 arrest. Lack of annexin A2 caused loss of JNK-regulated c-Jun expression followed by p53 increase transcriptionally. Treatment of cisplatin caused annexin A2 cleavage accompanied by p53 increase, whereas annexin A2 knockdown increased cisplatin-induced p53 expression as well as apoptosis while annexin A2 overexpression reduced such effects. These results demonstrate a novel role of annexin A2 in lung cancer cell proliferation by facilitating cell cycle through maintenance of JNK/c-Jun-inhibited p53.

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PA28γ is required for effective initiation of apoptosis signaling by p53.

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The tumor suppressor p53 regulates a wide array of biological processes including cell cycle arrest, DNA repair, and apoptosis to maintain genomic integrity. Due to p53's role in these critical cellular processes, persistent regulation is important. Cell stress typically results in increased concentration of p53, subsequently promoting cell cycle arrest, apoptosis, or senescence. In order to maintain low levels of p53 in normal cells, negative regulation of p53 is accomplished by MDM2-mediated proteasomal degradation. The proteasome activator, PA28γ, has been shown to play a critical role as a cofactor by facilitating MDM2-dependent degradation of p53 by proteasomes. Additionally PA28γ has been identified as an important component in regulating the cellular distribution of p53. Murine embryonic fibroblasts (MEF) that lack PA28γ, exhibit high levels of p53 expression but lack the expected increased sensitivity to apoptotic stimuli, measured by caspase 3/7 activity. This led us to hypothesize that our PA28γ-deficient MEFs express a dysfunctional p53 in order to compensate for lack of PA28γ expression and to maintain viability. Upon sequence analysis of cloned cDNA, it was determined that PA28$\gamma^{-/-}$ MEFs have a Met to Thr mutation at the 120th amino acid residue. The location of this mutation is in the DNA binding domain of p53, and is flanked by Ser residues that represent possible phosphorylation regulation sites. To examine the possible effects of the M120T mutation, we investigated p53's ability to transcribe the gene Noxa. Our data suggest that the M120T mutation does not affect the transcriptional transactivation of p53 on Noxa, under basal conditions or when cells are treated with UVR where a 9-fold increase in Noxa concentration is observed in both control and PA28 $\gamma^{-/-}$ MEFs. There are several post-translational modifications including phosphorylation and acetylation that affect p53 stability, localization, and function that could explain the phenotype we observe. Currently, no significant differences in p53 modifications have been detected in PA28 $\gamma^{-/-}$ cells. We confirm that elevated p53 levels in PA28

γ$^{-/-}$ MEFs are related to proteasome degradation rates; however, we conclude that PA28γ has additional roles in regulating the initiation of the caspase cascade and promoting apoptosis unrelated to its regulation of p53 concentration.

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Inhibitor of Growth 1 (ING1) Stabilizes Tumor Suppressor p53 by Blocking Polyubiquitination.

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Introduction: The INhibitor of Growth tumor suppressors (ING1-ING5) affect aging, apoptosis, DNA repair and tumorigenesis. Plant homeodomains (PHD) of ING proteins bind histones in a methylation-sensitive manner to regulate chromatin structure. ING1 and ING2 contain a polybasic region (PBR) adjacent to their PHDs that binds stress-inducible phosphatidylinositol monophosphate (PtIn-MP) signaling. ING1 induces apoptosis independently of p53, but several studies suggest proapoptotic interdependence of ING1 and p53 leaving their functional relationship unclear. Here we identify a novel ubiquitin-binding domain (UBD) that overlaps with the PBR of ING1 and shows similarity to previously described UBDs involved in DNA damage responses. **Results:** The ING1 UBD binds ubiquitin with high affinity (Kd~100 nM) and ubiquitin competes with PtIn-MPs for ING1 binding. ING1 expression stabilized wild-type, but not mutant p53 in an MDM2-independent manner and knockdown of endogenous ING1 depressed p53 levels in a transcription-independent manner. ING1 stabilized unmodified and six multimonoubiquitinated forms of wild-type p53 that were also seen upon DNA damage, but not p53 mutants lacking the six known sites of ubiquitination. We also find that ING1 physically interacts with herpesvirus-associated ubiquitin-specific protease (HAUSP), a p53 and MDM2 deubiquitinase (DUB), and knockdown of HAUSP blocks the ability of ING1 to stabilize p53. **Conclusion:** These data link ING1 to ubiquitin-mediated proteasomal degradation through the PBR/UBD of ING1 and further indicate that ING1 stabilizes p53 by inhibiting polyubiquitination of multimonoubiquitinated forms via interaction with and colocalization of the HAUSP-deubiquitinase with p53.

1345

EGFR-Stimulation in Breast Cancer Cells Causes Membrane Capacitance Changes Similar to a Neurosecretory Cell.

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Over-expression of the epidermal growth factor receptor (EGFR) is responsible for many cancers including breast, which is the most common cancer diagnosed among women in the United States. Although EGFR endocytosis has been extensively studied, the molecular machinery and regulatory determinants required for EGFR internalization and recycling remains uncertain. The objective of this study was to directly measure in “real-time” EGFR membrane trafficking in breast cancer cell lines and noncancer cell lines by using state-of-the-art electrophysiological membrane capacitance (Cm) measurements. Membrane capacitance allows detection of small changes in membrane surface area that are the result of incorporation and or retrieval of transport vesicles containing EGFR. We have discovered using this ‘high-resolution technique’ never before applied to cancer cells that EGFR stimulation with EGF and related ligands causes extremely fast, kinetically and mechanistically distinct pathways of EGFR membrane incorporation, endocytosis, and receptor recycling. Importantly, these pathways do not exist in noncancer breast cells. Based on our preliminary data, we propose that breast cancer cells possess cancer-specific EGFR pathways to efficiently maintain the cells high

functional demands and self-sufficiency characteristic of malignant breast cancer. (This work was supported by NIH grant GM068813)

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Phosphatase SHP-1 supports mammary epithelial cell acinus formation by limiting EGFR activity, phosphorylation and recycling.

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Primary epithelial cells harvested from mid-pregnant mouse mammary glands form acini when supported on a laminin-rich EHS extracellular matrix. We have previously shown that such acinus formation is dependent on JNK MAP kinase activity. Here we have used a small molecular weight JNK inhibitor to disrupt acinus formation (it blocks cell polarisation and lumen clearance) and so define the contribution of epidermal growth factor receptor (EGFR) signalling to acinus formation. In EGF is not added to the culture medium perfect acini form from dispersed primary mammary epithelial cells supported on the EHS matrix but they are significantly smaller than normal. If acinus formation is disrupted by inhibiting JNK activity, acinus formation can be rescued by inhibiting EGFR activity. Thus limited EGFR activity is required to support acinus formation. We have addressed a possible mechanism of how EGFR activity is curtailed in this context. As normal acini form we show that EGFR levels remain constant, the EGFR is retained on the plasma membrane and is under auto-phosphorylated on tyrosine. If acinus formation is disrupted by JNK inhibition total EGRF levels fall, EGFR levels on the plasma membrane fall (suggestive of receptor recycling) and the remaining EGFR is hyper-autophosphoylated. During normal acinus formation a small isoform of the EGFR phosphatase, SHP-1, was detectable; SHP-1 was phosphorylated on its Src-dependent activational site and SHP-1 activity was high. If acinus formation was disrupted with the JNK inhibitor, a bigger SHP-1 isoform was detected, low levels of SHP-1 phosphorylation on the activational site was detected and SHP-1 activity was low. Over-expression of two dominant negative SHP-1 mutants both impaired acinus formation by MCF10A human mammary epithelial cells and reduced total EGFR levels. Over-expression of a dominant negative JNK mutant, which impairs MCF10A acinus formation, reduced SHP-1 phosphorylation on the activational site. Thus, SHP-1 shows the properties necessary for a regulator of EGFR activity during acinus formation. Interestingly, we could show, in a large cohort of human breast cancer patients, that high SHP-1 expression was significantly associated with a good outcome in lymph node negative patients.

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Aberrant DNMT3B proteins alter E-cadherin expression and cell proliferation in breast cancer cells.

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With breast cancer responsible for more than 39,000 female deaths in 2010, it is the second most common cause of cancer death in American women. Tumor progression is regulated by changes in gene expression and one mechanism to regulate gene expression is DNA methylation. DNA methylation occurs when one of three DNA methyltransferases (DNMT1, DNMT3A, or DNMT3B) adds a methyl group to a CpG dinucleotide causing DNA to become tightly coiled. As a result, transcription of methylated DNA is prevented and affected genes become silenced. Previous studies have shown abnormal methylation in cancer cells. Furthermore, aberrantly spliced versions of DNMT3B have been indentified in several cancer cell lines, including breast cancer. Therefore, we hypothesize that aberrant DNMT3B isoforms alter protein expression leading to changes in cell proliferation in breast cancer cells. Our

studies indicate that the breast cancer cell lines MCF-7 and MDA-MB-231 have been shown to express many aberrant DNMT3B isoforms including DNMT3B7 and ∆DNMT3B6. Interestingly, the invasive MDA-MB-231 cells express increased levels of DNMT3B7 and ∆DNMT3B6 compared to the poorly invasive MCF-7 cells. To further our studies, MCF-7 and MDA-MB-231 cells were stably transfected with aberrant DNMT3B expression constructs. Western blots were performed on cytoplasmic and nuclear lysates collected from the transfected cells and observed bands revealed a difference in E-cadherin expression between transfected and non-transfected cells. Additionally, cell proliferation was measured and indicates a correlation with E-cadherin expression. The novel results presented in this study suggest the need for additional studies to determine the role of aberrant DNMT3B transcripts in cancer. Ultimately, this work may lead to a better understanding of breast cancer progression and the development of novel therapeutics.

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Role of HSP90 in stability of PTK6 which is an oncoprotein overexpressed in breast cancer cells.

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PTK6 (also known as Brk) is a non-receptor tyrosine kinase containing the SH3, SH2, and catalytic domains. PTK6 is overexpressed in breast carcinomas, but not in normal mammary tissues. PTK6 induces proliferation, anti-apoptosis, migration, and anchorage-independent growth. To understand roles of an oncoprotein PTK6, proteins interacting with PTK6 were analyzed. Heat shock protein 90 (Hsp90) was identified as an interacting protein of PTK6. Hsp90 interacted with the kinase domain of PTK6 but its catalytic activity was not required for the interaction. A Hsp90 inhibitor, geldanamycin, decreased the protein level of PTK6 through proteasome-dependent degradation of PTK6. Treatment of geldanamycin also reduced phosphorylation of PTK6 substrates due to the decreased amount of PTK6. Moreover, overexpression of CHIP, which is a chaperone-dependent E3 ligase, enhanced the proteosomal degradation of PTK6. Silencing of endogenous CHIP expression in breast carcinoma cells abolished the geldanamycin-induced degradation of PTK6. Geldanamycin increased the interaction of PTK6 with CHIP while it decreased the interaction of PTK6 with Hsp90. These results demonstrate that Hsp90 plays an important role in stability of PTK6 by assisting the correct folding of PTK6 and suggest that Hsp90 inhibitor should be useful as a therapeutic drug for the PTK6-positive cancers including breast cancer.

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The HER2 amplicon includes several genes required for the growth and survival of HER2 positive breast cancer cells.

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Over 20 % of breast cancers are characterized by amplification and overexpression of the *HER2* oncogene. Although significant progress has been achieved for treating such patients with HER2 inhibitor Trastuzumab, more than half of the patients respond poorly or become resistant to such treatment. Since the HER2 amplicon at 17q12 is large and contains multiple genes, we wanted to systematically explore the role of the *HER2* co-amplified genes in breast cancer development and Trastuzumab resistance. Here, we integrated mapping data of the

HER2 amplicon size in 71 HER2 positive breast tumors and 10 cell lines with systematic functional RNA interference analysis of the 23 of the core amplicon genes with several phenotypic endpoints in a panel of Trastuzumab responding, non-responding and HER2 negative breast cancer cells. Silencing of *HER2* caused a greater growth arrest and apoptosis in the responding compared to the non-responding cell lines. Several other genes in the amplicon also showed effects when silenced, indicating that expression of several *HER2* co-amplified genes may be needed to sustain the growth of breast cancer cells. Importantly, co-silencing of the *PPP1R1B (DARPP-32), STARD3* and *PERLD1* together with *HER2* led to a synergistic inhibition of cell viability as well as AKT and S6K phosphorylation. Taken together, these studies indicate that breast cancer cells may become addicted to the amplification of several genes that reside in the HER2 amplicon and that simultaneous targeting of these genes may increase the efficacy of the anti-HER2 therapies and possibly also counteract Trastuzumab resistance. Taken together with literature evidence, our data also suggest that many gene products in the HER2 amplicon also signal through the PI3K-AKT complex, supporting the ongoing efforts to develop inhibitors for these pathways for combinatorial treatment of HER2+ breast cancers.

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WNT5A Overexpression Promotes Breast Cancer Progression in the Presence of VANGL1.

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The 21T cell lines have previously been shown to mimic specific stages of breast cancer progression (21PT, Atypical Ductal Hyperplasia; 21NT, Ductal Carcinoma *in situ*; 21MT-1, Invasive Mammary Carcinoma). Expression profiling has revealed differences in WNT5A and VANGL1 expression among these three cell lines. WNT5A expression levels are moderate in 21PT, low in 21NT, and high in 21MT-1 cells, whereas VANGL1 levels are low in 21PT and high in 21NT and 21MT-1 cells. WNT5A and VANGL1 both play roles in the non-canonical Wnt/planar cell polarity (PCP) signalling pathway. The precise role of this pathway in breast cancer progression has yet to be determined, as activation of the pathway via Wnt5a has been shown to promote either differentiation or progression. This dual effect may be due to the presence or absence of other PCP components such as VANGL1. Based on this and the expression patterns of WNT5A and VANGL1 in the 21T cell lines, we hypothesized that WNT5A will promote breast cancer progression only in the presence of elevated levels of VANGL1. Here, we have characterized an *in vitro* model of breast cancer progression using the 21T cell lines to measure 3D colony morphology when grown in reconstituted basement membrane. Using this model and transwell migration and invasion assays we show that overexpression of transfected WNT5A in the 21NT cells (high endogenous VANGL1 expression), but not the 21PT cells (low endogenous VANGL1 expression) promotes cancer progression. Specifically, WNT5A overexpression had a profound effect to increase invasion and migration of the 21NT cells, but only had a modest effect on cell migration of the 21PT cells, and had no effect on invasion. WNT5A overexpression also increased the percentage of non-spherical colonies in 21NT cells but had no effect on 21PT cells. In addition, VANGL1 overexpression promoted progression of the 21PT cells (which have moderate levels of endogenous WNT5A expression). Specifically, VANGL1 overexpression increased cell migration and invasion and also decreased the percentage of extracellular lumen formation, polarized structures, and increased the number of non-spherical colonies. Taken together, these findings implicate a role for the PCP pathway in promoting breast cancer progression and this role may be dependent on VANGL1 expression.

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Tumor Suppressor Gene p16/INK4A Dependent Regulation of Cell Cycle Exit in a Spontaneous Canine Model of Breast Cancer.

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p16/INK4A is an important tumor suppressor gene which arrests cell cycle in early G1 phase inhibiting binding of CDK4/6 with cyclin D1 leaving the Rb tumor suppressor protein unphosphorylated and E2F bound and inactive. We have previously shown that p16 is frequently mutated in canine mammary cancer comparable to human breast cancer. Because p16 expression persists following cell cycle exit, we hypothesize that p16 has a role in exit from cell cycle that becomes defective in cancer cells and that p16 has alternate binding partners other than CDK4/CDK6 in quiescent or differentiated cells. Well characterized p16-defective canine mammary cancer cell lines (CMT28, CMT27, and CMT12), normal canine fibroblasts (NCF), and p16-transfected CMT cell clones (CMT27A, CMT27H, CMT28A, and CMT28F) exhibiting a rescued phenotype, have been used to investigate expression of p16 after serum starvation into quiescence followed by re-feeding to induce cell cycle re-entry. CMT cells lack p16 expression either at mRNA or protein expression levels, while p27, CDK4, CDK6, cyclin D1, and Rb, appear to be expressed at normal levels. We have successfully demonstrated cell cycle arrest and synchronous cell cycle re-entry in parental CMT12, CMT28 and NCF cells as well as p16 transfected CMT27A, CMT27H, CMT28A, and CMT28F cells and confirmed this by 3H-thymidine incorporation and flow cytometric analysis of cell cycle phase distribution. p16 transfected CMT27A and CMT27H exited cell cycle post serum-starvation in contrast to parental CMT27 cells. NCF, CMT27A, and CMT28F cells expressed up-regulated levels of p27 mRNA coincidently with elevated expression of p16 mRNA as cells exited cell cycle and entered quiescence. To find alternating binding partners of p16, co-immunoprecipitation was performed in quiescent CMT27A cells which resulted in unique co-immunoprecipitation of the p53-associated and putative tumor suppressor 14-3-3σ protein only in quiescent CMT27A cells in comparison to exponential cells. Levels of 14-3-3σ mRNA expression also rose along with p16 in quiescent NCF cells. Because quiescence and differentiation are associated with decreased levels of cyclin D1 and/or CDK6 our data demonstrating that p16 is up-regulated during quiescence suggests the presence of alternative binding partners for p16, such as 14-3-3σ, in promoting and maintaining the quiescent and/or differentiated phenotype (funded by NCI/NIH).

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The Sphingosine 1-Phosphate Receptor S1P2 Maintains Homeostasis of Epithelium.

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The cells comprising epithelia turn over via cell death and division at the highest rates in the body. Because most solid tumors, or carcinomas, originate from epithelia, misregulation of this balance of epithelial cell death and division, or homeostasis, is likely at the heart of tumor formation. Surprisingly little is known, however, about what regulates the balance between cell division and death in epithelia in vivo. We have found that epithelia use a process termed “apoptotic cell extrusion” to remove apoptotic cells from a layer while maintaining an intact barrier. Specifically, an early apoptotic epithelial cell triggers its neighboring cells within the epithelium to form and contract an actin and myosin ring, which squeezes the dying cell out. We recently found that the signal that induces extrusion is sphingosine-1-phosphate (S1P) and it signals through S1P2 in neighboring cells to bring about actin assembly and extrusion. One surprising finding is that when extrusion is blocked by S1P2 antagonist or anti-S1P mAb, there is a marked decrease of induced cell death. Interestingly, reduced cell death mediated by

blocking S1P2 is not observed in cells that cannot extrude, such as NIH3T3 cells, suggesting that extrusion is a necessary process for epithelial cell death. Overgrown human bronchial epithelial (HBE) cells with S1P2 knockdown not only display reduced naturally-occurring cell death but also accumulate up to three layers. Mixed culture of normal and S1P2 knockdown HBE cells shows that only S1P2 knockdown cells accumulate up to multiple layers and continue to proliferate (by BrdU incorporation assay) after a long period of culture. In addition, zebrafish that carry a loss-function mutation in S1P2 are defective in epidermal cell extrusion and develop epidermal cell clumps. Taken together, these data support a conclusion that S1P2 maintains epidermal homeostasis, likely through mediating cell extrusion.

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Molecular and Signalling Functions of Tumor Protein D52 in Cancer Cells.

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A large number of studies have shown that the *tumor protein D52 (TPD52)* gene is amplified and/or overexpressed in different types of cancer. Independent studies have demonstrated increase Akt phosphorylation in response to TPD52 overexpression in prostate cancer cells, suggesting that TPD52 represents a signalling intermediate in the PI3-Kinase pathway. Despite the increasing recognition of the significance of TPD52 overexpression in cancer, the basis of TPD52's oncogenicity is poorly understood, in part due to a lack of binding partners which might explain TPD52's oncogenic properties. The present study aimed to identify novel TPD52 partners with relevance to cancer through a large-scale yeast two-hybrid screening approach. A full-length human TPD52 bait was screened against a human breast carcinoma cDNA expression library in Hf7c cells. This screening identified 4 candidate partners for TPD52, which are all integral membrane or membrane-associated proteins. Our further study focused on two of these candidates, proteolipid protein 2 (PLP2) and Rab5c, as both proteins have been linked with PI3-Kinase and/or Akt functions. GST pull-down assay confirmed interactions between PLP2 or Rab5c and TPD52. Moreover, we also examined the binding activity of a panel of deleted TPD52 proteins with PLP2 and Rab5c in both the yeast two hybrid system and pull-down assays. Interaction domain mapping showed that these partners bind within TPD52 amino acids 111-131, outside the coiled-coil motif. Confocal-based immunofluorescence analyses in breast carcinoma cell lines showed that TPD52 and flag-tagged PLP2 co-localised towards the plasma membrane. Currently, we are examining the subcellular localisation of TPD52 and CFP-tagged Rab5c. In summary, we identified 2 novel binding partners for TPD52, PLP2 and Rab5c. These two partners have different structures and functions; however they bind similar regions of TPD52. Further functional studies of these two partners could provide a better understanding of TPD52 functions in cancer cells.

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Ha-Ras^{V12} induced cancer cell softening facilitate tumor formation.

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Large amount of evidence about cancer cell biology has been accumulated in the past with the advancement of genetic and biochemical approach. However, less was known about the mechanical or physical property of a cancer cell. We previously demonstrated that collagen gel

induced apoptosis in epithelial cells, but not transformed or cancer cells, due to low stiffness (lower than 100Pa) nature of the gel (1). We hypothesized that normal epithelial and cancer cells might exhibit different physical property which provided the advantage for cancer cell to evade low-substratum rigidity induced apoptosis. In order to explore the physical properties of cancer cells, we established the technology to explore the stiffness of a single cell with Bio-Atomic Force Microscope. We found that cancer cells were softer than normal epithelial cells in general. To further elucidate the underlying mechanisms that cancer cells are softer than normal cells, we established the IPTG inducible Ha-Ras^{V12} expression system in NIH3T3 and MDCK because oncogenic Ras has been implicated in carcinogenesis of various types of cancer. Ha-Ras^{V12} overexpression conferred the resistance to anoikis and facilitated foci formation, anchorage independence growth, cell proliferation on low rigidity as well as invasiveness ability in both NIH3T3 fibroblast and MDCK epithelial. Interestingly, those Ha-Ras^{V12} overexpressing cells exhibited lower cell stiffness earlier than obtaining the transformation phenotypes. MEK-1/2 inhibitor U0126 inhibited Ha-Ras^{V12} induced-cell softening and anchorage independence growth. Dominant negative Ras and mutant Raf reversed both cell stiffness and Ras-transformed phenotypes. Antitumor drugs such as Ras inhibitor trans-farnesylthiosalicylic acid (FTS) and Tricostatin A abolished Ha-Ras^{V12} overexpressing induced-cell softening. Taken these data together, Ha-Ras^{V12} induced-cell softening might confer the advantage of cell proliferation or survival on soft substratum as well as malignant phenotypes. Further investigation on the cell biological roles of lowered cell stiffness in cancer cells should facilitate our understanding of the development of cancer.

Reference:
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Adenomatous Polyposis Coli Mutation Leads to Increased Incidence of Head Tumors in Zebrafish.

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The most common mutation in colon cancer in humans is inactivation of adenomatous polyposis coli (APC), a key inhibitor of the Wnt/β catenin pathway. In human carriers of an APC inactivating mutation, the risk of colorectal cancer by age 40 is almost 100%. Truncation of the tumor suppressor APC constitutes the primary contributor to sporadic colon cancer in humans. In the zebrafish system, the same truncated mutation causes digestive tract neoplasia. However, the incidence of tumors outside the intestinal system in the APC mutant has not been reported. In order to determine if other tumors arise at high frequency in the APC mutant, we monitored our population of heterozygous APC fish on a weekly basis over a one-year period. Specimens displaying evidence of tumors or general signs of declining health were euthanized, photographed, tail clipped for genotypic analysis, and then fixed and embedded in Paraplast for histological examination. Analysis of the data showed that 1.2% of the APC mutant zebrafish within our facility had abnormally enlarged and distended abdomens (possibly from intestinal neoplasia), and 1.8% of the mutants developed head tumors. We have yet to detect a single head tumor in the non-APC carriers in our facility. Overall, the information collected reveals that APC mutant zebrafish exhibit remarkably increased incidence of externally visible head tumors when compared to incidence in wild type fish. Pathological investigation of tumor-stricken fish uncovered the highly invasive nature of these head tumors. Furthermore, relative to other zebrafish mutants, APC zebrafish begin developing tumors early in the life cycle. The APC mutant zebrafish is particularly susceptible to aggressive head tumors, providing new opportunities for cancer research in the zebrafish system.

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Role of 14-3-3 gamma in centrosome duplication and neoplastic progression.

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Cells, to achieve neoplastic transformation, need to proceed through a phase of genomic instability towards the genomic stability. Down-regulation of a pSer/Thr-binding protein 14-3-3gamma, increases aneuploidy and causes early tumor formation in SCID mice. These observations raise general questions that how the depletion of 14-3-3gamma causes genomic instability and how the unstable cells gradually achieve stability afterwards. One of the major reasons for genomic instability is unequal segregation of chromosomes during cell division. Unequal chromosome segregation is caused by asymmetric spindle organization. Spindle is generated in mammalian cells from a spindle organization centre known as centrosome. Depletion of 14-3-3gamma by short hair-pins causes multiple centrosome formation, a state that disrupts the bipolar chromosome segregation system during mitosis. Multiple centrosome foci correspond to the multiple spindle foci which in turn cause asymmetric segregation of chromosomes. To understand the fate of asymmetrically divided cells, the knockdown cell line was studied from an early passage to a very high passage. Cell death was found to be high at lower passage but the death rate gradually decreased with the increase of passage. Moreover, cells in higher passage showed an effective bi-polar spindle orientation in spite of having multiple centrosomes. This leads towards the stability and transformation.

Mechanistically, depletion of 14-3-3gamma activates Cdc25 phosphatases which in turn activate CycB1/CDK1 and CycE/CDK2 complexes. Active CDK1 and CDK2 phosphorylates NPM1 at T199 residue. Upon phosphorylation, NPM1 gets separated from the mother centriole and the licensing of daughter centriole formation begins at the G1/S checkpoint. Therefore, knockdown of 14-3-3gamma disrupts the centrosome duplication cycle causing centrosome amplification which leads to genomic instability and neoplastic progression.

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SFRP1 and IGFBP3 are secreted mediators of senescence.

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Cellular senescence is widely believed to play key roles in tumor suppression, but signaling pathways mediating senescence are incompletely understood. By analyzing the proteins secreted from senescent cells, we identified two novel mediators of senescence.

1) Employing the quantitative proteomic analysis of secreted proteins, we determined that the Wnt signaling inhibitor SFRP1 (Secreted Frizzled-related Protein 1) is over-secreted from IMR-90 human primary fibroblasts induced to senesce by DNA damage (etoposide treatment). Downregulation of SFRP1 by shRNA or by neutralizing antibody inhibited etoposide-induced senescence while recombinant SFRP1 by itself was able to induce senescence, indicating that SFRP1 is necessary and sufficient for senescence induction. SFRP1 induced senescence through inhibition of Wnt signaling and activation of the Rb pathway. Interestingly, cancer-associated SFRP1 mutants were defective for senescence induction.

2) In addition to normal cells, cancer cells also undergo senescence upon chemotherapeutic drug treatment. We observed that non-senescent MCF-7 breast cancer cells become senescent upon addition of the conditioned medium from senescent MCF-7 cells induced to senesce by

doxorubicin treatment, which suggested the presence of secreted mediator(s) of senescence. Proteomic comparison of protein secretion from senescent and non-senescent MCF-7 cells revealed that IGFBP3 (insulin-like growth factor binding protein 3) is over-secreted from doxorubicin-treated senescent MCF-7 cells. We found that IGFBP3 can be cleaved and inactivated by t-PA (tissue-type plasminogen activator). t-PA is negatively regulated by another known secreted mediator of senescence, PAI-1 (plasminogen activator inhibitor 1). We have discovered a cascade whereby PAI-1 induces senescence through the elevation of IGFBP3 levels. IGFBP3 is known to modulate the activities of insulin-like growth factors and display anti-proliferative effects on some cancer cells. RNAi suppression of PAI-1 or IGFBP3 inhibited senescence induction upon doxorubicin treatment of MCF-7, indicating that PAI-1 and IGFBP3 mediate doxorubicin-induced senescence in these cells.

These studies identified extracellular components of senescence signaling. Senescence mediators secreted from senescent cells may amplify the senescence response and provide a non-cell autonomous tumor suppression mechanism in precancerous cells as well as in chemotherapy-treated tumor cells.

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Effect of Androgen Receptor (AR) Expression Levels and Proteasome Inhibitors on AR Activity in Prostate Cancer Cell lines.

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Prostate cancer is a cancer of the male reproductive system and is one of the most common types of cancer occurring in older men. Androgen ablation therapy is the most common form of treatment for metastatic prostate cancer. Typically, prostate cancer grows back in an AR dependent but castration resistant form, hence it is important to develop means to block this activity. The aim of our project therefore was to examine the effects of proteasome inhibitors and AR antagonists on AR activity and cell growth and to characterize the transcriptional activity and overall cell growth in AR overexpressing cell lines. We also tried to determine the dose dependence of AR expression and activity in stable LNCaP cell line variants by various doses of doxycycline treatment.

The cell lines used were LNCap, VCaP, LNCaP-ARFL, LNCaP-ARV7. Casodex and MDV3100 were the AR antagonists used while Bortezomib and MLN2238 were used as proteasome inhibitors. Techniques used were qRT-PCR, Luciferase assay, ß-gal assay.

Results indicate that AR activity is reduced by proteasome inhibitors in all cell lines used. AR antagonists (Casodex and MDV3100) inhibit AR activity at lower expression levels but Casodex begins to behave as an agonist at higher levels. Dose dependence studies showed that AR activity peaks at a certain dose of doxycycline. However more testing needs to be done to further refine these results.

Cancer Therapy I

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Cytotoxicity of novel ruthenium-ketoconazole complexes on a panel of human cancer cells lines.

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Emerging novel chemical compounds with selective anti-cancer cytotoxic properties may have important consequences in the general undergoing of anticancer therapeutics. Novel ruthenium-ketoconazole compounds were characterized for their cytotoxicity activity on a panel of cancer and non-cancer cells, measured by Differential Nuclear Staining (DNS), a highly reliable imaging assay that used to determine the percentage of cytotoxicity after compound treatment. The DNS assay utilizes Hoechst to label all the cell nuclei and Propidium iodide, which only stains nuclear content of dead cells. The fluorescence signal emitted by stained cells was captured by a high-throughput bioimager in two separate channels according to the dye emission requirements. Ketoconazole is a member of the azole family that has been widely used as antifungal drug, but is also being used as a treatment for hormone-refractory cancer. The main goal of this project was to determine if the ruthenium-ketoconazole complexes had selective cytotoxicity as compared with ketoconazole alone, on cancer cell lines, with lower toxicity on untransformed human cells. Cytotoxicity assays were performed using 96-well format on three prostate cell lines (DU-145, LNCap, and 22Rv1), one of cervical origin (HeLa), one leukemia (YT NK-like) and non-tumorigenic human keratinocyte cell line (HaCaT). Our results indicate that several of the complexes induced cytotoxicity in some but not all cancer cell types, as compared with non-cancerous cell lines. Our results demonstrate that ruthenium-ketoconazole complexes are more effective than ketoconazole alone in inducing cell death in prostate cancer cell lines when compared with the non-cancerous cell line at low concentrations. These results confirm that the novel ruthenium-ketoconazole compounds have significant anticancer activity and that they may selectively induce cell death on specific cancer targets.

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Efficient Elimination of Cancer Cells by Deoxyglucose-ABT-263/737 Combination Therapy.

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Genotoxin-based chemotherapies are most effective in treating tumors at early stages of development, while recently developed targeted therapies can be very effective on certain types of cancer at later stages. For most of the common cancer types, however, these targeted therapies are not available because potential targets are not even identified in most of cancer types, let alone have targeting drugs in development. Furthermore, the recent data from the Cancer Genome Atlas Project also suggested that targeting and inactivating just one protein would not be enough to kill cells in most cancer types, implying that multiple targeted drugs may be required to kill most cancer cells. Here we described a new therapy that is safe and effective

in eliminating many cancer cells, including checkpoint defective, genotoxin-resistant, PTEN-deleted, late stage cancer cells.

The new therapy is based on combining 2-deoxyglucose (2DG), a glycolysis inhibitor, with ABT-263/737 (ABT), molecules targeting the anti-apoptotic Bcl-2 family of proteins. As single agents, ABT-263 and ABT-737 (ABT), molecular antagonists of the Bcl-2 family, bind tightly to Bcl-2, Bcl-xL and Bcl-w, but not to Mcl-1, and induce apoptosis only in limited cell types. The compound 2-deoxyglucose (2DG), in contrast, partially blocks glycolysis, slowing cell growth but rarely causing cell death. Injected into an animal, 2DG accumulates predominantly in tumors but does not harm other tissues. However, when cells that were highly resistant to ABT were pre-treated with 2DG for 3 hours, ABT became a potent inducer of apoptosis, rapidly releasing cytochrome c from the mitochondria and activating caspases at submicromolar concentrations in a Bak/Bax-dependent manner. Bak is normally sequestered in complexes with Mcl-1 and Bcl-xL. 2DG primes cells by interfering with Bak-Mcl-1 association, making it easier for ABT to dissociate Bak from Bcl-xL, freeing Bak to induce apoptosis. A highly active glucose transporter and Bid, as an agent of the mitochondrial apoptotic signal amplification loop, are necessary for efficient apoptosis induction in this system. This combination treatment of cancer-bearing mice was very effective against tumor xenograft from hormone-independent highly metastasized chemo-resistant human prostate cancer cells, suggesting that the combination treatment may provide a safe and effective alternative to genotoxin-based cancer therapies.

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Development of DNA aptamers to CD44 that inhibit migration of breast cancer cells.

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Aptamers have several advantages over antibodies. One of such advantages is that they can be reproduced by automated chemical synthesis thus offering a wide variety of targeted modifications to introduce the resistance to biodegradation process and visualize the target molecules. Although antibodies could be generated against immunologic target molecules, aptamers are isolated by in vitro selections that are independent of the antigenicity of target molecules but dependent on specific affinity for the targets. Thus, DNA aptamers that recognize cancer cells will be easier to develop and have a great impact for the next generation of strategies for cancer detection, diagnosis, and therapy.
In this study we established a model system to develop DNA aptamers targeting cell adhesion receptors in triple negative (TN) breast cancer cells. Recent studies identified CD44 as a metastasis-related molecule with multiple functions such as promoting cell-cell and cell-ECM interactions and as cooperating with growth factor receptors (i.e. c-met), which lead to an enhancement of migration, invasion and growth of tumor cells. Although previous studies demonstrated that exon v10 of CD44 plays a key role in promoting breast tumor develop and progression, the biological functions of this exon was not entirely clear. In this study, we demonstrated that antibody against exon v10 of CD44 significantly inhibited triple negative (TN) breast cancer migration toward type I collagen. Previous studies using peripheral blood stem cells suggest that exon v10 plays a key role in promoting leukocytes adhesion to bone marrow cells and migration to disease sites in mice. Thus, it is possible that exon v10 of CD44 expressed on TN breast cancer cells would play a key role in facilitating tumor invasion and metastasis.
We, therefore, established screening systems to isolate DNA aptamers that specifically bind to exon v10 of CD44 using SELEX (systematic evolution of ligands by exponential enrichment). Using recombinant exon v10 expressed in E. coliBL21(DE3)PLysS as a substrate, we isolated 7

DNA aptamers that appear to bind to CD44+ breast cancer cells (HCC38) but not to CD44- breast cancer cells (SK-Br-3) by FACS analysis. We also identified 4 DNA aptamers that significantly inhibited alpha2beta1/alpha3/beta1-mediated TN breast cancer migration to type I collagen. Thus, developing of small molecules (i.e. DNA aptamer) that specifically recognize and antagonize the biological functions of cell adhesion receptors such as CD44 would be a promising approach for treatment of TN breast cancer patients.

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Novel Stilbene-based compound(s) demonstrate an anti-migratory effect against glioblastoma cells.

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Even with modern treatment options, glioblastoma multiforme (GBM), the most common type of brain tumor, is highly aggressive. Treatments include chemotherapy and surgery, but prognosis is usually low, with patients only surviving one to two years. Due to the fact that the brain is a sensitive organ with its own protective barrier, designing new chemical compounds to target GBMs is difficult. Therefore, we designed a study to investigate the antitumor capacity of a series of newly synthesized aromatic stilbene-based compounds (JKS001 – JKS014). We hypothesized that these compounds would be able to prevent both GBM cell proliferation and/or migration based on results from compounds synthesized with similar chemical properties and structures. To investigate the effect of these compounds on the proliferative capacity and migration rate of these compounds we utilized MTT and wound healing assays respectively. None of the JKS, stilbene-based compounds (4 µM – 400 µM) were able to significantly decrease GBM cell proliferation. However, one of the tested compounds (JKS014) demonstrated a stable decrease of GBM cell migration. Specifically, the addition of 4 µM of JKS014 resulted in significant changes in cellular morphology, represented by shortened cell extensions and less cell protrusions. These cell shape changes were accompanied by changes in actin, tubulin and cofilin. These data suggest that the novel, newly-synthesized stilbene-base compound JKS014 has the ability to alter GBM cell architecture and migration capacity as well as reduce the metastatic potential of GBM cells.

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Enantiomer specific inhibition of Rac1 and Cdc42 in Ovarian Cancer.

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Ovarian cancer is the 5th leading cause of cancer death for women in the United States and is frequently diagnosed at late stage with multiple secondary metastases. Ras-homologous (Rho) family GTPases contribute to metastatic dissemination through regulation of actin reorganization, cell motility, cell-cell and cell-extracellular matrix adhesion. Rho GTPases are altered in a number of human cancers and our work provides the first evidence for dysregulation of Rac1 and Cdc42 in ovarian cancer. These findings suggest that Rac1 and Cdc42 inhibitors represent potential therapeutics for ovarian cancer. Using high throughput screening we identified the R-enantiomers of ketorolac and naproxen as novel inhibitors of Rac1 and Cdc42. Although it has been noted that R-enantiomers of nonsteroidal anti-inflammatory drugs are poor inhibitors of cyclooxygenase (COX) activity, little is known about the pharmacologic activities or targets of the R-enantiomers. The objective of this study was to investigate inhibition of Rac1

and Cdc42, and associated functional activities by R-ketorolac and R-naproxen in an ovarian tumor model. Cheminformatics approaches predict that the R-enantiomers of naproxen and ketorolac bind Rac1 and Cdc42 in the inactive GDP-bound state whereas binding of the S-enantiomers was not favored and enantiomer-selective inhibition of Rac1 and Cdc42 was detected in cell-based assays. The R-enantiomers inhibited cell:cell adhesion as measured by formation of multicellular aggregates and cell migration. The cellular responses to R-naproxen and R-ketorolac were equivalent to those detected using the established Rac1 inhibitor NSC23766; the S-enantiomers were essentially inactive. An intra-peritoneal xenograft model of tumor implantation was used to determine the effects of drugs *in vivo*. Both R-naproxen and racemic [R,S]-ketorolac decreased the number of implanted tumors by 70% with little observed effect of S-naproxen or the structurally related metabolite of nabumetone 6-methoxy naphthalene acetic acid. Together, these findings indicate that R-naproxen and R-ketorolac are novel inhibitors of Rac1 and Cdc42, and inhibit cellular processes necessary for ovarian cancer metastasis. In the future, these drugs may offer benefit to ovarian cancer patients through inhibition of these GTPase targets.

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Insights into the Molecular Mechanism of a Novel Microtubule Stabilizer Taccalonolide A.

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The discovery and development of microtubule stabilizers represents one of the most useful advances in the field of cancer therapeutics. Despite their success, intrinsic and acquired resistance occurs, all of which limit their use. The taccalonolides are a class of mechanistically distinct microtubule-stabilizing agents. In cells they increase microtubule density, cause microtubule bundling and shift the equilibrium of tubulin towards the polymerized state and as a consequence, disrupt mitotic spindles, leading to mitotic arrest and cell death. The molecular events responsible for the formation of aberrant spindles and subsequent arrest and cell death are poorly understood. We hypothesize that taccalonolide A initiates aberrant mitotic signaling cascades leading to abnormal entry into mitosis and the formation of defective mitotic spindles. HeLa cells were used to probe for changes in protein expression upon treatment with taccalonolide A or taxol. Here we show that the expression and localization of proteins involved in centrosome maturation and mitotic spindle formation are altered upon treatment with taccalonolide A. Additionally taccalonolide A treatment leads to severe defects in centrosome separation which are not seen in taxol treated cells.

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Generation of high-grade spindle multipolarity by centrosome hyperamplification and declustering: A novel chemotherapeutic approach.

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Extra centrosomes pose serious organizational challenges to cancer cells. Although amplified centrosomes would be expected to compromise cell viability by compelling cells to form multipolar spindles resulting in chromosomal instability, cancer cells can turn things in their favor by employing clever mechanisms to suppress multipolarity by clustering supernumerary centrosomes. As a result, cancer cells are able to maintain bipolar spindle phenotypes with low grade aneuploidy, an edge to their survival. Since cancer cells rely on these clustering mechanisms for survival, interfering with centrosome clustering can severely jeopardize cancer cell viability. Disruption of centrosome clustering is thus an attractive tumor-selective

chemotherapeutic strategy. We have previously shown that a microtubule-modulating noscapinoid, 9-bromonoscapine (EM011) induces formation of multipolar spindles containing 'real' centrioles, indicating drug-induced centrosome amplification and declustering. We also showed that EM011 treatment dampened microtubule dynamics and impaired the association of plus-end tracking proteins like EB1 and CLIP-170 with microtubule plus-ends. Drawing from these findings, we are comprehensively exploring how EM011 induces centrosome declustering by investigating the intricate interplay between microtubule and actin cytoskeletons. Dampened microtubule dynamics accompanied by diminished articulation between microtubule plus-end binding proteins (CLIP-170 and EB-1) and actin cytoskeleton can disrupt crucial forces required to keep the centrosomes coalesced at the poles. On the other hand, within the spindle apparatus, diminished activity of minus-end-directed microtubule motors (dyenin and HSET) is responsible for impaired cross-linking and focusing of microtubules from different centrosomes to the center of the spindle apparatus. We observed that EM011 treatment caused reduced expression and activity of HSET, evidencing its multifaceted role in the disruption of centrosome clustering. In conclusion, understanding key centrosome clustering pathways will aid in identification and development of novel molecules that exacerbate spindle multipolarity, induce high-grade aneuploidy and subsequent death, selectively in cancer cells.

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Detection of a human anti-PDGFRα therapeutic antibody in a human GBM orthotopic rat model.

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Glioblastoma multiforme (GBM) is the most common malignant brain tumor in adults with median survival time of one year which presents a challenge for clinical treatment. The standard of care includes tumor resection, followed by radiation and chemotherapy with temozolomide (TMZ). Despite treatment, most GBM tumors develop resistance to treatment and outcome of patients remains dismal. GBM contains several genetic alterations affecting proliferation; most commonly upregulation of autocrine signaling of platelet-derived growth factor (PDGF) and its receptor PDGFRα, which in tumor cells acts as a receptor tyrosine kinase as well as a mediator of stromal support for cancer growth.
MEDI-575 is a human monoclonal antibody that selectively targets PDGFRα. To determine anti-tumor activity in GBM tumors expressing PDGFRα, MEDI-575 was administered at 1 mg/kg or 3 mg/kg (2x/wk) to athymic nude mice bearing three different human GBM ectopic tumors (U118, U251 and SNB-19). We found anti-tumor activity of 118%, 71%, and 78% tumor growth inhibition (dTGI), respectively, and drug exposure levels in mouse serum of 30 to 80 µg/ml of MEDI-575.
The blood brain barrier (BBB), a highly specialized zone of astrocyte and endothelial cells separating circulating blood from brain tissue, is an obstacle to delivery of targeted treatment to brain tumors. To determine levels of MEDI-575 in GBM, immunofluorescence of rat brains harboring orthotopic human GBM demonstrated staining of human antibodies in GBM of rats treated with 10 mg/kg of MEDI-575 (2x/wk, 5 doses) while normal brain tissue did not show any staining. Furthermore, immunohistochemistry of rat brains with human GBM confirmed tumoral expression of PDGFRα. Surprisingly, host stromal cells within the tumor mass as well as in normal brain tissue stained for PDGFRα.
Pharmacokinetic analysis of serum from rats with orthotopic GBM showed MEDI-575 did not drop below 100 µg/mL for 3 days after five doses of MEDI-575 at 10 mg/kg, while in the brain MEDI-575 was detected at 26.0 µg/mL 48 hours after the final dose. This indicated that the BBB of rats with implanted GBM allowed MEDI-575 to pass into the rat brain. The resulting exposure levels of MEDI-575 in the GBM tumor might be sufficient to elicit a response based on active

serum levels in mouse GBM xenograft studies (30 to 80 µg/ml). These observations support ongoing Phase 2 clinical development of MEDI-575 to treat patients suffering from GBM after relapse from treatment and surgery.

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Development of PTK6 inhibitor which inhibits tyrosine phosphorylation in vivo.

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PTK6 (also known as breast tumor kinase, Brk) is a member of a novel family of non-receptor protein tyrosine kinases that include PTK6, Srm, and Frk. PTK6, which is closely related with Src family, has an SH3, SH2 and catalytic domain but lacks myristoylation signal. PTK6 is found in more than 60% of breast tumors and breast cancer-derived cell lines but not in normal mammary tissues and benign lesions. Upregulation of PTK6 plays a role in proliferation, anti-apoptosis, migration, and anchorage-independent growth in diverse cell lines. Thus, development of PTK6 inhibitor would be important to control PTK6-positive hyperplasia including breast cancer. In an attempt to screen inhibitors of PTK6, a non-isotopic PTK6 assay system was established using GST-PTK6-Linker(ΔN)-Kinase, which is a minimal domain for catalytic activity. Using the assay system, chemicals which inhibited the catalytic activity of PTK6 were selected from a chemical library. Derivatives of a lead compound which has a novel scaffold were synthesized and further analyzed for the PTK6 inhibitory function in vitro. Then, selected compounds were examined for in vivo inhibition of PTK6 catalytic activity using PTK6-expressing HEK 293 cells and cytotoxicity to human foreskin fibroblasts using MTT assays. Finally, BC-223 (IC50 = 0.50 uM) showed the strongest inhibition to PTK6 in vivo and little cytotoxicity. BC-223 would be a valuable candidate for PTK6-inhibiting drug which can be applied for treatment of PTK6-positive carcinomas including breast cancer. [Supported from Seoul Research and Business Development grant and BK21 trainee program]

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Synthesis of Polyaza[N] Helicenes Potential Inhibitors of Telomerase.

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Telomeres consist of DNA and bound proteins located at the end of human chromosomes. Telomeric DNA are double stranded and contain the sequence (TTAGGG/CCTAAA). Telomeric DNA are known to form four-stranded complexes called G-quadruplexes in which four Guanine bases are linked to one another. The enzyme telomerase catalyzes the synthesis of telomeric DNA repeats and has been associated with cell immortalization. Because telomerase is over expressed in cancer cells, it makes a valuable target in oncology. G-quadruplex stabilization has been shown to directly inhibit telomerase catalytic activity. G-quadruplex formation at the telomeres can be promoted by natural proteins and small molecules and thereby interfere with gene expression. Such Potential inhibitors of telomerase have been found to be either large in size or helical in shape in order to effect binding. Helix-shaped molecules also known as [N]helicenes have emerged as potent ligands to G-quadruplex and therefore as telomerase inhibitors. We have synthesized polyaza[N]helicenes, compounds 7 and 9 as potential inhibitors to telomerase. Our synthesis was based on an iterative tandem of reactions, Ullmann coupling followed by diazotization first to produce substituted benzo[c]cinnoline and then to afford racemic polyaza[5]- and [7]helicenes 7 and 9.

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Anthracenedione-methionine conjugates as a novel class of topoisomerase II-targeting anticancer drugs with favorable resistance profiles.

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Mitoxantrone (MX) and ametantrone (AT) represent two anticancer anthraquinones inducing topoisomerase II cleavable complex (TOP2cc) and cell death. However, the contributions of two human isozymes (hTOP2α and hTOP2β)-mediated DNA breaks to cancer cell-killing and other biological responses remained unknown. We studied and cross-related the cell-killing, TOP2-targeting and DNA-interacting activities of 1,4-bis(2-amino-ethylamino) MX- and AT-amino acid conjugates (M/AACs) as a potential solution to address their structure-associated isozyme targeting and drug resistance. The cell-killing ability of M/AACs correlate with amounts of DNA breaks induced. Notably, the 1,4-bis-L/L-methionine-conjugated MAC (L/LMet-MAC) induces DNA breaks, cancer cell-killing, apoptosis and anti-tumor activity rivaling those of MX whereas D/DMet-MAC is largely ineffective. Roles of hTOP2α and hTOP2β in the M/AAC-induced cellular responses were studied: (i) MACs induced reversible DNA breaks which can be effectively antagonized by TOP2 inhibitors; (ii) DNA M/AAC-induced breaks, cytotoxicity and apoptosis were reduced in TOP2-deficient conditions; (iii) MACs induced hTOP2α/βcc formation in cells and TOP2-mediated DNA cleavage in vitro. Interestingly, all steric-specific L- and D-form-Met-M/AACs significantly compromise the DNA-unwinding activity associated with MX and AT. Additionally, Met-MACs are poor MDR-1 substrates. These results suggested that both enzyme- and DNA-drug interactions contribute to TOP2 isozyme-targeting and cell death induced by M/AACs and L/LMet-MAC represents a promising class of TOP2-based anticancer drugs with reduced side-effects.

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The Mitotic Parameters of HeLa Cells are Altered by Dehydroleucodine.

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Cancer is a set of diseases in which the body´s cells become abnormal and divide without control. There are countless efforts to find agents to block the uncontrolled cell proliferation. Many sesquiterpene lactones (SL) have been isolated from medicinal plants and present a wide spectrum of biological activity. Dehydroleucodine (DhL) is a SL isolated from Artemisia douglasiana Besser, a plant that grow up in the west of Argentina. Previous results from our laboratory showed that DhL delays the proliferation of HeLa cells, arresting them in G2/M phase of the cell cycle. In this work we analyzed whether DhL affects M phase of HeLa cells. Cells synchronized in G1/S by double thymidine treatment, were stimulated to proliferate with 10% fetal bovine serum in presence of 0-20 μM DhL. The cells were photographed since time 0h of treatment, every 15 min during 32h using a Nikon Eclipse TE 2000-U microscope. The treatment with 10 and 20 μM DhL delayed the entry of cells to mitosis (4.33 ± 1.85 and 4.52 ± 1.06 h respectively) and increased the time that the cells spend in division (7.01 ± 1.07 and 6.43 ± 0.58 h respectively). 20 μM DhL extended 0.17 ± 0.04, 5.07 ± 0.58 and 1.78 ± 0.24 h the time of prophase, metaphase-telophase and cytokinesis respectively. These results show that DhL generated a delay of HeLa cell to enter to mitosis and an increase of the time that spend in division.

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Cloning of a novel centrin target.

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The origin of many disease states has been linked to genetic mutation, defects in gene expression, nuclear excision repair and ribosome biogenesis. Centrin, a calcium binding protein, has recently been found to regulate some of these processes along other target proteins within the nucleus. One target, Krr1, contains a K homology (KH) domain; which has been identified as a nucleic acid recognition motif, required for proper processing of pre-rRNA, for synthesis of 18S rRNA, and for the assembly of the 40S subunit. Our initial findings have identified a putative centrin binding site located within the KH domain of Krr1 within the Homo sapiens (Hs) centrin 2 (Hscen2-HsKrr1 complex) using bioinformatics tools. In this study, the KH domain (192 bp) was amplified by PCR and then ligated to the expression vector pET100 which adds a His tag to the peptide. Colony PCR was performed to identify the E. coli colonies that have been transformed effectively with the desired recombinant. The KH domain was then expressed in E. coli cells. To identify the presence of this peptide in the bacteria, an SDS-PAGE was performed. The overexpressed KH domain peptide will be purified and used for interaction studies with Hscen2.

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Tissue Inhibitors of Metalloproteinase 2 Up-regulates Beta-catenin Activity in Melanoma Cells.

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The tissue inhibitor of metalloproteinases family including tissue inhibitor of metalloproteinases-2 (TIMP-2) regulates the activity of multifunctional metalloproteinases, which regulate the pathogenesis of melanoma and other diseases. Beta-catenin plays a critical role in cell proliferation and tumorigenesis. We have established stable melanoma cell lines: parental A2058 expressing, A2058T2 overexpressing and A2058T2R underexpressing TIMP-2. In the current study, we used these cell lines to examine the relationship between TIMP-2 expression and beta-catenin activity. The TIMP-2 regulation of the beta-catenin activity was investigated at different levels, including total and phosphorylated beta-catenin, cellular location of beta-catenin, beta-catenin transcriptional activity, target gene expression, and cell proliferation. We found that the beta-catenin significantly increased in the A2058T2 overexpressing TIMP-2. TIMP-2 overexpressed cells had the lower basal level beta-catenin compared to the parental A2058 cells. The distribution of beta-catenin was also altered by TIMP-2-over-expression. Beta-catenin showed strongest immunofluorescence in circumfrential rings around each cell at sites of cell-cell contact in A2058T2, weaker staining in A2058T2R. Functionally, the cells growth was slower in TIMP-2 under-expression cells compared to A2058 cells and A2058T2 overexpressing TIMP-2. Our data demonstrate that the expression level of TIMP-2 protein can directly modulate the beta-catenin pathway in human melanoma cells. We explored the mechanisms of TIMP and beta-catenin in regulating proliferation in the cancer cells. Insights in the molecular mechanisms of TIMP in cancer will provide promising opportunities for therapeutic intervention.

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A novel ATM inhibitor that can target cell cycle checkpoints and increase radiosensitivity.

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The ATM kinase is one of the essential DNA damage response elements that play critical roles in regulation of cell cycle checkpoints in the presence of DNA damage. The enzymatic activity of ATM, enhanced after ionizing radiation (IR), is responsible for ample phosphorylation of downstream targets. The functional role of phosphorylation is to activate cell cycle arrest in order to coordinate DNA repair. Specific inhibitors that can interference with the kinase activity of ATM deem to be valuable for studying DNA damage response mechanisms as well as to be explored as sensitizing agents to cancer radiotherapy. Here we report that Quercetin (3, 3 ', 4', 5, 7 - Five-flavonoids) , a main component of flavonoids, with multifunctions on immune function, anti-oxidation, anti-viral, anti-inflammatory, and cardiovascular protection, possess properties to inhibit ATM in the DNA damage response. We show that quercetin abrogates the activation the G2/M checkpoint in response to IR. Further we show that ATM activation is diminished after IR when treated with quercetin. We also tested the *in vitro* radio-sensitization activity of quercetin in DLD1, HeLa and MCF-7 tumor cell lines by colony formation assays. We find that quercetin can significantly increase tumor radiosensitivity. The *in vitro* Sensitization Enhancement Ratio in DLD1, HeLa and MCF-7 cells were 1.87, 1.65, and 1.74, respectively. However, ATM-deficient cells failed to be sensitized by quercetin, indicating a specific inhibitory effect of quercetin on ATM. We also assessed *in vivo* activity in the DLD-1 human colorectal cancer xenograft model in nude mice. We find that the mean doubling time of tumor xenografts is significantly increased in irradiated mice treated with quercetin. Taken together, we have identified that quercetin is a novel ATM inhibitor that can target ATM-mediated pathways and enhance tumor radiosensitivity.

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Enhancement of Hepatic Phase I and Phase II Enzyme in the Carcinogenesis Prevention by an Anti-inflammatory Sesquiterpene α-Humulene.

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Hepatocytes play a primary role in carcinogen detoxification through the functions of "phase I" and "phase II" drug-metabolizing enzymes, which also work in other cell systems and organs. Cancer chemoprevention is suppression of carcinogenesis by means of naturally occurring or synthetic chemicals and, as the molecular mechanism, those enzymes are considered to protect cells from various xenobiotics. In the present study the effects of α-humulene (HUM) on the expression and activities of hepatic phase I and phase II enzymes have been analyzed. HUM is a sesquiterpene compound found in the essential oils of various plant species and is suggested to be anti-inflammatory and anticarcinogenic. First, the antitumor activities of HUM were confirmed by two-stage mouse skin carcinogenesis test. Skins were exposed to a tumor initiator 7,12-dimethylbenzo[a]anthracene and one week later were treated repeatedly with HUM followed by a tumor promoter 12-O-tetradecanoylphorbol 13-acetate for 20 weeks: HUM suppressed tumor formation by 75%. Next, hepatic enzymes were analyzed by using the tissues from mice treated orally with HUM once a day for 4 successive days. The contents of total cytochrome P450 and cytochrome b5 increased by up to 54% and 32% due to HUM. The activities of hepatic phase I enzymes 7-ethoxycoumarin O-deethylase, 7-ethoxyresorufin O-deethylase and 7-propoxyresorufin O-depropoxylase increased by up to 92, 47 and 885%, respectively. As to hepatic phase II enzymes, glutathione S-transferase (GST) activities toward 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene increased by up to 67% and 178%,

respectively, with western blotting analysis showing slight elevation of the GST subtype contents. Moreover, HUM significantly elevated the GST activity toward potent carcinogen 4-nitroquinoline 1-oxide, NAD(P)H:quinone reductase activity and hepatic glutathione contents. Taken together, HUM has been demonstrated to be a bifunctional inducer, which enhances both the phase I and phase II enzymes of the liver. The present results suggest that the carcinogenesis suppression by such anti-inflammatory or anticarcinogentic molecules as HUM involves modulations of the phase I and/or II drug-metabolizing enzymes.

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The Effects of Chitosan on Localization, Expression and Secretion of Osteopontin in an Ovarian Cancer Cell Line, SKOV-3.

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The most fatal of the gynecological cancers, epithelial ovarian cancer has a high recurrence rate despite current treatments of surgery, followed by chemotherapy and radiation. Although elevated levels of osteopontin (OPN) are always present in the ovaries, recent studies have implicated this protein as a novel biomarker for ovarian cancer. Novel treatments with fewer adverse effects than current treatments are currently being investigated. Chitosan, a natural polysaccharide that is biodegradable and biocompatible, may serve as a potential alternative treatment for ovarian cancer, and may function by regulating OPN expression and secretion. Therefore, the objective of this study was to determine the effects of chitosan on the localization, expression, and secretion of OPN in SKOV-3 cells. We hypothesized that the expression and secretion of OPN protein will be down-regulated in a dose-dependent manner. To test this hypothesis, SKOV-3 cells were treated with 0, 50, 100, 250, and 500, ng/mL of chitosan for 48 hours. Cells were harvested; total protein isolated and then subjected to SDS-PAGE and Western blot analysis. Immunocytochemistry was also used to determine localization of OPN in SKOV-3 cells, while an ELISA assay was used to determine OPN secretion. Osteopontin (OPN) was localized on the membrane of SKOV-3 cells. The expression and secretion of OPN was significantly decreased in chitosan treated-SKOV-3 cells in a dose-dependent manner. These data suggest that chitosan may inhibit ovarian cancer cell growth by decreasing the expression and secretion of OPN.

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A Natural Product, Schweinfurthin A, Inhibits the Growth Kinetics of Malignant Peripheral Sheath Tumor Cells with Quantitative Effects on Actin Cytoskeleton Morphology.

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Malignant Peripheral Nerve Sheath Tumors (MPNST) are a frequent cause of morbidity and mortality in Neurofibromatosis type 1 patients. One of the driving forces in the development of these tumors is loss of function of the NF1 gene. We previously found that NF1-deficient astrocytoma and MPNST tumor cell lines derived from both patients and a Nf1-/+;Trp53-/+ (NPcis) mouse model are highly sensitive to a natural product Schweinfurthin A (SA). Conversely, when the NF1 Ras Regulatory domain was re-expressed in these cells, they became resistant to SA. (Turbyville, et al 2011). In addition to effects on proliferation, this small molecule has unique effects on the actin cytoskeleton mediated, at least in part, by the Rho pathway, which is downstream of NF1. The objective of this study is to better characterize the

effect on proliferation and on the actin cytoskeleton, and to see whether these effects are related or merely epiphenomena. Here we show that two independently isolated MPNST cell lines from the NPcis mouse are differentially sensitive to SA, that the more sensitive cells have slower growth kinetics and are unable to recover log growth kinetics as quickly as the more resistant cells. In order to elucidate further the cytoskeleton changes we attached SA and DMSO treated cells to concave fibronectin micropatterns on coverslips. We observed differences in the ability of the cell lines treated with DMSO control to maintain convexity on the non-adhesive edges of the cells, which is masked by the random morphology observed when cells are grown on regular coverslips. Specifically, quantification of cortical actin curvature between the two untreated cell lines was significantly different ($p < 0.01$) implicating a functional relationship between membrane tension and proliferation. Treatment of cells on micropatterns with 100 nM SA revealed remarkable morphological differences that were not observed in normal culture. On micropatterns the more sensitive cells showed greater concavity as compared to the more resistant cells, which were markedly more convex. Taken together these results suggest a functional relationship between cell shape, membrane tension, adhesion and proliferation. Future studies will attempt to elucidate these findings using quantitative mathematical modeling.

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Crude Rosehip Extracts Prevent AKT/mTOR and MAPK Signaling in Glioblastoma Cells Which Results in Cell Cycle Arrest.

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Glioblastoma multiforme (GBM) are aggressive malignant tumors that develop in the brain. GBMs demonstrate increased rates of cell proliferation, a lack of apoptosis as well the propensity to migrate to distal sites in the brain. The clinical management of glioblastoma consists of surgical resection followed by radiotherapy and chemotherapy. However, this treatment regimen is not very effective and more effective therapies need to be developed. Therefore, we investigated the use of natural products as anti-tumor agents against brain tumors. We tested the antiproliferative capacity of extracts from the Rosehip (*Rosa* canina) plant. Rosehip extracts have been used as dietary supplements to relieve symptoms associated with diarrhea, gastritis, rheumatoid arthritis and have recently been shown to have anti-oncogenic properties. We tested the efficacy of rosehip extracts in three human GBM cell lines A-172, U-251MG and U-1242MG. All of the cell lines treated with Rosehip extracts (1 mg/ml - 25 µg/ml) demonstrated a decrease in cell proliferation. The Rosehip extract-mediated decrease in cell proliferation was equal or greater than the decrease of cell proliferation observed when LY294002 (20 µM) or U0126 (10 µM), known Akt and Mek inhibitors respectively, were utilized. Additionally, pretreatment of the U-1242 MG cells with the Rosehip extracts (1 mg/ml - 25 µg/ml) decreased Akt and p70S6K as well as Erk and Fra-1 phosphorylation, suggesting these extracts prevent GBM cell proliferation by blocking Akt/mTOR and MAPK signaling mechanisms. Results from colorimetric apoptotic assays and flow cytometry studies demonstrate that rosehip extracts are inhibiting cell proliferation by promoting cell cycle arrest but does not elicit apoptosis. Taken together these data suggest that Rosehip extracts are capable in slowing GBM cell proliferation by altering the cell cycle. More importantly, rosehip extracts may serve as an alternative or supplement to current therapeutic regimens for GBMs.

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Cytotoxic Properties of Natural Plant Extracts on Various Human Malignant Cell Lines.

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OBJECTIVE: This work aims to compare the cytotoxic properties of various natural plant extracts (phytochemicals) on human malignant tumor cell lines, and to investigate the mechanisms through which phytochemicals exert the cytotoxic effect on tumor cells. METHODS: 10 natural plant extracts (cinnamon leaf oil, its constituents including eugenol, methyl eugenol, o-eugenol, isoeugenol and trans-cinnamaldehyde, resveratrol, luteolin, zerumbone, oleanolic acid) were screened for cytotoxicity against human malignant melanoma cell line (WM1552C) by using a MTS proliferation assay in vitro. The two plant extracts that showed the highest cytotoxicity were tested on three additional more invasive human malignant melanoma cell lines (A-375, SK-MEL-5,SK-MEL-28), three human breast cancer cell lines (MDA-MB-468, BT-20, T47D) and a human prostate cancer cell line (PC-3). The apoptosis induction of WM 1552C cells was examined by Annexin V- FITC/PI staining followed by flow cytometry, Hoechst 33342 stain, pan-caspase inhibitor and determination of caspase 3 expression by RT-PCR and Western Blot. RESULTS: All natural plant extracts except o-eugenol, isoeugenol and methyleugenol showed strong inhibitory effect on the proliferation of maglignant melanoma WM1552C cells in both time and dose-dependent manners, with IC50 values at 48h of the range between 10.8 - 39.2 μM. Zerumbone exhibited significant cytotoxicity on all four malignant melanoma cell lines, three breast cancer cell lines and prostate cancer cell line PC-3. Zerumbone significantly increased the percentage of Annexin V- FITC positive WM1552C cells, and caused apoptosis-characteristic fragmented nuclei in WM1552C cells. The expression of apoptosis executor caspase 3 were elevated at both mRNA and protein levels in response to zerumbone. In addition, pan-caspase inhibitor Z-VAD at 0.5, 1 or 5 μM significantly alleviated the cytotoxicity of zerumbone at 5, 10 or 25 μM, and decreased the percentage of Annexin V- FITC positive WM1552C cells. Furthermore, pre-treatment with reducing agent glutathione significantly reduced the cytotoxicity of zerumbone on WM1552C cells. CONCLUSION: Zerumbone exhibited strong cytotoxicity to various human malignant cell lines. Zerumbone exerts its cytotoxic effect on malignant melanoma WM1552C cells by inducing apoptosis and oxidative stress. Zerumbone and its derivatives may be promising chemo-preventive and chemotherapeutic agents for cancers.

TUESDAY, DECEMBER 6

Regulation of Actin Dynamics II

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Drosophila Nurse Cell Dumping: Providing New Insights into Prostaglandin-Dependent Actin Remodeling.

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Prostaglandins (PGs) are small bioactive lipids that function as paracrine/autocrine hormones that have been shown to mediate actin cytoskeletal remodeling in a cell-type dependent manner. Previously, we have shown that PGs temporally and spatially regulate actin cytoskeletal dynamics during *Drosophila* nurse cell dumping, providing a novel, developmental model in which to study PG-dependent actin remodeling. Lasting just thirty minutes, *Drosophila* nurse cell dumping is a highly dynamic process requiring spatial and temporal coordination of active actin cytoskeletal remodeling in order for the nurse cells to supply their cytoplasmic contents to the oocyte. Specifically, disruption of PG signaling results in temporal, spatial, and structural defects in cytoplasmic actin filament bundles. Using both fixed and live imaging, we are developing quantitative parameters to define precise roles for PG signaling in mediating actin remodeling. In particular, we are interested in how filament length, width, number, density, elongation rate, and overall structure, are altered upon pharmacologic and genetic perturbation of PG signaling. In order to visualize real-time actin dynamics *in vivo*, we have generated transgenic flies expressing either Lifeact::mEGFP or Ftractin::mEGFP/tdTomato under the control of the UAS/Gal4 system. We have observed that germline expression of Lifeact results in disruption of normal actin remodeling during follicle development, leading to female sterility; both actin remodeling and fertility appear normal when Ftractin::tdTomato or Utophin::GFP (gift from Thomas Lecuit) is expressed within the germline. In addition to quantitatively characterizing the morphological defects associated with loss of PG signaling, we are working to uncover the mechanism by which PG signaling converges upon the actin cytoskeleton. In order to address this question, we are using an ongoing pharmaco-genetic interaction screen to identify actin binding proteins and regulatory molecules acting to mediate actin cytoskeletal dynamics downstream of PG signaling during late-stage *Drosophila* oogenesis. By combining quantitative morphometric analysis with robust genetic tools, we can begin to elucidate the mechanisms by which PG signaling mediates actin cytoskeletal remodeling.

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Drosophila nurse cell dumping reveals a novel interaction between prostaglandin signaling and Fascin.

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While actin cytoskeletal dynamics are known to be regulated by prostaglandins, lipid signals produced downstream of cyclooxygenase (COX) enzymes, the mechanisms by which PGs mediate this remain unknown. *Drosophila* oogenesis provides a model for studying how prostaglandin signaling affects actin remodeling. During oogenesis, a process called nurse cell dumping occurs. This process requires active remodeling of the actin cytoskeleton to allow the nurse cells to squeeze their cytoplasmic contents into the growing oocyte. Using this model, we have previously shown that prostaglandins are required for actin remodeling during nurse cell dumping, and that Pxt is the *Drosophila* COX-like enzyme. A screen utilizing our *in vitro* follicle

maturation assay (Spracklen, Meyer, and Tootle, unpublished data) identified Fascin (*singed* in *Drosophila*) as a downstream target of prostaglandin signaling. Fascin is an actin bundling protein that has previously been shown to be required for actin filament formation during oogenesis and nurse cell dumping. Here, we show that *fascin* and *pxt* mutants display similar actin remodeling defects in nurse cells. Reduced Fascin levels enhance the nurse cell dumping, and thus actin remodeling, defects of both COX inhibition and reduced Pxt levels. Additionally, over-expression of Fascin in the germline, using the UAS-Gal4 system, suppresses the effects of COX inhibition. Importantly, Fascin levels, both mRNA and protein, are not affected by alterations in prostaglandin signaling. Prostaglandin signaling does not appear to globally affect actin bundling, as another actin bundling protein Villin fails to interact with Pxt or COX inhibition. These data indicate that one role of prostaglandin signaling in regulating actin remodeling is to modulate Fascin activity. Current efforts are focused on determining the mechanism by which prostaglandins regulate Fascin. This novel interaction between prostaglandin signaling and Fascin has implications beyond nurse cell dumping, as Fascin is required for filopodia formation and cell migration, including during cancer cell migration.

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The role of F-actin in vesicular secretion.

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Acute pancreatitis is an inflammatory process that leads to auto-digestion of pancreatic tissue by premature activation of digestive enzymes within pancreatic acinar cells. One of the prominent cellular events in acute pancreatitis is formation of vacuoles and rapid disruption of the actin cytoskeleton surrounding the lumen of pancreatic ducts. Many observations have shown that secretory granules are coated with F-actin in response to secretagogue stimulation in healthy pancreatic acinar cells however the role of F-actin remodeling during granular secretion has remained unclear. Therefore, this study investigates the regulatory role of F-actin coating of secretory granules in pancreatic acinar cells. It is hypothesized that F-actin coating stabilizes the fused granule during exocytosis and this F-actin framework resists the force of granule content expansion.

Experiments were performed using clusters of acini, freshly isolated from the pancreas of 6-8 week-old male CD-1 mice. Acini were bathed in extracellular dye, sulforhodamine B before two-photon exicitation imaging was performed. The diameter of fusing granules was measured over a short period of time as they appeared after stimulation with the agonist, acetylcholine (600 nM). Under normal conditions, the granule size transiently increased at 5 seconds compared to the time of appearance and it remained constant after transient granule expansion. After treatment with Latrunculin B, actin perturbing drug, the granule diameter increased 1.25 fold of the initial diameter after 20 seconds of fusion. Also our preliminary data examined in confocal microscopy has shown that Latrunculin B inhibited F-actin coating of secretory granules. To determine the time-course and kinetics of F-actin coating of secretory granules in real time, we employed transgenic mice which express pEGFP-Lifeact (Riedl et al. 2008), which is a short peptide that has low affinity binding to F-actin. Two- photon live imaging of acinar cells from the transgenic mice was performed. A number of regions of interest were selected on the granule fusion site and the F-actin signal during granule fusion was tracked. It was observed that F-actin coating forms 6.48 +/- 0.94 s (n=55 granules) after the point of granule fusion followed by a monotonic rise as the granule becomes fully coated.

In conclusion, the results of this study suggest that loss of F-actin during secretion weakens the secretory vesicle membrane and destabilizes granules. This supports the idea that impaired F-actin polymerization leads to retention of enzymes in the cytosol which may contribute to auto-digestion of the vacuolar membrane in acute pancreatitis. Also it is further hypothesized that F-

actin granule coating will be delayed in models of pancreatitis that allows swelling of the retained content to drive dramatic increase in granule volume. We are currently working on examining the kinetics of F-actin coating during granule swelling to define the potential relationship between F-actin coating and acute pancreatitis.

Riedl, J., A. H. Crevenna, et al. (2008). "Lifeact: a versatile marker to visualize F-actin." Nat Methods 5(7): 605-607

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Timing of endocytic actin patch assembly in fission yeast.

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Yeast actin patches assemble at the sites of clathrin-mediated endocytosis, where actin assembly by the Arp2/3 complex helps promote endocytic internalization using mechanism conserved from yeast to man. The Arp2/3 complex is controlled by several nucleation promoting factors including Wiskott-Aldrich Syndrome protein Wsp1 and myosin-1 Myo1. Although previous work has shown that the presence of Myo1 and activation of the Arp 2/3 complex by Wsp1 are essential for patch internalization, there is limited information on the mechanisms responsible for Wsp1 and Myo1 localization and regulation. To dissect these mechanisms, we determined the timing of localization for actin patch components in a model organism, fission yeast Schizosaccharomyces pombe, and dependencies of their localization on Wsp1 or Myo1. We selected 24 proteins that either were expected to arrive at patches early and might assist in localizing Wsp1 to the patch, or were homologs of proteins that had previously been shown to interact with WASp in other systems, such as budding yeast. We used genetic crosses and tetrad analysis to combine each specific protein tagged with Green Fluorescent Protein (GFP) either with mCherry-tagged actin-binding protein Fim1 or with myo1 and wsp1 deletions. We imaged the strains by spinning disk confocal microscopy and carried out detailed image analysis to classify proteins into four different classes. The first class included Chc1, Ede1, End3, Ent1, Sla1, Pan1, and End4. These early proteins arrived at the patch earlier than Wsp1 by at least 10 seconds. The second class included Hob1, Hob3, Bzz1, Lsb1, Lsb4, Lsb5, and Ldb17. These potential regulating proteins arrived at either the same time as Wsp1 or after Wsp1. The third class included two proteins, Cdc15 and Bbc1, that assembled into patches and remained at the plasma membrane with Myo1, suggesting that they function together with Myo1 rather than Wsp1. Analysis of genetic dependencies of localization confirmed this classification. The fourth class included Lsb6, Nak1, Ucp8, and Ent3. These proteins did not localize to patches despite prior reports that Lsb6 and Nak1 interact with WASp in vitro. Some of the examined markers also localized to other cellular structures in addition to the patches (Cdc15, Bbc1, Lsb1) or instead of the patches (Nak1, Ent3). This classification has allowed us to concentrate on proteins that potentially recruit and regulate Wsp1 and Myo1 during endocytosis, in particular BAR domain proteins that may directly link membrane curvature with regulation of actin assembly.

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Multifunctionality of WH2 domains : lessons from Spire, Cordon-Bleu and VopF.

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WH2 domains are ~ 30 residues long, intrinsically disordered, actin binding modules, functionally evolved from ß-thymosins, that are present in a large number of proteins involved in motility. In spite of a poor signature, they all bind actin identically. Their acknowledged functional

versatility is amplified in tandem repeats found in proteins like Spire (4 WH2) and Cordon-Bleu (3 WH2), which play a role in early development and axis patterning processes, and in the bacterial effectors VopF from Vibrio cholerae and VopL from Vibrio parahaemolyticus. Their multiple functions in the regulation of actin assembly include G-actin sequestration, participation in filament barbed end assembly, filament nucleation, regulation of barbed end dynamics and filament severing. We are interested in understanding the structural basis for the multifunctional regulation of actin and how diverse profiles of regulation emerge from different selections of these multiple activities. Comparative biochemical analysis of Spire, Cordon-Bleu and VopF allows to propose simple models for filament nucleation and severing by WH2 domains.

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Fimbrin organizes the fission yeast actin cytoskeleton by competing with tropomyosin and crosslinking actin filaments.

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Cells simultaneously coordinate the assembly and maintenance of different actin filament-based structures to facilitate diverse fundamental processes. Fission yeast builds actin filaments with precise architectures and dynamics for Arp2/3 complex-dependent endocytic patches, and formin-dependent polarizing actin cables and the cytokinetic contractile ring. Partially overlapping sets of actin-binding proteins are required for the establishment, maintenance, and disassembly of the actin filaments within these structures. A major challenge is to determine how specific subgroups of actin-binding proteins localize to a particular structure and collectively influence actin filament organization and kinetics. Through a combination of in vitro and in vivo approaches, we discovered a novel paradigm for controlling actin filament dynamics in fission yeast. A primary role of the actin filament crosslinker fimbrin Fim1 is as a ‘gatekeeper’ that controls the access of other actin-binding proteins to filaments. Specifically, we found that Fim1 prevents tropomyosin Cdc8 from binding to actin filaments, permitting cofilin Adf1-mediated filament disassembly. Therefore, the balance between Fim1 and Cdc8 is important for both endocytic actin patches and contractile ring assembly. On the other hand, filament crosslinking by Fim1 is also critical. Examination of cells exclusively expressing a truncated version of Fim1 that can bind but not crosslink actin filaments revealed that the crosslinking activity of Fim1 is important for all three actin structures. Therefore fimbrin Fim1 has diverse roles as both a filament ‘gatekeeper’ and as a filament crosslinker.

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Cytoskeletal tension modulates cellular responses to mechanical stimuli.

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The ability to measure real-time mechanosensitive events at sub-cellular level in response to discrete and physiologically relevant mechanical stimulation is the critical component in understanding mechanically-induced cellular remodeling. RhoA activation increases vascular smooth muscle cell (VSMC) contraction by promoting actin polymerization and focal adhesion (FA) formation. It has been shown that the RhoA/Rho-kinase pathway is up-regulated in animal models and also in human patients with hypertension and atherosclerosis, and treatment with Y-27632, a specific Rho-kinase inhibitor, has a protective effect inducing a decrease in VSMC proliferation and contraction. Our data show that RhoA-T19N induces low cytoskeletal tension measured by the absence of stress fibers in the cell body with actin fibers present only at the cell edges, while RhoA-Q63L induces FA and F-actin fiber formation, hence high cytoskeletal tension. The F-actin area measurements present the highest F-actin in RhoA-Q63L expressing

cells and lowest F-actin in RhoA-T19N expressing cells. Moreover, cell stiffness is directly dependent upon the cytoskeletal tension. Thus, the cells expressing RhoA-T19N are indeed much softer than the ones expressing RhoA-wt or RhoA-Q63L, while the RhoA-Q63L expressing cells are stiffer than both other treatments, respectively. This result independently verifies the decrease in the cytoskeletal tension when RhoA/Rho-kinase pathway is inhibited and the increase in the cytoskeletal tension when it is activated. Thus, our imaging data correlate well with the stiffness measurements. Further, we tested whether the cell response to tensile stress induced by AFM tips modified with glass beads coated with fibronectin depends upon cytoskeletal tension. Cells expressing RhoA-T19N are detaching from the AFM tip at a low force level because the FA formed around the AFM tip is weak due to absence of F-actin network. However, RhoA-Q63L expressing cells are found to resist to the tensile stress and exhibited a high reactive response with time. By contrast, cells treated with ML7, a potent myosin inhibitor in VSMC, show a saturation curve due to the blocking of the actomyosin contractile apparatus, hence a reduced cytoskeletal tension without F-actin disassembly. Taken together, these data show that cell-adaptive response to the applied force is modulated by the cytoskeletal tension.

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Mechanism of the actin cortex and shape stability during cytokinesis.

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The formation and ingression of the cleavage furrow during cytokinesis relies on a controlled reorganization of the actin cortex. Starting from anaphase, the cortical network redistributes from the poles to the cell’s equator and gives rise to the cytokinetic ring, which separates the two daughters cells. Strikingly, cleavage does not only result from localized constriction of the ring, but also requires coordination of cortical activities at the poles of the cell. Here, we demonstrated that the presence of contractile polar cortex makes cytokinesis an inherently unstable process, where any imbalance in contractile forces between the two poles compromises the accurate positioning of the constriction ring. We show that limited asymmetric polar contractions occur during cytokinesis, and perturbing the polar cortex by number of treatments leads to oscillations in cell shape, resulting in furrow displacement and aneuploidy. A theoretical model based on a competition between cortex turnover and contraction dynamics accurately accounts for the oscillations. We further propose that blebs, membrane protrusions that commonly form at the poles of dividing cells and whose role in cytokinesis has long been enigmatic, stabilize the position of the cleavage furrow by acting as valves releasing cortical contractility. Our findings reveal an inherent instability in the shape of a dividing cell and unveil a novel, spindle-independent mechanism ensuring the stability of cleavage furrow positioning.

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Formation of stress-fiber arrays in fibroblasts spreading on circular adhesive islands.

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Well-organized system of focal adhesion-associated actomyosin cables (stress fibers, SFs) plays a major role in the maintenance of cell shape, generation of cell traction forces, and regulation of cell migration. To study the process of the organization of SF arrays, we plated human fibroblasts onto microfabricated fibronectin-coated circular adhesive islands. Cells spread isotropically over the entire area of such islands, acquiring discoid shape but not being

able to elongate. Despite isotropic morphology, many of these cells eventually form a system of parallel SFs, thus developing an intrinsic polarity. This process takes about ten hours and usually proceeds through several distinct stages. The first SFs appear at the cell periphery and form circumferential ring adjacent to the cell edge. Then the radial SFs begin to grow from the peripheral focal adhesions towards the cell center, while circumferential ("tangential") SFs move centripetally in between the radial SFs, simultaneously undergoing shortening. At this "radial stage" that can persist for several hours, the SF system still preserves the radial symmetry. A prominent transient stage that often precedes the formation of parallel SFs array is synchronous turn of the radial SFs ("cytoplasmic swirl" phenomenon). With some few exceptions, the swirl was oriented anti-clockwise, demonstrating intrinsic chirality of the SF system. Critical size of the adhesive island is required for the proper SF organization. While all the aforementioned stages in the process of SF array organization could be observed on the islands with the area of 1600 μm^2 or larger, the cells on smaller islands (1100 and 700 μm^2) are usually arrested at the stage of circumferential ring or at radial stage, which never succeeded by swirling, and were not able to organize the system of parallel SF. The unifying physical model explaining the main aspects of the SF array organization is presented.

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Model of Actin Turnover in the Lamellipodium that Reconciles Prior Experiment Results.

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The structure of the lamellipodium is a brush-like network of actin filaments interacting with many regulatory proteins. While the role of actin in the lamellipodium is functionally well understood, the local rate of actin turnover appears different in different experiments. Prior FRAP experiments suggested that the fluorescence of the F-actin network rapidly recovers at the leading edge but away from the leading edge the recovery is very slow. Single-molecule fluorescence microscopy (SMFM), however, shows turnover of labeled actin monomers far into the lamellipodium. To investigate this apparent discrepancy we developed models that simulate actin turnover using parameter values and rates obtained from SMFM. We simulate actin in the lamellipodium using a reaction diffusion model where actin changes between F-actin and G-actin. We use two techniques: an approximate PDE method and a stochastic simulation of individual monomers. In the first method concentrations of actin species are considered. The PDE model calculates bulk properties of the actin network, demonstrates time scales for gross changes and aids in initialization of our second approach: simulating individual actin monomers that can transition between G-actin and F-actin. In the stochastic model actin monomers diffuse freely in the lamellipodium; if they transition to F-actin then they stop diffusing and undergo retrograde flow until they transition back to G-actin. This technique produces simulated images to compare to images acquired from experiments. We find that including a range of diffusion coefficients (that effectively simulates oligomers and actin binding to protein complexes) can explain both sets of experimental data. With this assumption: (i) fast diffusing monomers contribute to an overall treadmilling-type turnover with a majority of the polymerization happening at the leading edge and depolymerization happening away from the leading edge (ii) slowly diffusing monomers appear and disappear throughout the lamellipodium and contribute to local remodeling. By making these assumptions we can reproduces FRAP recovery curves and speckle statistics.

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Dynamic instability of actin-like ParM proteins: Theory and experiment.

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Although microtubule dynamic instability was discovered more than 20 years ago, no analytical theory has succeeded in connecting the phenomenology of catastrophe and rescue to underlying biochemical parameters (e.g. rate constants for elongation and GTP hydrolysis). Attempts to formulate such a theory are complicated by the fact that: (1) microtubules probably do not grow by linear, helical assembly; (2) key rate constants, including the rate of GTP hydrolysis within a microtubule, have never been measured; and (3) structural transitions at the ends of growing and shortening microtubules are not well understood. We previously showed that the bacterial actin-like protein ParM, whose assembly drives segregation of large plasmids, is also dynamically unstable. ParM filaments are simpler than microtubules and provide an ideal system for quantitative analysis. In this study we develop an analytical theory of ParM dynamic instability based on a minimal set of assumptions and compare its predictions to stochastic simulations and quantitative experiments. To study ParM assembly dynamics we created stable, chemically crosslinked ParM filaments and used them as seeds to initiate assembly of wildtype ParM. By TIRF microscopy we observed repeated rounds of growth and catastrophe from these stable seeds. Using this system we confirmed that both ends of ParM filaments elongate at the same rate and discovered that they also switch to catastrophic shortening at the same rate. Consistent with a simple cap model, we found that both the filament length and lifetime are exponentially distributed and that the major catastrophe rate decreases with filament elongation rate. Comparing measured parameters to those predicted by theory indicate that the minimal ATP cap is 3-4 protomers and is the same for both ends of the filament. Finally, we observed that 3% of all major catastrophic shortening events experienced ‘rescue’ and switched back to steady elongation after significant periods of shortening.

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F-actin dynamics and migration of human oral keratinocytes are affected specifically by aspartyl proteases 4-6 secreted by *Candida albicans*.

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Cell migration of human oral keratinocytes is affected when the cells are exposed to the pathogenic fungus *Candida albicans*. In a previous study, we showed that the virulence factors, secreted aspartyl proteases (Saps), produced by *C. albicans* target the actin cytoskeleton and stimulate migration of keratinocytes by ~ 2-fold. The mechanism by which Saps affect cell migration is not known. Sap proteins are encoded by a family of 10 *SAP* genes; eight of which (Sap1-Sap8) are secreted into solution by the fungus. To determine the effect on migration of individual Sap family members, we treated keratinocytes with individual GFP-*SAP* promoter strains (*SAP4-SAP6*) and a *SAP4-6*-deficient triple mutant. We used OKF6/TERT-2 cells, an immortalized cell line of human oral keratinocytes co-cultured with hyphae-forming *C. albicans*. Rates of keratinocyte migration and the cellular redistribution of actin-binding proteins (ABP’s) vinculin and the Arp2/3 complex were determined at 0, 3 and 6 hours by multi-mode high resolution microscopy and quantitative image analysis.
OKF6/TERT-2 cells exposed to wild-type *C. albicans* showed that protrusion velocities exhibited migration rates, which were significantly greater than in untreated cultures. The changes in cell migration correlated with changes in the actin filament dynamics and the distribution of ABP’s. Vinculin was localized to newly formed focal adhesions (FAs) after 3 hours of treatment and was

excluded from the leading edge of the lamellipodia that formed after 6 hours of treatment. In contrast, Arp2/3 was absent from actin stress fibers and FAs that formed at 3 hours, but localized at the leading edge of newly formed lamellipodia at 6 hours. Fluorescence intensity profiles illustrated both the coordinated redistribution of ABP's and their colocalization with the actin cytoskeleton. Exposure of OKF6/TERT-2 cells to individual Sap4-6 proteins secreted by *C. albicans* promoter strains also induced actin cytoskeleton alterations and keratinocyte responses appeared similar to those that were co-cultured with wild-type *C. albicans*. These, Sap-induced cellular changes were not observed when OKF6/TERT-2 cells were co-cultured with the *sap4-6Δ/sap4-6Δ* mutant. We conclude that the specific virulence factors Sap4-6 increase cell migration through the activation of a signaling pathway that leads to the simultaneous reorganization of the actin cytoskeleton and its associated proteins. The effect of Sap4-6 on the actin cytoskeleton of oral keratinocytes may be part of fungal invasion strategies.

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LIM-kinase Dually Regulates Lamellipodium Extension by Decelerating Both the Rate of Actin Retrograde Flow and the Rate of Actin Polymerization.

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Actin filament dynamics play a fundamental role in cell migration, spreading and morphogenesis. During migration, cells extend F-actin-dense lamellipodia at the leading edge. Structurally, lamellipodia consist of polarized networks of actin filaments, with barbed ends toward the plasma membrane and pointed ends toward the rear. Whereas polymerization of actin monomers at the tip of lamellipodia is essential for lamellipodium protrusion, it also causes an actin retrograde flow. The speed of actin retrograde flow is controlled by the rate of actin polymerization, the stiffness of cortical membranes, the stability of actin filaments, and the rigidity with which actin filaments are anchored to the substratum. The rate of lamellipodium extension is determined by the balance between the rate of actin polymerization and the rate of actin retrograde flow. However, the cellular mechanisms regulating the rate of lamellipodium extension are not well understood. Cofilin is a key regulator of actin filament dynamics by stimulating actin filament disassembly near the pointed ends of actin filaments. LIM-kinase-1 (LIMK1) regulates actin dynamics by phosphorylating and inactivating cofilin. In this study, we examined the roles of LIMK1 and cofilin in lamellipodium extension by measuring simultaneously the rates of actin polymerization, actin retrograde flow and lamellipodium extension, using time-lapse imaging of fluorescence recovery after photobleaching (FRAP) of YFP-actin. In the non-extending lamellipodia of active Rac-expressing N1E-115 cells, LIMK1 expression decelerated and LIMK1 knockdown accelerated the rates of actin polymerization and actin retrograde flow, while cofilin expression accelerated and cofilin knockdown decelerated these rates, indicating that LIMK1 has a role in decelerating actin turnover by inhibiting cofilin. In the extending lamellipodia of neuregulin-stimulated MCF-7 cells, LIMK1 knockdown accelerated both the rate of actin polymerization and the rate of actin retrograde flow, but the accelerating effect on retrograde flow was greater than the effect on polymerization, thus resulting in a decreased rate of lamellipodium extension. These results indicate that LIMK1 has a dual role in regulating lamellipodium extension by decelerating both the rate of actin retrograde flow and the rate of actin polymerization and that in MCF-7 cells, endogenous LIMK1 contributes to increase the rate of lamellipodium extension by decelerating actin retrograde flow more effectively than decelerating actin polymerization.

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Remodeling and Generating the Cortical F-actin Array.

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Dynamic remodeling of cortical actin is essential for polarized growth in plant cells. To investigate factors that drive cortical actin dynamics, we imaged F-actin in cells lacking particular actin binding proteins. We found that proteins that mediate actin depolymerization, not polymerization, are essential for driving rapid remodeling. For example, RNAi of actin depolymerizing factor (ADF)/cofilin inhibits polarized growth and results in a cortical F-actin array that has dynamics similar to cells treated with the actin stabilizing drug, Jasplakinolide. Knock out of Actin Interacting Protein (AIP) 1 results in an intermediate phenotype. Although detailed analysis of cortical F-actin dynamics did not reveal a difference in severing between ΔAIP1 and wildtype plants, we did observe that ΔAIP1 plants had three-fold less dynamic filament ends as well as a dramatic increase in bundling. These data suggest that AIP1 is a negative regulator of actin bundling *in vivo*.
To study the contribution of proteins that promote polymerization, we investigated the role of formins in the cell cortex. Formins are evolutionarily conserved and promote actin polymerization by nucleating and elongating actin filaments. In plants, class II formins are essential for polarized growth. Class II formins have an N-terminal phosphatase tensin (PTEN) domain that binds $PI(3,5)P_2$ and is essential for interacting with the cell cortex. Using variable angle epifluorescence microscopy, we show that cortical formin localizes to discrete spots, suggesting that there are $PI(3,5)P_2$ rich islands on the membrane. 29% of cortical formin spots move rapidly in and out of the imaging field, remaining for less than one second on the cortex. The remaining cortical formin trajectories grouped into three main categories: linear, random, and stationary (no movement greater than 0.4 µm from the origin). Treatment of cells with latrunculin B reduced cortical formin dynamics, increasing the stationary population from 58% to 91%. The random population was reduced 2.8-fold and the linear population was eliminated. These data suggest that cortical formin movement depends on actin. Importantly, actin is essential for the linear trajectories. Based on formin's ability to processively polymerize actin filaments while remaining associated with the growing barbed end, we hypothesized that the cortical formin spots moving in linear trajectories were generating actin filaments. We used simultaneous imaging of actin and formin to test this and found that cortical formin generates actin filaments *de novo* and along pre-existing filaments. Formin-mediated actin polymerization along pre-existing filaments provides a novel mechanism for bundling and organization of the cortical array.
Taken together, we have found that actin depolymerization via ADF and AIP1 promote rapid remodeling of the cortical array. In contrast, formins do not appear to drive global dynamics, rather they remodel the cortical actin array via polymerization of new filaments. Both generation and rapid remodeling of the actin array are essential for polarized growth.

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Excitable Actin Dynamics in Leading Edge Protrusion and Retraction.

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Periodic patterns of protrusion and retraction have been observed along the leading edge of various cell types during spreading and motility, with many cells exhibiting wave-like propagation of protrusions along the cell membranes. Research has surged in this area as the reproducibility of these patterns offers opportunities to better quantify the regulatory mechanisms of actin polymerization at the leading edge. Previous works suggest possible roles

for membrane-curvature sensing proteins, actin filament nucleators, Rho signaling, myosin contraction, and severing in this process. We imaged the lamellipodia of XTC cells using Lifeact as marker for actin, and analyzed the position of the leading edge using active contours. Constant retrograde flow indicated that protrusions and retractions were driven by fluctuations of the actin polymerization rate. We developed a 1D model of these actin dynamics as an excitable system in which a diffusive, autocatalytic activator causes free barbed end formation and actin polymerization; F-actin accumulation in turn inhibits further activator accumulation. According to the model, the leading edge protrusion rate spikes before the maximum of the free barbed end concentration. To gain mechanistic insight into how polymerization is converted into protrusion, we extended the model in 2D and compare models of free barbed end generation to experiment, using spatiotemporal correlations of leading edge velocity with concentrations of Arp2/3 complex, capping protein and VASP, as a function of distance from the leading edge. Our findings suggest that the Arp2/3 complex participates in an activation mechanism that includes additional diffusive components.

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Numerical analysis of dendritic actin networks formation in relation to cell movement.

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Actin dynamics is essential mechanism for cell movements. Recent studies have revealed a number of proteins that control actin dynamics in cells: for example, G-actin for assembly and disassembly, Arp2/3 for filament branching, ADF/cofilin for filament severing, and capping protein for termination of filament elongation. Regulatory control of these proteins causes dendritic actin networks to form close to the edge of the cell, which mediates cell protrusion, the first step in cell movements. However, because of the difficulty in measuring actin network dynamics in a living cell, it is unclear how the actin network is dynamically remodeled. Therefore, in this study, we developed a computational stochastic model of actin network dynamics based on the dendritic-nucleation hypothesis using Gillespie's algorithm. Because the protruding edge of the cell is thin, and to avoid complicating the model, we assumed growth of the cell edge to be two dimensional. As a first step, velocity of a single actin filament was analyzed based on the reaction rate equations for G-actin polymerization and depolymerization, aging of F-actin, Pi release from F-actin, and nucleotide exchange between G-actin-ADP and G-actin-ATP. As a result, the rate constant of Pi release from F-actin greatly affected its velocity. In addition, from the comparison of the velocity of the single actin filament and that of the protruding edge of the cell, it can be expected that the concentration of free G-actin should be locally high at the protruding edge. We are currently developing a dendritic actin model which includes branching of F-actin via Arp2/3, severing of F-actin by ADF/cofilin, and capping of the barbed end of F-actin by capping protein. Collaboration of ADF/cofilin and capping protein could be an important factors for actin dynamics mediating the local supply of free G-actin.

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Dynamic Localization of Actin Monomers Drives Actin-based Membrane Protrusion and Directional Steering of Nerve Growth Cones.

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Nerve growth cones of axons use actin-based motility to extend and navigate to their specific targets for brain wiring. Dynamic assembly and disassembly of actin filaments (F-actin) from monomeric globular actin (G-actin) generate lamellipodia and filopodial protrusions that, coupled with selective adhesion, steer and drive the growth cone forward. Very little is known about the

role of G-actin in spatiotemporal regulation of actin dynamics underlying growth cone motility. Here we report that dynamic localization of G-actin to the leading edge of growth cones creates regions of high G-/F-actin ratio that promote membrane protrusions for growth cone extension and guidance responses. Using various specific probes for actin monomers, we first showed the local enrichment of a pool of endogenous G-actin at the leading edge of membrane protrusions of the growth cone. Ratiometric live imaging also confirmed the localization of G-actin and further revealed its dynamic nature and association with membrane protrusions of the growth cone. We consistently observed an elevated level of G-/F-actin ratio at the leading edge of protruding lamellipodia and filopodia, which was lost when the protrusion ceased and started to retract. Importantly, the observed localized high G-/F-actin ratio was abolished when pharmacological agents were used to alter the actin dynamics. Finally, a gradient of attractive guidance molecules was found to induce an asymmetric elevation of G-/F-actin ratio that was followed by preferential membrane protrusion and growth cone extension. Together, our results indicate that dynamic localization of actin monomers may represent an important mechanism to create spatiotemporally-restricted regions of high G-/F-actin ratio to promote site-directed actin assembly and membrane protrusion during growth cone migration and directional steering in response to extracellular signals.

1396

Dynamics of actomyosin bundles in migrating keratocytes of goldfish.

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Fish epidermal keratocytes constitute multi-cellular layers on the outside of scales. When fish skin is injured, the wound is rapidly closed up by multilayered epidermal keratocytes migrating from the wound margin. Individual keratocytes have been often studied as a model system of cell migration. During migration, solitary keratocytes maintain a relatively constant shape with extended broad, fanlike lamellipodia at the front. It was previously reported that actomyosin bundles parallel to the leading edge were located at the boundary between the lamellipodia and the cell body. In this study, we found that thick filament bundles could exist at the rear of the cell body, utilizing a special polarized light microscope, called LC-PolScope, developed by R. Oldenbourg. Furthermore, our fluorescence microscopic studies confirmed that signal of actin filaments and phosphorylated myosin II overlapped the bundles in the birefringence patterns, but distribution of β-tubulins didn't. We also investigated the dynamics of the actin bundles with the LC-PolScope. It was revealed in the time-lapse observations that the transverse bundles form alternately at both the front and back of the cell body during the migration, and the thick actin bundles at the rear often compressed the nucleus. These results suggest that the actomyosin bundles at the rear push the heavy nucleus, contributing to the forward movement of the cell body.

1397

Investigation of the Actin Binding and Bundling Properties Of Fission Yeast Alpha-actinin Ain1.

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The actin cytoskeleton is vital for many cellular processes in eukaryotes. It provides the forces for movement during cell migration, cleaves the cell during cytokinesis, and maintains cell polarity. Multiple actin-binding proteins work together to establish a dynamic and regulated actin network that must be assembled at the right time and place for specific cellular functions. One class of actin-binding proteins consists of actin filament crosslinkers. We are focused on the

activities and cellular functions of the crosslinking protein alpha-actinin Ain1 in fission yeast Schizosaccharomyces pombe. Ain1 is thought to be the main actin crosslinker required for cytokinesis in fission yeast, since it is found specifically at the contractile ring and deletion of Ain1 results in cytokinesis defects. Alpha-actin functions as a homodimer that dimerizes via spectrin repeats. Compared with animal alpha-actinin, fission yeast Ain1 contains only two spectrin repeats instead of four. We expect that Ain1 may dimerize weakly, which may have important consequences on binding and bundling actin filaments. Therefore we have expressed and purified full length and fragments of Ain1 from bacterial and insect expression systems, and are utilizing a range of bulk and single filament assays to characterize their actin binding and bundling properties.

1398

Dynamic imaging of free barbed ends in living cells.

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The regulation of polymerization from the barbed (+)-end of the actin filament is essential for motility, cytokinesis, cell polarity and endocytosis. The actin protomer at the barbed-end is unique among the ~2000 protomers within a typical filament, as it is the site of rapid polymerization, it interacts with a unique set of actin binding proteins and is juxtaposed to the plasma membrane. Understanding the mechanisms that control free barbed ends in living cells is made difficult owing to the rarity of the (+)-end protomer and transient (seconds) bursts of actin polymerization. Here we demonstrate high-contrast imaging of free barbed ends in living cells by using Kabiramide C (KabC), linked to tetramethylrhodamine (TMR). TMR-KabC binds selectively and tightly (nM) to the “barbed-end" of the actin protomer. Live cell imaging of TMR-KabC reveals transient accumulation at sites of membrane protrusion in particular within lamellipodia and on motile vesicles. On the other hand, TMR-KabC is absent from older, established filament assemblies that are brightly stained with fluorescent phalloidin, including stress fibers and focal contacts. The high quantum yield of TMR-KabC allows for few-single molecule imaging of free barbed-ends in complex samples.

1399

Effect of antioxidants on cadmium-induced actin glutathionylation and actin dynamics in rat mesangial cells (RMC).

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OBJECTIVES: Cadmium (Cd) is a nephrotoxic metal that accumulates in the proximal tubule and the glomerulus, disrupting normal hemodynamics. In particular, Cd has been shown previously to disrupt F-actin and shift the equilibrium towards a G-actin state in rat mesangial cells (RMC). Additionally, Cd induces oxidative stress by binding to thiol groups with high affinity thereby inhibiting redox enzymes and depleting intracellular glutathione (GSH). Glutathione is a tripeptide antioxidant capable of post-translational modification of protein thiols during oxidative stress, potentially protecting cells from irreversible protein damage. We hypothesized that i) Cd can induce actin glutathionylation in RMC, ii) the antioxidants *N*-acetyl-L-cysteine (NAC) or *tert*-butyl-4-hydroxyanisole (BHA) can attenuate this effect, iii) changes to total GSH (tGSH) levels are correlated with actin glutathionylation, and iv) actin glutathionylation affects actin dynamics *in vitro*. METHODS: RMC were serum-starved prior to treatment with 0.5-10 μM $CdCl_2$. Cells were pre-treated for 1 hr with either 50 mM NAC or 5 μM BHA. Actin polymerization was detected using pyrene-labeled actin, treated with GSH and diamide to induce glutathionylation. Finally, total GSH (tGSH) was measured at 412 nm using the DTNB GSH recycling assay. RESULTS: Cd induces glutathionylation of a 45 kDa protein in RMC in a dose- and time-

dependent manner that is reversible upon DTT treatment. By immunoprecipitation and LC-MS, we identified this glutathionylated protein to be actin. Inhibition of GSH synthesis by buthionine sulfoximine (BSO) resulted in a subsequent decrease in actin glutathionylation. Co-treatment with Cd and either NAC or BHA significantly decreased actin glutathionylation compared to Cd treatment alone, despite significant increases in tGSH. Furthermore, investigation into the effects of glutathionylation on actin dynamics *in vitro* showed that actin glutathionylation decreased the efficiency of actin polymerization. CONCLUSIONS: Cd induces actin glutathionylation in RMC and this can be attenuated by co-treatment with antioxidants BHA and NAC, though this was not correlated with tGSH levels. The shift in actin towards a depolymerized state with actin glutathionylation suggests that glutathionylation may be a potential mechanism of Cd-induced F-actin disruption in RMC.

1400

Role of CaMK-IIδ in cadmium-induced actin cytoskeleton disruption.

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OBJECTIVES: Cadmium (Cd) is a toxic metal with significant environmental impact. We have shown that Cd activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) and initiates CaMK-II-dependent apoptosis in rat renal mesangial cells (RMC). Cd also causes a selective disruption of the F-actin cytoskeleton, and inhibition of CaMK-II with KN93 partially prevents this effect. Additionally, Cd induces translocation of gelsolin to the cytoskeleton, and gelsolin is involved in Cd-induced F-actin disruption. The present study was undertaken to investigate the influence of Cd on the role of CaMK-IIδ in RMC cytoskeletal dynamics. METHODS: RMC were treated in serum-free conditions with up to 40 μM $CdCl_2$ with or without KN93. Actin polymerization was followed *in vitro* by changes in pyrene-labeled actin fluorescence. Implication of CaMK-IIδ's involvement was achieved by overexpression and silencing. RESULTS: CaMK-IIδ associates with monomeric G-actin and forms a complex with gelsolin in RMC. Partially purified recombinant CaMK-IIδ suppresses actin polymerization, suggesting it sequesters actin monomer. Cd also causes a dose-dependent increase in CaMK-IIδ association with the cytoskeleton. The cytoskeletal fraction from Cd-treated cells was found to decrease actin polymerization; KN93 significantly suppressed CaMK-IIδ cytoskeletal translocation and restored the Cd-dependent decrease in actin polymerization. The cytoskeletal fraction from Cd-treated cells overexpressing CaMK-IIδ showed a decrease in actin polymerization compared to transfeced-control cells, or compared to vector-transfected cells treated with Cd and KN93. On the other hand, silencing of CaMK-IIδ resulted in increased actin polymerization compared to the cytoskeletal fraction from control-transfected cells. Furthermore, both depletion and inhibition of CaMK-IIδ prevented translocation to the cytoskeleton of both full length gelsolin and its cleaved fragment. CONCLUSIONS: CaMK-IIδ plays a critical role in Cd-dependent disruption of the actin cytoskeleton in RMC, both through its association with actin and its influence on gelsolin translocation.

1401

Stoichiometry of Actin Regulatory Mechanisms Involving the Nck Adaptor Protein.

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Regulation of actin dynamics through the Nck/N-WASp/Arp2/3 pathway is essential for invadopodia formation during metastasis, development of various organ systems, and pathogen

infection. While upstream and downstream binding partners for Nck are known, the mechanism by which Nck activates nucleation promoting factors (NPFs), such as N-WASp, remains unknown. To examine the mechanism by which the Nck/N-WASp/Arp2/3 pathway induces actin polymerization, we employed a two-stage strategy involving both computational modeling and experimentation. First, we adapted the previously developed and publicly available “dendritic model of actin nucleation” using The Virtual Cell software system to test the effect of various proposed mechanisms by which Nck activates N-WASp and Arp2/3 on actin polymerization. Secondly, we tested the model predictions by implementing precise experimental manipulation in which we use antibody-induced aggregation of membrane-bound Nck SH3 domains to study the effect of the number of molecules in and the density of Nck aggregates on actin polymerization. The experimental results demonstrate that at a given Nck density, the amount of actin polymerization is only weakly dependent on the size of the Nck SH3 cluster. Rather, actin polymerization increases in a non-linear manner as local Nck SH3 density increases. This suggests that cellular responses to external stimuli involving Nck-dependent actin polymerization may be tunable simply by increasing or decreasing the density of Nck at sites of actin polymerization. In addition to this observation, our model predictions coupled with our experimental results revealed a previously unappreciated mechanism by which Nck molecules can activate N-WASp, which then binds and activates Arp2/3. The results of this study indicate that Nck-induced local actin polymerization is dependent on the density of Nck SH3 domains upstream of N-WASp and Arp2/3 and that the Nck/N-WASp/Arp2/3 pathway functions through a 4:2:1 Nck:N-WASp:Arp2/3 stoichiometry. (Supported by NIH Grants P41RR013186 and R01 CA82258)

1402

Extremely low polymerizability of a highly-divergent *Chlamydomonas* actin (NAP) as examined by ectopic expression in amphibian cultured cells.

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Novel actin-like protein (NAP), a highly divergent actin expressed in *Chlamydomonas*, shares only 65% amino acid identity with conventional actin. It is scarcely expressed in wild-type cells but is abundant in *ida5*, a mutant that lacks the conventional actin gene. Because *ida5* fails to produce actin-containing fertilization tubules, it is uncertain whether NAP can polymerize into filaments. Here I assessed the polymerizability of NAP by ectopically expressing enhanced green fluorescent protein–tagged NAP (EGFP-NAP) in cultured amphibian cells. EGFP-NAP was excluded from stress fibers but partially co-localized with endogenous actin in the cell periphery. Fluorescence recovery of NAP after photobleaching was not directional, and its turnover rate was similar to the estimated diffusion rate of monomeric actin. Therefore, EGFP-NAP likely accumulates by diffusion, not by addition to existing actin filaments. These findings suggest that NAP has extremely poor ability to polymerize and is not as effective as the conventional actin in forming actin meshwork in the cell periphery.

1403

Regulation of stereocilia length by myosin XVa and whirlin depends on the actin-regulatory protein Eps8.

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Proper hearing and balance depend on the staircase shaped bundle of inner ear hair cell stereocilia, which are the actin-based protrusions or “hairs” inside our cochlear and vestibular organs which move in response to sound vibrations and head movement. In comparison to other actin protrusions (e.g. microvilli), stereocilia are extraordinary in at least 2 ways: 1) Differential regulation - in each hair cell there are rows of stereocilia with lengths that increase in height by several micrometers, but stereocilia within the same row vary in height by no more than several nanometers. 2) Length – normal epithelial microvilli are ~500 nanometers long, while stereocilia are up to 120 micrometers long. A large number of mutations that cause deafness affect proteins involved in regulating stereocilia length. Our recent work has revealed that stereocilia are dynamic structures undergoing constant renewal and regulation via the activities of numerous myosin motor proteins and their actin-regulatory cargoes. Myosin XVa (MyoXVa) and its cargo whirlin are implicated in deafness (DFNB3 and DFNB31, respectively) and have been shown to localize at stereocilia tips and to be essential for stereocilia elongation. Given that whirlin is a scaffolding protein with no actin-regulatory activity, it remains unclear how these proteins work together to elongate stereocilia. Here we show that the actin-regulatory protein Eps8 interacts with MyoXVa and whirlin, and that mice lacking Eps8 have very short stereocilia similar to MyoXVa- and whirlin-deficient mice. We also show that Eps8 localizes to stereocilia tips in concentrations directly proportional to length, showing for the first time a relationship between the amounts of an actin-regulatory protein and stereocilia length, revealing a biochemical mechanism for differential stereocilia elongation. We show that Eps8 fails to accumulate at the tips of stereocilia in the absence of MyoXVa, that overexpression of MyoXVa results in both elongation of stereocilia and increased accumulation of Eps8 at stereocilia tips, and that the exogenous expression of MyoXVa in MyoXVa-deficient hair cells rescues Eps8 tip localization. We also found that both MyoXVa and Eps8 appear in reduced amounts at the tips of whirlin-deficient stereocilia, which suggests that whirlin plays an integral role in the efficient accumulation of the MyoXVa:Eps8 complex at stereocilia tips, perhaps via its scaffolding activity. Our data demonstrates that MyoXVa, whirlin, and Eps8 are integral components of a stereocilia tip complex, where Eps8 is a central actin-regulatory element transported by MyoXVa to stereocilia tips for elongation of the stereocilia actin core. This work provides insight towards DFNB3 and DFNB31 pathologies, and identifies EPS8 as a candidate deafness gene.

1404

Adult hair cell stereocilia actin cores undergo dynamic renewal.

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Inner ear hair cell (HC) stereocilia contain parallel, uniformly polarized and crosslinked actin filaments. In earlier studies we showed that in developing neonatal HC stereocilia actin filaments are continuously polymerizing and depolymerizing in a treadmilling process that results in dynamic renewal while maintaining steady-state lengths. Whether stereocilia actin filaments are dynamically turning over throughout the lifetime of the HC, and whether mature stereocilia have any structural plasticity have remained important open questions in the hearing

research field. Furthermore, structural plasticity in HC stereocilia may be an important consideration for the development of therapeutic interventions for both inherited and acquired hearing losses. Here we report the successful culturing and transfection of adult vestibular HCs with GFP-beta- and gamma-actin. Our experiments revealed that actin filaments in mature HC stereocilia (P30) are continuously polymerizing, albeit at a much slower rate than developing HC stereocilia. Similar to what has been shown in earlier studies on neonatal tissue, the actin polymerization rate was proportional to the stereocilia length; i.e., longer stereocilia had a higher rate of actin polymerization than shorter stereocilia. Using immunogold labeling, we also show that both the beta- and gamma-actin isoforms colocalize in stereocilia actin filaments as early as embryonic day 16.5, with 40% more gamma-actin than beta-actin. In contrast, in adult mouse stereocilia, beta- and gamma-actin are homogeneously distributed in a 1:1 ratio. Interestingly, stereocilia from aging 2-year-old mice presented an almost 3:1 ratio of beta- to gamma-actin, suggesting that the expression levels of each actin isoform may be modulated throughout the lifetime of the organism. Furthermore, we show that stereocilia in aging mice often vary in size and shape, including extremely long stereocilia, revealing intrinsic plasticity in the dynamics and structure of stereocilia actin cores. Notably, these very long stereocilia are similar to the stereocilia found in various mice with mutations in stereocilia proteins such as myosin VI, gelsolin, and PTPRQ. Overall, our data provide evidence for continuous structural plasticity of adult HC stereocilia actin cores, which may lend important insight towards future attempts at therapeutic treatment of acquired and inherited hearing loss.

Actin-Membrane Interactions

1405

Modulation of Beta Actin Filament Network along the Rat Seminiferous Epithelium Cycle.

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Junctional devices in Sertoli cells play a key role in maturation and differentiation of germ cells. However it is not totally understood the cellular and subcellular organization of these specializations. By combining light and electron microscopic techniques using β-actin with prosaposin and glutaredoxin antibodies (to stain Sertoli cytoplasm) we observed the structural (confocal microscopy) and ultrastructural (electron microscopy) organization of tight and adherent junctions, which are the morphologic substrate of the blood testis barrier (BTB). Beta actin also characterizes ectoplasmic specializations found at different level of the seminiferous epithelium. Actin was quantified (western blot technique) in relation to the spermiation period in different segments of the seminiferous tubules visualized by transillumination under stereomicroscopy. We analyzed by freeze fracture the characteristics of tubular bulbar complexes, a known component of ectoplasmic specializations.All together, these different approaches also allowed us to study the complex arrangement of actin cytoskeleton of Sertoli cells branches, which surround germ cells in different stages of the spermatogenic cycle. Overall, our results show a consistent labelling of β- actin before, during and after the release of spermatozoa in the tubular lumen (spermiation) suggesting a significant role of the actin network in spermatic cells differentiation. In conclusion, significant interrelations among the β-actin network, the junctional complexes of the BTB and the ectoplasmic specializations were detected at different stages of the seminiferous cycle.

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1406

Photoinactivation of dynamin reveals its essential role in invadosomes, actin-rich adhesion sites implicated in invasion.

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In addition to its role in endocytosis, dynamin has been shown to be associated with actin rich structures. To explore the specific role of dynamin in actin dynamics, we investigated its functions in invadosomes; 3-dimensional actin-rich adhesion structures, homologous to podosomes and invadopodia. Invadosomes are implicated in adhesion, motility and invasion and are constituted of actin cores, formed of long F-actin bundles maintained perpendicularly to the substratum, linked by F-actin fibers parallel to the substratum, the actin cloud. We show here that dynamin depletion by conditional gene knockout in v-Src-transformed fibroblasts triggers a complete disorganization of invadosomes and inhibition of their invasive properties. To regulate actin, dynamin acts not only as a simple adaptor protein for actin regulatory proteins but as a membrane interacting GTPase-mechanoenzyme. Using an optogenetic approach based on the photosensitizer KillerRed, we found that dynamin photo-inactivation leads to the disorganization of the actin cloud and decrease of cores height, but without affecting their initiation. Dynamin is therefore involved in the coupling these two actin components of invadosomes and is a regulator of their actin architecture.

1407

Antiparallel subunit contacts are present in supramolecular actin structures related to the endocytic compartment.

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The transient formation of an antiparallel actin dimer, also called 'lower dimer' (LD), was first described more than 20 years ago, when chemical crosslinking with 1,4-phenylenebismaleimide (1,4-PBM) of polymerizing actin resulted in two different dimers (Millonig et al., 1988). The ability of the actin-binding proteins gelsolin and toxofilin to stabilize two actin subunits in an antiparallel configuration (Hesterkamp et al., 1993; Lee et al., 2007) provided first evidence for a role of the LD in cellular actin patterning. However, a clear link of this unconventional actin species to a specific cellular function could not be established. Since antibodies represent powerful tools to detect different actin forms in the cell (Schoenenberger et al., 2005; Schroeder et al., 2009), we generated an antibody that selectively binds to LD and not to G-actin in actin assemblies. Using this antibody, we were able to unveil the presence of LD in several cell lines by immunofluorescence and immunoelectron microscopy. Co-labeling with different marker proteins suggested an association of LD with the endocytic compartment of the cell. Our results provide strong evidence that LD contributes to the supramolecular patterning of actin assemblies in mammalian cells.

1408

Dynamics of the Membrane Cortex in Cleavage Stage Sea Urchin Embryos.

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The cytoskeleton is involved in the establishment and maintenance of the non-uniform distribution of membrane domains in the plasma membrane. It has been previously reported

that the lipid raft marker ganglioside GM1 is preferentially localized to the free, apical surface of cleavage stage sea urchin embryos as early as the two cell stage (Alford, et al. 2009). We investigated the spatial and temporal regulation of this polarity as well as the actin cytoskeleton using FRAP analysis of zygotes and two cell stage sea urchin embryos. The mobility of GM1 was found to be dependent on myosin light chain kinase (MLCK) activity and cytokinesis. When myosin II phosphorylation was inhibited by treatment with the MLCK inhibitor ML-7, GM1 was found to become more mobile within the plasma membrane regardless of the cell cycle stage. Compounds that inhibit cytokinesis, such as cytochalasin D and nocodazole, led to a decrease in GM1 mobility. Contrary to previous studies, we found the rate of actin recovery to be similar between the cleavage furrow and the cell pole. In addition, the rate of actin mobility increased in two cell stage embryos compared to zygotes. These studies suggest that MLCK activity is involved in the organization of lipid rafts within the plasma membrane, and that actin is highly dynamic within the cell cortex during early sea urchin development.

1409

Cofilin is Excluded from Clusters of Influenza Hemagglutinin at the Host Cell Membrane.

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Viruses can hijack host-cell actin to aid in entry, assembly, and exocytosis. Cell membrane organization depends on the actin cytoskeleton, and influenza virus depends on cell membrane organization for entry and assembly. Influenza infectivity relies on high concentrations of the viral protein hemagglutinin (HA) for entry by fusion with the host cell endosomal membrane. In infected cells the host-cell plasma membrane contains HA clusters that accompany other viral components to form new viruses and bud. Despite interactions between actin and other viral components, no direct HA-actin interaction has yet been reported. The aim of this study was to characterize the association between HA and host cell actin, and investigate the mechanisms which underpin this association. Results using the super-resolution method fluorescence photoactivation localization microscopy (FPALM) in non-polarized cells show that HA clusters are correlated with actin, suggesting that HA associates with specific cortical actin structures. Disrupting actin affected HA cluster organization: treatment with the actin stabilizer Jasplakinolide or the actin barbed-end binding Cytochalasin D (CytoD) each resulted in larger HA cluster areas; treatment with CytoD or the actin monomer sequestering Latrunculin A each resulted in lower HA density in clusters as compared to controls. HA molecules were also found to move along actin-rich membrane regions. We hypothesize that HA drives structural changes in cortical actin. In support of this hypothesis, relationships between influenza virus infectivity and the mitogen-activated protein kinase (MAPK) and RhoGTPase pathways have been previously observed. To test this hypothesis, confocal and FPALM were used to image photoactivatable fluorescent protein (PAFP)-tagged versions of HA and the actin binding protein cofilin, and the relative distributions of each were determined at nanometer and micrometer length scales. Cofilin molecules are excluded from HA clusters on length scales of tens to hundreds of nanometers. The anti-correlation of HA molecules and cofilin suggests that HA may be influencing local actin structure through RhoA activation. Understanding these interactions could provide novel means to target HA-actin associations without disrupting the entire cytosolic actin pool, and thus identify specific new anti-viral therapies.

1410

Dynamic Super-Resolution PALM Imaging of the Actin Cytoskeleton in Living Mammalian Cells.

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The actin cytoskeleton plays a crucial role in the organization of the plasma membrane into segregated microdomains as well as in the regulation of membrane protein localization and trafficking. Standard diffusion-limited light microscopy fails to capture the structure of the cytoskeleton since the characteristic distance between actin filaments is below 100 nm. We imaged the actin network in the vicinity of the plasma membrane in living mammalian cells using photoactivated localization microscopy (PALM). This technique enabled the observation of actin motion and turnover with super-resolution. We transfected ND7/23 and HEK293 cells for the expression of Dendra2 labeled actin, a photoswitchable fluorophore suitable for PALM imaging. The cells were imaged in a custom-built total internal reflection microscope with the capability to maintain the cells under physiological conditions for prolonged times. Images of the actin cytoskeleton with a resolution of 50 nm were obtained within an imaging time of 5 seconds.
We further used live-cell PALM imaging to study the role of the actin cytoskeleton in the maintenance of Kv2.1 channel clusters on the cell surface. Kv2.1 channels form stable clusters in the plasma membrane of hippocampal neurons and transfected HEK cells. The mechanism by which the cell maintains these clusters of mobile proteins is still not well understood. By labeling voltage gated Kv2.1 channels with quantum dots, we were able to track individual Kv2.1 channels while simultaneously imaging the actin cytoskeleton with super-resolution. Kv2.1 channels were labeled with QD705 quantum dots, which are well separated from Dendra2, enabling two-color imaging. The combination of single molecule tracking with PALM imaging revealed a complex set of interactions between the cytoskeleton and the Kv2.1 channels by which actin is actively involved in localizing the channels to specific microdomains. This work shows that the ability of PALM to generate super-resolution imaging in live cells opens the way to probe the dynamic interaction between cellular components at nanometer length scales.

1411

Membrane tension in rapidly moving cells is determined by the balance between actin network assembly, adhesion and contraction.

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While the important role of membrane tension in cell motility is becoming apparent, little is known about how membrane tension is set and regulated. We measure membrane tension in rapidly moving fish epithelial keratocytes using a tether pulling assay. Membrane tension is found to range between 200-600 pN/µm in a population of keratocytes, values which are substantially higher than what has been measured for slower moving cells. To investigate the interplay between the motility machinery and membrane tension, we use various perturbations known to alter actin filament density, adhesion and contraction and study their effect. We find that decreasing the number of pushing filaments along the leading edge leads to a significant decrease in membrane tension. Moreover, we find that decreasing the adhesiveness of the substrate or increasing myosin-induced contraction also leads to lower membrane tension, whereas stronger adhesion or weaker contraction results in higher tension. These results suggest that tension is generated by growing actin filaments at the leading edge pushing against the inextensible membrane, and is relieved due to centripetal actin flow generated by myosin-powered contraction and mediated by the adhesion strength; stronger actin pushing

forces at the leading edge and/or a trailing edge which is harder to retract lead to higher tension. This notion is further supported by experiments in which large amounts of membrane are added to motile keratocytes, without changing their biochemical content, by fusion with giant unilamellar vesicles. In response to the significant increase in surface area (~25%) fused cells rapidly expand their lamellipodium, but continue to move with only minor changes in aspect ratio, speed and membrane tension. Together these results indicate that membrane tension is largely determined by the mechanical interplay between cytoskeletal forces and the membrane, and is not regulated through endo/exocytosis or other means, at least in rapidly and steadily moving cells such as keratocytes.

Microtubules and Associated Proteins

1412

Pac1p/Lis1 regulation via sumoylation.

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Microtubules are proteineceus polymers within the cell that make key contributions to cell motility and cell division. Microtubules are highly dynamic, continuously growing and shrinking. Because microtubules are fundamental for cell division, it is important to understand the molecular mechanisms that regulate them. Pac1p, the Lis1 homologue in yeast, is a microtubule plus-end-tracking protein. Mutations in the Lis1 gene have been correlated with Miller-Dieker lisssencephaly syndrome due to defects in neuronal migration. Spindle positioning in yeast is an essential process during cell division that is regulated by the KAR9 and dynein pathways. The KAR9 pathway guides cytoplasmic microtubule into the bud. Dynein pulls the spindle across the bud neck via the forces it exerts on the cytoplamic microtubule from the cortex. As part of the dynein pathway, Pac1p is important for recruiting dynein to the plus end of the microtubule. Pac1p interacts with other plus-end microtubule binding proteins including Bik1p, the CLIP-170 homologue in yeast. Although Pac1p plays vital roles for microtubule function, little is known about its regulation.

Sumoylation is a post-translational modification that covalently attaches a Small Ubiquitin-like Modifier (SUMO) protein to the target substrate. Sumoylation regulates many cellular processes such as cellular transport, protein stability, and cell cycle progression. Recently a few microtubule associated proteins including Kar9p have been shown to be conjugated to SUMO. Using a two-hybrid assay, Pac1p interacts with SMT3, the SUMO homologue in yeast, and other key players of the sumoylation pathway. Ubiquitin-Like Protein-1 (Ulp1) is a protease that specifically cleaves Smt3p from its protein conjugates. Using a temperature sensitive strain that inactivates the Ulp1p protease activity at 37°C, we observe an accumulation of higher molecular weight Pac1p bands. This suggests that the higher molecular weight forms of Pac1p are due to the accumulation of SMT3 conjugates. In contrast to ubiquitination, sumoylation is not a modification that tags the target substrate for direct degradation. However, SUMO-targeted ubiquitin ligases (STUbLs) can recognize a sumoylated substrate and promote its degradation of the substrate via ubiquitination. Using a two-hybid assay, we show that Pac1p interacts with the STUbL enzyme Nis1p/Ris1p. Strains deleted for RIS1 display an accumulation of higher molecular weight Pac1p conjugates, in comparison to WT. This suggests a novel model in which Pac1p is regulated via STUbLs.

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Structure-function Analysis of the Septin GTPase Interaction with Microtubules.

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The microtubule (MT) cytoskeleton is important for many cellular processes including vesicle transport and mitosis. MTs are highly organized structures and are tightly regulated. Besides well known regulators like microtubule associated proteins and plus end binding proteins, it was recently found that septins (SEPTs), a group of filament-forming GTPases, colocalize with MTs and affect MT organization and dynamics. However, how septins interact with microtubules is unknown. Mammalian septins are classified into 4 groups: SEPT2, SEPT6, SEPT7 and SEPT9. The building block of septin filaments is a SEPT9/7/6/2-2/6/7/9 octamer, in which each septin is replaceable by other members of the same group. By modeling the interaction of SEPT2/6 with microtubules, we identified a positively charged a-helix and loop domain within the SEPT2 structure. We hypothesized that these domains interact with the negatively charged C-terminal tails of tubulin. Alanine scanning mutagenesis of the positively charged residues in the a-helix of SEPT2 decreased the number of MT-associated septin filaments, while mutations in the loop region had no effect. Overexpression of the a-helix tagged with GFP led to less number of endogenous MT-associated SEPT2 filaments. This phenotype was rescued when the positively charged residues of the a-helix were mutated to alanine. These results indicated that the a-helix domain may mediate the interaction of SEPT2 with MTs. On going experiments aim at investigating whether SEPT2 interacts directly with MTs and how other septin subunits may contribute to the septin-MT interaction.

1414

Molecular Determinants for Regulation of Microtubule-Severing by Katanin.

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Microtubules are dynamic cytoskeletal polymers that form complex and highly organized arrays essential for cell motility, morphogenesis, and division. Microtubule-severing is a reaction that generates an internal break in the microtubule polymer and catalysis of microtubule-severing is required for the proper formation and function of microtubule-based arrays in a wide range of eukaryotic cells. The microtubule-severing enzyme Katanin is a conserved heterodimeric ATPase that severs and disassembles microtubules, but the regulatory mechanisms that control microtubule-severing by Katanin are poorly defined. To better understand the molecular regulation of Katanin, we conducted a structure-function study of the *Drosophila* Katanin catalytic subunit, termed Kat-60, using a combination of cell-based and cell-free approaches. To assess the contribution of each putative microtubule-binding region of Kat-60 to its microtubule-severing activity, we devised a quantitative assay to measure the relative loss of microtubule polymer in a large number of Kat-60 expressing cells using high-throughput microscopy. Using this assay, we found that certain microtubule-binding domains and residues of Kat-60 were more important than others for regulation of microtubule-severing. By expressing eGFP-tagged deletion derivatives and mutants of Kat-60 in living cells, we also discovered that the dynamic localization of Kat-60 on microtubules is dependent on both its microtubule-targeting and catalytic activities. Furthermore, we observed similar microtubule-severing and microtubule-targeting activities of purified eGFP-tagged full-length Kat-60 in cell-free assays compared to those found in cell-based assays, indicating that future in vitro studies of eGFP-tagged deletion derivatives and mutants of Kat-60 will be generally significant. Taken together, our results suggest that the putative microtubule-binding regions of the Katanin catalytic subunit cooperate to regulate its activity and dynamics in living cells.

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Single molecule studies of microtubule severing enzymes reveals their specific biophysical activities.

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The spatio-temporal dynamics of microtubules is finely tuned in cells by the orchestrated activity of microtubule-associated proteins (MAPs). Severing of microtubules in vivo is performed by microtubule severing enzymes of the AAA+ family of ATPases. The initial member of this novel class of microtubule-associated proteins is katanin-60, the catalytic subunit of katanin complex that regulates microtubule length and dynamics of cell during interphase and mitosis. Other members of microtubule severing enzymes include spastin in which mutations result in hereditary spastic paraplegia, and fidgetin, which is involved in mammalian development. We have performed the first single molecule characterization of katanin-60 and first biophysical characterization of fidgetin in vitro. Interestingly, at a low concentration both enzymes remove tubulin dimers from filament ends, which appear to depolymerize. Katanin-60 prefers the plus-end and fidgetin prefers the minus-end. At higher concentration both proteins sever microtubules, but they have different localization patterns. We find katanin-60 preferentially targets to and severs at lattice defects. Fidgetin preferentially removes GMPCPP stabilized tubulin and removes extended regions of protofilaments, an activity we call “protofilament stripping”. Our results indicate that katanin-60 and fidgetin are microtubule severing enzymes with specific biophysical abilities and targeting on microtubules.

1416

Defining the Critical Function of Tubulin Carboxy-terminal Tails.

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Microtubule tracks establish transport and signaling relationships between distant sites within the cell. Microtubules are navigated by motor proteins that use energy from nucleotide hydrolysis to drive a cycle of interactions, producing movement for force generation and cargo trafficking. Although these processes depend on interactions between tubulin subunits and motor proteins, the molecular features of tubulin subunits that contribute to these interactions are poorly understood. Important questions include whether different motors utilize distinct binding sites on tubulin, and how transport may be differentially regulated at the level of the microtubule. Here we employ genetics, quantitative live-cell microscopy, and biochemical assays to examine the role of the carboxy-terminal tail regions (CTTs) of α- and β-tubulin subunits. CTTs contain an abundance of negatively-charged residues that are thought to engage in electrostatic interactions with microtubule-associated proteins, are major sites of tubulin posttranslational modification, and exhibit high sequence variation amongst tubulin isoforms in humans. Our preliminary findings identify specific pathways that depend on CTTs for function, including the microtubule motor dynein. We explore the basis for this dynein phenotype in vivo and in biochemical assays. This work provides a foundation for the study of diverse motors and features on microtubules, and will offer new insights into the mechanics of evolutionarily distinct motors.

1417

Microtubule dynamics and maintenance of fission yeast cells morphology.

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Microtubules (MTs) are central to the organization of the eukaryotic intracellular space and are involved in the control of cell morphology. In fission yeast cells MTs transport polarity factors to poles where growth is located, thus ensuring the establishment and maintenance of the characteristic spherocylindrical shape. For this purpose, MT polymerization dynamics is tightly regulated. Using home-made automated image analysis software, we can measure the spatial dependence of MT dynamics in interphase fission yeast cells [1]. This systematic and quantitative analysis provides a tool to study the role of MT regulators. In combination with genetic modifications we aim to understand the function of +TIPs (plus-end tracking proteins) in the spatial regulation of MT dynamics. We analyze MT dynamics in round fission yeasts in order to observe the role of Tip1 during cortical contact of MT tips when generated compressive forces co-regulate MT dynamics [1]. In these mutated cells it is possible to resolve such contacts, while one cannot exclude the possibility of a contact in spherocylindrical cells [2]. Furthermore we try to decipher how the linear transport by MT interferes with the feedback circuitry that assures the correct spatial distribution of Tea1, the main polarity factor in fission yeast cells. Tea1 is delivered as packets of molecules at growing MT tips and is anchored at the membrane via a mechanism involving interaction with the membrane protein Mod5. This protein acts as a catalyst for the incorporation of Tea1 in a bipolar cluster [3]. In order to bypass this complex Mod5-mediated feedback mechanism we tagged Tea1 with a modified GFP allowing its attachment to the plasma membrane. The localization of the polarity factor at the cell pole is then expected to be due to MT-based transport only.

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1418

The structural basis of microtubule end recognition by EB proteins.

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The dynamic properties of the microtubule cytoskeleton are crucial for many cellular functions. Growing microtubule ends serve as transient binding platforms for a variety of essential proteins that regulate both microtubule dynamics and interactions with cellular substructures. End binding proteins (EBs) are crucial for the recruitment of most of these factors, as they have the unique ability to autonomously recognize an extended region at growing microtubule ends. The nature of this end region is unknown due to the lack of high-resolution structural information. Here we show, using cryo-electron microscopy (cryo-EM) and subnanometer single particle reconstruction, a pseudo-atomic model of how the calponin homology (CH) domain of the fission yeast EB Mal3 binds to GTPγS microtubules, a static mimic of the growing microtubule end region. We found that GTPγS microtubules have more extensive inter-protofilament lateral contacts than GDP microtubules. The Mal3 CH domain contacts 4 neighbouring tubulin subunits and also senses the additional, nucleotide-dependent inter-protofilament connection.
Single point mutational analysis and fluorescence imaging of microtubule end tracking in a reconstituted in vitro system confirm the relevance of the identified contacts for dynamic

microtubule end tracking in the presence of GTP. These results provide the first subnanometer resolution structure of a microtubule in a GTP-like state. They reveal the structural origin of selective binding of EBs to growing microtubule ends and strongly suggest that the entire EB binding region is an extended nucleotide-induced structural cap crucial for microtubule stability.

1419

+TIP interactions with EB1 are tuned by multisite phosphorylation and sequestration of distributed arginine residues.

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Microtubule (MT) plus-end tracking proteins (+TIPs) reversibly bind to growing MT ends through interactions of a SxIP motif with EB1. +TIPs are functionally diverse and implicated in the regulation of MT dynamic instability and in transient MT interactions with other proteins and intracellular structures. In cells, +TIP interactions are thus expected to be highly spatiotemporally controlled. Consequently, plus-end-tracking of several +TIPs is inhibited by phosphorylation but the underlying molecular mechanism is not understood. CLASPs, as representative +TIPs, contain a short tandem repeat of two SxIP motifs embedded in an arginine-rich basic region, and SxIP motifs are required for EB1-mediated plus-end-tracking in vitro. Either SxIP motif is functional and can mediate specificity of plus-end-tracking. However, the SxIP motifs alone are not sufficient. We demonstrate that in cells a major part of the binding energy results from electrostatic interactions that are negatively regulated by distributed multisite phosphorylation by GSK3β, and a large degree of flexibility exists in how these binding interactions are controlled. GSK3β phosphorylation requires prior phosphorylation by a priming kinase, and both interphase and mitotic cyclin-dependent kinases (CDKs) phosphorylate priming sites in the CLASP plus-end-tracking domain. CDK1-dependent CLASP hyperphosphorylation of CLASP2 completely inhibits plus-end-tracking in mitosis. Using an in vitro fluorescence anisotropy assay to measure equilibrium binding constants we show that priming site phosphorylation alone reduces affinity for EB1 only moderately. In contrast, complete phosphorylation of the GSK3β motif abolishes the interaction with EB1. To further elucidate the mechanism by which multisite phosphorylation regulates CLASP-interactions, we employed molecular dynamics simulations of EB1 binding to a minimal CLASP plus-end-tracking module. We find that arginine residues in non-phosphorylated CLASP form extensive bidentate H-bond networks with negatively charged glutamate residues predominantly in the unstructured C-terminal tail of EB1. This ‘molecular Velcro’ is largely disrupted when the CLASP GSK3β sites are phosphorylated, and arginines are tied up in intramolecular interactions with phospho-serines, which was further confirmed by NMR spectroscopy. This results in an overall reduction of H-bond formation between EB1 and phospho-CLASP indicative of a substantially reduced binding energy, and may represent an example of a more general mechanism by which multisite phosphorylation controls protein-protein interactions.

1420

Microtubule +TIPs protein, EB1 is critically involved in Paclitaxol mediated cell cycle arrest in cancer cells.

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Microtubule targeting agent, paclitaxol has been known to be most effective anticancer drug in cancer chemotherapy. It induces G2/M cell cycle arrest and kills cancer cells primarily by

interfering microtubule assembly-dynamics and associated signaling pathways. Despite its extensive clinical use, potentiality of paclitaxol is often limited duo to development of its resistance in tumor. Factors responsible for altered paclitaxol sensitivity and the mechanisms underlying remain poorly understood. Recent evidences suggest that the sensitivity of cancer cells to microtubule targeting agents is associated with the proteins involved in regulation of microtubules. In this study, we have found that paclitaxol mediated cell cycle arrest is regulated by microtubule plus end binding protein, EB1. EB1 regulates microtubule dynamics by localizing at microtubule plus end tips and has been implicated to be involved in tumorigenesis. We found that paclitaxol at sub-stoichiometric concentration increased the level of EB1 in MCF-7 breast cancer cells. SiRNA mediated knock down of EB1 resulted increased proliferation of cancer cells under paclitaxol treatment and released cells from paclitaxol mediated cell cycle arrest. Results suggest of an important role of EB1 in paclitaxol mediated mitotic arrest. Experiments are underway to uncover the molecular pathways associated with EB1 in paclitaxol mediated cell cycle arrest.

1421

Does EB1 bind to the Microtubule seam or to the lattice?

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EB1 is a highly conserved microtubule plus end tracking protein (+ TIP) involved in regulating microtubule dynamics, but the mechanisms of its +TIP behavior and MT stabilizing ability remain undefined. Microtubules consist of protofilaments associated in a cylindrical lattice that closes upon itself at a seam; this seam is generally believed to be weaker than the rest of the lattice. Previous electron microscopy has suggested that EB1 binds specifically to this seam, implying that EB1 promotes MT polymerization by stabilizing the seam (Sandblad et al., Cell 2006). Microtubule binding experiments in this manuscript were interpreted as supporting the idea that EB1 binds strongly to the MT seam, providing further evidence for this model (Sandblad et al., 2006). However, close inspection of the data suggests that the reported MT binding experiments are equally consistent with weak binding along the microtubule body. Moreover, the published work was performed with S. pombe Mal3, leaving open the question of how mammalian EB1 behaves. To resolve the question of whether mammalian EB1 binds primarily to the MT seam, we are using a combination of MT binding assays and theoretical modeling with MTBindingSim. Our results argue against strong seam binding for EB1, and suggest instead that binding of EB1 to stabilized MTs is best explained by weak binding along the lattice, perhaps with concomitant binding to the seam. These observations will help establish how EB1 interacts with microtubules, which in turn is essential for fully understanding how cells regulate the dynamics of the microtubule plus end.

1422

Acetylation-dependent regulation of kinetochore microtubule dynamics by PCAF acetyltransferase.

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Microtubule is a key cytoskeleton network essential for cellular plasticity and dynamics in all eukaryotes. Reversible acetylation of tubulin has been implicated in regulating microtubule stability and function. Here we show that PCAF acts as an intrinsic negative modulator of microtubule stability in mammalian cells. We show that acetylation of the EB1 on conserved lysine reduces the amplitude of microtubule plus-end growth by minimizing EB1 binding to the plus-end tracking proteins. PCAF associates with EB1 and functions as a cognate EB1 acetylase, which opposes the acetylation-induced microtubule stabilization at the kinetochore.

Using fluorescence resonance energy transfer (FRET)-based sensors in living HeLa cells, we show that kinetochore-associated PCAF activity peaks during chromosome congression and declines as sister chromatids segregate. Consequently, mitotic cells expressing acetylation-mimicking EB1 are delayed in metaphase alignment, resulting in impaired checkpoint silencing, thereby promoting a chromosome instability phenotype. Our findings identify reversible acetylation of EB1 as a molecular mechanism to orchestrate the dynamics of kinetochore signaling, and indicate that PCAF acts as rheostat to fine-tune microtubule dynamics in mitosis.

1423

Augmin promotes Meiotic Spindle Formation and Bipolarity in Xenopus Egg Extracts.

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Female meiotic spindles in many organisms form in the absence of centrosomes, the organelle typically associated with microtubule (MT) nucleation. Previous studies have proposed that these meiotic spindles arise from RanGTP–mediated MT nucleation in the vicinity of chromatin; however, whether this process is sufficient for spindle formation is unknown. Here, we investigated whether a recently proposed spindle–based MT nucleation pathway that involves augmin, an 8-subunit protein complex, also contributes to spindle morphogenesis. We used an assay system in which hundreds of meiotic spindles can be observed forming around chromatin-coated beads after introduction of Xenopus egg extracts. Spindles forming in augmin-depleted extracts showed reduced rates of MT formation and were predominantly multipolar, revealing a new function of augmin in stabilizing the bipolar shape of the acentrosomal meiotic spindle. Our studies also have uncovered an apparent augmin-independent MT nucleation process from acentrosomal poles, which becomes increasingly active over time and which appears to partially rescue the spindle defects that arise from augmin depletion. Our studies reveal that spatially and temporally distinct MT generation pathways from chromatin, spindle microtubules and acentrosomal poles all contribute to robust bipolar spindle formation in meiotic extracts.

1424

Spatial organization of self-organized microtubule arrays: role of MAP65.

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In most differentiated cells, including muscle, epithelial and neuronal cells, as well as in most fungi and plant cells, microtubules (MT) are not organized radially from the centrosome but form bundles that assume different configurations depending on the cell type. Although the mechanisms responsible for the formation of these self-organized MT arrays are not yet well understood, we outline the general features for their initiation and organization. These basic mechanisms include (i) MT nucleation, (ii) the release of the MTs from their nucleation sites, (iii) their transport in the cytoplasm by MT motor or treadmilling, (iv) their co-alignment with resident MTs leading to bundle formation (i.e. MTs present in already formed bundles). In vivo observations in acentriolar plant cells show that collisions between mobile and resident MTs can result for the mobile MTs in crossover, depolymerization or co-alignment depending on the encounter angle. In this study, we address the question of the self-organization of MT bundles induced by MT cross-linkers MAP65-1/Ase1/PRC1. Note that MAP65-1 (in plants), and its orthologs Ase1 (fungi) or PRC1 (vertebrate) are members of the non-motor MAP65 family. We systematically analyzed MT-MT (or MT-bundles) encounters of growing MTs in the presence of MAP65-1 and Ase1 in biomimetic assays using TIRF microscopy. In addition, we developed a biophysical model to determine the persistence length of single/bundled MT in the presence of

MAP65-1. Our data show that physical collisions of growing MT bundles have different outcomes depending of the encounter angles: (i) at shallow angle (<45°), MAP65-1 triggers MT zippering; (ii) steep-angle collisions (>45°) promote MT catastrophe, MT-MT crossing-over or MT-buckling. Bending stiffness being a crucial parameter to understand deformation resulting from MT-zippering, we measured the persistence length (Lp) of MTs in the presence of MAP65-1. Using active hydrodynamic flow or thermal fluctuations methods, we demonstrated that MAP65-1 has a strong softening effect for single MTs (the persistence length of single MT in the presence of MAP65-1 is six times smaller than that of single MTs without MAP) and lowers the global rigidity of MT bundles. In addition, this effect does not depend of the MT length. These data show how the key protein MAP65-1, by modifying the mechanical properties of MTs, controls the issue between MT-MT encounters resulting into the spontaneous formation of ordered MT bundle arrays.

1425

Spatial Localization Regulated By the Microtubule Network.

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The commonly recognized mechanisms for spatial regulation inside the cell are membrane-bounded compartmentalization and biochemical association with subcellular organelles. We use computational modeling to investigate another spatial regulation mechanism mediated by the microtubule network in the cell. Our results demonstrated that the mitotic spindle can impose strong sequestration and concentration effects on microtubule-binding molecules, especially dynein-associated cargoes. We applied our model to recapitulate the essence of three experimental observations on different organizations of the microtubule network: the sequestration of germ plasm components by the mitotic spindles in the *Drosophila* syncytial embryo, the asymmetric cell division initiated by the time delay between the centrosome maturation in the *Drosophila* neuroblast, and the diffusional block between neighboring nuclei in the *Drosophila* syncytial embryo. Our model thus suggests that the microtubule network provides an spatially extensive docking platform for the microtubule-binding molecules and gives rise to a “structured cytoplasm”, in contrast to a free and fluid environment. The cell cycle-dependent changes in the microtubule network is critical for achieving differential spatial regulation effects.

1426

Doublecortin specifies 13-protofilament microtubules by wrapping around the microtubule lattice.

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Neurons, like all cells, face the problem that tubulin forms microtubules with too many or too few protofilaments (pfs). Cells overcome this heterogeneity with the γ-tubulin ring complex, which provides a nucleation template for 13-pf microtubules. Doublecortin (DCX), a microtubule-associated protein expressed in developing neurons, also nucleates 13-pf microtubules *in vitro*. Using fluorescence microscopy assays, we show that the binding of DCX to microtubules is optimized for the lateral curvature of the 13-pf lattice. We discovered that this sensitivity depends on a cooperative interaction wherein DCX molecules decrease the dissociation rate of their neighbors. Mutations in DCX found in patients with subcortical band heterotopia disrupt the specificity for 13-pf microtubules and weaken cooperative interactions. Using truncation constructs, we argue that these interactions "wrap around" the microtubule. These results support a mechanism for stabilizing 13-pf microtubules that, *in vitro*, allows DCX to template new microtubules through lateral associations that arrange tubulin dimers into a 13-pf nucleus.

1427

Regulation of minus-end directed microtubule transport of membrane organelles by the microtubule-associated protein 4 (MAP4).

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Microtubule (MT)-based transport of organelles is driven by opposing MT motors, plus-end directed kinesins and minus-end directed dynein, simultaneously bound to the organelle surface. The direction of MT-based transport is regulated through changes in the lengths of plus- and minus-end runs, via as yet poorly understood mechanisms. Regulation might involve reversible binding to the MT wall of microtubule-associated proteins (MAPs), which has been shown to differentially affect the movement single kinesin and dynein molecules along MTs *in vitro*. However, whether binding of MAPs to MTs control the direction of organelle transport in cells remains unknown. Here, we tested the regulation of MT transport by MAPs using *Xenopus* melanophores as an experimental system. The main function of these cells is transport of melanosomes, which move along MTs to the cell center (aggregation) or to the periphery (dispersion) by means of cytoplasmic dynein and kinesin-2, respectively. The direction of transport is regulated by changes in the levels of cAMP, which induce opposite changes in the lengths of plus- and minus-end MT runs. We compared phosphoprotein profiles of cells stimulated to aggregate or disperse melanosomes and found that phosphorylation of *Xenopus* MAP4 (XMAP4) at Thr752 and Thr756, which has been shown to inhibit binding of MAP4 to MTs in mammalian cells, was significantly increased (~5.5-fold) in cells induced to aggregate melanosomes. Overexpression of wild-type XMAP4 or the XMAP4 Thr752A/Thr756A dephosphomimetic mutant substantially inhibited melanosome aggregation (~3-fold) but did not have a significant effect on their dispersion. This effect was explained by the shortening of MT minus-end runs. In contrast to the Thr752A/Thr756A mutant, overexpression of a Thr752E/Thr756E MAP4 phosphomimetic mutant had a weaker inhibitory effect. Our data are consistent with a model for the regulation of MT transport in melanophores in which binding of MAP4 to MTs stimulated to disperse melanosomes selectively suppresses their minus-end directed runs. Phosphorylation at Thr752 and Thr756 during aggregation reduces binding of MAP4 to MTs thus stimulating minus-end directed melanosome movement.

1428

MAP65-1 and MAP65-2 promote axial growth and cell proliferation in plant roots.

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We investigated the role of MAP65-1 and MAP65-2 in root growth in the model plant, *Arabidopsis thaliana*. Native promoter, fluorescent reporter fusions to genomic sequences revealed that both MAP65-1 and MAP65-2 accumulated in the actively growing regions of the root: the tip and the elongation zone. Similar expression patterns were found using a constitutively active promoter, which suggested that strong post-transcriptional control restricted MAP65-1/2 accumulation to growing portions of the root. We observed a complete overlap in MAP65-1 and MAP65-2 localization in the root interphase cortical arrays, preprophase bands, and phragmoplasts. Genetic analysis of insertion mutant alleles showed that MAP65-1 and MAP65-2 proteins play a functionally redundant role in etiolated (dark grown) root growth. Plants deficient for both proteins exhibited stunted root growth due to a drastic reduction in the total cell number and to a defect in the elongation of the mature root cells. We did not observe obvious defects in cytokinesis, such as wall stubs and misplaced cell walls, in the double mutant roots. The overall morphology of the double mutant roots was not drastically altered, beyond

being short. Overall, these data suggest that MAP65-1 and -2 are important for cell growth and proliferation in the *Arabidopsis* root.

1429

Genetic and physical characterization of Chlamydomonas reinhardtii mutant strain cmu1-1.

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Chlamydomonas mutant strain *cmu1-1* was initially identified based on its unusual cell shape. Cells tend to be tear-drop shaped rather than ovoid like wild-type cells. Further investigation revealed the cell body microtubules in *cmu1-1* are unorganized and frequently longer than those seen in wild-type cells, providing a possible explanation for the altered cell shape. Current studies show this change in microtubule organization also leads to changes in the distribution of organelles such as the pyrenoid. To determine pyrenoid position, the midplane of the cell was defined as the plane running from the base of the flagella at the anterior end of the cell to the cell's posterior end, thus bisecting the cell. In 66% of wild-type cells the pyreniod is centered within 10 degrees either side of the midplane. In contrast, only 30% of *cmu1-1* cells position the pyrenoid within 10 degrees either side of the midplane. *cmu1-1* was created by insertional mutagenesis to facilitate gene identification. The plasmid used was derived from pUC119 and contained *nit1* and *nar1* genes. Investigations into the identity of the defective gene in *cmu1-1* revealed several rearrangements occurred during insertion. Consistent with the ability of *cmu1-1* to reduce nitrate, almost all of *nit1* was inserted. However, the 3' UTR became attached to the 5' regulatory region. All of *nar1* was inserted except for part of the 3' UTR. None of the pUC sequences were inserted. Investigations of the insertion site region reveal that two adjacent genes were knocked out by the insertion. Interestingly, both genes code for divergent cytoskeletal proteins. Current investigations are focused on using complementation to determine the relative contributions of the two knocked out genes.

1430

Depletion of Spindle Pole Protein, CKAP2, Increases Chromosomal Instability in Colorectal Cancer Cells.

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Introduction: Cytoskeletal-associated protein 2, *CKAP2*, is a microtubule-associated protein that colocalizes with spindle pole and aids in microtubule stabilization. CKAP2 is over-expressed in a variety of cancers, including colorectal, but the exact function and mechanism of action are poorly understood. Experimental changes in CKAP2 expression results in spindle abnormalities, and thus, we hypothesize that CKAP2 could play an active role in aneuploidy, a common feature of colorectal cancers.

Methods: RNAi technology was utilized to determine the extent to which the over-expression of CKAP2 plays a role in chromosomal segregation using a diploid (DLD1) colorectal cell lines as a model. Endogenous expression and localization of CKAP2 was confirmed with immunocytochemistry and Western blot analysis. Knockdown efficiency was determined using QRT-PCR and Western blot. Aneuploidy was assessed by chromosome counts and structural aberrations were assessed by spectral karyotyping (SKY) analysis.

Results: We found that in DLD1, CKAP2 colocalizes with the spindle but not the centrosomes. Analysis of the cell cycle profile, after mitotic block, showed that CKAP2 protein levels are dramatically reduced post-mitosis, indicative of degradation by APC/C. Transfection with shRNA against CKAP2 results in a 15% increase of multi-polar spindles, 30% increase of centrosomal aberrations, and lack of centrosome clustering. Transfected cells also have an increase of lagging chromosomes and micronuclei, suggestive of errors in chromosome attachment or segregation. This finding is validated by an increase of average chromosome number in 50% of cells where expression of CKAP2 is inhibited.

Conclusion: We propose that CKAP2 depletion negatively impacts spindle tension, by affecting either the microtubule kinetochore attachments or microtubule cross-linking, ultimately resulting in a decreased capability to cluster extra centrosomes into a bipolar spindle.

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The minus-end-directed motor Kar3p drives nuclear congression through spindle pole body-cytoplasmic microtubule interactions.

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After cell fusion during mating in Saccharomyces cerevisiae the two nuclei are pulled towards each other through a microtubule-dependent process called nuclear congression. This process has been intensively studied using genetics and live cell fluorescence microscopy analysis of various mutants displaying specific defects in the mating process. However, the organisation and the interaction between cytoplasmic microtubules nucleated from opposite spindle pole bodies (SPBs) as well as the mode of force generation for nuclear migration remained unclear (Rose, 1996; Molk et al., 2006; Molk and Bloom, 2006). To approach this question we resolved the three-dimensional microtubule organisation during nuclear congression using electron tomography carried out on mating cells at different stages in the process. The models show no particular connections between microtubules emanating from opposite SPBs, for instance no microtubule overlaps or microtubule-end interactions. Strikingly, in these models we always observed long microtubules passing the opposite SPB with an average distance between the SPB and these microtubules compatible with the size of a molecular motor. The minus end-directed kinesin Kar3p is known to be essential for nuclear congression (Meluh and Rose, 1990; Endow et al., 1994). Therefore, we used a fully functional GFP labelled Kar3p for live cell imaging and confirmed its localization at the cytoplasmic side of the SPB. We also validated the significance of long cytoplasmic microtubules during nuclear congression. Subsequent work using genetics and live cell microscopy demonstrated that in fact no inter-microtubule interaction between mating partners is necessary for efficient nuclear congression. We identified the molecular anchor of Kar3p at the cytoplasmic side of the SPB and found an additional dependency on Cik1p, one of the two light chains of Kar3p. Altogether, our results support a model in which Kar3p drives nuclear congression by directly connecting SPBs through microtubules nucleated at the opposite SPB.

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Fission yeast Rad26^{ATRIP} and Ase1^{PRC1} delay spindle pole body separation in response to interphase microtubule damage.

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Interphase microtubules position organelles and transport cellular components. We report that damage to interphase microtubules in fission yeast activates a checkpoint that delays spindle pole body separation, an early event of mitotic entry. This interphase microtubule damage checkpoint is dependent upon Rad26^{ATRIP}, a regulatory subunit of Rad3^{ATR} that occupies central positions within DNA checkpoint pathways. Specifically, we find that mutation of a putative nuclear export sequence in Rad26^{ATRIP} disrupts this microtubule checkpoint without affecting DNA checkpoints. This Rad26^{ATRIP} mutation also compromises cellular morphology and mini-chromosome stability, suggesting that this interphase microtubule checkpoint facilitates proper morphology and chromosome segregation. Double mutant and overexpression analyses show that Ase1^{PRC1} also operates in this pathway. We discuss how Rad26^{ATRIP} and Ase1^{PRC1} may function in this checkpoint-response to interphase microtubule damage.

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A Role for SUMO-Targeted Ubiquitin Ligases in Spindle Positioning.

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Asymmetric cell divisions are crucial during development of multicellular organisms as well as to control cell fate between stem cells and differentiating cells. The crucial step in asymmetric cell division is orientation of the mitotic spindle along the axis of cell polarity. One mechanism to ensure spindle positioning is orientation of spindle microtubules (MTs) along the actin cytoskeleton by protein complexes that link astral MTs with cortical actin. In budding yeast, a complex consisting of the MT plus end-interacting protein Bim1, the linker protein Kar9, and the myosin motor Myo2 ensures spindle orientation along the mother – bud axis of the dividing cell.
Function of Kar9 as a crucial player in this complex is regulated by posttranslational modifications, i. e. phosphorylation, SUMOylation, and ubiquitylation. Specifically, phosphorylation and SUMOylation regulate asymmetric Kar9 localization on the spindle. Ubiquitylation targets Kar9 for proteasomal degradation and is dependent on actin-based transport to the bud neck and on the septin ring. Localized Kar9 degradation at the bud neck seems to be required to destabilize the Kar9 – Bim1 – Myo2 complex. Ubiquitylation of Kar9 depends on prior phosphorylation, unphosphorylated Kar9 is highly stable and stabilizes MT interactions with the bud neck.
Here, we investigate the interplay of SUMOylation and ubiquitylation. Kar9 interacts with the SUMO-targeted ubiquitin ligases (STUbL) Slx5 and Ris1 in a yeast two hybrid analysis. Deletion of Slx5, Slx8 (which acts in a complex with Slx5), and Ris1 leads to a reduction in Kar9 ubiquitylation and to Kar9 stabilization, suggesting that these enzymes are required for Kar9 ubiquitylation. In support of this idea, Kar9 is also partially stabilized upon inactivation of SUMO protein (*smt3-331* strain). Furthermore, in an *slx5slx8ris1Δ* strain, overall Kar9 levels are significantly increased, suggesting that the protein accumulates due to a defective degradation mechanism.
Thus, we propose that Kar9 SUMOylation promotes subsequent ubiquitylation and that Kar9 degradation is at least partially dependent on the STUbL Slx5 – Slx8 and Ris1. To our knowledge, this is the first demonstration of a cytoplasmic target for SUMO-targeted ubiquitin ligases.

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Proteomic and morphology-based approaches to microtubule nucleation in meiotic spindles.

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Microtubules nucleate throughout Xenopus egg meiosis-II spindles by an unknown mechanism, and spatial regulation of this nucleation is central to spindle morphogenesis. We addressed the mechanism by which Ran.GTP promotes microtubule nucleation using proteomic and morphology-based approaches. We curated a database of Xenopus laevis proteins, and used it to enable differential proteomics in Xenopus egg extracts. We showed previously that Ran-dependent microtubule nucleation factors could be captured on beads coated with antibody to XRHAMM, a spindle pole protein (Curr Biol. 2004 Oct 26;14(20):1801-11.). We were able to repeat this reaction in high-speed supernatant of egg extract, provided glycogen was added (see Groen et al, MBC, 2011, for glycogen effects). We applied differential proteomics to investigate factors that were recruited to XRHAMM coated beads that do, and do not, nucleate microtubules. In parallel, we investigated the spatiotemporal regulation of TPX2, a key intermediate between Ran.GTP and microtubule nucleation. Using a novel immuno-EM approach in egg extract, we localized TPX2 to microtubule bundles in the center of spindles and well as to poles. We hypothesize TPX2 may trigger microtubule nucleation from these bundles.

Cilia and Flagella III

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Three-dimensional analysis of airway cilia motility.

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Cilia and flagella are evolutionally-conserved organelles and observed in a variety of organisms ranging from protozoa to mammals. Cilia and flagella show highly coordinated motility. The ciliary and flagellar motilities have been well investigated for several decades, for example, as to the beating frequency, the beating amplitude, or the beating pattern. These have been examined so far with normal microscopy. The data obtained by the normal microscopy have only two-dimensional information. Thus, it is very difficult to analyze clearly the three-dimensional motility. In addition, the fluid flow generated by millions of cilia hampers the precise analysis of cilia motility as it can interfere with the ciliary motility through fluid dynamics. To overcome these two problems, we isolated ciliary axonemes from mouse tracheal cilia, reactivated them with ATP in vitro, and recorded the motion of fluorescent bead attached to the axonemal tip with recently-developed three-dimensional tracking microscope. The reactivated cilia showed three-dimensionally different trajectories in preparative and effective strokes. In particular, majority of cilia moved slightly counterclockwise when they were observed from top. In conclusion, we were able to observe and analyze the pure motion of ciliary strokes.

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The importance of *Ccdc42* in motile cilia function in *Tetrahymena thermophila* and flagella formation in *Mus musculus.*

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The motile cilium, referred to as a flagellum in certain cells, is a highly conserved microtubule based structure found in a diverse array of eukaryotic organisms. For example, homologous genes essential for the assembly of motile cilia are found in both the unicellular alveolate *Tetrahymena thermophila*, as well as in *Mus musculus* (house mouse). As motile cilia are essential to human development and fertility, the study of genes necessary for their function can advance our understanding of this organelle's importance. To help achieve this, we have embarked on a study of the cilia gene *Ccdc42* in both *T. thermophila* and in *Mus musculus*. This work has yielded a somatic knockout of *Ccdc42* in *T. thermophila* which results in defective motility and a defect in motile cilia assembly. This data strongly suggests the gene is necessary for proper motile cilia function in this organism. Strikingly, when we created a mouse homozygous for a null allele of *Ccdc42*, we found that the mutants do not display hydrocephalus or female infertility, phenotypes that are typically associated with defective motile cilia. However, further analysis revealed that male *Ccdc42* mutants were sterile due to a defect in flagellum assembly during spermatogenesis, in addition to displaying abnormal placement of the head-tail coupling apparatus, a spermatogonia structure homologous to the basal body. These results indicate an evolutionarily conserved function of *Ccdc42* between *T. thermophila* cilia and mouse spermatogonia that does not extend to all other mammalian cells possessing motile cilia.

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The cytoskeleton interprets PCP cues to promote establishment of cilia polarity and metachronic synchrony in multiciliated cells by connecting individual basal bodies.

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Motile cilia driven directed fluid flow plays an important role in a variety of different biological processes such as mucus clearance from the respiratory tract, oocyte migration, movement of cerebral spinal fluid through the ependyma and establishment of left-right axis during development. Ciliated epithelia rely on planar cell polarity (PCP) signaling to establish cilia polarity, as demonstrated for the mammalian embryonic node and ependyma as well as the skin of *Xenopus* tadpoles; however the mechanistic details downstream of PCP signaling remain largely unknown. Remarkably, both actin and microtubules interact with basal bodies in multiciliated cells and have been linked to PCP signaling in other systems. Using multiciliated cells in the skin of *Xenopus* tadpoles, we have investigated the role of cytoskeletal dynamics during the establishment and maintenance of cilia polarity. Multiciliated epithelia have to evenly space and orient individual cilia within a single cell to establish rotational polarity. Using fluorescent markers for basal bodies and rootlets we can score the position and orientation of individual cilia during establishment of cilia polarity. High resolution confocal imaging reveals that both actin and microtubule networks at the apical surface of ciliated cells undergo structural rearrangements during establishment of rotational polarity connecting individual basal bodies to their neighbors as they become oriented. These connections reflect the polarization state of the cell and pharmacological disruption of both actin and microtubule dynamics differentially perturbs the establishment of rotational polarity without affecting cilia structure or beat

frequency. In addition to establishment of rotational polarity, multiciliated cells have to temporally coordinate cilia beating in order to achieve metachronic synchrony. We show that actin filaments connecting individual rootlets to their neighboring posterior basal bodies along the axis of cilia beating are required to maintain the organization of the metachronic wave. Taken together our data suggest that the cytoskeleton is essential for the spatial interpretation of polarity cues downstream of PCP signaling as well as the temporal coordination of cilia beating. More specifically while cytoplasmic microtubules coordinate polarity between neighboring cilia, a distinct subset of cytoplasmic actin filaments are required for proper cilia spacing, integration global polarity cues and coordination of metachronic cilia beating.

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Flagellar Levels of Lis1 are Dynamically Modulated in Response to Alterations in Beat Frequency.

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Lis1 is a key regulator of cytoplasmic dynein and is also present in motile mammalian cilia and the flagella of wildtype Chlamydomonas. Lis1 associates with the outer dynein arms and is missing from flagella of mutants which lack this entire structure or just the outer arm alpha heavy chain. We have now found that Lis1 levels are significantly enhanced (5-10 fold) in flagella of impaired-motility or paralyzed mutants lacking various dyneins or motor units, the radial spokes or central pair microtubule complex. Analysis of double mutants defective for both outer arms and radial spokes (oda1pf14) or the central pair (oda1pf18) indicate that outer arms are required for localization and/or retention of these enhanced levels of Lis1 within the flagellum. Extraction of wildtype and pf14 mutant axonemes with increasing amounts of K acetate and NaCl revealed that Lis1 is more tightly bound to pf14 axonemes than to wildtype suggesting that the interaction is specifically modulated. As Lis1 levels were increased in motility-deficient mutants, we also tested whether the amounts varied with the ability of wildtype flagella to beat normally. Therefore, we reduced the beat frequency of wildtype flagella by placing the cells under high viscous load (16% Ficoll) and found that the flagellar levels of Lis1 increased significantly. We observed a similar (~5-fold) response when beat frequency was reduced from ~50 Hz to 20 Hz by placing the cells under high reductive stress. Within cytoplasm, Lis1 localized to the base of the flagella and was also detected in a second punctate structure towards the opposite end of the cell. Fractionation of wildtype cell body extracts identified Lis1 within three major cytoplasmic complexes with masses of ~1.1 MDa, 158 kDa and 40 kDa. In contrast, oda6 extracts which do not assemble intact outer arms contained mainly the 40 kDa peak which likely represents monomeric Lis1. These data suggest that Lis1 levels within the flagellum are dynamically modulated in response to alterations in flagellar motility; i.e. the cell responds to an imposed reduction in beat frequency (through mutation, viscous load or reductive stress) by increasing flagellar Lis1.

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Axoneme distortion in sea urchin sperm flagella evaluated by a geometric analysis of bending and shear.

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When the axoneme of a flagellum or cilium bends, it is an accepted maxim that the resulting interdoublet shear can be found by multiplying the interdoublet spacing in the bending plane by the shear angle. Shear angle is the difference in angle of the flagellum (radians) from the flagellar base to the point of interest. This relationship rests on the assumption that the outer

doublet microtubules of the axoneme are relatively inextensible and incompressible under physiological loading; an assumption that was tested by Brokaw (1991, J. Cell Biol. 114: 1201-1215) and found to be essentially correct. Due to the presence of interdoublet elastic linkages (nexin links) in an intact sea urchin flagellum, the unrestrained passive flagellum can only be straight when the interdoublet shear is zero. Sea urchin sperm that are treated with 50 μM sodium metavandate in the presence of 0.1 mM Mg-ATP are rendered passive by inactivation of the dyneins. When sperm in this condition are bent with a glass microprobe, the flagellum distal to the probe develops a “counterbend”, which is a bend of opposite curvature to the imposed bend (Pelle et al., 2009, Cell Motil. Cytoskeleton 66: 721-735). When bends were imposed on passive sea urchin flagella, the resulting counterbend was generally insufficient to bring the tip of the flagellum back to the shear angle of the flagellar base. Most often, when the flagellum was bent in the middle or basal region, the tip of the flagellum was straight; this indicates that there is no residual interdoublet shear at the tip. At the same time, the shear angle of the tip was as often as much as one radian different from the base angle. Under these circumstances, either the assumption that the doublets are incompressible and inextensible is incorrect, or the interdoublet spacing must be reduced in the bent region. Since the imposed bends were similar in magnitude to normal physiological bending during the beat cycle, the assumption of incompressibility/inextensibility of the doublets is likely valid. Therefore, we must conclude that the interdoublet spacing of the axoneme distorts appreciably (as much as 40 %) under physiological loading. Supported by grant MCB-0918294 from the National Science Foundation.

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Localization of TGFβ signaling components and Wnt receptors to primary cilia during neurogenesis.

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Transforming growth factor (TGF)-ß and Wingless/Int (Wnt) signaling controls various physiological and pathophysiological processes of the brain, e.g. through the regulating of proliferation and differentiation of neural stem cells, neuronal progenitor cells and mature neurons that possess primary cilia, which play a role in neuronal signaling, adult neurogenesis, and brain tumor formation. Here we investigated the link between primary cilia and regulation of Wnt and TGFβ signaling in NT2 cells, a human embryonic carcinoma stem cell line, which can be differentiated into neurons using all-trans-retinoic acid (ATRA). Undifferentiated NT2 cells have primary cilia and are positive for stem cell markers OCT2/3, Nanog and Sox2. Upon cell differentiation in the presence of ATRA, expression of stem cell markers is gradually replaced by increased expression of neuronal markers, PAX6, NEUROD1 and ß III tubulin. ß III tubulin marks mature neurons from day 12 of ATRA stimulition. Since TGFβ signaling inhibits neural cell differentiation, we initially studied the localization of TGFß-receptor 1 (TGFßR1) to primary cilia in non-differentiated NT2 cells and after stimulation with ATRA. Our studies show that TGFßR1 uniquely localize to the ciliary tip and at the ciliary base of non-differentiated NT2 cells, and that ciliary localization is abolished during the early steps of differentiation and prior to formation of mature neurons. Similarly, the Wnt receptor, Frizzled-3, localizes along the entire length of the primary cilium in non-differentiated cells. In line with this observation, activation of TGFßR1 signaling through phosphorylation of the transcription factors, SMAD2/3, was reduced at the ciliary base during the differentiation of NT2 cells. This is the first report on the localization of TGFβ signaling components and Frizzled-3 to primary cilia in neuronal stem cells. We hypothesize that primary cilia take part in coordinating TGFß and Wnt signaling to regulate maintenance of pluripotency and prevent neural cell differentiation, and when aberrantly regulated by defects in ciliary assembly or by mislocalization to primary cilia may lead to developmental brain defects and/or glioblastoma development from neural stem cells.

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Flagellar adhesion and cilium-generated signaling in *Chlamydomonas* trigger flagellar mobilization and shedding the adhesion receptor of SAG1.

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The cellular and molecular mechanisms that specify the concentrations of membrane proteins in cilia and flagella at steady state and during cilium-generated signaling are poorly understood. Our laboratory uses the biflagellate green alga *Chlamydomonas* as a model system to study regulated movement of the flagellar adhesion receptor SAG1 from the cell body to the flagella. Binding between the mating type + (mt+) flagellar adhesion receptor (agglutinin) SAG1 to its cognate receptor SAD1 on flagella of mt- gametes induces flagellar adhesion and activation of an IFT-requiring, cilium-generated signaling pathway ultimately leading to cell-cell fusion and zygote formation. Previous work from our laboratory and others using bioassays for SAG1 suggested that pathway activation triggers mobilization of a pool of SAG1 from the cell body to the flagella. Our work indicated that the cell body form was on the external side of the plasma membrane in an inactive form that became active upon recruitment to the flagellar membrane. Here, we report use of new tools to study regulation and properties of SAG1. We show that the *SAG1* gene bearing a C-terminal HA tag and driven by its endogenous promoter rescues flagellar adhesion and cell fusion in the flagellar adhesion mutant, *sag1-5*. Consistent with previous bioassays, immunofluorescence studies of resting *SAG1-HA-sag1-5* mt+ gametes show that the protein is present at the cell body and the flagella. As has been shown for a small number of other ciliary membrane proteins, we determined that SAG1 undergoes cleavage soon after its synthesis, and we detect only the HA-tagged, 65 kDa, C-terminal portion (SAG1-HA-C65). The full length, non-glycosylated form is predicted to be ~330 kDa, and the glycosylated form likely is much larger. SAG1-HA-C65 is not detected in vegetative *SAG1-HA-sag1-5* cells. Consistent with predicted C-terminal transmembrane domains, solubilization of SAG1-HA-C65 requires detergent and is not achieved upon mechanical disruption, high salt or high pH treatments. Cell fractionation demonstrates that in resting gametes the majority of SAG1-HA-C65 is present in cell bodies and only a small portion (~7%) is in flagella. Within minutes after cilium-generated signaling is triggered by flagellar adhesion, however, pre-existing SAG1-HA-C65 on the cell body is mobilized to the flagella. In cells undergoing prolonged flagellar adhesion and signaling, we detect larger molecular weight precursors in the cell body, but only the 65 kDa form is detected in the flagella. Finally, we demonstrate that flagellar adhesion and cilium-generated signaling leads to loss of SAG1-HA-C65 from the flagella and concomitantly we find that large amounts of the protein appear in particulate form in the medium. Our work suggests that *Chlamydomonas* controls the flagellar amounts of the membrane protein SAG1 by at least two mechanisms. In resting cells, movement into flagella is restricted by an uncharacterized barrier; and in cells undergoing flagellar adhesion and signaling, SAG1 levels are reduced by release or shedding of SAG1. Such a model could explain regulated increase and loss of other ciliary proteins such as Patched and Smoothened in vertebrate cells. Experiments are underway to determine whether the SAG1 released into the medium is in the form of large non-membranous aggregates or if it is in membrane vesicles.

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Evidence for G Protein-coupled receptor dimerization in neuronal ciliary signaling.

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Primary cilia are specialized organelles that perform important sensory and signaling functions and are required for cellular homeostasis and proper development. Defects in the formation or function of primary cilia have been implicated in the pathogenesis of many human

developmental disorders and diseases, collectively termed ciliopathies. Although great progress has been made in elucidating the functions of primary cilia on some cell types, the precise functions of most primary cilia remain unknown. The importance of neuronal cilia function is highlighted by the fact that ciliopathies can be associated with neurological phenotypes, such as cognitive deficits. Interestingly, disruption of certain signaling molecules which normally localize to neuronal cilia, such as adenylyl cyclase type III (ACIII) and the G protein-coupled receptors (GPCRS) Melanin concentrating hormone receptor 1 (Mchr1) and Somatostatin receptor 3 (Sstr3) is associated with learning and memory deficits in mice. This suggests neuronal ciliary signaling is required for proper learning and memory. For the first time, we have detected Mchr1 and Sstr3 colocalizing within the same cilium in distinct brain regions. In addition, we have evidence suggesting Mchr1 and Sstr3 heterodimerize. GPCR dimerization can affect ligand binding properties as well as down stream signaling. Therefore, our findings add an additional layer of complexity to neuronal ciliary signaling. Understanding the functional relevance of ciliary GPCR heterodimerization could elucidate the roles neuronal cilia play in non-synaptic signaling and could provide insight into potential therapeutic targets for ciliopathies.

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Hedgehog signaling regulates the myelination of peripheral nerve axons through the primary cilia.

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Myelin formation is an essential prerequisite for the nervous system to transmit an impulse efficiently by a saltatory conduction. In the peripheral nervous system (PNS), Schwann cells (SCs) engage in the myelination, whilst a detailed mechanism underlying the myelination is still an open question. In this study, we assume that the primary cilium of Schwann cells is a regulator of myelination from the following reasons; (1) Our previous results on Kif3B hypomorph mutants suggested that primary cilia may be responsible for the myelination of PNS, since these mutants showed severely disorganized PNS (Takeda et al., 44th ASCB, 2004). (2) Previous study has shown that Dhh-null mutant mice showed abnormal development of perineurium, and reduced number of myelinated axon as well as leanness of myelin sheath. (3) Molecular machineries for Hh signaling are specifically localized on the primary cilia. Taking these lines of evidence into account, we hypothesize that Hh signaling received and transduced by a primary cilia play an important role in modulating the myelination of the PNS.

To testify our hypothesis, we used the co-culture system of mouse dorsal root ganglion (DRG)/SC, wherein the behavior of SCs can be analyzed by maintaining the interaction of SCs with DRG neurons intact. Under this condition, SCs had primary cilia, and Hh signaling molecules were accumulated on the primary cilia. When the SCs were stimulated by an addition of Dhh or SAG, a Smo agonist, formation of myelin segments on the DRG axons was facilitated. On the contrary, upon administration of cyclopamine (CPN), an inhibitor of Hh signaling, myelin segments became comparable to those of controls. Of noteworthy, the ratio of SCs harboring the primary cilium reached highest during the pre-myelination stage. Furthermore, strongest effects of Hh on the myelination were encountered during the same stage. These results collectively indicate that the Hh signaling regulate the myelin formation through the primary cilia in the PNS.

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Cilia in Neural Progenitor Cells in the Adult Hippocampal Subgranular Zone: Relation to Cell Cycle.

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Primary cilia are critical for the genesis of the mammalian brain, as evidenced by the marked reductions in neural cell populations of cilia-deficient transgenic mice. New neurons are generated throughout adulthood in the hippocampal subgranular zone, where cilia-mediated sonic hedgehog signaling promotes proliferation. In the present immunohistochemical study of the mouse subgranular zone, cilia were characterized in the neuronal lineage, which includes neural stem cells, progressively more committed progenitor types, neuroblasts, and neurons. Both the length and incidence of cilia were lower in the more proliferative populations, consistent with the need for cilium withdrawal before mitosis. Cilia were seen in a substantial percentage of Ki67+ cells (engaged in the active cell cycle), but rarely in cells expressing phospho-histone 3 (late G2, S). Thus, primary cilia may be retained through much of the cell cycle. Cilia of Ki67+ cells were distinctive in having faint ACIII immunostaining despite strong Arl13b immunoreactivity; ciliary ACIII levels were notably higher in Ki67- cells, which are post-mitotic or non-cycling. The differential expression of these two proteins might be important for cilium resorption and cell cycle progression. Data from the present study were incorporated into a model of cilia assembly and disassembly in relation to the cell cycle during neurogenesis.

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NHE1 trafficking in fibroblasts with primary cilia.

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We previously showed that Platelet-Derived Growth Factor Receptor alpha ($PDGFR_{\alpha}$) is upregulated and targeted to the primary cilium in growth arrested wt mouse embryonic fibroblasts (MEFs)and that $PDGFR_{\alpha}$ signaling is coordinated by the cilium to control directional cell migration and chemotaxis [Schneider et al., 2010, Cell Physiol. Biochem. 25:279-292]. The $PDGFR_{\alpha}$-mediated signaling cascade activated by its specific ligand, PDGF-AA, runs through the Mek1/2-Erk1/2 and Akt pathways in the cilium and via the Na+/H+ exchange protein, NHE1. Directional cell migration and chemotaxis in response to PDGF-AA is blocked in mutant Tg737orpk MEFs that have no or very short cilia. SEM reveals that wt MEF primary cilia extend from a ciliary pocket positioned at the apical cell surface above and close to the nucleus. In migrating cells the cilium orients towards the leading edge and parallel to the path of migration, where NHE1 at the leading edge alkalinizes the cytoplasm resulting in assembly of the actin cytoskeleton. Consequently, specific inhibitors of Akt, Mek1/2 or NHE1 destroy PDGF-AA-mediated directional cell migration. Preliminary evidence has indicated that NHE1 is present in cytoplasmic vesicles before stimulation and that localization at the leading edge is blocked by Akt inhibition. This implies that the ciliary $PDGFR_{\alpha}$ signaling cascade coordinates the trafficking of NHE1-containing vesicles through the cell to the forming leading edge for directional migration. To visualize NHE1 transport directly, we have stably transfected GFP-NHE1 into wt MEFs that form primary cilia. Fluorescent NHE1 is seen in the Golgi and in small vesicles. Video imaging shows that vesicular trafficking of NHE1 is occurring as the cell moves. We suggest that NHE1 vesicle transport will occur in a direction determined by a PDGF-AA gradient to help establish actin-rich lamellopodia at the leading edge of migrating cells when primary cilia are present.

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The Primary Cilium is Regulated By and Plays a Critical Role in Epithelial-Myofibroblast Transition.

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Epithelial-myofibroblast transition (EMyT), a key process in fibrogenesis, involves the activation of a myogenic program (-smooth muscle actin (SMA) expression) in the injured epithelium. EMyT is an important pathologic feature of polycystic kidney disease, the prototypic ciliopathy. However, the impact of EMyT on the cilium or conversely the role of the cilium in EMyT remains largely unknown. Previously we have developed a two-hit model of EMyT, wherein the injury (uncoupling) of adherens junctions (AJs) induced by scratch wounding or low calcium medium (LCM) and the presence of the fibrogenic cytokine TGFß are both necessary to induce SMA expression in kidney tubular (LLC-PK1) cells. These inputs activate myocardin-related transcription factor (MRTF) and Smad3 respectively, the interplay of which elicits SMA expression. In this study we followed ciliary changes in this two-hit EMyT model and tested the effect of deciliaition on SMA expression. We show that EMyT is associated with a biphasic change in the primary cilium, characterized by initial growth followed by dramatic ciliary loss. Specifically, LCM-induced AJ uncoupling or siRNA-mediated downregulation of E-cadherin triggered a 2-fold growth in ciliary length at 24 h. TGFß alone had little effect on the cilium, while complete EMyT, induced by LCM and TGFß, led to significant ciliary lengthening at early times (6-24 h), followed by dramatic deciliation at 48 h (< 10% ciliated cells vs. 80% in the control). The loss of the cilium is Smad3-dependent since downregulation of Smad3 suppressed the LCM+TGFß-provoked deciliation. While the emergence of myofibroblasts is eventually associated with ciliary loss, the primary cilium itself is not dispensable for EMyT. Deciliation prior to induction of EmyT by Chloral Hydrate or the Hedgehog Pathway Inhibitor-4 (HPI-4) abolished the TGFß+LCM-triggered SMA-promoter activation and SMA protein expression. These deciliating agents caused substantial reduction in MRTF and Smad3 protein levels (or immunoreactivity), concomitant with a robust increase in cytosolic acetylated tubulin. Cyclopamine, a hedgehog pathway inhibitor, which does not affect ciliation, reduced SMA expression and had no effect on MRTF, Smad3 and cytosolic acetylated tubulin. Thus we conclude that the primary cilium is both a target and a mediator of EMyT. The cilium is regulated by AJ integrity and it participates in the regulation of SMA expression via hedgehog pathway-dependent and independent mechanisms. Regarding the latter, cilium integrity seems to control protein acetylation and the expression/stability of MRTF and Smad3.

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Targeting of PDGFRα to the primary cilium.

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Primary cilia are microtubule-based organelles that extend from the surface of most mammalian cells in growth arrest. The ciliary membrane is continuous with the plasma membrane but contains cilia-specific receptors and ion-channels like polycystin-1 and -2, components from the Hedgehog and Wnt signaling pathway, and the Platelet Derived Growth Factor Receptor (PDGFR)α. PDGFRα is up regulated during growth arrest and localizes to the primary cilium in NIH3T3 cells; this localization is essential for activating the receptor through the ligand PDGF-AA (Schneider et al., Curr Biol, 2005). The molecular mechanism that transports PDGFRα to the primary cilium is unknown.

Studies on other ciliary proteins showed that specific amino acid sequences function as Ciliary Localization Signals (CLSs). Polycystin-2, rhodopsin, somatostatin and serotonin receptors all

contain one or more CLSs, but currently no clear consensus CLS has been found. However CLSs have generally been localized to the cytoplasmic region of the proteins, and some CLSs resemble classic Nuclear Localization Signals (NLSs) that can confer nuclear as well as ciliary targeting, depending on Ran-GTP concentrations (Dishinger et al., Nat Cell Biol, 2010). PDGFRα and PDGFRβ localize to the cilia- and plasma membrane, respectively, suggesting that these closely related receptors contain different targeting signals. PDGFRαand PDGFRβ are almost identical in amino acid sequence (92% identity), except for the last ca. 146 amino acid residues that are localized in the C-terminal tail. Since PDGFRα localizes to primary cilia whereas PDGFRβ does not (Schneider et al. Curr Biol, 2005), the C-terminal tail of PDGFRα likely contains a specific CLS. To test this hypothesis, we cloned the cytoplasmic C-terminal fragment of PDGFRα and PDGFRβ, respectively, into the pEGFP-C1 vector (Clontech), and expressed these fragments in Inner Medullary Collecting Duct (IMCD) 3 cells. The transfected cells were grown to confluence, serum-starved to induce ciliogenesis, and examined by immunofluorescence microscopy with antibodies against acetylated alpha tubulin (cilia marker) and GFP. Interestingly, we found that GFP-tagged, C-terminal PDGFRα localized predominantly to the nucleus whereas GFP-tagged C-terminal PDGFRβ was localized to the cytosol. Furthermore, we observed that in a few cells the C-terminal PDGFRα fusion protein also localized to the primary cilium. We are currently trying to further define the CLS of PDGFRα by mutagenesis of full-length GFP-PDGFRα, and are also investigating the potential role of known cilia vesicle transport proteins (Arf4, IFT20, Rab8) in targeting of PDGFRα to the ciliary membrane.

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A role of the primary cilium in regulating TGFβ signaling in cell cycle control and differentiation.

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The transforming growth factor (TGF)-β signaling pathway plays a critical role in cell cycle control, migration and differentiation and when aberrantly regulated leads to developmental defects and cancer. TGFβ signaling is regulated through the endocytosis and internalization of TGFβ receptor (TGF$\tilde{\beta}$R) via clathrin-coated pits (CCPs) and vesicles (CCVs) at which the receptor activates transcription factors, Smad2/3 and modulates the downstream signaling pathways, PI3K-Akt and Mek1/2-Erk1/2. Here we investigated the relationship between regulation of TGFβ signaling and primary cilia, which are major sensory organelles in developmental processes and in tissue homeostasis. In many cell types a large fraction of the cilium is localized deep in the cytoplasm within an invagination of the plasma membrane known as the ciliary pocket, which is an endocytic domain for CCV formation.

Initially we used cultures of fibroblasts to show that TGFβ signaling components (TGFβ-RI/RII and Smad2/3/4/7) localize at the base and/or along the lower region of the primary cilium in hFF and MEF cells. TGFβ stimulation induced phosphorylation of Akt, Mek1/2, Erk1/2 and Smad2/3 and increased the localization of TGFβ-RI and phospho-Smad2/3 at the base and lower part of the primary cilium, which had high numbers of CCVs and early endosomes. These events were associated with phosphorylation of Retinoblastoma protein (Rb) marking G_1/S-phase transition and cell cycle entry. In cilia deficient MEFs, *Tg737^{orpk}*, there was a major reduction in TGFβ-induced Smad2/3 and Rb phosphorylation and formation of CCVs at the base of stumpy cilia. These results indicate that the primary cilium coordinates TGFβ signaling and partly through the ciliary pocket via CCV formation to control cell cycle entry in fibroblasts. Similar results were

observed during the differentiation of EC P19.CL6 stem cells and human embryonic stem cells (hESC) into cardiomyocytes. During the early stages of differentiation, TGFβ-R and Smad2/3/4 were highly up-regulated at both mRNA and protein levels. Localization of TGFβ-RI and p-Smad2/3 was highly increased at the ciliary base during differentiation, which was accelerated with TGFβ-R agonist, TGFβı, and blocked in the presence of antagonists. Further, knockdown of IFT20 required for transport of Golgi-derived vesicles to the ciliary base blocked TGFβ signaling and differentiation of P19.CL6 cells. Our results support the conclusion that TGFβ signaling is regulated by primary cilia, via the ciliary pocket to regulate cell cycle control and differentiation.

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A Role for the Primary Cilium in Notch Signaling and Epidermal Differentiation During Skin Development.

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Ciliogenesis precedes lineage-determining signaling in skin development. To understand why, we performed shRNA-mediated knockdown of seven intraflagellar-transport-proteins (IFTs), and conditional ablation of Ift-88 and Kif3a during embryogenesis. In cultured keratinocytes and embryonic epidermis, all eliminated cilia and many (not Kif3a) caused hyperproliferation. Surprisingly and independent of proliferation, ciliary-mutants displayed defects in Notch-signaling and in commitment of progenitors to differentiate. Notch-receptors and Notch-processing-enzymes colocalized with cilia in wild-type epidermal cells. Moreover, differentiation defects in ciliary-mutants were cell-autonomous and rescued by transgenic expression of activated Notch (NICD). In contrast, Shh-signaling was neither operative nor required for epidermal ciliogenesis, Notch-signaling or differentiation. Rather Shh-signaling defects in ciliary-mutants occurred later, arresting HF morphogenesis in the skin. These findings unveil temporally and spatially distinct functions for primary cilia at the nexus of signaling, proliferation and differentiation: a novel, early role in epidermis, whose morphogenesis relies upon Notch-signaling; and a later role in HFs, reliant upon Shh-signaling.

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Primary Cilia in Appetite and Satiety.

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Primary cilia are required to regulate certain signaling pathways during mammalian development. In adult tissues, cilia are also important as evidenced by the spectrum of phenotypes observed in mouse models and human patients with ciliary defects (ciliopathies); however, their roles in tissue physiology and homeostasis are poorly understood and the mechanisms underlying the ciliopathies remain enigmatic. We are addressing this issue using conditional tissue-specific cilia mouse mutants and show that disruption of cilia in the hypothalamus results in hyperphagia and obesity. A similar obesity phenotype was reported in mouse models of Bardet-Biedl Syndrome (BBS). BBS mutants have defects in cilia receptor localization and are thought to be unable to properly localize the receptor for the anorexigenic peptide leptin. Here we assess the leptin signaling axis in cilia mutants and demonstrate they are sensitive to leptin prior to becoming obese, are leptin insensitive when obese, and then regain leptin sensitivity after caloric restriction to bring mutants back to wild type body weight. Thus leptin resistance is a secondary consequence of the obesity and not causal, indicating that

pathways other than the leptin-melanocortin axis are involved. Data indicate that one of these pathways could be the melanin concentrating hormone (MCH) axis. Previous data indicate that MCH receptor localizes in the cilium of control but not of BBS mutants. Further, we show that MCH binds MCHR1 in the cilium and that in the absence of the cilium, the MCH signaling pathway is altered. Pharmacological and genetic studies are being conducted to assess whether defects in MCH signaling are directly associated with the obesity phenotype in cilia mutants. This work will provide novel insights into how dysfunction of the cilium contributes to the onset of obesity and more generally the importance of the cilium in regulating normal neuronal activity and energy balance.

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Ciliary trafficking of polycystin-1 is regulated by distinct components of the BBSome.

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Polycystin-1 (PC1), whose deficiency causes autosomal dominant polycystic kidney disease, localizes to the primary cilia of renal epithelial cells where it mediates mechanosensation and signaling through several pathways. It remains elusive, however, how PC1 is targeted to cilia. We generated an N-terminal YFP-tagged full-length PC1 expression construct (YFP-PC1), which successfully traffics to primary cilia in IMCD3 cells. Removal of the entire C¨Cterminal cytoplasmic tail (C-tail, ~198 aa) abolishes this ciliary trafficking, however. Further deletion analyses of the PC1 C-tail narrowed the region responsible for ciliary trafficking to the middle 100 amino acid of the C-tail spanning the coiled-coil (CC) motif. Notably, deletion of the most C¨Cterminal fragment of PC1 containing the VxPx motif, which has recently been reported to bind ARF4 and is required for the ciliary trafficking of a mini-PC1 construct, does not have any effect on the targeting of full-length PC1 to the primary cilia. Through a yeast two-hybrid screen, we identified that BBS8, a gene mutated in Bardet Biedl Syndrome (BBS), interacts with PC1. Disruption of the CC motif in the PC1 C¨Ctail abolishes this interaction. We also tested the interaction of PC1 with 7 other BBS proteins and found that PC1 interacts with distinct BBS proteins. shRNA depletion of BBS1 but not BBS8 is sufficient to reduce PC1 trafficking to the primary cilia. Furthermore, we found that PC1 is able to interact with the wild type, dominant negative, and constitutively active Rab8 with similar affinities, but the ciliary localization of PC1 relies on functional Rab8. Taken together, these data suggest that the ciliary trafficking of PC1 is subject to the control of the BBSomal pathway.

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CRMP-2 in non-neuronal cells: Identification of a novel cilium targeting signal.

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The primary cilium is a microtubule-based structure. Previously, we showed that lithium induces cilium elongation in a number of cell types. Lithium inhibits several protein kinases, including GSK-3beta. To identify protein components that are involved in lithium and GSK-3beta-related cilium length regulation, we focused on collapsin response mediator protein-2 (CRMP-2). CRMP-2 binds tubulin dimers and microtubules, promoting microtubule assembly. In neuronal cells, CRMP-2 plays a key role in axonal development by regulating microtubule dynamics. GSK-3beta phosphorylates CRMP-2, subsequently regulating axon elongation. In addition, inhibition of GSK-3beta, or expression of the nonphosphorylated form of CRMP-2, induces the formation of multiple axon-like neurites in hippocampal neurons. In non-neuronal cells, CRMP-2 has been found to play a role in regulating trafficking of endocytic components. However,

whether CRMP-2 in non-neuronal cells participates in any microtubule-based functions remains unclear.

In this study, we show that CRMP-2 is a centrosomal protein in cycling cells, with prominent expression at the centrosomes of early interphase cells. In cilium-expressing cells, CRMP-2 shows enhanced association with the basal body and is also present at the primary cilia, although at a barely-detectable level. Lithium treatment of cultured cells could increase CRMP-2 levels at the cilium. Preliminary knockdown of CRMP-2 by shRNA expression interfered with cilium elongation. Expression of CRMP-2-GFP fusion protein confirms that CRMP-2 is expressed at the primary cilia. Deletion analysis of CRMP-2 indicated that CRMP-2 is targeted to the cilia via a small domain close to the C-terminus of CRMP-2. Intriguingly, this domain is a major site of GSK-3beta phosphorylation and contains two VxPx motifs which were shown to be cilium targeting signals found in some cilium proteins. Mutation of the GSK-3beta phosphorylation sites abolished the targeting of the fusion protein to the cilia. However, mutation of both VxPx motifs had no evident effect, suggesting that CRMP-2 contains a novel cilium targeting signal. At present, we are pursuing which protein(s) interacts with this novel cilium target signal to bring CRMP-2 to the primary cilia.

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A transition zone complex of Meckel and Joubert syndrome proteins is required for ciliary assembly and trafficking.

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Primary cilia are cellular antennae that receive and transduce signals critical for embryonic development and adult tissue homeostasis, and whose malfunction leads to a group of human diseases known as ciliopathies. Many ciliopathy proteins localize to a region of the ciliary base known as the transition zone, but the functions of this ciliary compartment remain poorly understood. Here, we found that Tectonic1 (Tctn1), a regulator of mouse Sonic Hedgehog (Shh) signaling, physically interacts with a number of proteins involved in Meckel and Joubert syndromes, two human ciliopathies. Furthermore, we demonstrate that these proteins are part of a large molecular weight complex that localizes to the transition zone. Interestingly, mice lacking components of this complex fail to form cilia in some tissues, but not in others. In the tissues where ciliogenesis is not affected, ciliary membranes display compositional and functional anomalies, as evidenced by the absence of proteins involved in ciliary signal transduction, including the calcium channel Pkd2, Adenylyl Cyclase III, Inositol 5-phosphatase-E and the Shh signaling regulators Smoothened and Arl13b. Taken together, our data reveal a dual role for the transition zone in ciliary assembly and trafficking, and suggest that transition zone dysfunction underlies Meckel and Joubert syndromes. Consistent with this, we have identified a TCTN1 mutation that causes Joubert syndrome in human patients.

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Primary Cilia Loss Occurs Early in Prostate Cancer Progression.

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Prostate cancer is the most prominently diagnosed cancer and the second leading cause of cancer related deaths in men in the U.S. Little is known about the role of primary cilia in prostate cancer progression. Primary cilia are microtubule based organelles present on many mammalian cell types and help the cell sense the extracellular environment. Various signaling pathways important in development involve primary cilia, such as the Hedgehog (Hh) and Wingless/Int (Wnt) pathways, and these pathways are also known to be abrogated in prostate cancer. Dysfunction of primary cilia is the cause of numerous ciliopathies, such as polycystic kidney disease and Bardet-Biedl syndrome, and has been linked to cancers including basal cell carcinoma and medullablastoma. We hypothesize that primary cilia loss promotes prostate cancer by altering cell signaling pathways like Hh and Wnt. We looked in human prostate cancers at cilia, Hh and Wnt signaling. Human prostate tissue from 75 prostate cancer patients was stained with antibodies that recognize acetylated and ¦Ã-tubulin to visualize primary cilia. The expression of these proteins was analyzed and compared between adjacent normal, hyperplastic, prostatic intraepithelial neoplasia (PIN), malignant and perineural invasion areas of each patient's tissue. As a control, normal prostate tissue from 10 patients with bladder cancer (but no prostate cancer) was used. A decrease in the percentage of ciliated cells in cancer (median=1.85%) and PIN (median= 5.67%) was observed compared to normal prostate tissue (median =8.88%). Higher grade cancer (Gleason sum ¡Ý7) also had fewer cilia (median=1.42%) than lower grade (Gleason sum = 6) (median = 2.16%).We are currently investigating Hh and Wnt signaling activity in these tissues by staining serial sections to look for the expected increase (Wnt) and decreased (Hh) as a result of loss of cilia. These results indicate that cilia are lost early in the progression of cancer and that severe cilia loss is increased in the most aggressive forms of prostate cancer. Based on these results, we propose that primary cilia play a role in promoting prostate cancer progression.

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Primary cilia are decreased during breast cancer progression.

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Primary cilia are microtubule-based organelles that protrude from the cell surface. A primary cilium consists of a centriole-based basal body and an axoneme nucleating from the basal body. Intensive research within the last decade demonstrates that the primary cilium plays crucial roles in development and disease through regulation of signaling pathways such as the Hedgehog (Hh), Wingless/Int (Wnt) and platelet-derived growth factor (PDGF) pathways. Recent studies have also linked ciliary dysfunction to cancer. The goal of the present study is to investigate the role of primary cilia in breast cancer progression. To accomplish this goal, we examined the expression of cilia in tissues from 85 breast cancer patients by counting and measuring cilia in four different cell types: the basal and luminal epithelial cells, stromal cells and also cancer cells associated with pre-malignant and malignant lesions. In addition, we examined histologically normal cells that are found adjacent to breast cancer lesions. In order to examine the occurrence of primary cilia in these patients, the tissue was stained with anti-ã tubulin (basal body) and anti-acetylated tubulin (axoneme) antibodies. Cilia were visualized using a Leica Confocal Microscope and the median number and length of the detected cilia was determined. We observed a decrease in the percentage of ciliated cells on pre-malignant lesions (grades 1&2: 0.92%, grade 3: 0.18%) as well as in invasive cancers (grades 1 & 2:

0.16% and grade 3: 0.00%) when compared to basal cells of adjacent normal tissue (22.33%). It is possible that cilia are lost on cancer cells due to their highly proliferative state. However, in cancer cells stained with Ki67 we found no correlation between decreased cilia and high Ki67. Interestingly, the percentage of ciliated stromal cells was decreased in both pre-malignant lesions (grades 1&2: 4.1%, grade 3: 5.9 %) and invasive cancers (grades 1&2: 4.8%, grade 3: 1.4 %) when compared to stromal cells associated with normal adjacent tissue (11.8%). This data suggests that primary cilia are lost very early in breast cancer progression. Future research will include the investigation of the Hh and Wnt signaling pathways in serial sections of these tissue samples to determine if loss of cilia is associated with the anticipated misregulation of these pathways. We propose that loss of cilia works through misregulation of Hedgehog and Wnt pathways to promote breast cancer progression.

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Smurf1 is involved in cardiac development and congenital heart defects.

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The transforming growth factor β (TGF-β) superfamily controls important events in heart development, including cell migration, cardiomyocyte proliferation, chamber formation, endocardial cushion formation, development of smooth muscles and vessel stabilization. The concentration and expression of TGF-β components are tightly controlled with respect to tissue, concentration and time during normal heart development. Congenital heart defects (CHDs) are characterized by a large spectrum of structural anomalies of the heart, and since TGF-β is highly implicated in cardiogenesis, the different TGF-β components are candidate genes for CHDs.

An arrayCGH screening identified a 486 kbp de novo duplication of 7q22.1 in a patient with coarctation of the aorta. This region contains three genes; *TMEM130*, *TRRAP* and *SMURF1*. Smurf1 is an E3 ubiquitin ligase involved in targeting TGF-β components for degradation. SMURF1 interacts with six known heart proteins and is highly expressed in human embryonic and fetal heart. Immunohistochemical analysis of human embryonic and fetal hearts revealed that the SMURF1 protein has a spatially and temporally restricted expression pattern during cardiogenesis, which suggests that SMURF1 plays a specific role in chamber formation. *Smurf1* expression is up regulated during differentiation of P19.CL6 mouse stem cells into mature beating cardiomyocytes, and the expression peaks at day seven post differentiation induction. The same pattern is observed on protein level. Knockdown of Smurf1 results in a delay in differentiation of P19.CL6. Surprisingly, knockdown of Smurf1 with subsequent TGF-β stimulation had a negative effect on the TGFβ/BMP pathway, visualized by decreased phosphorylation of Smad2, which localizes to the base of primary cilia and. In the same knockdown study it was shown that the overall level of Smad2 seemed to be unaffected, indicating an effect upstream of Smad2 phosphorylation. During differentiation, the localization of Smurf1 shifts from being nuclear to cytosolic – a pattern similar to Smad7 localization – suggesting an interplay between these two proteins in regulation of the TGF-β pathway. Since TGF-β receptors localize to primary cilia of P19.CL6 cells to control Smad2 phosphorylation during cardiomyocyte differentiation, it is possible that Smurf1 may act as a positive regulator of ciliary TGF-β signalling in P19.CL6 cells.

Our results suggest that SMURF1 has important functions during heart development, specifically during cardiomyogenesis and chamber formation and potentially through interaction with TGF-β signaling at the primary cilium.

Mitotic Spindle and Kinetochores

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Identification of a TPX2-like protein in Drosophila.

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TPX2 is highly conserved and is one of the best studied spindle microtubule-associated proteins. Characteristic intracellular dynamics and several molecular activities have been reported for this protein, such as nuclear localisation during interphase, poleward movement in the metaphase spindle, microtubule nucleation, microtubule stabilisation, Aurora A activation, kinesin-5 binding, and kinesin-12 recruitment. This protein has been shown to be essential for spindle formation in every cell type analysed so far. However, Drosophila homologues of TPX2 have not yet been identified. In this study, I identified DPX2, a Drosophila protein that has limited but significant homology to TPX2. Sequence conservation was found to be limited to the putative spindle microtubule-associated region of TPX2, and direct binding of DPX2 to microtubules was seen in vitro. Intriguingly, DPX2 lacks the Aurora A- and kinesin-5-binding domains; these domains are highly conserved in other animal and plant species, including many insects such as ants and bees. The contribution of DPX2 to spindle assembly seems to be minor and differs in this regard from other systems. Live cell imaging of microtubules and chromosomes after RNAi knockdown of DPX2 in S2 cells showed significant delay in chromosome congression in only 18% of the cells. Thus, the Drosophilidae family does possess this conserved spindle protein but also possesses other mechanisms that largely compensate for its function.

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CENP-T proteins are conserved KMN-independent centromere receptors of the Ndc80 complex.

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Transmission of genetic information in eukaryotes depends on kinetochores which drive chromosome segregation by providing a dynamic link between centromeres and spindle microtubules. All kinetochores contain multiple copies of the four-protein Ndc80 complex whose microtubule binding activity is essential for chromosome segregation in all systems tested. A fundamental question is therefore how this complex is anchored to centromeres. Here we show that the conserved histone-fold protein Cnn1^{CENP-T} is a direct centromere receptor of the Ndc80 complex. Using bioinformatic, proteomic and biochemical reconstitution approaches we demonstrate that centromere-binding proteins are highly conserved between yeast and humans. We identify the novel budding yeast protein Wip1 as a CENP-W homolog that interacts with the CENP-T-like protein Cnn1. The N-terminus of Cnn1 contains a conserved peptide motif that mediates stoichiometric binding to the Spc24-25 globular domain of the Ndc80 complex. Cnn1 and the Mtw1 complex are mutually exclusive binding partners of Spc24-25, demonstrating the presence of distinct Ndc80 populations at yeast kinetochores. Artificial tethering of Cnn1 but not of the Ndc80 complex itself is sufficient to promote acentric mini-chromosome segregation depending on the Ndc80 interaction motif. Our results reveal the conserved molecular function

of CENP-T proteins and identify a kinetochore attachment fiber that combines microtubule-binding with DNA-binding activities.

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Structural cell biochemistry of a novel histone fold vertebrate kinetochore complex: CENP-TW and CENP-SX form a heterotetramer.

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Kinetochore is a conserved multi-protein structure required for segregation of sister chromatids during mitosis. It functions as an interface with spindle microtubule. Without a functional kinetochore, the cell fails to segregate chromosomes and this results in aneuploidy or even cancer. Recent proteomic analyses have identified components of the Kinetochore ensures proper segregation of the replicated sister chromatids during mitosis. It connects the sister chromatids and the spindle microtubules emanating from the two daughter centrosomes. With the progress of proteomic analyses, we now know that the vertebrate kinetochore complex comprises of more than 80 proteins and the numbers are still increasing. On the other hand, structural and functional analyses are progressing at a much slower rate.
To elucidate the structure-function relationships of the vertebrate kinetochore components, we are taking two approaches: high-speed atomic force microscopy (AFM) and X-ray crystallography. High speed AFM enables direct visualization of the protein architecture such as globular, flexible or coiled- coil regions at nanometer resolution and its solution dynamics in millisecond intervals. On the other hand, crystal structure reveals the details of protein ultrastructure at atomic resolution. Following these structural analyses, we can perform various biochemical and cell biological analyses.
We have measured the high-speed AFM movies and determined the crystal structures of CENP-T/W and CENP-S/X complexes. Both were histone fold and they formed dimer and tetramer, respectively. The structures were similar between each other. Surprisingly, mixture of the two complexes resulted in a new complex that formed a heterotetramer. Biochemistry and cell biology suggest that the complex formation is required for functional kinetochore.

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Using Electron Tomography to Understand the Organization of the Microtubules in the *Xenopus laevis* Spindle.

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Understanding the organization (polarity, length and spacing) of the microtubules (MTs) in the vertebrate spindle is important for extending our understanding of the spindle functionality including chromosome segregation. Light microscopy studies done on spindles formed in the *Xenopus laevis* egg extract system propose a complex array of MTs with different dynamics that are cross-linked and focused during bipolar spindle assembly. However, the fundamental three-dimensional (3D) organization of the vertebrate spindle at electron microscopy resolution is still unknown and many questions remain about the detailed organization of the MTs in the spindle. This work aims to address those knowledge gaps. We are using the *Xenopus laevis* egg extract system to assemble cycled meiotic spindles. The spindles are assessed using light microscopy and well-formed, isolated spindles are high-pressure frozen, freeze substituted and processed into plastic for electron tomography. Samples are then aligned relative to the long axis of the spindle, serial sectioned, post-stained and screened on a transmission electron microscope. For spindles that are properly oriented, high magnification montage tilt series are acquired on a FEI

Tecnai F30 transmission electron microscope, and reconstructed into tomograms. Serial tomograms are then stacked together into a large 3D volume before structures of interest are modeled. To date, a partial volume of the spindle midzone has been acquired, reconstructed, stacked and partially modeled. We are currently trying to overcome acquisition challenges that are limiting our x,y resolution for such a large area. We will present our current model of spindle subzones. Furthermore, we will discuss the technical approach we are now taking, and the lessons we have learned while acquiring the largest sample yet to be attempted by our laboratory.

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Determining the nanoscale distribution of kinetochore proteins around the microtubule tip.

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Chromosome segregation during cell division relies on the activity of the kinetochore as a microtubule (MT) plus-end tracking motor. As a macromolecular complex, the kinetochore executes highly sophisticated plus-end driven motility that cannot be entirely explained from the relatively simple biochemical properties of the MT-binding kinetochore proteins Ndc80, Spc105/KNL-1, and Dam1 (in fungi). This emergent behavior of the kinetochore results from the combined activity of multiple copies of each of these proteins cooperating in a specific geometry with respect to the MT plus-end. Although much is known about the biochemical and structural properties of Ndc80, Spc105/KNL-1, and Dam1, the nanoscale geometry relative to the plus-end in which they operate remains virtually unknown. We have developed a systematic approach for determining the nanoscale distributions of kinetochore proteins using live-cell fluorescence microscopy. By establishing the correspondence between FRET efficiency (using donor quenching and fluorescence lifetime methods) and sensitized emission measurements, we can exploit the sensitivity of a fluorescence microscope for indirect, but accurate FRET efficiency measurements. We also incorporate fluorescence polarization data to ensure that the assumption of isotropic rotation of the fluorophores is valid. Using this approach, we are measuring the average separation of adjacent Ndc80 molecules within a kinetochore-MT attachment. Our data reveals that the N-terminal MT-binding domains on adjacent molecules are in close proximity to each other, while the centromeric side of the complex is further apart on average. We are also measuring the position of various Dam1 complex subunits with respect to the MT lattice, each other, and the Ndc80 complex. This work will reveal whether the Dam1 complex forms oligomeric rings in vivo.

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The Ska1 and Ndc80 complexes form an integrated kinetochore-microtubule interface.

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The macromolecular kinetochore connects the mitotic spindle with chromosomes to facilitate faithful chromosome segregation in mitosis. Force generated by microtubule depolymerization drives chromosome movements and generates tension across sister kinetochores. How the kinetochore couples chromosome movements to microtubule depolymerization remains poorly understood. The Ndc80 complex and the Ska1 complex have both been individually implicated in this process. In the absence of either complex, robust kinetochore-microtubule attachments fail to form. Using biochemical, single-molecule, and cell biological approaches, we demonstrate that the Ndc80 complex and the Ska1 complex bind to microtubules cooperatively, an activity conserved from human to C. elegans. This synergy is required to produce tension across sister

kinetochores. In vitro, the Ska1 and Ndc80 complexes readily diffuse on microtubules and couple bead movement to microtubule depolymerization. We propose that the combined activities of the Ndc80 and Ska1 complexes allow the kinetochore to utilize the energy of microtubule depolymerization to generate tension across sister kinetochores and drive chromosome movement.

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Kinetochore-microtubule attachments slip dependent on the phosphorylation of Ndc80.

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Accurate segregation of mitotic chromosomes is a prerequisite for the maintenance of genomic integrity. To prevent aneuploidy, the interaction between kinetochore and kinetochore-microtubules (kMTs) has to be regulated to ensure the timely correction of erroneous attachments. Although most of the kinetochore components interfacing with kMTs have been identified, very little is known about the actual mechanics of their interactions. To address this question, we have expanded previous single-chromosome tracking and modeling methods in budding yeast to differentiate the effects of kMT-interfacing kinetochore components on transient kinetochore-kMT binding versus on regulation of kMT dynamics. Here we report the construction of yeast strains with symmetrically-tagged Chromosome IV, where the fluorescent signal accurately represents the location of centromeric DNA (CEN), and tagged spindle pole body (SPB). We propose that the dynamics of CEN relative to SPB can be subdivided into two components: (1) the global extension and shortening of the CEN-SPB distance are controlled by the growth and shrinkage of kMT, respectively; whereas (2) the local adjustment of the distance between CEN and kMT plus end is fine-tuned by kinetochore and can be quantified by using the previously published framework of autoregressive-moving-average (ARMA) models. Attempts to reproduce experimentally observed ARMA models of chromosome dynamics with simulation of kMT dynamics strongly suggest that the kinetochore constantly slides about the kMT end, defining an interface of transient bindings and releases. To further investigate the origin of this slippage we began to analyze the changes in the dynamics induced by ndc80 phosphorylation site mutants (kindly provided by Dr. Biggins). We find that ndc80 mutants with un-phosphorylatable N-terminal domains exhibit dampened dynamics between CEN and kMT plus end, indicating the crucial role of ndc80 in fine-tuning the kinetochore-kMT interaction.

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Importin beta regulates the RANBP2/RANGAP1 complex at mitotic kinetochores.

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The GTPase Ran regulates nuclear transport in interphase and plays important roles in mitosis. The underlying mechanism in both processes is based on RanGTP ability to bind importin beta, the major nuclear transport vector, thereby releasing importin beta cargoes in a free active form. We previously investigated how the system operates in mitosis, when nuclear transport ceases, and found that Ran and importin beta co-localize at mitotic spindle poles and microtubules. Inducing an imbalance between Ran and importin beta yields the formation of abnormal spindles that drive chromosome mis-segregation.

To further investigate how importin beta acts in human mitotic cells, we have combined deletion mapping, functional assays, and proteomic analysis of importin beta mitotic partners. We find that importin beta functional domains regulate diverse aspects of mitosis via different classes of target proteins. The C-terminal domain (interacting with importin alpha and hence nuclear cargoes in interphase) regulates spindle pole organization in early mitosis. The central domain, harboring nucleoporin binding sites, regulates microtubule dynamic functions and interactions with kinetochores. Importin beta interacts through this region with NUP358/RANBP2 and SUMO-conjugated RanGAP1 even after nuclear pore disassembly. We found that this interaction is required to regulate RanGAP1 localization on microtubule-attached kinetochores and hence the local hydrolysis of RanGTP after microtubule attachment.
Together these data reveal a novel interplay between importin beta and RanBP2 at KTs and, more generally, indicate that importin beta domains regulate distinct temporal and spatial aspects of mitosis in a highly regulated manner. Overall, our results highlight the role of importin beta as a master regulator of mitotic progression.

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The human Ndc80 complex is a microtubule rescue factor regulated by Aurora B kinase.

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During mitosis, kinetochores couple chromosomes to the dynamic tips of spindle microtubules. Since microtubule dynamics are stochastic in nature, the precise movement of chromosomes is dependent on the regulation of these dynamics. Several accessory factors, regulated by the Aurora B kinase, localize to the kinetochore and can affect microtubule dynamics. However, the role of core kinetochore components is not well understood. Using electron microscopy and single-molecule biophysical techniques, we show that the human Ndc80 complex directly modifies microtubule dynamics by stabilizing the tips of disassembling microtubules and promoting microtubule rescue (the transition from microtubule shortening to growth). Mutations in the Ndc80 complex that mimic its phosphorylation by Aurora B inhibit the ability of the complex to influence microtubule dynamics, but do not prevent the complex from forming stable attachments to disassembling microtubule tips. Our results indicate that Aurora B can regulate microtubule dynamics through phosphorylation of the Ndc80 complex, a conserved and essential microtubule-binding component of the kinetochore.

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Autoactivation of Aurora B Kinase Can Generate a Mobile Phosphorylation Switch in Mitotic Cells.

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Aurora B kinase is one of the major regulators of proper chromosome segregation during cell division. Its critical role is to phosphorylate kinetochore proteins, thereby changing their affinity to the microtubules. When Aurora B kinase activity is inhibited, the inappropriate, merotelic kinetochore-microtubule attachments become stabilized, leading to lagging chromosomes in Anaphase. Current views of the kinetochore bi-orientation and error-correction imply that the activity of Aurora B kinase is differentially distributed across the kinetochore. Strikingly, Aurora B kinase is concentrated not at the kinetochore but in the centromeric chromatin. It is thought that

the active kinase propagates spatially via diffusion to reach its targets at the outer kinetochore plate, which is located hundreds of nanometers away. However, the molecular mechanisms that determine the precise positioning and shape of this phosphorylation gradient are still unknown. Here, we consider a mathematical model in which Aurora B kinase is present as centromere-bound and soluble pools. Within each pool there are two forms of the kinase with different activities; a partially active form can be activated by trans-phosphorylation giving rise to a fully active form. This auto-activation creates a positive feedback loop, which is counteracted by a cytoplasmic phosphatase. We show that such previously unexplored, coupled kinase-phosphatase system exhibits a highly non-linear behavior and can generate a variety of complex spatio-temporal regimes, including bi-stability, propagation of autowaves and generation of stable long-range gradients. We test the predictions of this in silico model by visualizing in real time the spatial phosphorylation gradients in mitotic HeLa cells using a FRET-based phosphorylation sensor. By varying the concentration of a kinase inhibitor, we observed that sensor phosphorylation changes in a highly non-linear, step-wise manner with slight hysteresis. Furthermore, we observed the constant rate of propagation of phosphorylation waves, which arise following the removal of kinase inhibitor, implying that these waves are self-sustained. Together, these results support a model in which a vitally important long-range pattern of Aurora B activity at the kinetochore is established via the formation of a stable dissipative structure, which arises dynamically around the centromeric region where the binding sites for Aurora B kinase are clustered.

1468

The Microtubule-Associated Protein TPX2 contributes to interspecies spindle scaling in *Xenopus.*

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The mitotic spindle is a dynamic macromolecular assembly comprised mainly of microtubules (MT) and microtubule associated proteins (MAPs) that must react to cell size in order to properly organize and separate chromosomes during cell division. However, physiological determinants of spindle size control are poorly understood. Taking advantage of spindles formed *in vitro* in egg extracts from the frog *Xenopus laevis* and its smaller relative *Xenopus tropicalis*, we are investigating the role of spindle assembly pathways that likely contribute to the observed cytoplasmic, dose-dependent regulation of spindle size. Spindle assembly in *X. laevis* egg extracts relies heavily on RanGTP, which is locally generated by its nucleotide exchange factor RCC1 bound to chromatin, creating a spatial gradient of active Spindle Assembly Factors (SAFs) released from the cargo carrying proteins importin α/β. SAFs regulate aspects of MT organization and dynamics that could contribute to spindle size, including nucleation and bundling. Interestingly, whereas adding hydrolysis-deficient Ran(Q69L)GTP caused MT spontaneous nucleation and formation of self-organizing bipolar structures in *X. laevis* egg extracts, MTs formed asters but not bipolar structures in *X. tropicalis* extracts under the same conditions. Adding the dominant-negative RanT24N, which inhibits RCC1, resulted in strong spindle disruption in *X. laevis*, with mislocalization of SAFs including the spindle MT-associated protein (MAP) TPX2 (Targeting Protein for Xklp2). In contrast, *X. tropicalis* extracts formed bipolar spindles even at 20-fold higher RanT24N concentrations, and strong TPX2 staining persisted. Furthermore, TPX2 levels appeared approximately three-fold higher in X. tropicalis egg extracts by Western blot, whereas importin α levels are three-fold lower. These results suggest that *X. tropicalis* depends less on the Ran pathway for spindle assembly and that TPX2 is a candidate spindle scaling factor. Consistent with this model, addition of TPX2 to *X. laevis* reduced spindle length. Experiments are underway to test whether TPX2 levels affect the activity of the kinesin-5 spindle motor Eg5 in egg extracts, thereby modulating the outward sliding of antiparallel MTs and steady state spindle length.

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Regulation of Kif2a by Importin α Contributes to Spindle Size Scaling during *Xenopus* Development.

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During the cleavage divisions of *Xenopus laevis* embryos, mitotic spindle size must adjust to decreasing cell size to ensure proper segregation of replicated chromosomes to daughter cells, but mechanisms underlying changes in spindle size and morphology during development are not understood. We observed increased recruitment of the microtubule depolymerizing kinesin-13 Kif2a to the spindle later in development, a phenomenon that could be recapitulated *in vitro* with spindles assembled in embryo extracts prepared from stage 8 embryos (~4000 cells) compared to those in stage 3 (4 cells). Whereas inhibiting Kif2a in stage 3 reactions did not significantly affect spindle size, stage 8 spindles increased in area by 34 +/- 5%, appearing wider and lantern-shaped due to an increase in microtubules. Interestingly, although Kif2a localizes to centrosomes and not the nucleus during interphase in human cells, it contains a canonical Nuclear Localization Signal (NLS) in its motor domain, suggesting regulation by importins, and we detected importin α (impα) in Kif2a immunoprecipitates. Although Kif2a levels do not change significantly during *X. laevis* early development, impα levels drop 50% by stage 8, and addition of excess impα increased the size of stage 8 spindles. We hypothesize that impα sequesters Kif2a in early stage embryos thereby increasing steady state spindle size. In support of this model, we have found that impα inhibits Kif2A microtubule depolymerization activity *in vitro*. Experiments are underway to test direct binding of the two proteins and its dependence on the Kif2a NLS, and to evaluate the effects on spindle size of altering Kif2a and impα *in vivo* during *Xenopus* development.

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Microtubule Destabilizing Activities Scale Spindle Size in Xenopus.

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Bipolar spindles must separate chromosomes the appropriate distance during cell division, but mechanisms determining spindle size are poorly understood. Using two Xenopus-based systems to study physiological mechanisms of spindle scaling, we have identified microtubule (MT) destabilization as a major spindle size controlling activity. Based on predictions from a 2-D meiotic spindle simulation, we identified katanin-dependent MT severing as an activity reduced in egg extracts from X. laevis compared to the smaller frog X. tropicalis, which forms smaller spindles in vitro. Interestingly, X. tropicalis lacks an inhibitory Aurora B phosphorylation site in the p60 catalytic subunit of katanin found in X. laevis at Ser131. P60 depletion increased spindle length and disrupted spindle structure in X. tropicalis, as kinetochore fibers protruded through spindle poles. Spindle morphology and size defects were reversed by addition of a recombinant X. laevis p60Ser 131Ala mutant that cannot be phosphorylated, but not by the phosphomimetic mutant Ser131Glu. Thus, phosphoregulation of p60 dependent MT destabilization scales meiotic spindle size between closely related species and coordinates stability of multiple MT populations in order to generate a robust steady state structure. MT destabilization also correlated with mitotic spindle size changes during X. laevis embryogenesis. By establishing an embryo extract system to examine intrinsic spindle scaling during development, we could compare extracts from stage 3 to stage 8 embryos that contain 4 large or thousands of small cells and exhibit a ~2.5 fold difference in spindle size. We observed increased MT catastrophe frequency from centrosomal MTs, a shift away from chromatin mediated spindle assembly, and increased recruitment of both p60 katanin and the MT

destabilizing protein kif2a in stage 8 spindles. Thus, in both systems appropriate spindle size appears to depend on defined levels of tightly regulated MT destabilizing activity, establishing this mechanism as a physiologically utilized means of spindle scaling.

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IFT88 and Ciliary IFT B Complex Members in Mitosis.

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Intraflagellar transport (IFT) is a bidirectional transport system required for the generation and maintenance of primary and motile cilia in noncycling cells. IFT proteins form multimeric complexes, A and B, which transport ciliary components from the cell body to the ciliary tip, and back to the base, while riding on microtubule motors. Cilia protein disruption results in multiple disorders collectively termed ciliopathies. Interestingly, IFT88, a conserved IFT component required for ciliogenesis, also localizes to mitotic centrosomes/spindle poles where it functions in spindle orientation and astral microtubule organization (Delaval et al, Nature Cell Biology, 20ll). Like in cilia, where IFT complexes carry pre-assembled ciliary components along the microtubule axoneme, mitotic IFT88 carries peripheral microtubule bundles containing spindle pole proteins along microtubules to spindle poles. However, it is currently unknown if IFT88 functions in combination with the ciliary IFT B complex during mitosis, as it does within cilia. To answer this question we characterized two additional IFT proteins: IFT57 and IFT20. Like IFT88, these proteins are members of the ~14 protein IFT B complex that localize to mitotic spindle poles. As with IFT88, IFT57 and IFT20 depletion led to disrupted spindle poles and an increased mitotic index. Furthermore, IFT57 and IFT20 co-fractionated with IFT88 in mitotic lysate, suggesting that like in cilia, some or all IFT B complex members may form a mitotic complex. Consistent with this model, IFT20 depletion partially mislocalized IFT88 from spindle poles, demonstrating interdependence amongst IFTs for spindle pole localization. Together our data suggest that like in cilia, IFT88 functions along with additional IFT B complex members during mitosis. It also raises the important possibility that mitotic defects induced by mutations in IFT proteins could contribute to the phenotypes associated with ciliopathies. In this regard, it may be the centrosome, an integral cilia and spindle pole component, which is the underlying source of ciliopathies.

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An inducible RNAi screening system reveals augmin-mediated spindle microtubule generation in plant cells.

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The moss *Physcomitrella patens* is an emerging land plant model to which several key molecular techniques, such as homologous recombination or RNA interference (RNAi), can be applied. The caulonemal cells in the protonemata (a filamentous structure arising from spore germination) have stem cell characteristics and less interference by autofluorescence, and therefore are suitable for live imaging with fluorescent protein-tagged markers. Recently, it was reported that the γ-tubulin ring complex (γ-TuRC) and augmin (an 8-subunit protein complex) are required for centrosome-independent microtubule generation within the mitotic spindle in fly and human cells possessing centrosomes. However, the contribution of augmin to spindle microtubule generation in cells that naturally lack centrosomes has not been elucidated. In this study, we developed an inducible RNAi system in *P. patens* to evaluate the mitotic phenotype

after augmin knockdown in caulonemal cells that naturally lack centrosomes. After RNAi, ~50% longer bipolar spindles were initially formed with reduced microtubule intensities and misaligned chromosomes, and were eventually collapsed during prolonged mitosis. Cytokinesis was also incomplete owing to failure in phragmoplast expansion. Furthermore, fluorescence recovery after photobleaching analysis for green fluorescent protein (GFP)-tubulin revealed that the recovery of GFP intensity within the spindle is significantly delayed. Indistinguishable phenotypes were observed after RNAi of γ-TuRC subunits. These results show that γ-TuRC and augmin play a critical role in spindle microtubule generation during metaphase and cytokinesis in *P. patens* caulonemata. We also found unanticipated dynamics during caulonemal mitosis, such as asymmetric spindle formation in prometaphase or midzone accumulation of γ-tubulin, augmin, and EB1 in anaphase, which may represent the unique mechanism of non-centrosomal cell division developed by land plants.

Kinesins

1473

The Nucleotide-Binding State of Microtubules Modulates Kinesin Processivity and Tau's Ability to Inhibit Kinesin Mediated Transport.

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Tau's ability to act as a potent inhibitor of kinesin motility in vitro suggests it may actively participate in the regulation of axonal transport in vivo. However, it remains unclear how kinesin-based transport could then proceed effectively in neurons, where tau is expressed at high levels. One potential explanation is that tau, a conformationally dynamic protein, has multiple modes of interaction with the microtubule, not all of which are inhibitory for kinesin motility. Thus, if tau can bind microtubules in distinct conformations or at unique binding sites that no longer inhibit kinesin, transport would proceed unhindered along the axon. Previous studies support the hypothesis that tau has at least two modes of interaction with microtubules, but the mechanisms by which tau adopts these different conformations and their functional consequences have not previously been investigated. In the present study we have used single molecule imaging techniques to demonstrate that tau inhibits kinesin motility in an isoform dependent manner on GDP-microtubules stabilized with either paclitaxel or glycerol, but not GMPCPP-stabilized microtubules. Furthermore, the order of tau addition to microtubules before or after polymerization has no effect on tau's ability to modulate kinesin motility regardless of the stabilizing agent used. Finally, the processive run length of kinesin is reduced on GMPCPP-microtubules relative to GDP-microtubules, and kinesin's velocity is enhanced in the presence of 4RL-tau but not the 3RS-isoform. These results shed new light on tau's potential role in the regulation of axonal transport, which is more complex than previously recognized.

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The length of the neck linker domain controls processivity across diverse N-terminal kinesin families.

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Consistent with their diverse intracellular roles, the processivity of N-terminal kinesin motors varies considerably between different families. Gating mechanisms that control kinesin processivity involve inter-head tension that must be transmitted through the neck linker domains of each head. The objective of this study is to determine the degree to which unloaded

processivity is controlled by the length of the neck linker, as opposed to kinetic differences in the core motor domains. The motor and neck linker domains of Kinesin-2, 3, 5 and 7 were fused to the neck-coil and rod domains of Kinesin-1 and run lengths of GFP-tagged motors visualized by total internal fluorescence microscopy. When the neck linkers were shortened to the 14 amino acids found in Kinesin-1, all motors had similar run lengths, and when the neck linkers were extended the run lengths fell. These results suggest that inter-head tension, which is regulated by the mechanical properties of the neck linker, controls processivity in a conserved manner across diverse kinesins. Monte Carlo modeling of the kinesin hydrolysis cycle conclude that the results can be quantitatively accounted for by differences in either front head or rear head gating.

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The loop 5 element structurally and kinetically coordinates dimers of the human kinesin-5, Eg5.

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Eg5 is a homotetrameric kinesin-5 motor that generates outward force on the overlapping, antiparallel microtubules (MTs) of the mitotic spindle. Upon binding a MT, an Eg5 dimer releases one ADP molecule, undergoes a slow (~0.5 s-1) isomerization, and finally releases a second ADP, adopting a tightly MT-bound, nucleotide-free conformation. This conformation precedes ATP binding and stepping. We have used mutagenesis, steady-state and presteady-state kinetics, motility assays, and electron paramagnetic resonance (EPR) spectroscopy to examine Eg5 monomers and dimers as they bind MTs and initiate stepping. We found that a critical element of Eg5, loop 5 (L5), accelerates both basal and MT-stimulated ADP release and ATPase rates. Furthermore, L5 mediates the slow isomerization by preventing Eg5 dimer heads from binding the MT until they release ADP. Structurally, the slow isomerization is a transition of the forward head from an ADP-bound, MT-unbound state into an APO, MT-bound state having an undocked neck-linker. Taken together, these properties of L5 completely explain the kinetic effects of L5-directed inhibition on Eg5 activity and may direct further interventions targeting Eg5 activity.

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Measuring the cytosolic drag experienced by molecular motors *in vivo.*

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Molecular motors are responsible for facilitating many vital cellular processes. By generating forces to haul various cargo to their destinations over the necessary time scales, these motor proteins help achieve the complex yet organized net distributions of vesicles and organelles that are essential for the cell's functionality and survival. Motors such as kinesin-1 and cytoplasmic dynein have been extensively studied *in vitro* where viscous drag has negligible effects on the motion of cargos. However, the issue of whether cytosolic drag *in vivo* is large enough to significantly slow cargo motion has remained controversial. To probe the importance of viscous drag on cargo motion, we tracked lipid droplets being hauled by kinesin motors in *Drosophila* embryos. An optical trap was used to apply a force on these cargos sufficient to make them stall and then fall back to the trap center. The magnitude of the cytosolic drag force determines how quickly the lipid droplets recoil back to the trap center. We use high speed detection to study the details of the recoil and its dependence on the size of the cargo and cytoskeletal content. Our

data enable us to estimate the drag force experienced by the motors and relate it to the how multiple motors cooperate in hauling cargos.

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Role of kinesin-mediated transport of VEGFR2 to the cell surface in mediating angiogenesis.

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Although VEGF/VEGFR2 ligand-receptor system is known to play a central role in angiogenesis, the underlying mechanism(s) regulating transport of VEGFR2 to the cell surface is not well understood. The pan-inhibitor of kinesins and dyneins, Rose Bengal lactone, prevented VEGF-mediated migration of human umbilical vein endothelial cells (HUVECs) and tube formation in Matrigel. Based on an RNAi screen, we focused on investigating the role of the molecular motor KIF13B/GAKIN (guanylate kinase-associated kinesin) in mediating VEGF-induced angiogenesis. KIF13B is known as a PIP3 transporter in neurons. In our studies, KIF13B-knockdown inhibited VEGF-mediated migration of HUVECs, as well as tube formation in Matrigel in vitro, and in Matrigel plugs in mice. PIP3 and VEGFR2 were co-localized in VEGF-stimulated endothelial cells, and interestingly, accumulated at the leading edge of migrating cells. These responses were abolished by KIF13B depletion. To address whether KIF13B mediates transport of VEGFR2, we detected KIF13B-VEGFR2 complex by co-immunoprecipitation. After VEGF stimulation, VEGFR2 is known to internalize, which mediates angiogenic signaling from the intracellular signaling compartment. VEGF also induces de novo synthesis and translocation of VEGFR2 from intracellular pool to the cell surface to receive second peak of receptor phosphorylation subsequent to VEGF binding to VEGFR2. We observed that KIF13B-knockdown inhibited translocation of VEGFR2 from intracellular pool to the cell surface and second peak of phosphorylation. These data demonstrate the crucial role of KIF13B as a VEGFR2 transporter to the endothelial membrane and in thereby mediating angiogenesis.

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Probing Molecular Motor Coordination via Thermal Fluctuation Analysis.

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Lipid droplets in *Drosophila* embryos are transported along microtubules by kinesins and cytoplasmic dyneins, each of which moves mainly unidirectionally along the track. Lipid droplets exhibit local bidirectional motion while globally moving in one direction or the other. The mechanism of direction switching is largely unknown. Here we present an *in vitro* study of the dynamics along the microtubule of endogenous cargo-motor complexes, i.e. lipid droplets purified from *Drosophila* embryos. In contrast to their transport characteristics *in vivo*, the majority of purified lipid droplets exhibit short-range transport. We combine Thermal Fluctuation Analysis via Photonic Force Microscopy, fluorescence and bright field microscopy to study the interaction of opposite-polarity motors bound to the same cargo near an immobilized microtubule. Using this approach, we can resolve individual motor binding events and motor run-lengths that would otherwise be masked by Brownian motion.

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Mechanical Tug-of-war Models Explain In Vitro Unidirectional but not In Vivo Bidirectional Multiple Kinesin-Dynein Transport.

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Intra-cellular transport via the microtubule motors kinesin and dynein plays an important role in maintaining cell structure and function. Often, multiple kinesin or dynein motors move the same cargo. Because their collective function depends critically on the detachment kinetics of motors under load, we experimentally measure these kinetics for both kinesin and dynein. This experimental constraint—combined with other experimentally determined parameters—is then incorporated into theoretical stochastic and mean-field models. Comparison of modeling results and in vitro data shows good agreement for the stochastic, but not mean-field, model. In many cases, both kinesin and dynein function on the same cargo, which then moves bi-directionally, frequently reversing course. One popular hypothesis explaining this behavior is that opposite polarity motors engage in unregulated stochastic tug-of-wars and that the properties of cargo motion are determined entirely by the outcome of these opposite-motor competitions. We use fully calibrated stochastic and mean-field models to test the tug-of-war hypothesis. Neither model agrees well with our in vivo data, so we conclude that such models are not consistent with the experiments, suggesting that in addition to inevitable tug-of-wars between opposite motors, there is an additional level of regulation not included in the models.

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Microtubule Motor Traffic Jams.

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Microtubules are polarized filaments that function as transport tracks for motor proteins. These filaments are arranged such that their plus ends reach towards the cell periphery. Kinesin-1 is a motor protein that walks towards the plus ends of these filaments, carrying cargoes to the cell periphery. Numerous studies have revealed that cargo transport is often achieved through the cooperation of multiple motors carrying a single cargo. However, these studies are often carried out in a dilute environment, where factors such as crowding along the microtubule and motor exchange on a single cargo are eliminated. The objective of this study is to better understand how crowded conditions along the microtubule and motor exchange on cargoes affects cargo transport. We use quantum dots (Qdots) to create self-assembled cargoes. Qdots are known to non-specifically bind kinesin-1. When Qdot cargoes encounter excess motile kinesin-1 on the microtubule, they self-assemble cargoes with many motors, creating a system that allows for an exchange of motors on a single cargo. We used Total Internal Reflection Fluorescence (TIRF) microscopy to track individual self-assembled cargoes in varying degrees of crowded conditions. We found that while the velocity of cargoes decreased as conditions became more crowded, the run length and total association time of cargoes increased. We also found that cargoes paused more frequently in more crowded conditions. Interestingly, we observed cargo reversal events during runs, which were more likely to occur in crowded conditions. We believe

that these reversals occur when a cargo bound to multiple motors is blocked from moving forward, resulting in the release of the front kinesin. When this occurs, a kinesin in the back will most likely bind the microtubule, resulting in a rock backwards. Using statistical mechanics simulations, we can recapitulate velocity reduction, longer run lengths, increased pausing, and reversals *in silico*. From this study, we conclude that transport of self-assembled cargoes is affected by levels of crowding along the microtubule.

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Experimental and theoretical analyses of the mechanism and function of Kinesin-1-dependent cytoplasmic flows.

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We study two types of motion that depend on the motor protein Kinesin-1: the transport of material along microtubules and cytoplasmic streaming. One context in which streaming occurs is the establishment of Drosophila body axes, when Kinesin-1 transports the axes determinants and drives ooplasmic streaming. Although Khc is essential for flows, neither the mechanism by which Khc induces streaming nor the impact of these flows on transport are known.

Using an interdisciplinary approach we are deciphering the biophysical behavior of streaming as the oocyte polarizes. Firstly, we have succeeded in the quantitative measurement of the statistical properties of streaming by Particle Image Velocimetry (PIV, a method used in fluid dynamics), allowing us to understand the link between the motivate force of streaming and its macroscopic consequences. Secondly, we have determined the rheological properties of the ooplasm. Since Khc drives flows measured viscous energy dissipation can be related to the number of motors contributing to fluid deformation. Calculation of the viscous energy dissipation requires knowledge of the flow at all points in space (by PIV), and the fluid viscosity which we have measured using microrheology (by injecting beads into khc mutant oocytes that lack flows and tracking their Brownian motion). The calculated viscosity (1 Pa.s, 1000 x water) makes the cytoplasm a low Reynolds number fluid, meaning that the energy required to deform fluid equals the energy dissipated by Khc. Using a value of 10 kBT per motor step, and an stepping rate of 10Hz, then the number of Kinesins contributing to flow is ~ 2500.

To analyze the impact of flows on motor function, we are currently correlating the flow rate with the flux of oskar RNP (a Khc cargo). With these parameters and theoretical analysis of the impact of streaming on transport, we aim to learn whether streaming assists or hinders the asymmetric distribution of cargoes. Ultimately, we would like to reconstitute streaming in vitro in controlled geometries.

In addition, a functional/structural study of Khc has shown that the tail of Khc is not required for streaming, since a tailless Khc supports flows in oocytes that lack endogenous Khc. This truncated motor is able to localize Dynein to the posterior of the oocyte, another function of Khc. These findings suggest that Khc has domain-specific functions, which is further supported by the fact that the tail is essential for oskar RNA localization but only partially contributes to the Khc-dependent positioning of the oocyte nucleus

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Binding studies of the TRAK/kinesin-1 trafficking complex.

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Little is known regarding the molecular interactions between kinesin motor proteins and kinesin adaptor proteins. Trafficking kinesin proteins (TRAK) are a family of kinesin adaptor proteins that facilitate transport of cargoes in neurons via the microtubule network. There are two TRAKs

in vertebrates, TRAK1 and TRAK2. TRAKs bind to kinesin-1 molecular motors and to their cargoes. The best characterised cargo transported by TRAKs is mitochondria. Mitochondrial transport occurs via the binding of TRAKs to the mitochondrial outer membrane protein, Miro. In vitro experiments using the kinesin-1, KIF5C, showed that TRAK proteins bind to the motor via the C-terminal cargo binding domain. However, in the brain, TRAK2 associates predominantly with the kinesin-1, KIF5A. In this study we have designed experiments to refine further the TRAK binding site within the cargo domain of KIF5A. A series of KIF5A C-terminal truncations were generated. The cargo binding domains of the kinesin-1 family share an overall ~ 85% amino acid identity but KIF5A has an additional 75 C-terminal amino acids compared to KIF5B and KIF5C. The KIF5A C-terminal deletions were designed to exploit similarities and differences between the members of the kinesin-1 sub-family. Wild-type KIF5A (1-1032) and nine KIF5A truncations (including KIF5A (1-825) that contains the motor and stalk domains only), were each co-expressed in parallel with FLAG-tagged TRAK2 in mammalian cells. The binding of KIF5A truncations to TRAK2 was determined by quantitative immunoblotting following immunoprecipitation. Similarly to KIF5C, TRAK2 was shown to bind to the KIF5A cargo binding domain. Deletion of the KIF5A C-terminal tail appeared to enhance the binding of TRAK2. Further truncations resulted in a reduction in KIF5A immunoreactivity in immune pellets. These studies yield new insights into kinesin/kinesin adaptor interactions which may impact in the future on a better understanding of neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease which have both been linked to deficiencies in neuronal transport mechanisms of mitochondria.

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Probing CENP-E function in central spindle plasticity using chemical inhibitor syntelin.

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Mitotic kinesin CENP-E plays key roles in chromosome congression, spindle checkpoint satisfaction and central spindle plasticity. We have identified and characterized syntelin, a novel selective CENP-E inhibitor (Ding et al., 2010. Cell Res. 20, 1386-1390). Cells treated with syntelin progress through interphase, enter mitosis normally with a bipolar spindle and lagging chromosomes around the poles. Chromosomes in syntelin-treated cells frequently have both sister kinetochores attached to spindle microtubules from the same pole (syntelic attachment). Our biochemical study shows that syntelin is an allosteric inhibitor which tightens CENP-E-microtubule interaction by slowing inorganic phosphate release. Given the spatiotemporal dynamics of CENP-E localization to the central spindle during anaphase onset and accurate inhibition of CENP-E with syntelin, we probe for a role of CENP-E in the organization of interpolar microtubules into an organized central spindle. Inhibition of metaphase synchronized cells does not perturb interpolar microtubule assembly but abrogates the anti-parallel microtubule bundle formation. Inhibition of CENP-E motor activity leads to an aberrant localization of PRC1 and CLASP1 to the central spindle. Real-time image shows that CENP-E inhibited cells undergo central spindle splitting and exhibits chromosome instability phenotypes. These findings reveal a previously uncharacterized role of CENP-E in orchestrating central spindle plasticity essential for the initiation and execution of cytokinesis.

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A tethering mechanism controls the processivity and kinetochore-microtubule plus-end enrichment of the kinesin-8 Kif18A.

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The alignment of chromosomes at the metaphase plate prior to segregation is an essential step towards ensuring genomic stability. This alignment process critically depends on the activity of Kif18A, a kinesin-8 motor that shows a graded distribution along K-MTs, peaking at their plus-ends where it suppresses their dynamics. The significance of this graded enrichment to the control of chromosome movements is not understood. By engineering Kif18A mutants that retain the ability to suppress MT dynamics but fail to become enriched at K-MT plus-ends, we identify a mechanism that allows Kif18A to concentrate at K-MT plus ends to a level required to suppress chromosome movements. The graded localization of Kif18A along K-MTs depends on the activity of its C-terminal tail domain, while the ability of Kif18A to suppress MT growth is an intrinsic activity of the motor. Kif18A's C-terminal tail contains a second MT-binding domain that makes diffusive movements along the MT lattice, suggesting that it effectively acts as a tether to prevent motor dissociation from the MT track. In support of this idea, the tail significantly enhances the processivity of single Kif18A molecules and is crucial for Kif18A to accumulate at K-MT plus-ends in cells. These data support a model in which the Kif18A tail confers unusually high processivity to the motor via a tether-like activity. This increased processive movement, in turn, promotes concentration of the Kif18A motor at K-MT plus-ends, where it suppresses their dynamics to control chromosome movements.

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Regulation of *C. elegans* MCAK by Aurora Kinase Phosphorylation.

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Microtubules are required for multiple cellular processes including mitosis, cytokinesis, and vesicle transportation. Factors that regulate microtubule dynamics in the cell help determine the final form and precise cellular role of many cytoskeletal structures. Among the known modulators of microtubule dynamics, we are specifically interested in the microtubule-depolymerizing kinesins of the kinesin-13 family, such as KLP-7 (CeMCAK).
In *C. elegans*, KLP-7 locates to the kinetochore and the centrosome. It has been implicated in regulating microtubule outgrowth at the centrosome (Srayko *et al.*, 2005, Schlaitz *et al.*, 2007). Extensive work on the vertebrate homologues of KLP-7 indicates that they are negatively regulated through phosphorylation by the Aurora kinases (Andrews *et al.*, 2004, Lan *et al.*, 2004, Ohi *et al.*, 2004, Schlaitz *et al.*, 2007, Zhang *et al.*, 2007 and 2008). In order to understand how KLP-7 is regulated in the cell, we tested the possibility that Aurora kinases are directly involved. 2D gel-electrophoresis revealed an alteration in the ratio of potential KLP-7 phosphorylation variants in lysates from either Aurora A-depleted or Aurora B-depleted embryos, compared to wild type. Furthermore, we found that both Aurora A and B kinases phosphorylated KLP-7 *in vitro*. We used a combination of *in vitro* kinase assays, similarity to data on the vertebrate homologues, and an Aurora kinase phospho-site prediction algorithm to identify potential in vivo phosphorylation sites within KLP-7 (Zhou *et al.*, 2004). We are currently performing a structure-function analysis to determine which putative Aurora sites are required for KLP-7's intracellular location and/or its depolymerase activity at the centrosome. Results obtained thus far indicate that mutating one of the C-terminal Aurora sites from serine to

glutamic acid (to mimic constitutive phosphorylation) or to alanine (to mimic non-phosphorylation) interferes with KLP-7 function but not its ability to target to centrosomes or kinetochores.

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In Vitro and In Vivo Single-Molecule Imaging Experiments Provide Mechanistic Insight into the Role of the Arabidopsis FRA1 Kinesin in Cell Wall Assembly.

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The interphase microtubule cytoskeleton at the cortex of plant cells drives directional cell expansion by regulating the assembly of the cellulosic wall. Genetic analysis has indicated that the *Arabidopsis* FRA1 kinesin, a member of the kinesin-4 family, mediates the cytoskeletal control of cellulose patterning. To understand the mechanism of FRA1 function, we analyzed the motor properties of FRA1 both in vitro and in vivo using single-molecule imaging. We found that recombinant GFP-tagged FRA1 consisting of the motor and coiled-coil domains moves towards the microtubule plus-end as a dimer. Notably, the processivity of FRA1 is at least twice the processivity of conventional kinesin in vitro, making FRA1 the most processive kinesin to date. To study the motility of full-length FRA1 in vivo, we introduced a full-length FRA1-3GFP construct, driven by the native *FRA1* promoter, into a *fra1* null mutant. The FRA1-3GFP construct fully complemented the *fra1* mutant phenotype indicating that the encoded protein is functional. We used highly inclined and laminated optical sheet (HILO) microscopy for single-molecule imaging of FRA1-3GFP in living *Arabidopsis* seedlings. We found that FRA1-3GFP localizes to the cell cortex as discrete particles that bind to cortical microtubules. A subset of the cortical microtubule-bound FRA1-3GFP particles move highly processively while others dissociate quickly and do not show processive movement. Interestingly, the motile fraction of FRA1-3GFP correlates with the cell expansion rate. These observations reveal that FRA1 motility is tightly regulated in cells and probably contributes to altering the cell wall composition and/or structure during cell expansion. Pharmacological experiments showed that FRA1 motility is independent of cellulose synthesis. In addition, the velocity of full-length FRA1 in vivo is at least 60-fold greater than the velocity of the plasma-membrane-embedded cellulose synthase complexes, which synthesize and extrude cellulose microfibrils in the extracellular space. Therefore, FRA1 is unlikely to regulate cellulose patterning by guiding the directional movement of cellulose synthase complexes. Instead, FRA1 may transport vesicles containing cell wall matrix polysaccharides along cortical microtubules. Together, our data provide new insights into how the FRA1 kinesin mediates the cytoskeletal control of cell wall assembly.

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Mechanisms of Fast Axonal Transport of Prion Protein Vesicles and Infectious Prions.

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Movement and positioning of vesicles inside axons is accomplished by the regulated active transport of kinesin and dynein motor proteins, which move along microtubules in anterograde and retrograde directions, respectively. The normal mammalian prion protein (PrPC) is a GPI-anchored protein that follows the secretory pathway inside vesicles toward the cell surface by an unknown mechanism. Once at the cell surface, PrPC is thought to interact with a number of ligands, including infectious scrapie (PrPSc), if present. Thus, trafficking of PrPC to the plasma membrane via an intact transport system might be a relevant mechanism for initiation of neurodegenerative conditions. Using imaging, biochemical, and genetic approaches, we show differential transport of the prion protein along axons by Kinesin-1 and cytoplasmic dynein. We

show that anterograde and retrograde motors associate to PrPC vesicles regardless of directionality or speed of movement, suggesting that the activity of these motors is uncoupled from their association to these vesicles and that regulatory factors instead of structural changes to motor-vesicle associations dictate the transport mechanics of these vesicles in axons. Furthermore, we show evidence for the fast axonal transport of infectious prions, and how prion infection might lead to the poisoning of an intact transport system in axons.

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A kinesin-mediated linkage between centrosomes and the nuclear envelope.

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Interphase centrosomes in Dictyostelium cells are located in the cytoplasm, adjacent to and tightly connected with the nucleus (hence the name, nuclear-associated bodies). Deletion of the M-type kinesin isoform, Kif9, disrupts the centrosome-nuclear linkage and enables the centrosome to move in the cytoplasm independent of the nucleus. This disruption leads to an accumulation of supernumerary centrosomes and produces multiple mitotic defects. Kif9 null cells are viable but they accumulate in suspension at an exceedingly slow rate, indicating that a physical association between these two organelles is required for normal cell growth. Kif9 contains a C-terminal transmembrane/tail domain that appears to function as a nuclear envelope anchor. Kif9 localization reveals that the motor is restricted to a perinuclear region close to the centrosome position. Removal of the C-terminal domain distributes the truncated motor into the cytosol, whereas a Kif9 fragment containing only the C-terminal tail is nuclear envelope localized similar to the wild type protein. The solubilities of expressed full length and truncated polypeptides are consistent with the localization data. These results indicate that the C-terminus of Kif9 contains an important nuclear targeting motif. Dual labeling of Kif9 and the nuclear envelope linker protein, Sun1, demonstrate significant overlap in polypeptide distribution. Similar to Kif9, Sun1 staining is enriched on the side of the nucleus facing the centrosome. Interestingly, in the absence of Kif9, Sun1 distribution is more uniform over the nuclear surface. Kif9 also shows ATP sensitive binding to microtubules. Taken together, we postulate that Kif9 functions as a KASH protein, and directly couples Sun1 to cytoplasmic microtubules that are, in turn, anchored to the Dictyostelium centrosome. If Kif9 functions as a microtubule depolymerase activity similar to other M-type kinesins, it would draw the centrosome toward the nucleus and thus act to maintain the normal tight association between these two organelles. A close proximity between these two organelles likely facilitates centrosome insertion into the nuclear envelope for division and minimizes multiple centrosome-nuclear interactions in multinucleated cells. We are currently testing this hypothesis.

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Functional Interaction between phosphatidylinositol 4-phosphate 5-kinase α and KIF2A in Neurite Remodeling.

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Neuronal morphology is regulated by the cytoskeleton. Kinesin superfamily protein 2A (KIF2A) depolymerizes microtubules (MTs) at growth cones and regulates axon pathfinding. The factors regulating KIF2A in neurite remodeling remain elusive. Here, using immunoprecipitation with an antibody specific to KIF2A, we identified phosphatidylinositol 4-phosphate 5-kinase (PIPK) as a candidate membrane protein that regulates the activity of KIF2A. Yeast two-hybrid and biochemical assays demonstrated direct binding between KIF2A and PIPKα. Partial colocalization of the clusters of punctate signals for these two molecules was detected by

confocal microscopy and photoactivated localization microscopy. Additionally, the MT-depolymerizing activity of KIF2A was enhanced in the presence of PIPK in vitro and in vivo, suggesting a novel PIPK-mediated mechanism controlling MT dynamics in neurite remodeling.

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Preferential binding of a kinesin-1 motor to GTP-tubulin-rich microtubules underlies polarized vesicle transport.

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Polarized transport in neurons is fundamental for the formation of neuronal circuitry. A motor domain-containing truncated KIF5 (a kinesin-1) recognizes axonal microtubules, which are enriched in EB1 binding sites, and selectively accumulates at the tips of axons. However, it remains unknown what cue KIF5 recognizes to result in this selective accumulation. We found that axonal microtubules were preferentially stained by the anti-GTP–tubulin antibody hMB11. Super resolution microscopy combined with EM immunocytochemistry revealed that hMB11 was localized at KIF5 attachment sites. In addition, EB1, which binds preferentially to GMPCPP(guanylyl-methylene-diphosphate)-microtubules in vitro, recognized hMB11 binding sites on axonal microtubules. Further, expression of hMB11 antibody in neurons disrupted the selective accumulation of truncated KIF5 in the axon tips. In vitro studies revealed approximately three times stronger binding of KIF5 motor head to GMPCPP-microtubules than to GDP-microtubules. Collectively, these data suggest that the abundance of GTP-tubulin in axonal microtubules may underlie selective KIF5 localization and polarized axonal vesicular transport.

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Transgenic mice study of cargo binding domain mutants of KIF17 reveals a mechanism for NMDA receptor transport regulation in synaptic plasticity and spatial memory in vivo.

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CaMKII phosphorylates the tail domain of KIF17 to control NR2B transport by modulating the interaction between KIF17 and Mint1 *in vitro*. However, the mechanism of regulation of NR2B transport *in vivo* has been largely unknown. Here, we generated transgenic mice of cargo binding domain mutants of KIF17, and show that altering KIF17–Mint1 binding leads to a disturbance of neuronal plasticity and memory formation *in vivo*. KIF17 with the S1029A mutation does not release cargo and KIF17 with the S1029D mutation does not bind cargo. Consequently, *TgA/kif17-/-* and *TgD/kif17-/-* mice showed significant reductions in the amounts of synaptic NMDA receptors. Accordingly, hippocampal LTP, CREB activation and spatial memory were severely impaired. These results suggest that not only binding of NR2B to KIF17 but also release of NR2B from KIF17 is a critical step for NR2B transport and regulation of learning and memory *in vivo*.

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Kinesin Motor Eg5 Functions during Protein Synthesis to Promote Ribosome Transit.

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Kinesin microtubule motors are important for the transport of specific intracellular cargoes along microtubules and are critical for cell division. Eg5 is a plus-end directed motor essential for spindle assembly during mitosis. Eg5 is believed to function exclusively in mitosis and as such

is an anti-cancer drug target. Inhibitors to Eg5 are currently undergoing stage I and stage II clinical trials and new clinical trials of Eg5 inhibitors are ongoing. However, unlike some other mitotic proteins, mitotic motors can be detected throughout the cell cycle and anti-Eg5 antibodies robustly label interphase cells. Such constitutive expression led us to investigate potential interphase functions for Eg5. We performed sucrose gradient fractionation of mature ribosomes and immunoprecipitation of RPE1 cell lysates and determined that during interphase Eg5 predominantly associates with mature ribosomes in the cytoplasm. In vitro microtubule binding assays demonstrated that the 80S ribosome association with microtubules requires Eg5. In addition, inhibition of Eg5 function, by knockdown or small molecule inhibition, resulted in a marked ~40% decrease in nascent protein synthesis in multiple human cell lines. This decrease in protein synthesis was reversible and was not due to mitotic arrest or apoptotic cell death. In order to identify which step in translation Eg5 could be participating in, polysome profiling was completed to observe the distribution of ribosomes within cells. After Eg5 inhibition by monastrol, S-Trityl-L-cysteine, or dimethylenastron, inhibitors of the ATPase activity of Eg5, a reduction in the 80S ribosomes and an increase in polysomes were observed, typical for an elongation/termination defect phenotype. Eg5 function in translation elongation/termination was corroborated by completing ribosome transit time assays, which measures the time it takes one ribosome to traverse a single mRNA. After Eg5 inhibition, a ~2.5-fold increase was observed in the transit rate demonstrating the requirement of Eg5 for elongation/termination. When Eg5 was knocked-down, polysome profiling analysis demonstrated an increase in 80S ribosomes and Eg5 function was more essential for translation of longer polypeptides than shorter polypeptides. Therefore, we propose that Eg5 links 80S ribosomes to microtubules during translation and is required for optimal efficiency of the elongation step of translation. The ribosomes processivity, the distance the ribosome can travel on the mRNA, appears to be enhanced by its association with microtubules through Eg5. This suggests a novel role for the mitotic microtubule motor Eg5 and the microtubule cytoskeleton as processivity factors for protein synthesis.

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Dissecting the cellular functions of *Drosophila* kinesin 5 using STLC-sensitive KLP61F chimeras.

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The *Drosophila* kinesin 5, KLP61F, is a homotetrameric motor that slides apart interpolar microtubules within mitotic spindles, thereby driving poleward flux and controlling mitotic spindle length by opposing Ncd and other force generators that pull the spindle poles together. Since KLP61F is not inhibited by known chemical inhibitors of kinesin 5, most of our findings have relied on the microinjection of function-blocking antibodies into embryos. Here, we created KLP61F chimeras containing an engineered S-trityl-L-cysteine (STLC) binding site similar to that found in the human kinesin 5, Eg5, and expressed it in *Drosophila* Schneider S2 cells and flies. We find that mitosis proceeds normally in cells expressing the chimeric motors, but is severely impaired in the prescience of STLC. Low concentrations of STLC cause a significant decrease in metaphase spindle length. The results so far are highly concordant with our published antibody inhibition results. We are analyzing the effects of STLC in syncytial embryos from transgenic flies. The ability to use the small molecule inhibitor, STLC will greatly facilitate the study of loss of kinesin-5 function on multiple aspects of mitosis and other processes, such as neuronal cell function.

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Molecular mechanism of kinesin-1 activation by Alcadeinα.

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Objectives: We previously reported that Alcadeinα (Alcα) functions a novel cargo to kinesin-1, which comprises two heavy (KHC) and two light (KLC) chains (*EMBO J*. [2007] 26, 1475-1486). Association of Alcα to KLC evoked the vesicle association of functional kinesin-1 resulting in the fast anterograde transport of Alcα-vesicles, while the kinesin-1 was inactive with an autoinhibition state in the absence of Alcα. We dissected the function of WD motif which is the region required for binding to the tetratricopeptide repeats (TPR) region of KLC1, and determined whether the binding itself to KLC is required for activation of kinesin-1. It is known that KLC binds to C-terminal 11-amino-acids of c-Jun N-terminal kinase-interacting protein 1 (JIP1C11) and KHC associates with fasciculation and elongation protein ζ 1 (FEZ1). Those interactions activates kinesin-1 in vesicular transport (*J. Cell Biol*. [2007] 176, 11-17). Herein we analyzed the regulatory mechanism of kinesin-1 activation by WD motif of Alcα in comparison with known mechanism.
Methods: Artificial membrane proteins possessing a WD-motif of Alcα were expressed in a mouse CNS catecholaminergic cell line CAD cells with EGFP-KLC1 in the presence or absence of EGFP-KHC, and CAD cells were subjected to differentiation by serum depletion. Vesicular formation and anterograde transport of EGFP-KLC1 and EGFP-KHC were examined using a total internal reflectance fluorescence (TIRF) microscopy system.
Results: Artificial membrane proteins possessing a single WD-motif, which is enough to bind KLC1, could induce vesicle association of EGFP-KLC1 and EGFP-KHC, and these vesicles were transported anterogradely. The 1st and the 6th tetratricopeptide repeats of KLC1 are dispensable for WD motif-dependent kinesin-1 activation *in vivo*. The C-terminal 11-amino-acid sequence of JIP1 can recruit KLC1 to vesicles, but does not activate kinesin-1 for vesicular transport.
Conclusion: Our previous and current studies indicate that either of two WD motifs in Alcα cytoplasmic region is necessary and sufficient to initiate kinesin-1 activation. Moreover, Alcα may initiate the activation of kinesin-1 on its own, that differs from a manner by JIP1 which required for an assistance of FEZ1 binding to KHC.

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Kinesin-1 and dynein transport Dense Core Vesicles in axons.

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Microtubule based transport is essential for proper cellular function, particularly in neurons, which have a highly asymmetric structure. In the neuron, there is a constant flux of newly synthesized material from the cell body toward the axon terminal (anterograde) while excess material and endocytic organelles are returned toward the cell body (retrograde). Anterograde axonal transport is carried out mainly by kinesins 1-3, while retrograde transport is achieved by cytoplasmic dynein. To understand how multiple types of motor proteins contribute to the intracellular distribution of organelles, we are studying Dense Core Vesicles (DCV) loaded with the neuropeptide ANF::GFP in the axons of Drosophila. Using this system our lab previously found that mutations in unc104, which encodes for kinesin-3, inhibit DCV flux, run velocity and run length in both directions. To test for contributions by other motors , we are doing live

imaging of DCV transport in intact kinesin-1 (Khc) and dynein (Dhc64C) mutant larvae. Hypomorphic Khc mutations cause a 4 fold reduction in anterograde and a 10-fold reduction in retrograde DCV flux. Anterograde vesicles have reduced net velocity, reduced run length and they have increased pause time. Hypomorphic Dhc64Cmutations also cause ~4- and 10-fold reductions respectively in anterograde and retrograde flux, slower run velocities, shorter run lengths and spent more time paused compared to DCVs from the control animals. Anterograde DCVs in the Dhc64C mutants have shorter run lengths and mildly reduced net velocity compared to those from the control animals. These results show that:
1) cytoplasmic dynein is the retrograde DCV motor, 2) kinesins-1 and -3 both are important anterograde motors for DCV and 3) anterograde transport by one or both kinesins is somehow dependent on dynein. These findings suggest important coordination and interdependence for the three vesicle motors. To gain insight into the cooperative mechanisms, we are pursuing further genetic and biochemical tests.

Neuronal Cytoskeleton

1496

Growth Cone Traction Forces in Regenerating *Aplysia* Neurons.

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The structure and dynamics of the growth cone cytoskeleton is central to developement and regeneration of neurons. The present study addresses the mechanical engagement between the growth cone cytoskeleton and the extracellular environment. We have used laminin-coated flexible PDMS (polydimethyl siloxane) substrates to measure traction force and strain energy in regenerating *Aplysia* bag cell growth cones. Traction force vectors are oriented perpendicular to the leading edge and parallel to the direction of retrograde flow. Traction force measurements also give insight into the mechanical organization of the neuron. Integrating force vectors over the growth cone reveals a net nano-Newton scale force in the direction of the axon shaft, implying a mechanical connection between the growth cone and other parts of the cell. Highest forces were measured in the transition zone coincident with previously reported myosin II localization. Growth cone traction is myosin II dependent: treatment with myosin II inhibitor significantly reduced total growth cone strain energy.

1497

The Axonal Translation of Cortactin Contributes to the Formation of Axonal Filopodia and Collateral Branches.

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Axon collateral branches allow individual neurons to make connections with multiple targets. In vitro and in vivo collateral branches arise through the maturation of transient axonal filopodia. In turn, axonal filopodia emerge from axonal patches of actin filaments. The formation of axonal actin patches requires the actin nucleation Arp2/3 complex. Cortactin is a multifunctional protein that can regulate the stability of Arp2/3 nucleated actin filaments and promote complex activity.

We investigated the role of cortactin in the formation of axonal filopodia and branches using chicken sensory neurons. As determined by immunocytochemistry and live imaging of RFP-cortactin and eGFP-actin, cortactin is recruited to actin patches. In vivo and in vitro over-expression of cortactin increased the number of axonal filopodia and branches along sensory axons in the spinal cord. In vitro over-expression of cortactin increased the duration of actin patches and the frequency of the emergence of filopodia from patches, but did not affect the rate of patch formation. Expression of the NTA fragment, which prevents cortactin binding to Arp2/3, decreased the duration of patches but did not affect filopodial emergence. These data indicate that cortactin regulates the duration of actin patches through Arp2/3 but promotes formation of filopodia in an Arp2/3 independent manner. Treatment with nerve growth factor (NGF) promotes branching and increases the rate of formation of axonal filopodia and patches without affecting the rate of filopodial emergence from patches. In the absence, but not presence, of NGF over-expression of cortactin increased filopodial formation. Cortactin and Arp2 mRNAs have been previously reported in axons, and using a purified axonal preparation we have detected cortactin mRNA in axons. NGF induced a cycloheximide sensitive increase in the levels of cortactin and Arp2 in axons severed from the cell body, suggesting cortactin and Arp2 are locally translated in axons. Cycloheximide also blocked the NGF-induced increase in filopodia and patch formation. Collectively, the data indicate that the promotion of axonal filopodia by NGF requires the intra-axonal translation of cortactin, which acts to promote the emergence of filopodia from actin patches in the context of the NGF-induced increase in the rate of patch formation.

1498

Mitotic motors co-regulate microtubule patterns in axons and dendrites.

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Vertebrate neurons extend two distinct types of processes, axons and dendrites, which differ in morphology, composition and function due, in part, to distinct patterns of microtubule (MT) polarity orientation. MTs in the axon are nearly uniformly oriented with their plus end distal to the cell body, whereas MTs in the dendrites are non-uniformly oriented. Studies to date suggest a scenario for establishing these MT patterns whereby MTs are transported into the axon and nascent dendrites with plus-ends-leading, and then additional MTs of the opposite orientation are transported into developing dendrites. If this is correct, molecular motor proteins with assigned roles would presumably tightly regulate these MT transport events. Kinesin-6 (also called CHO1/MKLP1), a molecular motor best known for its role in mitosis, is present in cell bodies and dendrites but is absent from the axon. We found that depletion of kinesin-6 by siRNA from cultures of rat neurons significantly reduces the number of processes with dendritic characteristics, which is accordant with our previous work in neurons using antisense oligonucleotides. Those processes that did display some dendritic characteristics were nevertheless longer than control dendrites, and thinner in their more proximal regions. In addition, such processes displayed a higher proportion of plus-end-distal MTs than control dendrites, as indicated by live-cell imaging with GFP-tagged EB3. Interestingly, there was no effect of kinesin-6 depletion on axonal branch formation or growth cone turning, which is consistent with the motor protein being absent from axons. Surprisingly, however, depletion of kinesin-6 caused axons to grow faster, and there was a notable increase in the frequency of transport of short MTs compared to control axons. These effects on axonal length and MT transport are similar to those observed in previous studies in which we depleted either kinesin-5 or kinesin-12, two other molecular motors mainly known for their roles in mitosis. Based on these observations, we propose that all three of these mitotic motors act within the cell body to

attenuate the transport of plus-end-distal MTs into the axon while driving the transport of minus-end-distal MTs into dendrites. In this manner, we speculate that mitotic motors co-regulate MT polarity patterns in axons and dendrites.

1499

Kinesin-6, a motor protein essential for cytokinesis, plays a key role in neuronal migration.

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The development of the mammalian brain requires the orderly migration of neurons from their sites of origin to their final destinations. Neuronal migration is critically dependent upon both microtubules (MTs) and actin-based motility. Here we focused on a potential role in neuronal migration of kinesin-6 (also called CHO1/MKLP1), a molecular motor protein best known for its role in mitosis. We recently published that migratory neurons maintain their bipolar morphology when depleted of kinesin-5 (another mitotic motor protein, also called Eg5), but migrate notably faster than controls. Here we found that neurons depleted of kinesin-6 were typically multi-polar, and displayed continuous alterations in their direction of movement, resulting in no net movement. Interestingly, kinesin-6-depleted neurons displayed numerous outgrowths along their processes, which were reminiscent of the actin-rich protrusions that often appear on developing axons. Such outgrowths were rarely observed in control migrating neurons or migrating neurons depleted of kinesin-5. This observation is particularly intriguing as kinesin-6 has two splice variants, one of which has an actin-binding domain not found in kinesin-5. Consistent with our previous report using fluorescence microscopy, we have now obtained results with electron tomography demonstrating that not all of the MTs in the migratory neuron converge on the centrosome. These studies reveal the presence of a sub-population of MTs that extend into the leading process but have free minus ends posterior to the centrosome. Such MTs intermingle with MTs of the opposite orientation, resulting in regions of antiparallel organization behind the centrosome, ideally suited to interact with molecular motors such as kinesins 5 and 6. The very different phenotypes obtained from depleting each of these motors indicates, however, that the unique functions of each motor are not explicable entirely on the basis of interactions with antiparallel MTs. In mitotic cells, kinesin-6 signals to the actin cytoskeleton while interacting with MTs in the spindle midzone, to concentrate cortical movements to the cleavage furrow during cytokinesis. We hypothesize that kinesin-6 functions analogously in migrating neurons to constrain process number and restrict protrusive activity to the tip of a single leading process. Together, these two effects result in a bipolar neuron able to move in a concerted fashion.

1500

Glutamate-Induced Incorporation of Cofilin into Rods Depends on Disulfide Inter-molecular Bonds: Implications for Actin Regulation and Neurodegenerative Disease.

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Rod-shaped aggregates (“rods”), containing equimolar actin and the actin dynamizing protein cofilin appear in neurons following a wide variety of potentially oxidative stress: simulated microischemia, cofilin overexpression, and exposure to excess glutamate, peroxide, or the dimer/trimer forms of amyloid beta peptide (Aβd/t), the most synaptotoxic species. These rods are initially reversible and neuroprotective, but if they persist in neurites, the synapses

degenerate without neurons dying. Herein we report that rod formation depends on the generation of inter-molecular disulfide bonds in cofilin. Of four Cys-to-Ala cofilin mutations expressed in rat E18 hippocampal neurons, only the mutant incapable of forming inter-molecular bonds (CC39,147AA) has significantly reduced ability to incorporate into rods. Rod regions show unusually high oxidation levels, and rods can be reversed by treating cells with reducing agent. Rods, isolated from stressed neurons, contain dithiothreitol-sensitive multimeric forms of cofilin. Oligomerization of cofilin in cells represents one more mechanism for regulating cofilin's actin dynamizing activity and probably underlies synaptic loss.
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1501

A new model for cofilin's action in morphological plasticity in dendritic spines of neurons.

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Long-term depression (LTD) is a form of plasticity at excitatory synapses characterized by decreased synaptic strength and shrinkage of dendritic spines. A widely cited model has proposed that cofilin becomes activated during LTD and mediates spine shrinkage by severing and depolymerizing actin filaments. However, we observed, using fluorescence resonance energy transfer, that the interaction between F-actin and cofilin actually decreases in spines following LTD, and that the concentration of free barbed ends of actin filaments also decreases. These results are therefore inconsistent with an LTD-induced increase in cofilin activity. Furthermore, enhancing endogenous cofilin activity by overexpressing the protein phosphatases chronophin or slingshot not only attenuated the LTD-induced decrease in cofilin-actin binding, but also inhibited LTD-induced spine shrinkage. Sequestration by PIP2, reduced cofilin concentration and an increase in the fraction of phospho-cofilin within the spine all may contribute to the LTD-induced inhibition of cofilin activity. These results suggest that constitutive F-actin severing by cofilin is essential for normal spine maintenance, and that a decrease in cofilin activity, not an increase, significantly contributes to a net loss of F-actin and consequent spine shrinkage during this form of synaptic plasticity.

1502

Myosin-X and its Motorless Isoform Differentially Regulate the Formation of Dendritic Filopodia and Spines, which are Actin-rich Structures in Neurons that Resemble Leading Edge Protrusions in Migrating Cells.

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Filopodia are dynamic, finger-like protrusions that are thought to guide directed cell migration and to promote cell motility. Similar protrusive structures known as dendritic filopodia are found in neurons and later mature into dendritic spines, which comprise the postsynaptic sites of excitatory synapses. Alterations in spine formation are mediated by actin rearrangement and are crucial for cognitive functions such as learning and memory; however, the roles of actin-based motors in this process are not well understood. Myosin-X (Myo 10), an unconventional myosin motor, promotes the formation of filopodia in non-neuronal cells, leading us to hypothesize that it regulates dendritic filopodia dynamics and spine development in neurons. Here, we use cultured hippocampal neurons to show that full-length Myo 10 and its motorless isoform (headless Myo10) play important, but distinct, roles in regulating the formation of dendritic spines and their precursors, dendritic filopodia. Full-length Myo10 localizes to the tips

of dendritic filopodia; in contrast, headless Myo10 is enriched at the heads of dendritic spines. While most headless Myo10 puncta co-localize with presynaptic terminals, the majority of full-length Myo10 puncta do not make stable contacts. Interestingly, dendritic filopodia in full-length Myo10 expressing neurons exhibit robust movement. Elevated expression of full-length Myo10 results in a significant increase in the density and length of dendritic filopodia and a decrease in the density of spines and synapses. Importantly, knockdown of full-length Myo10 also reduces the number of spines and synapses, suggesting that full-length Myo10 is required for spine formation via the regulation of dendritic filopodia. Expression of headless Myo10, unlike the full-length form of this protein, does not affect the formation of dendritic filopodia, but does induce the expansion of spine heads. Collectively, our data indicate that full-length and headless Myo10 differentially modulate the formation and maturation of dendritic spines.

1503

The Rac and Cdc42 Guanine Nucleotide Exchange Factor Asef2 Mediates Dendritic Spine Formation in Cultured Hippocampal Neurons.

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The formation of dendritic spines, actin-rich protrusions from dendrites that form synapses with presynaptic axons, is dependent upon the activity of molecular switches, such as the Rho family of small GTPases. Once activated, these proteins are thought to play vital roles in regulating actin cytoskeleton dynamics within dendritic spines; however, the function of upstream activator proteins, known as guanine nucleotide exchange factors (GEFs), in spine formation is not well understood. Here, we show that the Rac and Cdc42 GEF Asef2 is critical for the development of dendritic spines in hippocampal neurons. Expression of EGFP-tagged Asef2 causes a significant increase in the density of spines and synapses, while knockdown of Asef2, using an siRNA approach, leads to a loss of dendritic spines and synapses. To determine if the GEF activity of Asef2 is critical for mediating its effects on spine formation, point mutations that disrupt GTPase activation were generated in the DH domain. Abrogation of Asef2's GEF activity results in a decrease in mature spines as well as an increase in filopodia-like protrusions, which are thought to be the precursors of dendritic spines. Collectively, our data suggest that Asef2 utilizes its GEF activity to regulate the development of dendritic spines. This underscores the need for precise control of GTPase activation, which is necessary to properly modulate the actin cytoskeleton in order to promote spine formation.

1504

Functional analysis of Dysbindin, a schizophrenia risk factor, in dendritic spine formation.

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Dysbindin (dystrobrevin-binding protein, DTNBP) is a multi-functional adaptor protein thought to be involved in cell growth, neuronal apoptosis, intracellular vesicle traffic and neurotransmitter release. Genetic variations in dysbindin are one of the most commonly reported variations associated with schizophrenia. Since schizophrenia could be regarded as a neurodevelopmental disorder resulting from abnormalities of synaptic connectivity, we attempted to clarify the function of dysbindin in neuronal development. We examined the developmental change of dysbindin in rat brain by Western blotting and found that a 50 kDa isoform is highly expressed during the embryonic stage while a 40 kDa one is detected at postnatal day 11 and increased thereafter. Immunofluorescent analyses revealed that dysbindin is enriched at the spine-like structure of primary cultured rat hippocampal neurons. Knockdown

of dysbindin led to the generation of abnormally elongated immature dendritic protrusions. To elucidate dysbindin function at the molecular level, we screened dysbindin-binding proteins, and consequently identified an actin cytoskeleton regulator, WAVE2, and an adaptor protein, Abi-1. Abi-1 is known to associate with WAVE2 and be involved in spine morphogenesis. Interestingly, dysbindin promoted the binding of WAVE2 to Abi-1, and the phenotype of dysbindin-knockdown was rescued by overexpression of Abi-1. Collectively, the present results indicate possible function of dysbindin at the postsynapse in the regulation of dendritic spine morphogenesis through the interaction with WAVE2 and Abi-1. These results also support the "neurodevelopmetal hypothesis" for the mechanism of schizophrenia symptoms.

1505

Microtubule and Protein Kinase A Interaction is Important for Neurite Morphogenesis.

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Neurite morphogenesis is a process through which neurons generate their widespread axon and dendrites. During neurite morphogenesis, the microtubule cytoskeleton plays a crucial role. Our proteomic study indicated that the amount of protein kinase A (PKA) on microtubules significantly increased after neuronal differentiation and neurite morphogenesis. PKA has been show to play essential roles in various cellular processes. However, its function on microtubule organization during neurite morphogenesis remains elusive. Since PKA is essential for various cellular functions, knocking down PKA genes will causes a plethora of phenotypes unrelated to neurite morphogenesis. As a result, we developed two different methods to alter the amount of PKA on microtubules without changing the overall PKA level. This allows us to determine the specific function of PKA on microtubule.

MAP2c, a neuron-specific microtubule-associated protein, is also a PKA anchoring protein in neurons. It has been shown that overexpressing MAP2c can produce cellular protrusion in non-neuronal cells. A MAP2c deletion construct (MAP2c-ΔRII) is generated in which the PKA binding domain is removed; this allows us to reduce PKA localization on microtubules without changing the overall PKA level. Here we show that overexpressing MAP2c-ΔRII in P19 cells resulted in shorter protrusions than overexpressing MAP2c. In addition, overexpressing MAP2c-EEE in mouse primary hippocampal neurons resulted in shorter neurite length than MAP2c-EEE-ΔRII. These results indicated that PKA's association with microtubules is important for generating cellular protrusions and neurites.

Another approach to investigate the function of PKA on neuronal microtubules is by using an artificial protein-protein interaction method. In this method, we can force the PKA complex from the microtubule cytoskeleton onto the actin cytoskeleton. Currently, we have proved that our method is feasible by diverting a fluorescent cytoplasmic protein onto the cell membrane.

Keywords: protein kinase A, PKA, cAMP-dependent kinase, microtubule, neurite morphogenesis, MAP2

1506

Pro- and mature neurotrophins differentially regulate neuronal morphology.

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The establishment and refinement of neural circuits during development depends upon a dynamic process of outgrowth and branching of axons and dendrites that leads to synapse

formation and connectivity. However, the mechanisms that translate extracellular signals into axonal branch formation are incompletely understood, as are the mechanisms that negatively regulate the arborization of processes. The neurotrophin BDNF plays an essential role in the outgrowth and activity-dependent remodeling of axonal arbors in vivo. We recently reported that the MAP kinase phosphatase-1 (MKP-1) controls BDNF-dependent axon branching. MKP-1 expression induced by BDNF signaling exerts spatio-temporal deactivation of JNK, which negatively regulates the phosphorylation of JNK substrates that impinge upon microtubule destabilization. Indeed, neurons from mkp-1 null mice were unable to produce axon branches in response to BDNF. On the other hand, dendritic growth is affected negatively by the BDNF precursor, proBDNF. Mice impaired in proBDNF processing display markedly reduced dendritic complexity in vivo, suggesting that proneurotrophins negatively influence neuronal morphology. Both proBDNF and proNGF acutely cause collapse of growth cones in vitro, a retraction that is mediated by two discrete signaling pathways: Inactivation of the actin-bundling protein fascin and dissociation of the Rac activator Trio from p75NTR, resulting in decreased activation of Rac. These results identify a bifunctional signaling mechanism through which proneurotrophins and their mature forms differentially modulate neuronal morphology in the nervous system.

1507

Tropomyosins induce neurite formation in B35 neuroblastoma cells and control neurite branching.

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Fundamental mechanisms of neuronal differentiation and neurite formation are controlled through the actin cytoskeleton. Dynamic and structural properties of the actin cytoskeleton are regulated by a large pool of actin-associated proteins including tropomyosins, a family of actin-associated proteins controlling actin filaments stiffness in an isoform dependent manner. In neurons products from three tropomyosin genes are found (α-, γ- and δ-tropomyosin). The current study aims to understand the role of α- (TmBr1, TmBr2, TmBr3) and δ-tropomyosin (Tm4) gene products in early neuronal development.
Using B35 neuroblastoma cells overexpressing individual tropomyosin isoforms we investigated the impact of the actin cytoskeleton on neuronal morphology and early stages of neuritogenesis. We show that the overexpression of tropomyosins is sufficient to induce the formation of neurites which is associated with an upregulation of markers of neuronal differentiation including MAP2c.
Tropomyosins differentially control the branching of neurites in db cAMP stimulated B35 cells. While we observed no changes in the neurite branching in TmBr2 overexpressing B35 cells, overexpression of TmBr1 attenuated and overexpression of TmBr3 and Tm4 increased the degree of branching. This was associated with an isoform dependent change in growth cone size and number of filopodia along these neurites. Overexpression of TmBr3 and Tm4 resulted in larger growth cones and increased number of filopodia while TmBr1 led to smaller growth cones and no change in filopodia number as compared to control B35 cells.
Our data suggest a central role for the actin-associated protein tropomyosin in neuronal differentiation and establishing of complex neuronal networks.

1508

Kinesin-5 regulates microtubule organization in dendrites.

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Kinesin-5 (also termed kif11 or Eg5) is a homotetrameric motor protein that plays an essential role in mitosis. Its best recognized mode of function is to generate forces between oppositely oriented microtubules (MTs). We reported previously that kinesin-5 is expressed robustly in developing neurons, and that it plays key roles in axonal elongation and turning of the growth cone. These effects are explicable, at least in part, by kinesin-5 acting as a kind of "brake" on the transport and sliding of MTs relative to each other. The precise mechanism by which this occurs remains mysterious, however, because nearly all of the microtubules in the axon have the same plus-end-distal orientation. By contrast, dendritic MTs are non-uniformly oriented, and hence would presumably be better suited for interacting with kinesin-5. We have now endeavored to determine whether kinesin-5 plays an important role in regulating the dendritic MT array. Treatment of cultured rat sympathetic neurons with monastrol, a potent and specific inhibitor of kinesin-5, resulted in dendrites that were thinner and longer than control dendrites, and which contained far fewer minus-end-distal MTs (as assessed by live-cell imaging with GFP-tagged EB3). Thus, the dendrites were much more similar to axons with regard to both morphology and MT polarity orientation. Immunostain analyses indicate a relatively uniform distribution of total kinesin-5 in axons and dendrites, but an enrichment in dendrites of a variant of kinesin-5 phosphorylated at a site required for it to interact with MTs. Additional studies revealed that kinesin-5 binds more avidly to tyrosinated MTs than detyrosinated MTs, which is provocative in light of the fact that dendritic MTs are known to be less detyrosinated than axonal MTs. Partial knockdown of tubulin tyrosine ligase resulted in an elevation of detyrosinated MTs in dendrites, a more homogeneous distribution of phospho-kinesin-5 in axons and dendrites, and morphological changes akin to those observed with monastrol treatment. Collectively, these results suggest that kinesin-5 is an important factor in establishing the distinct MT polarity pattern and morphology of the dendrite, and that kinesin-5's localization and functions are regulated in part by its phosphorylation and in part by its preference for tyrosinated rather than detyrosinated MTs.

1509

Phosphorylation by GSK-3β and subcellular Localization of Stathmin 3/SCLIP in Neurons.

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During development of the nervous system, neuronal growth, migration and functional morphogenesis rely on the appropriate control of the subcellular cytoskeleton. Among the numerous proteins involved in the control of microtubule dynamics, stathmin family proteins are involved in the control of diverse stages of neuronal differentiation, including axonal growth and branching, or dendritic development. An important issue is the deciphering of differences and specificities in the likely complementary roles of the various members of the stathmin family, as well as their underlying molecular and regulatory features. We have shown previously that stathmins 2 (SCG10) and 3 (SCLIP) fulfill distinct, independent and complementary regulatory roles in axonal morphogenesis. Although the two proteins have been proposed to display the characteristic four conserved phosphorylation sites originally identified in stathmin 1, we show that they possess distinct phosphorylation sites within their specific proline-rich domains (PRDs) which are differentially regulated by phosphorylation by proline-directed kinases involved in the control of neuronal differentiation. ERK2 or CDK5 display different site specificities for the two

proteins and, in contrast to stathmin 2, stathmin 3 is a substrate for GSK-3β which phosphorylates its serine 60 both in vitro and in vivo. Moreover, serine 60 phosphorylated stathmin 3 displays a specific subcellular localization at neuritic tips and within the actin-rich peripheral zone of the growth cone of differentiating hippocampal neurons in culture. Finally, pharmacological inhibition of GSK-3β induces a redistribution of stathmin 3, but not stathmin 2, from the periphery towards the Golgi region of neurons. Stathmin proteins can thus be either regulated locally or locally targeted by specific phosphorylation, each phosphoprotein of the stathmin family fulfilling distinct and specific roles in the control of neuronal differentiation.

1510

GSK3β activity and CNS axonal regeneration: insights from in vivo studies.

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The central nervous system (CNS) regenerates under certain conditions such as following a conditioning lesion (CL): the central branch of a dorsal root ganglia (DRG) neuron is capable of growing within the CNS inhibitory environment after its peripheral branch has been previously injured. We aimed at disclosing intrinsic mechanisms enabling CNS axonal regeneration using the CL as a model and used two proteomic approaches, iTRAQ and phosphoproteomic arrays. From the proteins identified in the DRG as differentially regulated following CL in comparison with spinal cord injury (SCI), the Glycogen synthase kinase 3β (GSK3β) pathway, involved in the regulation of microtubule dynamics, emerged as a key player. GSK3β activity was downregulated following CL. Significantly, despite the focus on the inhibition of GSK3β through Ser9 phosphorylation, our data shows that a decreased phosphorylation of Tyr216, the activator of kinase activity, both in DRG and in the spinal cord injury site is the regulatory event leading to gain of regenerative capacity following CL. Preliminary results suggested that Fyn kinase is a candidate for the modulation of Tyr216 phosphorylation. In the spinal cord injury site the levels of phosphorylated CRMP2 (GSK3β substrate negatively regulated by the kinase) were decreased, while MAP1B (GSK3β substrate positively regulated by the kinase) phosphorylation levels were increased after CL. Concerning the role of GSK3β Ser9 phosphorylation, we observed that DRG neurons from transgenic mice expressing the GSK3β mutant Ser9Ala (GSK3βS9A knockin mice) have increased neurite outgrowth in vitro following CL. Moreover, in vivo retrograde tracing with cholera toxin B of dorsal column axons confirmed the conditioning effect of GSK3βS9A knockin mice thus showing that modulation of GSK3β activity through Ser9 is not needed to induce axonal growth. Further studies to clarify the role of GSK3β in axonal growth in vivo are currently being performed.

1511

Role of GSK3 in regulation of growth cone microtubule dynamics.

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Emerging evidence indicates that glycogen-synthase kinase 3 (GSK3) proteins are fundamental components in neurodevelopmental processes including neurogenesis, neuronal migration, neuronal polarization, and axon outgrowth. Previous studies, including ours, have revealed that GSK3 signaling regulates neuronal polarization and axon growth via controlling reorganization of neuronal microtubules. However, how GSK3 controls the dynamics of neuronal microtubules remains unknown largely due to lack of technology that allows high-resolution live-cell imaging of microtubule dynamics in mammalian neurons. Leading advances in live cell imaging techniques and computer-based image analysis have now provided an opportunity to solve this problem, thus allowing us to perform more detailed investigation of the role of GSK3 in neuronal

microtubule dynamics. By using a newly developed approach that analyzes microtubule dynamics through detecting microtubule plus-end tracking proteins (+TIPs), we studied how GSK3 and its downstream microtubule regulatory proteins, such as APC and CLASP, control microtubule dynamics in nerve growth cones. Results show a surprising spatial difference in microtubule dynamics between distal axon shaft and the growth cones, consistent with the spatial distribution of GSK3 activities. We are now investigating if modulation of GSK3 activity or the function of APC and CLASP can affect such spatial distribution of microtubule dynamics. The results will reveal the molecular mechanism by which GSK3 regulate neuronal microtubules and its role in controlling neuronal function.

1512

The F-BAR protein CIP4 inhibits neurite formation by producing lamellipodial protrusions.

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F-BAR proteins are a large family of proteins that function in sensing and inducing membrane curvature. Cdc42-interacting protein 4 (CIP4), a member of the F-BAR family of proteins, regulates endocytosis in a variety of cell types. However, there is little data on how CIP4 may be functioning in neurons. Here we show that CIP4 plays a novel role in neuronal development by inhibiting neurite formation. Remarkably, CIP4 exerts its effects, not through endocytosis, but by producing lamellipodial protrusions. In primary cortical neurons, CIP4 does not localize to endosomes, but instead is concentrated at the tips of extending lamellipodia and filopodia. Overexpression of CIP4 results in lamellipodial protrusions around the cell body, resulting in delayed neurite formation and enlarged growth cones. Conversely, cortical neurons from CIP4-null mice initiate neurites nearly twice as fast as controls. These data demonstrate CIP4 is one of few proteins whose expression inhibits neurite formation. Moreover, it is the first to demonstrate that an F-BAR protein functions differently in neuronal vs. non-neuronal cells and induces lamellipodial protrusions instead of invaginations.

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Evidence for end-to-end annealing and breakage of neurofilaments in cultured neurons.

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We have shown previously that neurofilaments expressed in heterologous non-neuronal cell lines can lengthen by end-to-end annealing (Colakoglu & Brown, *J. Cell Biol.* 185:769-777, 2009). To test if this also occurs in neurons, we transfected cultured rat cortical neurons with neurofilament protein M fused to the photoconvertible fluorescent protein mEos2 (mEos2-NFM), or we co-transfected with neurofilament protein M fused to photoactivatable GFP (PAGFP-NFM) and neurofilament protein L fused to mCherry (NFL-mCherry). These fusion proteins all co-assembled with endogenous neurofilament proteins throughout the neurons. To test for end-to-end annealing, we used mEos2-NFM photoconversion, or we combined PAGFP-NFM photoactivation with NFL-mCherry photobleaching, to create distinct populations of red and green fluorescent neurofilaments within the cell bodies or axons. Over time, the red and green fluorescent filaments intermixed due to their rapid intermittent movement by axonal transport. After several hours, we observed the appearance of chimeric filaments consisting of alternating red and green segments, which is indicative of end-to-end annealing of red and green filaments. Time-lapse imaging of the axonal transport and Brownian motion of these chimeric filaments

demonstrated that the red and green segments were part of the same filament. Thus neurofilaments can lengthen by end-to-end annealing of preexisting filaments in cultured neurons. In addition to this evidence for end-to-end annealing, we also observed gradual shortening of the photoconverted or photoactivated filaments, suggesting that there are also mechanisms in nerve cells that can break or sever neurofilaments. In two rare instances, we were able to capture an annealing and a severing event "on camera" in time-lapse movies. We propose that the length of neurofilaments in nerve cells is regulated by a dynamic cycle of annealing and severing, and we speculate that this may also influence their transport within axons.

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Dynamic microtubules protect dendrites from degeneration triggered by poly-Q proteins or reduced levels of an axon transport motor.

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Dendrites and axons differ not only in their morphology and function, but also in their responses to injury. Using Drosophila dendritic arborization neurons as a model system, we previously showed that microtubule dynamics are up-regulated in response to axon, but not dendrite, severing (Stone et al. MBC, 2010).

We now show that similar increases in the number of growing microtubules are triggered by reducing levels of unc-104/imac, a kinesin-3 motor protein involved in synaptic vesicle trafficking in axons. This observation led us to hypothesize that increased microtubule dynamics are a general response to axon stress. In support of this hypothesis, expression of expanded poly-Q proteins that cause neurodegeneration, including SCA3tr-Q78 (Q78), increased the number of growing microtubules. Thus in addition to axotomy, reduced levels of a motor protein or overexpression of disease proteins can increase microtubule dynamics.

In a candidate screen to identify proteins required for the increased dynamics, we found that reducing the level of gamma-tub23C, the core nucleation protein in drosophila, suppressed dynamics induced by poly-Q proteins. Reducing levels of microtubule severing proteins did not have a strong effect. We conclude that microtubule nucleation is required to generate large numbers of growing microtubules in stressed neurons.

As the microtubule dynamics is increased throughout the cell, including in dendrites, we hypothesized that persistent microtubule growth in dendrites might help this compartment resist degeneration while the cell attempts axon repair. We first tested whether increased microtubule dynamics can delay short-term degeneration induced by injury. At 18h post dendrite severing, only 5% of the control neurons failed to remove the dendrites, whereas 30% and 60% remained in the presence of unc-104 RNAi and Q78, respectively. This supports the hypothesis that microtubule dynamics might be part of a protective response.

To test whether microtubule dynamics protects dendrites from longer term degeneration, Q78 was co-expressed with gamma-tub23C RNAi or a control RNAi, and dendrite morphology was monitored for four days. More dendrite degeneration was observed in neurons with reduced nucleation than in the control, supporting the idea that increased microtubule growth is a protective mechanism to combat dendrite degeneration.

In summary, elevation of microtubule dynamics represents a novel neuroprotective pathway that is intrinsically activated by a variety of axon stresses. Enhancing this protective pathway could be a strategy to prevent dendrite loss in neurodegenerative diseases.

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The microtubule severing protein spastin is required for regeneration after axon injury.

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Most neurons are irreplaceable, yet they are susceptible to many types of injury ranging from trauma and disease to stroke and immune attack. Neurons typically have many dendrites and a single axon. This axon often extends long distances from the cell body and is especially vulnerable to injury. Axon injury induces a number of cytoskeletal changes thought to be involved in the degeneration of the distal region and subsequent regeneration. Using live imaging of whole Drosophila larvae and cell type-specific RNAi, we found that *spastin* is required for regeneration following axon injury. Mutations in the gene that encodes *spastin*, a microtubule severing protein containing a transmembrane domain, have been shown to be a primary cause of hereditary spastic paraplegia in humans. In many cases, loss of a single copy of *spastin* will result in this degenerative disease. To determine whether axon regeneration is sensitive to *spastin* gene dosage, we assayed axon regeneration in animals heterozygous for a *spastin* null allele. Surprisingly we found that axon regeneration, following both distal and proximal axotomy, is extremely sensitive to *spastin* dosage. In control neurons, axons extended an average of 250 microns from dendrites after proximal axotomy. This number was reduced to 50 microns in *spastin* heterozygotes. After distal axotomy, 100% of control neurons initiated axon regrowth from the stump, but only 50% regrew in *spastin* heterozygotes. To test whether this represented a general requirement for *spastin* in neurite growth, we assayed developmental outgrowth of photoreceptor and mushroom body axons and dendrite regrowth after pruning. Both of these types of neurite growth were normal in *spastin* heterozygotes. We conclude that *spastin* plays a crucial role in axon regeneration and that this function of *spastin* is sensitive to *spastin* dosage, while other types of neurite growth are not.

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Myelin-derived inhibitors suppresses axon regeneration by PIR-B/SHP-mediated inhibition of Trk activation.

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Paired immunoglobulin-like receptor B (PIR-B) mediates certain regeneration-inhibiting effects of the myelin-derived proteins-Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp). Here, we report that inhibition of the PIR-B enhanced axon regeneration in the central nervous system (CNS). Binding of MAG to PIR-B led to the association of PIR-B with tropomyosin-receptor kinase (Trk) neurotrophin receptors, which were known to promote neurite growth in neurons. Src homology 2-containing protein tyrosine phosphatase (SHP)-1 and SHP-2, which were recruited to PIR-B upon MAG binding, functioned as Trk tyrosine phosphatases. Further, SHP-1 and SHP-2 inhibition reduced MAG-induced dephosphorylation of Trk receptors, and abolished the inhibitory effect of MAG on neurite growth. Thus, PIR-B associated with Trk to downregulate basal and neurotrophin-regulated Trk activity through SHP-1/2 in neurons. Moreover, in vivo transfection of siRNA for SHP-1 or SHP-2 induced axonal regeneration after optic nerve injury in mice. Our results thus provide a new molecular target to enhance the regeneration of the injured CNS.

1517

A specialized tubulin in the nervous system of *C. elegans.*

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Microtubules are self-assembling tube-shaped polymers composed of heterodimers of the GTP-binding alpha- and beta-tubulin. Microtubules play a fundamental role in multiple cellular structures and processes including cytoplasmic organization, intracellular transport and the mitotic spindle. Multicellular organisms have multiple genes that encode for highly conserved alpha- and beta-tubulins, which only significantly diverge at their C-termini. This raises the question of whether paralogues are redundant or specialized. In many organisms, tubulins are often redundant in the mitotic spindle. In other contexts, mutations in certain tubulins cause specific developmental and neurological conditions. *C. elegans* has nine alpha- and six beta-tubulins. TBA-9 was originally identified as a ciliary/axonemal alpha-tubulin. While it is expressed in multiple ciliated sensory neurons, it is also expressed in certain motor neurons and neuronal support cells that are non-ciliated. Mutations in *tba-9* cause a number of behavioral abnormalities in nematodes including altered locomotion, increased roaming behavior and reduced slowing upon encountering food. The sum of these mutant phenotypes suggests defects in dopamine signaling. A beta-tubulin, BEN-1, was originally identified in a screen for mutations that provide resistance to the microtubule depolymerizing drug benomyl, which causes paralysis in *C. elegans*. It is coexpressed with TBA-9 in a set of motor neurons that also express dopamine receptors. To determine where TBA-9 functions in dopamine signaling and how it might interact with BEN-1 and play a role in locomotion, we have undertaken multiple approaches. First, we have analyzed the structure of motor neurons in single and double mutants for these tubulins. Second, we used behavioral assays to assess the point of disruption of dopamine signaling in *tba-9* mutants. Single mutants show no gross abnormalities in the structure of the nervous system, and the defect in dopamine signaling appears to be during synthesis, transport or secretion as opposed to reception. These results suggest that TBA-9, a highly specialized tubulin in *C. elegans*, plays a unique role in the nervous system.

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Cellular damage in brain of rat with endotoxic shock induced by LPS.

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Bidirectional communication between the immune system and brain allow a regulation in response to infections. Endotoxic shock is caused by lipopolysaccharides (LPS) of gram-negative bacteria, followed by overproduction of diverse molecules that cause damage on diverse tissues; such molecules modify neuroimmune functions. Enolase and GFAP are cellular damage markers in brain. We studied brain cell damage in rats with endotoxic shock induced by LPS (*E. coli* 0111:B4). Windstar adult males were used, five animals/group received intraperitoneal injections as follows: (i) Control, 500 µl of normal saline. The remaining groups were inoculated with 12 mg/Kg of LPS dissolved in 500 µl of distiller water. Brains were obtained at: (ii) Group, 60 min after LPS. (iii) Group 120 min after LPS. (iv) Group 240 min after LPS. The brain was cut at 4µm. In each cut antibody anti-GFAP, anti-enolase and anti-TNFa were added for 30', washed down with PBS and added anti-IgG conjugate fluorescein for 30', washed again and observed under a microscope. Our results show that **Enolasa** in (ii), there are fluorescent deposits in the periphery of the cell, increased in (iii), in (iv) decreased slightly. **GFAP** in (ii) dim deposits on the periphery of the cell and dotted deposits in the center of the cell

are seen; in (iii) deposits and number of positive cells increased; in (iv) more dispersed continued increase all over the tissue. **TNF-α**, show faint deposits in (ii). In (iii) more intense deposits, as well as more positive cells, decreased in (iv). In conclusion, the presence of TNFα was the factor that triggered the destruction of neuronal and astrocyte cells, which increased in relation to time elapsed after inoculation.

Mechanotransduction II

1519

The pulsating migration of single breast cancer cells mediated by the collective migration of normal breast epithelial cells.

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Whether normal epithelia cells actively participate in the early steps of cancer metastasis in epithelial sheets is unclear. A co-culture system of scattered, individual human breast cancer cells surrounded by dense, non-transformed human breast epithelial cells reveals that the non-transformed cells collectively mediate a dramatically enhanced, “pulsating” migratory phenotype of the individual carcinoma cells. Quantitative analysis correlates this enhanced migration with the intermittent crowding of individual cancer cells by the stiff nuclei of normal cells, which induces a cyclic stretch-shrink of the soft nucleus of the cancer cell. Enhanced pulsating migration is absent in single and confluent metastatic cancer cells and in non-metastatic cancer cells surrounded by normal cells. The pulsating migration of individual cancer cells is mediated by the motility of the normal cells and the differential strengths of homotypic and heterotypic cadherin-based cell-cell adhesions: Disruption of cortical actin filament bundles, or depletion of α-catenin in normal cells or the re-expression of E-cadherin in cancer cells all halted the pulsating migration of cancer cells. These results reveal a previously unknown, but critical, role played by normal epithelial cells in the enhanced migration of metastatic carcinoma cells, which might provide new avenues for cancer therapies where, in addition to cancer and stromal cells themselves, normal epithelial cells in the vicinity of a carcinoma tumor are targeted to reduce metastasis.

1520

Constraining Cell Motility Increases Sensitivity to Nocodazole via the Inhibition of Glu-Tubulin Formation.

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The production of traction forces – forces generated by the actin-myosin II cytoskeleton and exerted on the substrate – is vital to the proper regulation of many functions of adherent cells including adhesion and differentiation. Traction forces increase upon the depolymerization of microtubules, indicating intricate crosstalks between the actin and microtubule cytoskeletons. In addition, this interaction may be affected by the post-translational de-tyrosination of tubulin, which forms what is termed Glu-tubulin and imparts stability to modified microtubules. Based on the previous observations that Glu-tubulin forms preferentially at the leading edge of wounded monolayers, where cells migrate actively, we hypothesized that migration activities cause cells to form an increasing amount of Glu-tubulin, which may then weaken traction forces and adhesions, causing a positive feedback by decreasing substrate anchorage. To test this hypothesis, we applied time-lapse traction force microscopy to NIH 3T3 fibroblasts and NMuMG

mouse breast epithelial cells on micropatterned polyacrylamide hydrogels before and after the addition of 10 µM nocodazole. We found that 3T3 cells confined to 50x50 µm squares generated significantly higher traction stress and showed stronger responses to nocodazole than cells that were allowed to spread freely. Similar results were obtained using a teardrop-shaped pattern designed to mimic the shape of migrating cells. Immunofluorescence staining showed little or no Glu-tubulin in immobilized cells, but prominent presence of Glu-tubulin in motile 3T3 cells. The difference in nocodazole sensitivity between motile and non-motile cells was eliminated by limited serum starvation, which is known to inhibit the formation of Glu-tubulin while allowing cell migration. As Glu-tubulin has been found to accumulate in invasive cancer cells, we hypothesized that constraining the motility of post-EMT cells may reduce this response. Using NMuMG cells that were engineered to be in a pre- or post-EMT state, we found that constraining the motility of post-EMT cells significantly reduced the amount of Glu-tubulin, while pre-EMT cells showed no accumulation of Glu-tubulin in unconstrained or constrained cells. Thus, contrary to the common notion that genetic aberrations cause cancer phenotypes, we suggest that the stimulation of cell migration by a damaged basal membrane or other factors may be responsible for some of the invasive phenotypes, due to the ability of mesenchymal cells to sense their migration state and regulate their cytoskeletal structures and mechanical output to further enhance the motility.

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Megakaryoblastic leukemia-1 influences proliferation and cell migration by transcriptional regulation of tenascin-C and other cancer-relevant genes.

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The extracellular matrix protein tenascin-C (TNC) is highly over-expressed in cancer stroma and favors cell migration and invasion. Furthermore, TNC is up-regulated in processes influenced by mechanical stress, such as inflammation, tissue remodeling, wound healing, and tumorigenesis (1). Cyclic strain-induced TNC expression depends on RhoA-actin signaling, the pathway that regulates transcriptional activity of serum response factor (SRF) by its coactivator megakaryoblastic leukemia-1 (MKL1). Therefore, we tested whether MKL1 controls TNC transcription. We found that overexpression of MKL1 strongly induces TNC expression in mouse NIH3T3 fibroblasts and normal HC11 and transformed 4T1 mammary epithelial cells. An MKL1 mutant incapable of binding to SRF still strongly induced TNC, while induction of the SRF target c-fos was abolished. Cyclic strain failed to induce TNC in MKL1-deficient but not in SRF-deficient fibroblasts, and strain-induced TNC expression strongly depended on the SAP domain of MKL1. Promoter-reporter and chromatin immunoprecipitation experiments unraveled a SAP-dependent, SRF-independent interaction of MKL1 with the proximal promoter region of TNC, attributing for the first time a functional role to the SAP domain of MKL1 in regulating gene expression (2). Recent data from the literature have indicated that the stiffness or rigidity of the cancer stroma is a crucial cause for the invasive behavior of cancer cells and e.g. tissue stiffness is a bad prognostic factor in human breast cancer. We postulate that mechanical stimulation of cells induces similar cellular responses as the contact of cells with rigid matrices does. In a stiff microenvironment, the cytoskeleton will contract, actin polymerizes and G-actin levels fall. This will induce signaling by the actin sensor MKL1 and result in the expression of tenascin-C as well as other proteins co-regulated with tenascin-C that we were able to identify in our screen based on the use of cells overexpressing different MKL1 mutants. We could identify groups of transcripts that were specifically induced in cells that exhibited increased proliferation and migration. The expression of these genes was dependent on the presence of the SAP domain of MKL1 pointing to a crucial role for the SAP-domain in the control of proliferation and cell migration.

(1) Chiquet-Ehrismann and Tucker. 2011. Cold Spring Harb Perspect Biol 2011;3:a004960 (2) Asparuhova et al. 2011. FASEB J. Jun 24. [Epub ahead of print]

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Substrate stiffness regulates NF-kB expression and activity in cancer cells.

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Tumor tissues are stiffer than normal ones. The stiffer the extracellular environment is, the more invasiveness of cancer cells is induced. Cells can sense stiffness of the surrounding substrate; it is called mechanosensing. However, it is not clear if the cancer malignancy is induced by the mechanosensing process.
In this study, we focused on transcription factor NF-kB, which regulates the expression of many kinds of molecules in the immune system in response to multiple stresses and also induces proliferation and invasion of cancer cells. We make a hypothesis that tissue stiffness enhances NF-kB expression and/or activity in cancer cells, causing malignant transformation.
We cultured A549 lung carcinoma cells and observed the morphology of the cells cultured on stiff or soft substrates. For a stiff substrate, we used a glass or plastic plate coated with type-I collagen. And for a soft substrate, we used a type-I collagen gel (1.6 mg/mL). On the stiff substrate, A549 cells adhered with spreading morphology and showed high migrating activities. On the other hand, on the soft substrate, most of A549 cells adhered with round morphology and showed low migrating activities. Western blotting experiments revealed that expression of NF-kB (p65) on the stiff substrate was greater than that on the soft substrate. Furthermore, A549 cells showed a localization of NF-kB at nucleus sites within 60 minutes after adhering on the stiff substrate. By contrast, on the soft substrate, A549 cells did not show a localization of NF-kB at nucleus sites after adhesion.
These results indicate that cancer cells cultured on a stiff substrate enhance NF-kB expression and activation, which promote inflammatory reaction. Stiffer tumor tissues possibly induce cancer malignancy through the enhancement of NF-kB expression and activity.

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Forcing Tumor Cell Invasion: The Role of Extracellular Matrix Mechanics and Topology.

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Tumor progression is traditionally linked to the sequential accumulation of genetic mutations. However, we have shown that tumor development is also strongly influenced by physical cues such as extracellular matrix (ECM) organization and stiffness. To understand how ECM mechanics and architecture regulate tumor progression, we conducted a comprehensive analysis of the interplay between ECM tension and topology and tumor development. Second-generation harmonics (SHG) and structured-illumination polarization microscopy (SIM-POL) imaging revealed that ECM stiffening in developing mammary cancers is functionally linked to collagen linearization and cross-linking that generate heterogeneous oriented matrix bundles associated that globally influence tissue architecture. Moreover, AFM mapping of PyMT mammary tumors and human invasive triple-negative breast cancers revealed that the invasive front of these lesions frequently contained tracts of highly rigid oriented ECM. We hypothesize

that this aligned ECM architecture creates a mechanically anisotropic environment, which fosters directed tumor cell invasion. To explore the molecular mechanisms through which anisotropic ECM stiffness regulates tumor cell invasion, we performed experiments using mechanically heterogenous 2D and 3D gel systems. In 2D we demonstrated that ECM tension gradients significantly promote the directional migration, or durotaxis of oncogenically (EGFR, ErbB2, Ha-Ras, V12 Ras) transformed breast cells. To address this issue in 3D we engineered a mechanically anisotropic 3D organotypic gel system. SHG and SIM-POL imaging revealed that our mechanically and topologically heterogeneous (MTH) gels recapitulate the architectural heterogeneity of the ECM at the invasive front of breast tumors *in vivo.* Utilizing our MTH gel system and real time two-photon imaging we could show that the structure and organization of nonmalignant and oncogenically-transformed mammary organoids adjacent to mechanically loaded collagen fibers were consistently disrupted and that cells within the organoids invaded into the ECM along collagen fibers in response to epidermal growth factor (EGF) stimulation. Consistent with results from our 2D assays, we found that in 3D, persistent invasion was favored following oncogene transformation. We also noted that mammary epithelial cells (MECs) in a 3D context demonstrated heightened EGFR-induced Rac activity suggesting that a 3D ECM may potentiate invasion by modifying the growth factor and mechanosignaling behavior of MECs. Studies are now underway using our MTH gel system in combination with transgenic mouse models to explore how dimensionality and ECM architecture in combination with oncogenic transformation could modify MEC signaling to promote invasion. Work supported by: PSOC U54CA143836-01, R01 CA138818-01A1, DOD W81XWH-05-1-0330 to VMW.

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Focal adhesion kinase stabilizes the cytoskeleton.

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Focal adhesion kinase (FAK) is a central focal adhesion protein that promotes focal adhesion turnover, but the role of FAK for cell mechanical stability is unknown. We measured the mechanical properties of wildtype (FAKwt), FAK-deficient (FAK-/-), FAK-silenced (siFAK), and siControl mouse embryonic fibroblasts by magnetic tweezer, atomic force microscopy, traction microscopy and nanoscale particle tracking microrheology. FAK-deficient cells showed lower cell stiffness, reduced adhesion strength, and increased cytoskeletal dynamics. These observations imply a reduced stability of the cytoskeleton in FAK-deficient cells. We attribute the reduced cytoskeletal stability to a compensatory overexpression of *rho*-kinase (ROCK) in FAK-deficient cells that suppresses the formation of ordered stress fiber bundles, enhances cortical actin distribution, and reduces cell spreading. In agreement with this interpretation is that cell stiffness and cytoskeletal stability in FAK-/- cells are partially restored to wildtype level after ROCK inhibition with Y27632.

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Vinculin is required for cell polarization, migration and extracellular matrix remodeling in three-dimensional collagen matrices.

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Vinculin is an F-actin binding protein that localizes to integrin-based cell-extracellular matrix (ECM) adhesions. Studies in two-dimensional (2D) tissue culture models have suggested that vinculin is a negative regulator of cell migration via its role in cytoskeleton-ECM coupling to strengthen and stabilize adhesions, as well as in modulating leading edge protrusion dynamics. However, whether vinculin plays a similar role in regulating cell migration in more physiologically relevant 3D environments is not known. To address this question, we analyzed morphodynamics and ECM remodeling of primary murine embryonic fibroblasts (MEF) with cre/loxP-mediated vinculin gene disruption (Vcl-KO) in 3D collagen/fibronectin cultures. By spinning disk confocal and Bessel beam microscopy of plasma membrane or F-actin markers (tdTomato-Farnesyl or tdTomato-Ftractin), we found that control MEF exhibited an elongated morphology with extended, polarized, sheet-like lamellipodial protrusions, while Vcl-KO MEF were less extended and polarized, with lamellipodia being dominated by finger-like protrusions. These protrusions exhibited mEmerald-VASP at their extending tips, confirming them as filopodia. Analysis of protrusion dynamics revealed increased velocity of cell edge protrusion in Vcl-KO compared to control MEF. Thus, vinculin is required for cell polarization, regulating lamellipodial protrusion and suppressing filopodia in 3D ECM, similar to functions described in 2D culture. Surprisingly, long term time-lapse imaging showed that vinculin depletion decreased 3D cell migration velocity of control MEF, opposite to observations in 2D culture. However, Vcl-KO MEF showed an increased cell shape persistence compared to control MEF, demonstrating that vinculin may be important for effectively converting edge protrusion into cell shape change, and thus forward motion in 3D collagen. This suggested that vinculin may be required for effective interaction with 3D ECM. To test this further, we examined the ability of cells to remodel ECM. In a macroscopic gel contraction assay, Vcl-KO MEF exhibited decreased contraction of 3D collagen gels compared to control MEF. Consistent with this, live-cell polarization microscopy of collagen gels containing MEF revealed that while control cells induced a highly homogeneous, highly aligned collagen matrix, gels containing Vcl-KO cells displayed a more heterogeneous fiber orientation and a lower degree of fiber alignment, indicating defects in ECM organization. Our results suggest that vinculin promotes cell migration and matrix remodeling by effectively coupling cell morphodynamics to the ECM in complex 3D extracellular environments.

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Spatial distribution of protrusion, contraction and adhesion determines cell migration strategy in 3D matrices: a computational study.

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Cell migration on a two-dimensional flat surface has been extensively studied and is generally characterized by a front-protrusion-rear-contraction process. In a 3D environment, on the other hand, cells adopt multiple, not well understood, migration strategies depending on the cell type and the properties of the extracellular matrix (ECM). By using mathematical modeling and

computer simulations, we find that these migration strategies in 3D can be classified by various spatial arrangements of actin-based protrusion, contraction and actin-ECM adhesion. We show that when the ECM undergoes proteolysis, a cell can move by using a broad protrusion at the front followed by transient actin-ECM adhesions at the side and a strongly contracting actin-myosin network at the rear. The cell can also be pulled along thin protrusive extensions if there are transient adhesions near the tip and a contractive actin network along the extension. In the absence of proteolysis, the cell switches to an amoebae-like motion to squeeze through pores in the ECM. We propose that these migration strategies are triggered by mechanical sensing of ECM through cell signaling pathways.

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The actomyosin linkage and integrin-ligand interaction regulate adhesion dynamics and cell migration.

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The adhesive and migratory behavior of a cell depends on its type, its adhesion receptor repertoire and the substrate to which they are attached. Previous observations show the different MII isoforms and the level of phosphorylated RLC (pRLC) regulate adhesion maturation and turnover, protrusion rates, and polarity for cells migrating on fibronectin or vitronectin. We have asked whether adhesion and migration are similarly regulated in cells using other two other integrin receptors, α6β1 and αLβ2. We find that CHO.B2 cells expressing either α6β1 or αLβ2 integrins migrate and protrude faster on laminin or ICAM-1, respectively, than CHO.B2 cells expressing α5β1 migrate on fibronectin. However, changing the levels of MIIA, MIIB or pRLC do not have the large effects on migration, adhesion, and protrusion that are seen in α5β1-expressing cells on fibronectin. Despite the presence of large adhesions, the force on the substrate was also reduced in regions of protrusions in the α6- vs α5-expressing cells, and they showed robust tyrosine phosphorylation. Surprisingly, we observed a rapid retrograde flux of paxillin and integrin in the elongated adhesions in the protrusions of α6β1-expressing cells on laminin or αLβ2-expressing cells on ICAM-1, but not with α5β1-expressing cells on fibronectin. However, activation of α6β1, using Mn^{2+} inhibits this flux, as well as the cell migration and protrusion rates. Finally, we show that the migratory behavior of fast migrating leukocytes on Fn or ICAM-1 is largely independent of myosin II. Our data suggest the integrin-ligand interaction differs among integrin-ligand pairs and affects traction forces, signaling, adhesion, protrusion and cell migration.

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Septins are required for efficient epithelial cell-ECM adhesion and protrusion.

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Septins comprise an evolutionarily conserved family of guanine nucleotide-binding proteins that assemble into oligomeric filaments. Abnormal septin expression has been implicated in several disease states including cancer. Overexpression of septins has been shown to promote cell migration and invasion. In previous studies, pharmacological targeting and knock down of septins have resulted in attenuation of cell motility. Septins associate with the actomyosin and microtubule networks and effectors of Rho signaling, but the precise role of septins in cell motility is unknown. Since motility relies upon a dynamic interplay between membrane protrusion and adhesion to the extracellular matrix, we explored a role for septins relative to components of both processes. Here, we show that in epithelial cells (MDCK, HeLa), septin 2 (SEPT2) colocalizes with actomyosin stress fibers and microtubules, but also forms peripheral filaments and rings that localize at lamella, near sites of enlarging focal adhesions. Using an

alternative fixation technique, which extracts cytoplasmic proteins before paraformaldehyde fixation, we show that peripheral septins are associated with the cortical membrane. Knockdown of SEPT2 in HeLa cells resulted in smaller, more peripherally localized focal adhesions compared to control cells. Since focal adhesion enlargement is a force-driven process dependent upon myosin II-generated contraction, we examined myosin activity by staining for the phosphorylation of its regulatory light chain. More diffuse and reduced levels of phosphorylated myosin were observed in SEPT2-depleted cells compared to the control, suggesting that septins play a role in the mechanotransductive properties that regulate growth of focal adhesions. Additionally, preliminary data show that SEPT2 depletion decreases the width of cortactin-enriched lamellipodia, suggesting a role for septins in lamellipodial generation and/or maintenance. Our results indicate that septins are essential for the mechanochemical and protrusive activities that underlie cell motility. On-going studies aim at defining the molecular relationship between septins and actin in the leading lamellae and at the cortical membrane.

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Conformational Switch of Vinculin: a Prerequisite for Mechanical Force Transmission.

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Mechanical coupling of the actin cytoskeleton to the extracellular matrix via the integrin-focal adhesion (FA) link is crucial for proper cellular function. Within the focal adhesion complex, the mechano-coupling protein vinculin plays a fundamental role in transmitting cellular forces. Vinculin consists of a head and a tail domain and displays a bi-stable behavior, operating either in a closed auto-inhibited, or an open active state. In the open conformation, the head and tail dissociate, exposing numerous binding sites for different proteins. We developed a constitutively open vinculin mutant by inserting 3 point-mutations in the head domain (E28, 29, 31 → R). This mutant was compared to a constitutively closed vinculin (A50 → I). FRAP experiments revealed a decreased recovery time of constitutively open vinculin, suggesting that vinculin in the active (open) conformation inserts more stably into the focal adhesion complex. We next tested whether cell mechanical properties are also affected by vinculin head-tail interactions. Vinculin in the closed state displayed lower stiffness and reduced tractions compared to vinculin in the open state. Interestingly, cell stiffness and traction forces were even further reduced in a VinΔex20 mutant where vinculin was in the open (active) form but devoid of actin binding sites. Together, these data suggest that a conformational switch of vinculin strongly affects cellular mechanics through a concurrent modulation of force transmission to actin and to focal adhesion proteins.

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Mechanical Stimulation Induces an Actin Cytoskeletal Response and Recruitment of Zyxin, Mena/VASP and α-Actinin.

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Mechanotransduction occurs in organs that experience regular mechanical stress, such as lung, bladder, uterus and the vasculature. Stretch stimulation of cells in culture induces dramatic alignment and reinforcement of the actin cytoskeleton, visualized by phalloidin staining and fluorescence microscopy. To investigate mechanotransduction at the cellular and molecular level, we applied uniaxial cyclic stretch to fibroblasts expressing wild-type and mutant proteins then evaluated the stretch response. Indirect immunofluorescence microscopy studies showed that zyxin, α-actinin, and Mena/VASP proteins mobilized from focal adhesions to stretch-

stimulated actin stress fibers while canonical focal adhesion markers such as vinculin and tyrosine-phosphorylated proteins remained at focal adhesions. The actin cytoskeletal response was abrogated in zyxin-null cells, and was rescued by adding back wild-type zyxin, implicating zyxin in mechanotransduction. Furthermore, zyxin proteins mutated to disrupt binding partner interactions (e.g. for α-actinin and Mena/VASP) retained their mechanosensitive localization to stretch-induced stress fibers but the resulting actin failed to thicken normally. These results revealed that zyxin plays a key role in recruiting actin regulatory proteins to sites of dynamic actin remodeling. Interestingly, immunoblot analysis of a survey of mouse tissues detected the highest zyxin expression in lung. Subsequent immunohistochemistry detected zyxin in lung alveoli, sites of greatest inspiration induced tissue expansion. To test if zyxin activity is required for maintaining barrier function integrity, we compared lungs from wild-type and zyxin-null mice. Measurement of lung/body ratios and bronchoalveolar lavage for protein leak did not detect a zyxin-dependent difference. We conclude that in unstressed conditions, zyxin is dispensable for normal lung physiology. In future work it will be interesting to assess the impact that zyxin, α-actinin, and Mena/VASP have on actin dynamics that occur in lungs challenged by mechanical stress or pharmaceutical agents.

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Oscillatory Coupling between the Extracellular Matrix and the Actin Cytoskeleton is Essential for Cell Mechanosensing and Durotaxis.

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The ability of cells to sense mechanical properties of the extracellular matrix (ECM) and to exhibit durotaxis (directed migration toward stiffer environments) is thought to underlie many biological processes including angiogenesis, neurogenesis and cancer metastasis. Although it has been shown that both structural and signaling components of focal adhesions (FAs) are crucial to translate mechanical cues into cell behavior, the molecular mechanism of mechanosensing remains unknown. Here we demonstrate that the ‘molecular clutch’, an ensemble of links between the actin cytoskeleton and ECM-engaged integrins, is mechanoresponsive. We identify a functional module, composed of FAK, paxillin and vinculin, that is essential to enable mechanoresponsive behavior of the FA molecular clutch and is required for durotaxis over a wide range of ECM compliances.

By high-resolution traction force microscopy on polyacrylamide hydrogels of varying stiffness, we visualized traction distribution along individual FAs in migrating mouse embryonic fibroblasts. We revealed two distinct patterns of traction dynamics within individual FAs: static and oscillating over time. FAs with static traction exerted low traction stress, which did not change over time. FAs with oscillating traction were identified as exerting higher traction stress on average, but exhibited periodic centripetal movement of the peak position of traction within an individual FA from the distal tip of the FA towards the FA center, which was accompanied by a transient increase of traction magnitude. As ECM rigidity was increased, the traction peak movement was suppressed, signifying mechanoresponsiveness of traction oscillations. We found that perturbing paxillin phosphorylation by expressing Y31/118E- or Y31/118F-paxillin mutants, inhibiting FAK, or expressing a paxillin point mutant defective in vinculin binding declined traction stress in FAs, abrogated traction oscillations on soft ECM and switched adhesions to the non-mechanoresponsive static regime of traction dynamics. However, mechanoresponsive traction oscillations could be rescued by further decreasing either ECM stiffness or myosin II contractility, suggesting a shift in the ECM stiffness range in which FAs are mechanoresponsive. This finding allowed us to suppress either oscillating or static regimes of traction dynamics and to reveal the physiological importance of traction oscillations. We demonstrated that traction oscillations are essential for stiffness-dependent FA maturation and

durotaxis. Random cell migration and chemotaxis, assessed by a Boyden chamber assay, occurred independently of the dynamic state of FA traction. Our results suggest that not a certain protein within a FA, but the entire 'molecular clutch' may act as a mechanosensor in FAs, and that a FAK-Paxillin-vinculin module regulates the dynamic range of cell mechanosensing to guide durotaxis.

1532

Cellular Traction Forces are Regulated by the Cell Spread Area not Substrate Stiffness.

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Extracellular Matrix stiffness has been shown to impact numerous aspects of cell physiology including cell migration, proliferation and differentiation. Such cellular mechanosensation is thought to be driven by stiffness-dependent perturbations to actomyosin contractility and subsequent mechanotransduction pathways. However ECM stiffness simultaneously drives changes in cell spread area, lamellar actin organization and the magnitude of traction forces on the ECM, making it difficult to identify causal relationships. Here we focus on the traction forces exerted by the cell and explicitly investigate how changes in these forces vary with changes in cell spread area and lamellar actin organization on ECM of varied stiffness. To measure the actin organization we developed a novel quantitative Fourier image analysis technique. This technique measures the local direction and magnitude of alignment within the cytoskeleton and calculates an order parameter based on comparison with adjacent regions. Surprisingly, we found no correlation between the organization of the lamellar actin into bundles with the magnitude of cellular traction forces exerted on the ECM. Instead, we find that the total cellular force is directly proportional to the cell's spread area, with the slope defining a cellular contractile stress. This relationship is robustly observed among several different cell types, with each cell type exhibiting a unique level of contractile stress. Additionally, this contractile stress can be varied by titration of the pharmacological inhibitor of myosin II ATPase activity, blebbistatin. Quite unexpectedly, however, we find that the contractile stress is independent of substrate stiffness, despite large perturbations to the cell spread area. Cells that spread on soft substrates are able to generate equivalent amounts of force to similarly spread cells on stiff substrates. These results indicate that cellular contractile stress is not directly regulated by substrate stiffness, but rather indirectly impacted through the cell's spread area. Thus, these data suggest stiffness sensing occurs during the early spreading phase and is not a product of whole cell contractility.

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Spaceflight alters the migratory ability of stem cell derived keratinocytes resulting in decreased wound healing potential.

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Spaceflight can cause degenerative conditions in multiple tissues including incomplete or delayed wound closure. We hypothesize that somatic stem cells responsible for tissue regeneration require mechanical stimulation from gravity to regenerate tissues at normal rates and that spaceflight conditions, specifically microgravity, may interfere with their proliferation and differentiation. Previous experiments conducted in microgravity on shuttle mission STS-131 using stem cell derived embryoid bodies, resulted in many genes involved in stem cell pluripotency failing to turn off, and in the normal up-regulation of germ-layer markers being inhibited. Additionally, results from hypergravity experiments show an increase in proliferation of

mouse embryonic stem (mES) cells, suggesting gravity mechanical stimulation may regulate various stages of stem cell-based tissue regeneration. Here we specifically hypothesize that mechanical unloading in microgravity may alter the ability of mES cells to differentiate into keratinocytes. To test this hypothesis we induced keratinocyte differentiation from mES cells in microgravity through the addition of BMP4, retinoic acid and calcium to culture medium for 48 hrs prior to spaceflight. Cells were transferred to keratinocyte low serum medium and flown on the space shuttle STS-135 mission in the Cell Culture Module hardware using hollow fiber bioreactors. After 13 days orbital flight, cells were either fixed in-orbit for gene expression analysis, or returned to Earth for live wound closure assays. Keratinocytes isolated from bioreactors were allowed to attach to a collagen matrix before exposure to a migration exclusion zone. After a 48 hr period, 5.2% of cells exposed to microgravity had migrated into the exclusion zone whilst 9.3% of cells differentiated at 1g had migrated ($p=0.048$) indicating that spaceflight strongly inhibits their migratory ability. However, spaceflight did not affect the ability of keratinocytes to adhere to, and spread on a collagen matrix, with 37.3% of cells from ground controls and 34.7% of cells differentiated in microgravity attached and spread to a diameter >100 µm. These initial results suggest that exposure of keratinocyte progenitors to microgravity may alter their differentiation and reduce their ability to regenerate epidermal tissue during wound closure. (Supported by NASA NRA-NNH08ZTT003N)

Signaling Networks Governing Cell Migration II

1534

Live *in vivo* imaging of active RhoGTPase and cell behavior during neural crest EMT reveals a role for Rho in promoting EMT.

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Objective: Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells become motile. EMTs are critical events in normal development and are co-opted during pathological processes such as carcinoma invasion. To understand mechanisms controlling EMT, it is critical to establish *in vivo* models to study cells undergoing EMT in their natural environment. RhoGTPase has been implicated in EMT, although its precise function is controversial. We are investigating mechanisms of EMT using zebrafish neural crest cell (NCC) EMT as an *in vivo* model. We imaged the distribution of endogenous active Rho during cell behaviors associated with NCC EMT and tested how disruption of Rho signaling affects these behaviors.

Methods: We imaged endogenous active Rho distribution with a biosensor containing the Rho binding domain of Rhotekin fused to GFP driven by a NCC specific promoter. We expressed the biosensor in individual NCCs along with a volume marker, and performed ratiometric imaging to identify subcellular localizations of endogenous active Rho during EMT *in vivo*. We used small molecule inhibitors to disrupt Rho/ROCK signaling and examined the effects on NCC behaviors.

Results: Premigratory NCCs have an elongated morphology similar to other neuroepithelial cells, spanning from the apical midline to the basal surface of the neuroepithelium (NE). During EMT, the apical attachment retracts and NCCs move to the basal edge of the NE and begin blebbing. Active Rho levels spike just before the apical tail releases from neighboring cells and remain elevated during apical tail retraction. As retraction completes and NCCs exit the NE, apical active Rho levels decrease. Pharmacological inhibition of Rho/ROCK signaling results in

an increase in the number of NCCs that remain in contact with the apical midline and do not leave the NE, suggesting that active Rho is required for NCC tail retraction and EMT. We also observe active Rho in retracting NCC blebs, behaviors associated with NCC motility. NCC blebbing is blocked by inhibition of Rho/ROCK or myosin II. These results suggest Rho/ROCK signaling may drive contractile forces needed for motile NCC behaviors and EMT.

Conclusions: We have established zebrafish NCCs as an *in vivo* EMT model, and describe the first imaging of endogenous active Rho distribution during EMT in living embryos. Our data support Rho signaling as a source of contractile force that drives retraction of apical NCC tails and blebs to promote NCC motility and EMT.

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Oncogenic RABGAP EVI5 is a novel regulator of collective cell migration.

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Neuroblastoma (NB) is the most common form of extracranial solid tumors in childhood which develops from failure of neural crest cells to migrate and differentiate properly. Since few predisposition mutations have been identified in NB, very little is known about the molecular mechanism that define the role of those mutated genes in NB development. EVI5, a poorly characterized oncogene, is one of those mutated genes found in NB.

One of the main goals of our laboratory is to investigate the role of vesicular trafficking during cell migration. We study the migration of Drosophila border cells (BC) which migrate collectively during oogenesis. We published recently that Rab11 is essential to restrict guidance receptors at the leading edge of BC. Rab11 is a small GTPase involved in the recycling of cargos such as transmembrane receptors from intracellular endosomes back to the cell membrane. Rab proteins are cycling between an active GTP-bound state and an inactive GDP-bound state through the action of different regulators. Here, we have performed an RNAi screen to identify specific Rab11 regulators and we identified the Drosophila ortholog of EVI5 (dEVI5) as a potential GTPase activating protein (GAP) regulating Rab11 and BC migration.

We demonstrate that dEVI5 fulfill every criteria for a GAP proteins for Rab11. Biochemically, we show that dEVI5 interacts directly with Rab11. Moreover, the overexpression of dEVI5 increases the fraction of inactive Rab11 both in Drosophila S2 cells and in vivo, whereas the knockdown of dEVI5 has opposing effects. Furthermore, overexpressing dEVI5 phenocopies Rab11 loss of function in BC by blocking cell migration and disrupting the polarity of guidance receptors.

Our results suggest that EVI5 induces NB by disrupting the migration of neural crest cells. To test this hypothesis, we overexpressed EVI5 during zebrafish development and found that neural crest cell migration is strongly impaired.

In conclusion, we have identified dEVI5 as a novel regulator of collective cell migration through the regulation of Rab11 and we propose that ectopic activation of EVI5 induces NB by inhibiting neural crest cell migration.

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PI3-Kinase Regulation of Myosin-IIA Heavy Chain Phosphorylation Promotes Tumor Cell Invasion.

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EGF stimulation of MDA-MB-231 breast cancer cells results in the transient phosphorylation of S1943 on the nonmuscle myosin-IIA heavy chain (NMHC-IIA), which is known to promote myosin-IIA filament disassembly *in vitro*. We have extended these observations to show that EGF-stimulated NMHC-IIA S1943 phosphorylation occurs in multiple breast cancer cell lines, as well as in a glioma cell line. Using small molecule inhibitors and shRNA approaches, we are mapping signaling pathways downstream of the EGF receptor that modulate NMHC-IIA S1943 phosphorylation. An examination of the regulation of NMHC-IIA phosphorylation by PI-3 kinases in EGF-stimulated cells demonstrated that p110α-selective inhibitors block NMHC-IIA phosphorylation; whereas p110β-selective inhibitors enhance NMHC-IIA phosphorylation. Furthermore, inhibition of downstream PI3-kinase pathway components also blocks NMHC-IIA phosphorylation. To examine the cellular and functional consequences of S1943 phosphorylation, we are using lentiviral-mediated shRNA gene transfer and stable plasmid transfection to produce stable human breast cancer cells in which the expression of the endogenous NMHC-IIA is reduced and an exogenous GFP-tagged wild-type NMHC-IIA, NMHC-IIA phosphomimetic (S1943D/E) or NMHC-IIA non-phosphorylatable analog (S1943A) is expressed. One functional assay used to study tumor cell invasion is the 3D invasion assay, which investigates macrophage-stimulated tumor cell invasion through a collagen matrix. In breast cancer cells with a 50% reduction in NMHC-IIA expression, macrophage-stimulated 3D invasion is reduced 66.6% as compared to control siRNA transfected cells. A similar decrease in macrophage-stimulated 3D invasion was observed in stable shRNA NMHC-IIA knockdown cells where NMHC-IIA levels were reduced by 50-90%. Together, these observations suggest that NMHC-IIA is required for tumor cell invasion and S1943 phosphorylation is regulated by PI3-kinase. Future studies will be directed at examining the role of NMHC-IIA phosphorylation in modulating tumor cell invasion.

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The role of NEDD9 in oral squamous cell carcinoma invasion and metastasis.

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Using a phosphotyrosine proteomics screen we demonstrated that vascular endothelial growth factor (VEGF) action on human oral squamous cell carcinoma (OSCC) cell lines leads to the tyrosine phosphorylation of NEDD9 (neuronal expressed developmentally down-regulated9; human enhancer of filamentation1, HEF1; Crk-associated substrate in lymphocytes, CasL), cortactin, paxillin and focal adhesion kinase and an invasive phenotype (Oncogene 29, 4449-4459, 2010). NEDD9 is a scaffold protein implicated in the metastatic signatures of glioma, melanoma and lung cancers and regulated by tumor suppressor LKB1. NEDD9 knockdown decreased and overexpression increased cell migration, invasion and matrix metalloproteinase 2/9 (MMP) levels, respectively. To further define how NEDD9 regulates these events and in particular, the role of its tyrosine-rich substrate domain, we generated: F13 NEDD9, in which all CrkL motifs (YXXP) were mutated to F; Y629F, the site where Src binds to NEDD9 in order to phosphorylate the substrate domain; F14 NEDD9, representing the combined mutations in F13 and Y629F NEDD9. In addition, to determine NEDD9's role in HNSCC progression and metastatic spread NEDD9 null vs. wt mice were treated with the chemical carcinogen/tobacco-

mimetic, 4-nitroquinoline 1-oxide (4NQO; 50 µg/ml in the drinking water for 16 weeks). Analysis of conditioned medium from cells stably expressing F13, F14, or Y629F NEDD9 by zymography revealed a dramatic loss of MMP9 expression/secretion, while MMP2 expression/secretion was only slightly decreased. In contrast, cells stably overexpressing NEDD9 exhibited enhanced expression/secretion of both MMP2 and MMP9, consistent with NEDD9 silencing reducing MMP9 levels to a greater extent than MMP2. Complementary results were seen for invadopodia assays with NEDD9 silencing reducing and NEDD9 overexpression increasing invadopodia formation. In animal studies, NEDD9 null and wt mice all exhibited tumors/lesions on their tongues after 16 weeks of 4NQO treatment. The extent of tumor formation, progression and level of metastasis to cervical lymph nodes is currently being quantified. These findings suggest a role for the NEDD9 substrate domain in MMP9 regulation and signaling to HNSCC cell invasion and metastasis.

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Migration during metastasis: the cellular response to tunable cross-linked ECMs.

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The modalities of cell migration and persistence rely on integrin engagement with the extracellular (ECM), focal adhesion formation, protease secretion and numerous intracellular signaling cascades. Previous investigations utilizing 3D ECM environments have provided invaluable insights to cellular migration and mechanosensing; however, it is still unclear as to which parameters of the ECM a cell mechanically senses in order to elicit biochemical responses.

The exact role of matrix in controlling cellular fate, particularly during invasion and 3D migration remains elusive. To explore the impact of the ECM structure and mechanics on cell adhesion and signaling in native environments, we utilized 3D collagen gels and cross-linked them with naturally secreted tissue transglutaminase (tTG), which exhibits abherrant expression in native prostate cancer environments. The objective of this study was to investigate the molecular mechanisms affected by altering single variables of the ECM as they influence prostate cancer proliferation. Here we quantitatively provide mechanistic understanding of cell migration through 3D ECM substrates of constant ligand density but increased stiffness, implemented by a covalent chemical crosslinker. Our results demonstrate how these mechanisms regulate cellular migratory mechanisms.

Methods used to characterize such environments and cellular responses include Scanning Electron Microscopy (SEM), Confocal Microscopy, Flow Cytometry, Gelatin Zymography, and Immunoblotting. Current studies utilize siRNA and rtPCR quantitation to further elucidate cross-talk between cell-ECM adhesion stimulated signaling as it affects extracellular and intracellular activity of matrix associated MMPs. Our signaling studies of prostate cancer cells in tTG cross-linked systems exhibit a novel mechanism for sensitivity to substrate stiffness, in contrast to ligand density, and substrate cross-linking. We observe cooperative increased expression of the integrin family of adhesion proteins, Src and matrix metalloproteinases (MMPs) from cells cultured in environments of intermediate stiffness. The invasive ability of prostate cancer cells in native environments exhibits novel interactions between integrin-induced signaling pathways and MMP activity. Our data provides a systems level quantitative mechanism of mechanosensing of fibril density, cross-linking and ECM stiffness and its synergistic regulation of adhesion-based prostate tumor cell migration in native like environments.

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Cross-talk between matrix tension, PKA, and Ca^{2+} in ovarian cancer cell migration and invasion.

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The cAMP-dependent protein kinase (PKA) is spatially regulated during chemotaxis, with specific enrichment within the leading edge of migrating cells. However, the mechanism and consequences of this activation are not fully understood. Here, we show that PKA is activated at the leading edge of ovarian cancer (OvCA) cells during migration and during invasion of a three-dimensional extracellular matrix, and that the activity of both PKA and A-kinase anchoring proteins (AKAPs) are required for OvCA cell migration and matrix invasion. Furthermore, we provide strong evidence for cross-talk between PKA, extracellular matrix tension, and intracellular calcium (Ca^{2+}) in the regulation of leading edge dynamics and cell migration in OvCA cells. Increased matrix stiffness induces durotactic migration of individual OvCA cells and also promotes the disaggregation of multicellular OvCA spheroids. PKA is rapidly and locally activated by acute increases in matrix tension. PKA activity within the leading edge is blocked by inhibition of the stretch-activated Ca^{2+} channels, including TRPM7. Conversely, inhibition of PKA activity or AKAP function significantly decreases the appearance of Ca^{2+} flickers - leading edge Ca^{2+} transients that are known to be mediated by TRPM7 and play a role in chemotaxis. These data support a hypothesis in which cell-matrix tension locally controls PKA activity, which serves as both a regulator and an effector for stretch-activated Ca^{2+} currents and is important for leading edge dynamics, cell migration, and matrix invasion.

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Protein Kinase A activity and anchoring are required for ovarian cancer cell migration and matrix invasion.

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Epithelial ovarian cancer (EOC) is the deadliest of the gynecological malignancies, due in part to its clinically occult metastasis. Therefore, understanding the mechanisms governing EOC dissemination and invasion may provide new targets for antimetastatic therapies or new methods for detection of metastatic disease. The activity of the cAMP-dependent protein kinase (PKA) and its subcellular localization by A-kinase anchoring proteins (AKAPs) are important regulators of cytoskeletal dynamics and cell migration. Moreover, the expression of various PKA subunits and certain AKAPs positively correlates with advanced stage and/or more aggressive disease. Thus, we sought to study the role of PKA and AKAP function in both EOC cell migration and invasion. Using the plasma membrane-directed PKA biosensor, pmAKAR3, and an improved migration/ invasion assay, we show that PKA activity is up-regulated at the leading edge of SKOV-3 EOC cells during migration, and that this activity is mediated via anchoring of the RII subunit of PKA. Importantly, we demonstrate that inhibition of PKA activity or anchoring through either RI or RII subunits blocks SKOV-3 cell migration. Furthermore, we show that PKA activity is also up-regulated at the leading edge of EOC cells invading into a three-dimensional extracellular matrix and demonstrate the necessity of PKA activity and anchoring for functional matrix invasion. Specifically, invasive leading edge PKA is mediated specifically through AKAP-RII interaction, while both type I and type II PKA anchoring are required for invasion. These data are the first to demonstrate a requirement for PKA activity and anchoring during matrix invasion and suggest an important role for PKA and AKAPs in EOC metastasis.

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The Regulation of RhoA by Stard13 in Focal Adhesions is Essential for Astrocytoma Cell Motility.

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Malignant astrocytomas are associated with high mortality rates. These tumors are highly invasive into adjacent and distant regions of the normal brain rendering them medically unmanageable. Invasion is a multistep process, which ultimately requires the cell to actively migrate through the ECM. Rho-GTPases, mainly RhoA, Rac, and Cdc42, play a major role in the regulation of the processes that ultimately lead to cell migration. StarD13 is a RhoGAP that inhibits the function of RhoA and Cdc42.
Observations: We first aimed at determining the role of RhoA in the progression of astrocytic tumors, a topic that is still controversial in the literature. Looking at the activation of RhoA through the use of a FRET biosensor showed RhoA to localize to the tail as well as to the leading edge of astrocytoma cells undergoing random movement. Our results also showed that knocking down RhoA by siRNA transfection inhibited cell motility. Surprisingly, when we knocked down StarD13 using siRNA we saw an inhibition of cell motility as well. This was mimicked by transfecting the cells with a dominant active RhoA construct. StarD13 mainly localized to adhesion structures, thus RhoA needs to be inhibited at the sites of adhesion. StarD13 knock down cells were unable to move and showed a phenotype of an elongated tail. This showed us that Rho inhibition by StarD13 pays a role in the cell detaching its tail to move forward. Looking at the adhesion structures we also saw complete inhibition of focal complex formation in cells overexpressing active RhoA or where StarD13 was knocked down. This was also mimicked by Rac knock down, showing that StarD13 also inhibits RhoA at the leading edge of cells for Rac to get activated and focal complexes to form.

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hDlg Enhances SGEF/RhoG Activity and is Required for Invasion of HPV-18 Transformed Cells.

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The Discs Large (Dlg) tumour suppressor forms part of a tri-partite multi macromolecular complex that is integral to the regulation of cell polarity and diverse cell signaling pathways. In support of a tumour suppressive potential in human malignancy, expression of Dlg is frequently lost in many late stage cancers, and most intriguingly, it is also a target of the Human Papillomavirus (HPV) E6 oncoprotein, which is an essential viral protein required for the development HPV-induced cervical cancer. We have been interested in understanding why HPV E6 might interact with Dlg during the development of HPV-induced malignancy. Using a proteomic approach we now show that a strong interacting partner of Dlg is the RhoG-specific guanine nucleotide exchange factor SGEF. This interaction involves PDZ and SH3 domain recognition and this directly contributes to the regulation of SGEF cellular localisation. The interaction between Dlg and SGEF appears to be strictly controlled, with increased levels of association detected during active cell migration. Consistent with this, we show that Dlg is a strong enhancer of RhoG activity, which occurs in an SGEF dependent manner. In HPV transformed cells, a significant proportion of Dlg is subjected to proteasome degradation, in an HPV E6 dependent manner. However, we also show that a part of the E6-Dlg complex contributes to maintaining high levels of SGEF and RhoG activity in these cells. Furthermore, this E6-Dlg complex directly contributes to the invasive capacity of these cells, which occurs in an SGEF dependent manner. These studies demonstrate a novel pro-oncogenic activity for Dlg

in HPV induced malignancy, one of the outcomes of which is increased RhoG activity and increased invasive potential.

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Essential role of store-operated calcium entry in serum-induced lung cancer cell migration.

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The Ca^{2+} influx is essential for the migration of various cell types including cancer cells. Orai/STIM proteins mediate store-operated calcium entry (SOCE) which is important for cell migration and proliferation. However, the functional role of SOCE in tumor cell migration remains poorly understood. Here we investigated the molecular mechanism by which store-operated calcium (SOC) channels regulates cellular migration of lung cancer cell line H1693.
Ca^{2+} influx is critical for serum- or growth factor-induced cell migration. We identified that SOCE was increased by treatment of serum and/or diverse growth factors; epidermal growth factor, insulin-like growth factor 1, and platelet-derived growth factor. Interestingly, serum-induced H1693 cell migration was specifically blocked by SOC channel inhibitors such as SKF96365 and 2APB but not by voltage-gated calcium channel inhibitor, nimodipine. We identified expression of isoforms of Orai (1/2/3) and STIM (1/2) proteins in H1693 cells. In particular, serum treatment increased Orai1 mRNA expression. Orai1 is pore-forming unit of Ca^{2+}-release-activated Ca^{2+} (CRAC) channels. Serum treatment enhanced CRAC currents in both H1693 cells and HEK293 cells overexpressing Orai1. This suggests that SOCE might be critical for serum-induced H1693 cell migration.
Serum and growth factors are known to activate both phosphatidylinositol 3-kinase (PI3K)-Akt and/or Erk1/2 pathways. We questioned if the Orai1 associated cell migration is linked to these pathways. Indeed, serum-induced augmentation of SOCE and cell migration was blocked by treatment of wortmanin, a PI3K inhibitor. This data suggest that the serum-PI3K-Akt pathway is critical for SOCE-mediated cell migration. In addition, U0126, an inhibitor of Erk1/2 pathway also reduced serum-induced cell migration and SOCE.
Taken together, this study revealed the signal transduction pathway in lung cancer cell migration that serum and growth factors stimulate the Orai1-mediated SOCE via activating PI3K-Akt and/or Erk1/2 pathway.

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A Rab35/Arf6 recycling complex regulates cell adhesion and migration: implications for cancer biology.

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Integrins and cadherins control distinct cell adhesion properties leading to functionally opposite consequences. Specifically, integrins mediate migration over the extracellular matrix whereas cadherins mediate cell-cell adhesion, which inhibits migration. How surface expression and activity of these receptors are coordinated remains unclear. Here we uncover a sorting and recycling compartment that efficiently coordinates cadherin and integrin recycling. Cargo sorting through this compartment is defined by the small GTPases Rab35 and Arf6, which are mutually antagonistic. Knock down of Rab35 prevents E-cadherin recycling inhibiting intercellular adhesion, and promotes Arf6 activity, needed to stimulate beta1-integrin-dependent cellular motility. In fact, Rab35 knock down leads to a global increase in beta1-integrin levels, explained by a switch in trafficking that favours recycling over degradation. Furthermore, the activity of the

signalling kinases FAK, Erk1/2 and Akt downstream of beta1-integrin are strongly elevated. The phenotypes observed following Rab35 knock down are consistent with the epithelial-to-mesenchyme transition, and we find that Rab35 expression is significantly suppressed in surgically retracted breast, skin and brain tumors, cancers where Arf6 activity and levels are upregulated. Our data identify a Rab35/Arf6 molecular module that explains how cadherin and integrin trafficking is coordinated. The importance of this module is highlighted by its disruption in cancer.

1545

Phosphoinositide specificity determines which cytohesins promote β1 integrin recycling.

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Integrin based cell-to-matrix adhesions provide traction to convert contraction to movement during cell migration. Adhesion remodeling, the disassembly and reassembly of attachments to the substratum, is a critical step in cell movement. Remodeling of these adhesions is carried out by internalization and reinsertion of integrins from the plasma membrane. Stimulation of integrin exocytosis can initiate or promote cell migration. ARF small GTPases initiate vesicle budding during trafficking processes. Guanine nucleotide exchange factors (GEFs) are accessory proteins that turn on GTPases. We have recently determined that two closely related ARF-GEFs, cytohesin-2 and cytohesin-3, have opposing effects on stimulated β1 integrin recycling (Oh, S. J. and L. C. Santy (2010) J Biol Chem 285: 14610-14616.). Cytohesin-2 enhances adhesion, spreading and migration while cytohesin-3 inhibits these processes. Furthermore, only cytohesin-2 is required for β1 integrin recycling to the cell surface. We have now determined the specific sequence differences between these highly related proteins that underlie their differential effects on β1 integrin recycling. We have found that the ability of cytohesins to promote β1 integrin recycling to the plasma membrane depends upon the phosphoinositide specificity of their PH domains. Cytohesin-3 has 2 glycine residues at a critical point in its PH domain and therefore binds with high affinity and specificity to PI-(3,4,5)P_3. Cytohesin-2 on the other hand has 3 gylcine residues at this position and binds with lower and equivalent specificity to both PI-(3,4,5) $_3$ and PI-(4,5)P_2 (Klarlund, J. K. et. al. (2000). J Biol Chem 275: 32816-32821). Switching the number of glycines in the PH domains of cytohesin-2 and cytohesin-3 is sufficient to reverse their effects on adhesion and spreading. Furthermore cytohesin-3 with three rather than two glycine residues in its PH domain can promote β1 integrin recycling. We conclude that phosphoinositide specificity is the sole determinant of which cytohesins can promote recycling of β1 integrin recycling.

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R-Ras on recycling endosome membranes is transported to plasma membrane ruffles by endosomal trafficking which requires geranylgeranylation, microtubules and R-Ras activation.

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R-Ras, a member of the Ras family of small G proteins, has distinct cellular functions from K-, H-, and N-Ras; in particular, R-Ras regulates cell spreading and motility through unique signaling pathways. Recently, an activated pool of R-Ras was localized to the membranes of recycling endosomes (REs), specialized tubulo-vesicular organelles involved in receptor recycling and secretory pathways. An important function of REs is delivery of pro-migratory signaling cargo and membranes to the leading edge in migrating cells. In addition to REs, activated R-Ras has also been localized to plasma membrane ruffles at the leading edge, important cellular components participating in forward motility. We investigated a putative role of

REEs in R-Ras sub-cellular targeting. REs containing R-Ras aligned along microtubules, and microtubules were required for R-Ras-dependent cell spreading. Disruption of microtubules with nocodazole dispersed R-Ras vesicles from tubule-vesicle arrays to diffuse cytosolic patterns. We used real-time live cell confocal microscopy coupled with a fluorescence recovery after photobleaching (FRAP) scheme to track the dynamics of REs containing red fluorescent protein (RFP) fusions of R-Ras alongside GFP-Rab11, a marker for REs, in randomly migrating cells. Wild type R-Ras, R-Ras harboring a mutation rendering it constitutively active (38V), and dominant negative R-Ras (43N) all localized to Rab11-positive REs and additional Rab11-negative endosomal structures, as well as at the Golgi, and were concentrated at the endosomal membranes – this phenomenon could be more easily observed in live cells as opposed to fixed cells in which many of the endosomes are often collapsed and appear as dense puncta. R-Ras vesicles appeared to undergo dynamic sorting in the vicinity of the Golgi. In the cytosol, wild type and activated R-Ras engaged in anterograde trafficking in saltatory fashion towards the leading edge on both Rab11-positive and -negative vesicles in migrating cells. FRAP analysis demonstrated that wild type and activated R-Ras vesicles contributed to multi-phasic recovery of bleached fluorescence (subtracting recovery by diffusion) in zones anterior to the nucleus, with continual vesicle-derived peaks at 2.07 ± 0.15 peaks/min. These vesicles appeared in most cases to pause as they reached the actin-dense lamellipodia, and smaller R-Ras structures emerged from these vesicles and continued trafficking to the leading edge, where R-Ras concentrated in membrane ruffles. R-Ras was also recycled in Rab11-negative vesicles from the ruffles and trafficked in retrograde fashion towards the Golgi. Vesicles containing inactive R-Ras(43N) oscillated and also reached the plasma membrane, but with significantly slower dynamics, and these cells had many fewer membrane ruffles. Nocodazole halted R-Ras anterograde traffic, significantly delayed FRAP recovery times with diminished peak intensities, and prevented R-Ras from reaching ruffles. In addition, activated R-Ras mutated at C215 to serine, preventing geranylgeranylation of R-Ras, sequestered R-Ras diffusely in the cytosol, thus eliminating vesicle-derived FRAP recovery, and this R-Ras variant was completely absent from ruffles. However, R-Ras-negative Rab11 endosomes trafficked normally. Thus, we have described a mode of sub-cellular targeting for the pro-migratory protein R-Ras: R-Ras is targeted to the plasma membrane and ruffles by trafficking in endosomal vesicle membranes with the aid of microtubules, in a manner which requires R-Ras geranylgeranylation for membrane anchorage, and R-Ras activity to permit vesicle trafficking. This distinct mechanism of R-Ras targeting likely contributes substantially to its unique signaling and cellular functions.

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MARCKS phosphorylation relies on PKC isoform delta(δ) in human neutrophils.

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MARCKS (Myristoylated alanine-rich C kinase substrate) is a PKC (Protein Kinase C) substrate that is involved in mucin and neurosecretion, postnatal survival and regulation of cell morphology and migration. In most cell types, MARCKS is associated with the cell membrane in the resting state and is translocated into the cytosol when it is phosphorylated by PKC.
We previously determined that MARCKS plays an important role in neutrophil migration and adhesion using a cell permeant peptide derived from the MARCKS myristoylated aminoterminus (MANS). To unravel how MARCKS phosphorylation affects neutrophil migration and adhesion, we identified the PKC isoforms involved in MARCKS phosphorylation. We tested PKC isotypes alpha (α), beta (β), delta (δ), and zeta (ζ), known to be present in human neutrophils, using specific PKC isotype inhibitor Go6976, CG53353, rottlerin and ζ pseudosubstrate, respectively.

Human neutrophils were pretreated with inhibitors and MARCKS phosphorylation was measured after stimulation of cells with fMLF. Pretreatment of neutrophils with the pan-PKC inhibitor staurosporin blocked fMLF induced MARCKS phosphorylation in a dose dependent manner, as did specific inhibition of PKC delta; but other PKC isoforms including alpha, beta and zeta did not affect MARCKS phosphorylation. The IC50 of staurosporin and rottlerin were 1.43 μM and 8.14 μM respectively for the fMLF induced MARCKS phosphorylation. Subcellular fractionation assay showed that the PKC isotype delta, but not others, translocated from cytosol to membrane in response to fMLF and PMA stimulation. These results suggest that PKC delta plays a crucial role in fMLF induced MARCKS phosphorylation in human neutrophils.

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Comparative Analysis of Light-stimulated Motility Responses in Diatoms.

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Our lab is continuing to study the effects of light irradiation on diatom motility. Our results indicate that the orientation of diatom movement shows light sensitivity, to both irradiance levels and wavelength, that is characteristic for each species. By measuring the time for the leading and trailing ends of the cells to respond to rapid (1 sec) light irradiations, we have been able to compare the responses of three diatom species (*Craticula cuspidata*, *Stauroneis phoenicenteron*, and *Pinnularia viridis*). In contrast to the *Craticula* and *Stauroneis* cells, the *Pinnularia viridis* cells show virtually no sensitivity to red light (650 nm) irradiations. In comparison, at moderate light levels *Craticula cuspidata* are stimulated by light irradiations to move into blue light (450 nm) at moderate light levels while stimulated to move out of red light at the same levels. The *Stauroneis* cells show the strongest sensitivity to red light, showing an stimulated movement into red light even at very dim levels. These light-stimulated movements appear to be regulated by differential responses at the leading and trailing end of the diatom cells. For example, with 1 sec irradiations of high irradiations (10^5 μmol/m^2s) of blue light, the leading end of *Craticula* cells will change direction in 17±1 sec, while the trailing end has no response at all (161 ± 8 sec, compared to unirradiated control response times of 135 ± 7 sec), leading to an “out of the light” response. Such irradiations with more moderate blue light (attenuated with 1.0 OD neutral density filters) shows a faster response time with the trailing end (41 ± 4 sec) than the leading end (79 ± 11 sec) giving rise to an “into the light” response. Similar irradiations on *Craticula* with moderate level red light shows a continued “out of light” response (81 ± 9 sec response for leading end; 117 ± 14 sec response for trailing end). In contrast *Pinnularia* cells show virtually no red responsiveness at any light level, while *Stauroneis* cells shows a strong “into the light” response at low red light levels (198 ± 18 sec response for leading end; 115 ± 12 sec response for trailing end). Moreover, light irradiations on *Craticula cuspidata* and *Pinnularia viridis* show that the blue light irradiations not only stimulate direction changes but also repress further direction changes in the cell for 15-60 sec, helping to reinforce the light-directed changes. Such studies allow us to better understand the regulation of single-celled algae in establishing and maintaining healthy aquatic ecosystems. This work was supported by DePaul University Research Council, the DePaul College of Liberal Arts & Sciences, and equipment purchased through NSF Grant IBN-9982897.

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Activation of sperm motility by a seminal fluid protease in *C. elegans.*

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Sperm motility is controlled to promote the ability of sperm to navigate through the reproductive tract and fertilize an egg. In *C. elegans*, sperm move by crawling and their motility is acquired during a process termed activation, in which immotile round spermatids reorganize to become motile, polarized spermatozoa. Both males and hermaphrodites make sperm, and activation must be triggered at the appropriate time for each sex, or fertility suffers. Although activation is readily induced by a variety of treatments in vitro, the signals that trigger activation in vivo have been elusive. We have identified a serine protease, TRY-5, that is a component of seminal fluid and is required for sperm activation by *C. elegans* males. While misregulation of TRY-5 within males leads to premature activation, males lacking TRY-5 do not transfer activator and must rely on signals from the hermaphrodite to activate their sperm. TRY-5 is expressed in secretory cells of the male somatic gonad, and a TRY-5::GFP reporter is transferred along with sperm during mating. Thus, TRY-5 is a seminal fluid factor that couples the onset of motility in sperm to their transfer to a hermaphrodite. We propose that TRY-5 triggers activation by cleaving proteins on the sperm surface, and we are searching for sperm proteins that may be targets of TRY-5. We have identified a candidate for such a factor: the SLC6 family transporter SNF-10. Sperm lacking SNF-10 fail to activate in response to protease either in vitro or in vivo. Curiously, they show enhanced response to other activators. Thus, analysis of TRY-5 and SNF-10 may reveal how sperm cells integrate a variety of signals from their environment to ensure functional motility.

1550

Poly(vinylmethylsiloxane) elastomer networks as functional materials for cell adhesion and migration studies.

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Cell migration is central to physiological responses to injury and infection and in the design of biomaterial implants. The ability to tune the properties of substrate and relate those properties quantitatively to the dynamics of intracellular processes remains a definite challenge in the manipulation of cell migration. Poly(vinylmethylsiloxane) (PVMS) networks, a novel substrata for cell adhesion and migration, offers the ability to tune chemical functionality and elastic modulus independently. Importantly, PVMS networks are compatible with total internal reflection fluorescence (TIRF) microscopy, which is ideal for interrogating the cell-substratum interface; this latter characteristic presents a distinct advantage over polyacrylamide gels and other materials that swell with water. In addition, a higher ligand density can be achieved using a polymer brush-based approach, which potentially offers better control in making substrates with gradients of mechanical and chemical properties. To demonstrate these capabilities, different adhesive peptides were successfully grafted to the surface of PVMS network. Peptide-specific adhesion, spreading, and random migration of NIH 3T3 mouse fibroblasts show that presence of the synergy sequence (PHSRN) along with the binding motif (RGD) of fibronectin in peptide promotes more productive cell migration without markedly enhancing cell adhesion strength. Using TIRF microscopy, signal transduction dynamics through the phosphoinositide 3-kinase pathway were monitored in cells as they migrated on peptide-grafted PVMS surfaces. These approaches offer a promising avenue for studies of directed migration and mechanotransduction at the level of intracellular processes.

Cadherins and Cell-Cell Interactions

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E-cadherin-mediated cell-in-cell formation by compartmentalized Rho activity.

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Adherens junctions are major cell adhesion complexes that organize epithelia by coordinating cytoskeletal tension between neighboring cells. Dysregulation of adherens junctions is frequent in human tumors, and E-cadherin and alpha-catenin are tumor suppressors that can limit proliferation and inhibit invasion. Recently a mechanism of cell death was described, called entosis, which involves adherens junction-mediated engulfment of live cells. The cell structures formed by entosis directly resemble live cell engulfments found in human cancers, which are commonly referred to as ¡°cell-in-cell¡± or cell cannibalism. Here we find that the expression of epithelial cadherins in cancer cells correlates with their ability to form cell-in-cell structures, and introduction of E- or P-cadherin to tumor cells lacking expression is sufficient to induce cell-in-cell formation, which results in the death of internalized cells. We further show that imbalanced RhoA/ROCK signaling between cells underlies cell-in-cell formation, as actomyosin contraction at the cortex of internalizing cells, distal to E-cadherin, drives their engulfment, and cells with higher RhoA activity tend to be engulfed. Moreover, overexpression of another GTPase, Rac1, is able to transform cells into engulfers by inhibiting RhoA/ROCK activity. Mechanistically, junction-localized p190 RhoGAP is required for compartmentalizing Rho activity within and between cells. Finally, we show that E-cadherin-mediated cell-in-cell formation inhibits transformed growth in vitro. Together, these data demonstrate that cell cannibalism in human breast tumors may result from abnormal cell adhesion, which may underlie a heretofore unrecognized mechanism of tumor suppression by epithelial cadherins.

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Rapid Suppression of Activated Rac1 by Cadherins and Nectins during *De Novo* Cell-Cell Adhesion.

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Cell-cell adhesion in simple epithelia involves the engagement of E-cadherin and nectins, and the reorganization of the actin cytoskeleton and membrane dynamics by Rho GTPases, particularly Rac1. However, it remains unclear whether E-cadherin and nectins up-regulate, maintain or suppress Rac1 activity during cell-cell adhesion. Roles for Rho GTPases are complicated by cell spreading and integrin-based adhesions to the extracellular matrix that occur concurrently with cell-cell adhesion, and which also require Rho GTPases. Here, we designed a simple approach to examine Rac1 activity upon cell-cell adhesion by MDCK epithelial cells, without cell spreading or integrin-based adhesion. Upon initiation of cell-cell contact in 3-D cell aggregates, we observed an initial peak of Rac1 activity that rapidly decreased by ~66% within 5 minutes, and further decreased to a low baseline level after 30 minutes. Inhibition of E-cadherin engagement with DECMA-1 Fab fragments or competitive binding of soluble E-cadherin, or nectin2alpha extracellular domain completely inhibited Rac1 activity. These results indicate that cadherins and nectins cooperate to induce and then rapidly suppress Rac1 activity during initial cell-cell adhesion, which may be important in inhibiting the migratory cell phenotype and allowing the establishment of initially weak cell-cell adhesions. Future studies aim to investigate the GEFs and GAPs involved in establishing and maintaining initial cell-cell adhesion.

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Cell contact localized β-actin translation is required for E-cadherin anchoring during adherens junction assembly.

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Adherens junctions are multi-protein complexes critical during development for cell sorting and establishment of tissue polarity. Previously we demonstrated that *de novo* protein synthesis is required for adherens junction assembly by treating MDCK cells with the translational inhibitors, cycloheximide or puromycin following cell contact. Moreover, we demonstrated contact-localized β-actin translation was correlated with increased contact-localization of N-cadherin in myoblast cells. In contrast, mislocalization of β-actin translation sites reduced cellular polarity and decreased contact-localization of N-cadherin in myoblast cells. The objective of this project was to test the extent to which the location of β-actin translation sites correlated with sites of E-cadherin anchoring at epithelial cell contacts. MDCK cells stably expressing plasmids containing either the β-actin FL or β-actin without its zipcode sequence (Δ zipcode) were grown as monolayers and cell contact was stimulated using the calcium switch method. Pearson's correlation analysis of colocalization was used to quantify the extent of colocalization between E-cadherin and β-actin +/- zipcode at cell contact sites and within the cytoplasm. MDCK cells expressing full-length β-actin exhibited colocalization between E-cadherin and β-actin at cell contact sites following the calcium switch. By contrast, cells expressing Δ zipcode β-actin failed to reform a monolayer and had reduced colocalization between E-cadherin and β-actin. During wound-healing assays, Δ zipcode β-actin expressing cells failed to repair the wound which correlated with a reduced velocity of cell migration relative to MDCK cells expressing full-length β-actin. These results demonstrate that contact-localized β-actin translation is required for cadherin anchoring during the assembly of epithelial adherens junction complexes.

1554

A novel proteolytic processing of talin is associated with cadherin-mediated cell-cell adhesion.

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Talin is an essential cell adhesion molecule that plays a key role in integrin-mediated cell adhesion to the extracellular matrix, but has never been found to be involved in cadherin-mediarted cell-cell adhesions. Talin is a large rod-like molecule that scaffolds multiple components of the actin cytoskeleton. It is regulated by limited calpain-mediated proteolysis with a release of its N-terminal regulatory domain during integrin adhesion maturation and turnover. Other types of cleavages in vivo with functional significance have not been reported. Here we studied talin regulatory proteolysis in response to changes in cell density and found that the formation of cell-cell adhesions in a dense culture is accompanied by calpain-dependent generation of a novel 70 kDa talin fragment, which partially co-localizes with cadherin and accumulates in a dose-dependent manner in cells establishing cell-cell adhesions. Analysis of arginyltransferase (Ate1) knockout cells deficient in cell-cell adhesions revealed that these cells have little or no 70 kDa talin fragment, suggesting that arginylation knockout inhibits this fragment's generation, which in turn may be responsible for the adhesion defects in these cells. Phentype rescue experiments showed that reintroduction of the 70 kDa talin fragment into Ate1 knockout cells rescues their cell-cell adhesion defects, and that arginylation of this fragment further enhances the rescue effect in addition to regulating its turnover. Thus, we have discovered a novel proteolytic cleavage of talin that participates in its novel function in cadherin-mediated cell-cell adhesion and is regulated by a multi-level mechanism that involved cell density, calpain, and arginylation.

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Dissecting the Roles of Desmosomal Cadherins in Cell-Cell Adhesion and Epithelium Remodeling.

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During normal development, tissue regeneration and cancer, epithelia undergo dramatic morphogenetic processes, ranging from cohesive sheet migration, cell position changes within cohesive tissues, to individual cell migration after dissociation from tissues. Such cell movements require coordination between cell migration and intercellular adhesion. In epithelial cells, there are two types of intercellular adhesion complexes that involve the *trans*-membrane protein cadherin superfamily: desmosomes and adherens junctions (AJs). AJs contain E-cadherin, a classical cadherin that mediates intercellular adhesion by forming homotypic bonds between neighboring cells. Desmosomes, however, contain multiple isoforms of two types of cadherins (desmocollin/dsc and desmoglein/dsg). Mis-expression of desmosomal cadherin isoforms within the epidermis (a specialized epithelium) compromises tissue integrity. How different desmosomal cadherins form bonds within desmosomes and ultimately contribute to intercellular adhesion - thereby regulating cell migration and tissue integrity - is unknown. To understand desmosomal cadherin functions, we studied desmocollin and desmoglein recruitment and epithelial cell motile behavior by TIRF and live-cell microscopy on epithelial cells adhering to substrates patterned with alternating stripes of desmosomal cadherins and collagenIV. We find that cells assemble desmosomes on the substrate's patterns that are functionalized with dsc or both dsg and dsc, but not dsg alone. Additionally, cell adhesion to surfaces functionalized with dsc or dsc/dsg decreases the rate of cell migration. To address how desmosomal cadherins interact, we engineered a W2A mutation; the W2 is important for *trans*-binding of classical cadherins. The W2A mutation in desmocollin affects protein localization and turnover; DscW2A localizes in punctate spots at nascent cell-cell contacts similar to wild type protein, but within a few hours DscW2A staining becomes diffusely distributed throughout the plasma membrane and is highly mobile, as assessed by FRAP. Preliminary results suggest that there is increased lamellipodia activity in cells expressing DscW2A. Interestingly, the W2A mutation in desmoglein does not have a significant effect on adhesion or localization of DgW2A. These results show that desmocollin and desmoglein have unique binding properties, resulting in differences in their recruitment and stability at cell-cell contacts, and changes in collagenIV-based cell migration. Thus modulation of desmosomal cadherin interactions during development and cancer may contribute to the regulation of morphogenetic cell movements.

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M-Cadherin-mediated signaling attenuates GSK-3ß-dependent ß-catenin phosphorylation and promotes myogenic differentiation.

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β-catenin is an essential molecule in both cadherin-mediated cell-cell adhesion and canonical Wnt signaling. The phosphorylation of β-catenin by GSK-3β at residues Ser33/37/Thr41 regulates its stability and its role in Wnt signaling. Previously we reported that M-cadherin-mediated signaling protects myoblasts against apoptosis and promotes myogenic differentiation by suppressing GSK-3β activation. In this study, we further investigated if the GSK-3β-dependent phosphorylation of β-catenin will be regulated by M-cadherin and if this regulation mediates M-cadherin's role in promoting myogenic differentiation. We demonstrated that knockdown of M-cadherin expression in C2C12 myoblasts by RNA interference(RNAi) significantly decreased the protein abundance of serine37/Thr41-unphosphorylated β-catenin. M-cadherin RNAi also impaired the myogenic and fibrotic induction by LiCl treatment, which is

an established activator of canonical Wnt signaling. Knockdown of β-catenin expression in C2C12 myoblasts by RNAi also significantly impaired myogenic induction by LiCl treatment. On the other hand, forced expression of phosphorylation-resistant mutant form of β-catenin(S33Y-β-catenin) alone failed to induce myogenesis but partially rescued the phenotype caused by M-cadherin RNAi. We concluded that M-cadherin-mediated signaling attenuates β-catenin phosphorylation at Ser37/Thr41 by GSK-3β and thus has a positive effect on canonical Wnt signaling. Our findings provide a novel mechanism on M-cadherin's role in promoting myogenic differentiation.

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Kinesin-1 and Kinesin-2 Mediate Transport of the Desmosomal Cadherins, Desmoglein 2 and Desmocollin 2, and are Required for Keratinocyte Cell-Cell Adhesion.

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Desmosomes are intercellular junctions that appeared late in the evolution of multicellular organisms, and their proper assembly and function are critical for the morphogenesis and mechanical integration of tissues such as skin and heart. The desmosomal adhesive core contains two types of cadherin, desmogleins (Dsgs) and desmocollins (Dscs), both of which are required for desmosome function. However, mechanisms that coordinate desmosomal cadherin trafficking into intercellular junctions to regulate their proper ratio and distribution are unknown. Using live cell imaging we show that Dsgs and Dscs exhibit long range microtubule-dependent transport in epithelial keratinocytes but utilize distinct motors to traffic to the plasma membrane. Inhibition of kinesin-1 by gene silencing or expression of dominant negative (DN) kinesin heavy chain (KIF5) mutants completely blocked transport of vesicles containing Dsg2, resulting in a significant decrease in Dsg2 at cell-cell interfaces. By contrast, inhibition of kinesin-2 function with silencing of KIF3A or KAP3 (kinesin-2 associated protein) or by expression of DN KIF3A blocked transport of Dsc2 and reduced its localization on the plasma membrane without affecting Dsg2 trafficking. In biochemical interaction assays, we found that 14-3-3□ coimmunoprecipitated in a complex with kinesin-1 and Dsg2. Furthermore, 14-3-3□ silencing inhibited Dsg2 accumulation at the plasma membrane, suggesting this scaffolding protein regulates Dsg2 assembly into desmosomes through interaction with kinesin-1. Using a mechanical dissociation assay, we showed that either kinesin-1 or -2 deficiencies weakened intercellular adhesion by ~30- fold, despite the maintenance of adherens junctions and other desmosome components at the plasma membrane. Differential regulation of desmosomal cadherin transport could provide a mechanism to "tailor" adhesion strength during tissue morphogenesis and remodeling.

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P-cadherin is a direct Pax3-FOXO1A target involved in myoblast transformation and invasiveness of alveolar rhabdomyosarcoma.

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Alveolar rhabdomyosarcoma (ARMS) is an aggressive childhood cancer of striated muscle characterized by a chromosomal translocation which generates the chimeric oncogenic transcription factor Pax3-FOXO1A. Identification of Pax3-FOXO1A targets is therefore important to understand pathogenesis of RMS. Analysis of transcriptomic data sets of RMS samples

revealed that P-cadherin is correlated to Pax3-FOXO1A and Pax7-FOXO1A. Using ARMS cells, we showed that expression of Pax3 dominant negative construct inhibits expression of P-cadherin. Analysis of P-cadherin expression during mouse embryonic development revealed that it is expressed in epaxial dermomyotome. Using transgenic mouse embryo, we demonstrated that P-cadherin lies genetically downstream from Pax3. In vitro gel shift experiments and chromatin immunoprecipitation with ARMS cell extracts, show that the P-cadherin gene is a direct target for Pax3-FOXO1A through a conserved 160 bp sequence located -2kb from the transcription start site. We then assessed the role of P-cadherin in the development of RMS and showed that P-cadherin expression in normal myoblast results in myogenesis inhibition and induces myoblast transformation, migration and invasion. Conversely, inhibition of P-cadherin expression by small hairpin RNA decreases the transformation, migration and invasive potential of ARMS cells. Finally, as cadherin switching is a hallmark of metastatic progression, we analyzed if P-cadherin impacts on other skeletal muscle cadherin expression. We showed that P-cadherin controls N- and M-cadherin expression and/or localization in myoblasts and ARMS cells.
Our results show that P-cadherin is a direct Pax3-FOXO1A transcriptional target involved in transformation, migration and invasion of ARMS cells. Therefore, P-cadherin emerges as a new and attractive target for therapeutic intervention in this aggressive rhabdomyosarcoma.

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Flotillins are new cadherin partners that control cadherin stabilization and the formation of adherens junctions.

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Cadherins are essential in many fundamental processes such as tissue patterning during development, growth control during tumorigenesis, and in the maintenance of adult tissue architecture. At regions of cell-cell contact, cadherins assemble into large macromolecular complexes named adherens junctions. Full knowledge of proteins associated with cadherins is far from complete. Here we identify flotillin 1 and 2 as new partners of cadherins required for their stabilization at the cell-cell contacts. Flotillin 1 (Flot 1 ou reggie-2) and flotillin 2 (Flot 2 ou reggie-1) are ubiquitous and highly conserved proteins, located at the cytoplasmic face of the plasma membrane (PM), involved in the scaffolding of large complexes that signal across the PM thanks to their ability to form hetero-oligomeric complexes. The intracellular domain of cadherin appears not to be involved in the association with flotillins. Therefore, flotillins being proteins localized to the cytoplasmic face of the PM, this association is likely to be indirect. We initially identified flotillin 1 as a novel N-cadherin associated protein, by immunoprecipitation followed by mass spectrometry. We extended this association to cadherin N, E, , P, R and 11 indicating that flotillins probably associate with all classical cadherins. Knockdown of flotillins dramatically affect N- and E-cadherin recruitment at adherens junctions in both mesenchymal and epithelial cell types. At the molecular level, we show that in contacting cells, flotillins stabilize cadherins at the PM and prevent their degradation, by strengthening the coupling to p120 catenin, one of the main cadherin stabilizing partners. In conclusion, we report here for the first time, that flotillins are major regulators of cadherin assembly and stabilization at cell-cell contacts and are thus essential for the formation of adherens junctions.

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Biochemical Characterization of αE-Catenin Phospho-Mutant Conformations and Functions.

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αE-Catenin binds and bundles F-actin, and binds the cadherin/β-catenin cell-cell adhesion complex. Although it is assumed that αE-catenin links F-actin to the cadherin complex to regulate cell-cell adhesion, biochemical studies of mammalian αE-catenin show that it forms a monomer that binds cadherin/β-catenin but not F-actin, and a dimer that binds strongly to F-actin but not the cadherin/β-catenin complex. It has been suggested that the local concentration of αE-catenin in the cytoplasm (~0.5μM) is sufficient for dimerization of monomers that dissociate from the cadherin complex, yet how the conformation of αE-catenin monomers and dimers affects ligand binding and how dimers are derived from monomers are poorly understood. Clues to a possible regulatory mechanism comes from a proteomic analysis of different mouse tissues that mapped phospho-serine and -threonine residues in a non-α-helical domain between the "Head" and "Tail" domains of αE-catenin: ^{631}MIR**T**PEELDD**S**DFE**T**EDFDVR**S**R**TS**VQ**T**EDD^{661}. Interestingly, these sites are not conserved in species in which α-catenin does not form a dimer (*C. elegans*, *Dictyostelium*). The 7 serine/threonine residues in this region were mutated to alanine (αEcat7A; phospho-null) or glutamatic acid (αEcat7E; phospho-mimic), and the properties of mutant proteins were compared with wild-type αE-catenin (αEcatwt). Like αEcatwt, αEcat7A and αEcat7E formed monomers and dimers in solution. Limited proteolysis showed that αEcatwt and αEcat7E were conformationally similar, although the rates of proteolysis were different. In contrast, αEcat7A monomer and dimer generated similar patterns of proteolysis, that were most similar to those of αEcatwt dimer, indicating that the conformation of αEcat7A monomer was altered to the dimer state. αEcatwt and αEcat7A monomers were rapidly (<2min) converted to dimers at 0°C or 37°C at low concentrations (>0.3 μM), but αEcat7E monomers converted to dimers only at 37°C and at higher concentrations (1-2 μM). αEcatwt, αEcat7E and αEcat7A had similar distributions at MDCK cell-cell contacts, and current studies are investigating protein dynamics. These data show that mutations in these serine and threonine residues in αE-catenin have subtle effects on protein conformation (αEcat7A monomers and dimers have a similar conformation), and dimerization (αEcat7E dimerization is slower and requires a higher protein concentration than αEcatwt or αEcat7E). We speculate that these changes may be important in dynamic control of αE-catenin functions (dimerization, F-actin binding) at the plasma membrane.

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Cadherin-23 mediates heterotypic adhesion between tumor cells and stromal fibroblasts and may represent a key initial step in the metastatic cascade.

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Adhesive contacts between invading tumor cells and stromal fibroblasts have been previously observed in cancer pathology samples, but little was known about the molecular identity or function of these heterotypic adhesions. Here we have shown that an atypical cadherin, cadherin-23, is expressed by both the weakly metastatic MCF-7 breast cancer epithelial cells and by normal breast fibroblasts. When these two cell types are grown together in a co-culture model of the tumor microenvironment, cadherin-23 is recruited to sites of heterotypic adhesion. Adhesion assays with inhibitory antibodies to cadherin-23 or after RNAi of cadherin-23 suggest that this adhesion protein is significantly responsible for mediating heterotypic adhesion between tumor cells and stromal fibroblasts. This was particularly surprising as this cadherin with 27 extracellular cadherin domains and an atypical cytoplasmic domain is unlike the type I cadherins typically associated with cell-cell adhesion, and to date, has only been described in

the neurosensory epithelium. However, we find that cadherin-23 is expressed in normal human breast ductal epithelial cells, and is unregulated in both the epithelial cells and the surrounding stroma in cancer tissue samples in a pattern that suggests it may play a role in mediating the tumor-stroma interactions that initiate the metastatic cascade.

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Regulation of collective cell invasion of prostate cancer cells by N-cadherin.

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In the United States, prostate cancer is the second leading cause of cancer death in men with a death rate of 1 in 36 prostate cancer patients (American Cancer Society, 2010). This is partly due to recurrence of metastasized cancer after surgery, radiation or androgen deprivation therapy. The dissemination of cancer cells significantly decreases the patient survival rate. Thus, the importance of finding a way to fight metastatic cancer cannot be overstated. However, little is known about how cancer cells invade and initiate metastasis. Using a highly invasive prostate cancer cell line (PC3), we sought to investigate the mechanisms by which prostate cancer cells invade in a three-dimensional (3D) Matrigel. We found that prostate cancer cells did not migrate as single isolated cells, but rather, migrated persistently as multi-cellular clusters in a single file. These data suggest that cell-cell adhesion may promote collective migration. Immunostaining showed that N-cadherin and actin co-localized at cell-cell contacts, suggesting that N-cadherin junctions mediate collective cell invasion. Furthermore, the addition of purified N-cadherin extracellular domain inhibited collective cell invasion in 3D Matrigel, but the purified cadherin-11 extracellular domain did not. In addition, the presence of purified N-cadherin extracellular domain significantly decreased the speed of single cell migration on a 2D surface. Therefore, N-cadherin may regulate cell-cell adhesion between prostate cancer cells as well as cell migration potential of prostate cancer cells. Understanding the regulation of N-cadherin mediated collective cell migration will define unique therapeutic targets to effectively minimize cancer cell invasion, and therefore, metastasis.

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The Roles of N-cadherin in Three-dimensional Cell Invasion.

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Almost 80% of cancer originates from epithelial tissue. The high mortality rate of epithelial derived cancer is partly due to the ability of tumor cells to migrate away from the initial growth site and metastasize. To metastasize, cancer cells are thought to undergo a transformation in which they lose typical epithelial cell-cell junctions and develop an invasive phenotype. Surprisingly, some invasive cancer cells appear as a multi-cellular linear cluster, and often highly aggressive cancer cells up-regulate neural (N)-cadherin cell adhesion protein, but we know very little about the roles of N-cadherin in cancer cell interactions and migration. To gain a mechanistic understanding of cancer cell invasion, we analyzed the cell adhesion between invasive, transformed epithelial cells in a three-dimensional (3D) collagen matrix. Transformed epithelial cells were transfected with shRNA specific to N-cadherin, and the N-cadherin knockdown cells were transfected with N-cadherin mutants for phenotype rescue experiments. Using 3D cell invasion assays, transformed epithelial cells formed elongated multi-cellular structures, and migrated as a collective unit. The individual cells in cell clusters migrated faster and more persistently than single cells in isolation. Calcium depletion by EDTA treatment caused the multi-cellular cluster to dissociate and induced membrane extensions in resulting single cells. Furthermore, depletion of N-cadherin disrupted cell-cell contacts and these N-cadherin deficient cells no longer migrated as a collective unit, suggesting that N-cadherin is

required for calcium dependent cell-cell adhesion and multi-cellular invasion. Interestingly, the ectopic expression of the N-cadherin cytoplasmic domain enhanced the formation of membrane extensions in N-cadherin knockdown cells, but could not rescue multi-cellular cluster formation or coordinated migration. In contrast, ectopic expression of the extracellular domain of N-cadherin resulted in the formation of small clusters. Our data suggest that the cytoplasmic signaling of N-cadherin induces membrane extensions while the presence of N-cadherin mediated cell junctions may suppress membrane extensions. This unique role of N-cadherin may distinguish the leader cells that form membrane extensions at the free leading edge, apart from the follower cells with N-cadherin junctions that suppress membrane extensions. Together, our findings suggest that the extracellular and cytoplasmic domains of N-cadherin play an important role in regulating membrane extensions, cell cluster formation, and collective migration, thus N-cadherin up-regulation may be the key requirement for metastasis.

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Effects of Neural Cells on the Expression of Semaphorin4A in cocultured Retinal Pigment Epithelial Cells.

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The retina is a complicated network of nerve cells that changes light into nerve impulses that travel to the brain where they are interpreted as visual images. The most common disease of retina is age-related ,macular degeneration (AMD). And, the damage to the retina pigmental epithelial cells (RPE) at the macular regions is an early and crucial event in the development of AMD. But, its mechanism has not been studied.
Development of multicellular organisms requires interactions between cells and their local environment and between neighboring cells.
We recently established an in vitro model based on the coculture of human corneal epithelial cells, fibroblasts, or neural cells separated by a collagen membrane.
We have now examined the role of neural cells in retinal homeostasis, and effects of neural cell to the RPE. As a results, we show that presence of neural cells (PC12) resulted in upregulation of neural guidance protein, semaphorin (semaphorin4A) in retinal pigment epithelial cells (ARPE19). Furthermore, the amounts of inflammatory cytokines, interleukin-6 (IL-6) was decreased by the presence of neural cells.
Therefore, our findings indicate that some factor(s) released from neural cells may play an important role in the regulation of intercellular communication between retinal pigment epithelial cells as well as in the maintenance of retina structure and function.
In the future, these finding provide a useful model for understanding cell-cell interactions that occur between photoreceptors and the RPE.

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Triggering Receptor in Myeloid Cells (TREM)-Like Transcript (TLT) - 1 Mediates Platelet-Neutrophil Interaction.

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Hemostasis and inflammation are intimately linked. Platelets play an indispensable role in mediating this relationship, however the mechanisms that regulate this interaction are poorly understood presenting a gap in our knowledge. Platelet α-granules are secretory granules that house the adhesion receptor P-selectin. Upon activation P-selectin is exposed and mediates primary contact with neutrophils or monocytes through interaction with the P-Selectin Glycoprotein Ligand - 1. Studies with the psel$^{-/-}$ mice reveal increased hemorrhage in response

to inflammatory challenge, and delayed neutrophil migration, demonstrating that P-selectin mediates hemostasis and platelet regulation of neutrophils. Recently, our laboratory has cloned the Triggering Receptor Expressed in Myeloid (TREM)-Like Transcript (TLT)-1, which is also found in the platelet α-granules. Expression of TLT-1 strongly correlates with P-selectin expression ($p< 0.996$). Oddly enough, characterization of treml$^{-/-}$ mice also shows a phenotype similar to the psel$^{-/-}$ mouse with the addition to a severe hemorrhage associated with lipopolysaccharides (LPS) administration in mice. This hemorrhage seems to be mediated by neutrophil deregulation. We hypothesize that, TLT-1 functions in tandem with P-selectin to mediate platelet control over neutrophils. To test our hypothesis, we are developing in vitro and in vivo methods to evaluate TLT-1 influence of neutrophil function. We used a flow cytometric assay to measure platelet-neutrophil conjugates (PNC) in wild type and treml1$^{-/-}$ mice. PNCs are known to be mediated by P-selectin. Mice were treated with LPS nasally and blood was taken from the retro-orbital plexus at 0, 1, 2, and 12 hours and the amounts of PNCs were analyzed by flow cytometry. At base line, there was no significant difference in PNCs between wild type and treml$^{-/-}$ mice. However, after 1 and 2 hours of LPS treatment, PNC were significantly higher over base line in wild type mice compared to null mice. In conjunction with our previously published data, this data supports the hypotheses that platelet-leukocyte conjugates are affected by the presence of TLT-1, and suggest that TLT-1 and P-selectin work in tandem to modulate leukocyte function. To further these studies we are, evaluating if we can use the soluble form of TLT-1 (sTLT-1) to rescue the pulmonary bleeding phenotype seen in the lungs after LPS treatment of treml1$^{-/-}$ mice. We are attempting to introduce sTLT-1 protein into the mice via hydrodynamic injection of DNA into the tail vein before LPS treatment. Soluble TLT-1 is measured in the blood by capture ELISA and pulmonary bleeding is measured by flow cytometric measurement of bronchial lavage fluid. The status of these studies is reported here.

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Cell-cell contact dependent regulatory role of RFP in integrin beta1 expression.

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Integrins play central roles in the biology such as apoptosis, growth, differentiation and cell migration by controlling cell adhesion to the extracellular matrix. Although contribution of integrins on development of neoplasia has been well studied, the mechanism how the expression of integrins is regulated remains elusive. Here we report that a transcriptional regulator RET Finger protein (RFP) regulates integrin beta1 expression in a cell-cell contact dependent manner. First, we found that RNAi-mediated knock-down of RFP caused the reduction of integrin beta1 expression in less adhesive cell line MDA-MB-231, but not in adhesive squamous cell carcinoma cell line A431. Based on the difference between these two cell lines, we hypothesized that cell-cell adhesion inhibits the function of RFP on regulation of integrin beta1. Decrease of integrin beta1 by RFP knock-down in A431 was observed when E-cadherin was inhibited by knock-down or neutralizing antibody. These results indicate that cell-cell contact affects the regulatory role of RFP in integrin beta1 expression.

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A marker of "Self "extends the circulation of nanoparticles.

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A major challenge for injecting particles or implanting biomaterials into the body is that they activate immune cells such as macrophages, the cells that normally function to clear invading pathogens. Interestingly, macrophages have a surface receptor mechanism which prevents

them from phagocytosing our own cells. During initial macrophage engulfment, macrophages recognize foreign and self targets because they both have antibodies or plasma complement proteins on their surface. However, before the macrophage engulfs the target, cells are checked for the presence of the surface protein CD47. Whether the CD47 interaction is functional with small targets of phagocytosis is unclear and relevant perhaps to nano-sized targets. Here we quantified binding and signaling of RBCs as well as soluble-CD47 to its receptor, SIRPα, inhibition of phagocytosis of RBC or CD47 coated beads, and in vivo clearance within NSG mice. The level of CD47 on the surfaces of either virus-sized beads or antibody-blocked NSG-RBCs governs circulation time in vivo, and although the affinity of human-CD47 for NSG-SIRPα is weak, it is within the range of affinities that we measure for ten human variants of SIRPα.

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Evidence for the Pre-Metazoan Evolution of Teneurins, a Family of Transmembrane Proteins Necessary for Patterning and Neuronal Development.

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Teneurins are large type II transmembrane proteins that are found at sites of pattern formation, in some basement membranes and in interconnected populations of neurons. Teneurins can be processed such that the intracellular domain (ICD) is transported to the nucleus, the extracellular domain (ECD) is shed into the extracellular matrix, and the C-terminus converted into a neuropeptide. Knockout or knockdown of teneurins in *D. melanogaster*, *C. elegans* or mouse leads to abnormal patterning, disorganization of basement membranes, defasciculation of neurites and/or abnormal neuronal development. Here we have identified and analyzed teneurins from a variety of genomes for nuclear localization sequences (NLSs), furin cleavage sites and protein interaction domains. In all bilateria teneurins have the same basic organization: 1) an ICD with or without an NLS, and 2) an ECD with EGF-like domains, a cysteine-rich domain, and a series of NHL and YD-repeats. The ICD of teneurin-1 from amniotes is predicted to have a strong NLS, while teneurins-2 and -3 have furin cleavage sites that could release the ECD. Teneurins were not found in cnidarians, placozoa or sponges, but a teneurin with a domain organization identical to teneurins from bilateria was identified encoded in the genome of the choanoflagellate *Monosiga brevicollis*. Analysis of the predicted *M. brevicollis* teneurin reveals sequence similarity between the cysteine-rich domain and enzymes from diatoms and algae, and similarity between the carboxy half of the teneurin and prokaryotic YD-repeat proteins. Moreover, most of the ECD is encoded on a single giant exon. Thus, teneurins appear to have evolved via horizontal gene transfer to an ancestral choanoflagellate from one or more of its prey, perhaps to facilitate prey capture. The appearance of this hybrid protein in a choanoflagellate may have contributed to the evolution of multicellularity and complex histogenesis in metazoa.

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Teneurin homophilic interaction and brain specific functions.

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Many key developmental processes are regulated by transmembrane proteins mediating cell-cell interactions. Teneurins are a unique family of type II transmembrane proteins conserved from Drosophila melanogaster and Caenorhabditis elegans to vertebrates, where four paralogues exist called teneurin 1 4. Using Single-Cell Force Spectroscopy (SCFS) we were able to quantify adhesion forces between single cells expressing different teneurin proteins. We were able to show homophilic, but not heterophilic interaction between teneurin expressing

cells. This suggests that teneurins are target recognition molecules for a subset of neurons. In a primary neuron co-culture assay, we showed that teneurin expressing HEK293 cells are attractive for neurites. This finding might reflect the recently discovered role for the teneurin protein family in establishing arealization and patterning in the developing embryo. These processes are regulated by a network of transcription factors, which are expressed in gradients in the developing cortex. The homeobox containing protein Emx2 is the best studied protein in the network, although direct targets involved in patterning are not established yet. We identified a novel promoter upstream of the published transcription start of teneurin-1, which is conserved in chicken and mice. We show that Emx2 directly binds to and regulates human teneurin-1 expression at this alternative promoter contributing to the patterned expression of the different members of the teneurin family[1]. Taken these results together, we were able to establish teneurins as molecules defining target areas for subsets of neurons both on the transcriptional level as well on the level of their function.

1. J Beckmann, A Vitobello, J Ferralli, D Kenzelmann Broz, F Rijli, R Chiquet-Ehrismann: Human teneurin-1 is a direct target of the homeobox transcription factor EMX2 at a novel alternate promoter. BMC Developmental Biology, 11:35.

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Characterizing osteoclast precursor fusion: contribution of E-cadherin and migration.

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Osteoclasts are giant, multinucleate cells which perform a unique role within the vertebrate system. Osteoclasts actively resorb targeted areas of bone, which is an important part of the bone remodeling process. An important step in the maturation of osteoclasts is the early fusion between multiple precursor cells. Even though this is a key commitment step during osteoclast differentiation, little is known about the mechanisms and molecules involved. Our strategy to observe individual fusion events has been to utilize long-term imaging with an epifluorescent Zeiss AxioObserver equipped with a humidified, temperature-regulated CO_2 incubation chamber throughout the 6-day differentiation process. Live-cell movies revealed that RAW 264.7 cells, stimulated with RANKL, use multiple approaches for the generation of multinucleate osteoclasts. Early differentiation, post-48 hours of RANKL treatment, was characterized by simple fusion events between small multinucleate cells with mononuclear cells. However, by 96 hours of RANKL treatment, multinucleate cells fusing with other multinucleate cells were observed, which has not been previously documented. Further observation revealed fusion preferentially occurred at the leading edge of migrating precursor cells or at sites of active pseudopod extension. Fixed and live immunofluorescent localization of E-cadherin, a potential fusogenic molecule, confirmed these observations. Accumulation of E-cadherin-mCherry at sites of cell-cell contact and prior to the fusion event was captured using live epifluorescent and confocal techniques. Application of functional blocking antibodies against E-cadherin also caused a significant reduction in overall cell fusion. These results suggest an important role for E-cadherin during early stages of osteoclast differentiation, implicate migration as a critical component for successful fusion and demonstrate the utility of live-cell techniques to study the osteoclast fusion event.

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Intercellular force transmission through adherens junctions during collective cell migration.

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In a wide variety of migratory processes, including embryonic morphogenesis, wound healing, and some forms of epithelial tumor invasion, cells migrate while maintaining adherens junctions. Transmission of physical forces through these junctions has recently been implicated in mechanotransduction and collective cell guidance, but the mechanisms of intercellular force transmission remain poorly understood. Our goal is to study the role of the E-cadherin, beta-catenin and alpha-catenin complex in the regulation of force transmission and cell velocity during migration of an epithelial cell sheet.
We knocked down cell-cell adhesion proteins in human mammary epithelial cells (MCF10A cells) by using siRNA technology. By using Traction Force Microscopy, Monolayer Stress Microscopy and Particle Image Velocimetry, we mapped cell-matrix forces, cell-cell forces and cell velocities, respectively. To ensure robust initial conditions we developed a novel collective cell migration assay based on micropatterning technology. Control cells exhibited strong and punctate traction forces at the cell-substrate interface and long ranged transmission of forces through cell-cell junctions. Depletion of E-cadherin, alpha-catenin and beta-catenin did not affect the generation of traction forces but the transmission of intercellular forces reduced. Our results provide the first direct evidence that E-cadherin, alpha-catenin and beta-catenin transmit intercellular forces during collective cell migration.

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Deformation of Endothelial Cells during Leukocyte Rolling.

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During the immune response or the atherosclerotic lesion development, leukocytes first roll on the endothelium when they migrate out of the blood vessel. During this process, membrane tethers (i.e., membrane nanotubes) can be extracted from both leukocytes and endothelial cells that line the blood vessel. Tether extraction, which has been shown to stabilize leukocyte rolling, is preceded by surface protrusion, which dictates whether tether extraction can occur. Although surface protrusion of leukocytes has been characterized, surface protrusion of endothelial cells has not. In this work, we present a detailed study of surface protrusion of human umbilical vein endothelial cells (HUVECs). Using the micropipette aspiration technique, we measured the protrusional stiffness and the crossover force during HUVEC surface protrusion. We found that, compared with leukocytes, the protrusional stiffness and the crossover force of HUVECs were both larger at similar force loading rates. The values of these two parameters depended on temperature, the cytoskeletal integrity, asymmetric dimethylarginine, and whether CD31 or CD29 was used as the force handle. However, they did not depend on cell attachment state or intracellular calcium. These results show that similar mechanisms govern surface protrusion, hence also tether extraction in leukocytes and endothelial cells. This new understanding from a biophysical viewpoint is essential for us to eventually control this critical step of leukocyte migration.

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Epithelial Contact Expansion Requires *de novo* mRNA Translation.
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Epithelial adherens junctions are important for polarized tissue assembly during development and tissue homeostasis during adulthood. Junction assembly occurs in 3 distinct steps: contact initiation, contact expansion, and junction maturation. The epithelial-specific transmembrane protein E-cadherin regulates the initiation of junction assembly by acting as a molecular sensor, detecting epithelial cell-cell contact through homophilic binding. This initial E-cadherin-mediated engagement results in contact inhibition of movement via assembly of intercellular actin cytoskeleton-cadherin-catenin adherens junction complexes through the local activation of intracellular signal transduction pathways including the Rho family GTPases RhoA and Rac1, along with the tyrosine kinase Src. In mesenchymal cells the peripheral localization of β-actin mRNA relies upon RhoA activity while its local translation relies upon Src activity. We hypothesized that epithelial adherens junction assembly requires RhoA and Src-dependent β-actin translation at E-cadherin homophilic contact sites. To test this, sparse plating assays with and without the translational inhibitors cycloheximide or puromycin were performed using epithelial IAR-2 cells. These studies demonstrated that *de novo* mRNA translation was required for progression from contact initiation to contact expansion during adherens junction assembly. To further investigate protein synthesis inhibition during adherens junction assembly, Ca^{2+} switch experiments, performed with cycloheximide or puromycin, showed that inhibiting protein synthesis significantly delayed adherens junction assembly, correlating with E-cadherin mistargeting and reduced E-cadherin/F-actin anchoring at cell contact sites. Ca^{2+} switch experiments in MDCK cells expressing a RhoA biosensor were performed to investigate the spatial correlation between adherens junction assembly sites and RhoA activity. RhoA activity became plasma membrane-localized within 30 minutes of contact initiation and persisted at the plasma membrane for at least 4 hours following contact initiation. Intriguingly, Ca^{2+} switch experiments with cycloheximide completely prevented the localization of active RhoA to the plasma membrane at all time points. Together, these data suggest that epithelial cell-cell contact stimulates the contact-localized signal transduction activity required for β-actin mRNA targeting and translation and this localization is necessary for the transition from contact initiation to contact expansion during adherens junction assembly.

Focal Adhesions and Invadosomes

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FAK recruits talin to nascent adhesions, a paradigm shift in the control of cell motility.
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Cell migration is a dynamic process that involves the continuous formation, maturation, and turnover of matrix-cell adhesion sites. Nascent adhesions form at the protruding cell edge in a tension-independent manner and are comprised of integrin receptors, signaling, and cytoskeletal-associated proteins such as paxillin and talin. Integrins generate important signals controlling cell motility mediated in part by the recruitment of focal adhesion kinase (FAK) and talin to nascent adhesions. Canonical models propose that talin functions as an intermediary in binding both integrins and FAK. It is hypothesized that talin facilitates FAK localization and

activation at adhesions. However, how and when talin and FAK form a complex remains unresolved. To investigate the dynamics of protein recruitment to nascent adhesions, we replated FAK-/- fibroblasts and ovarian carcinoma cells (with FAK knockdown) on fibronectin (15 min) to synchronize nascent adhesion formation. As detected by immunofluorescent staining, paxillin was equally recruited in the presence or absence of FAK but talin localization to nascent adhesions was dependent on FAK re-expression. By evaluating cells expressing integrin cytoplasmic domain mutations, talin nascent adhesion recruitment occurred in the absence of direct talin binding to integrins. Combined in vitro pull down, mutagenesis, co-immunoprecipitation assays identified the direct binding site of the talin head domain to FAK C-terminal domain residues 1011-1042. E1015A point-mutation of FAK selectively disrupted binding to talin without affecting FAK-paxillin association. Re-expression of FAK E1015A in FAK /- fibroblasts prevented talin localization to nascent adhesions without altering FAK recruitment and activation at adhesions. Moreover, FAK E1015A inhibited ovarian carcinoma cell motility and proteolytic talin cleavage needed for efficient adhesion dynamics. Our results support a model whereby FAK association with talin at adhesions facilitates the recruitment of a protease such as calpain that would mediate talin cleavage, trigger focal adhesion turnover, and enable cell movement. This study defines a paradigm shift and a new FAK to talin hierarchical linkage controlling cell migration.

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Differential Requirement for Vinculin in Actin Engagement and Growth of Focal Adhesions.

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Cell migration is critical for various tissue morphogenic and homeostatic processes and requires the dynamic integration of cell adhesion to the extracellular matrix (ECM), and the actin cytoskeleton. Migrating cells form nascent ECM-adhesions within their leading edge lamellipodia in association with rapid filamentous actin (F-actin) polymerization and retrograde flow. Engagement of nascent adhesions to lamellipodium F-actin flow transmits cytoskeletal force to the ECM and establishes a flow velocity gradient between the lamellipodium and the lamellum. In the lamellum, nascent adhesions undergo myosin II-dependent maturation in which they grow and increase ECM traction force. Although cytoskeletal engagement, force transmission and growth of nascent adhesions require interactions with F-actin, it is unclear whether these processes are mechanistically coupled during adhesion maturation. Here we analyze the role of the adhesion protein vinculin in these distinct aspects of adhesion maturation. Cre/loxP-mediated vinculin depletion in primary murine fibroblasts inhibited nascent adhesion formation, perturbed the segregation of lamellipodium and lamellum, and reduced ECM traction. In contrast, vinculin depletion accelerated adhesion growth. Thus, vinculin promotes nascent adhesion assembly and engagement to lamellipodial F-actin flow for ECM traction generation, but inhibits adhesion growth. Our findings show that cytoskeletal engagement and growth of ECM-adhesions are mediated by distinct mechanisms of interaction with the actin cytoskeleton, which interdependently control adhesion maturation and actin cytoskeleton dynamics during cell migration.

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Cellular mechanosensing of dynamic force and stiffness signals.

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Mechanical signals from the cellular and tissue microenvironments have been implicated in the regulation of a number of cellular processes including differentiation, migration and proliferation. However, the mechanisms by which cells sense different types of mechanical cues, such as external force or extracellular stiffness, remain an active area of study. Here, we implement an atomic force microscopy (AFM)-based force and stiffness clamp technique to dynamically and independently modulate the external force and stiffness that is presented to a single cell as it spreads onto both the extracellular matrix-coated AFM cantilever on the top and a glass coverslip on the bottom. Cells studied with this technique experience a pseudo-three-dimensional microenvironment in which horizontal spreading on the two surfaces is resisted in the vertical direction by the force of deflecting the cantilever. We characterized the response of NIH 3T3 fibroblast cells to changes in external force and stiffness in the vertical direction by tracking cell spreading, cell contraction and focal adhesion dynamics. We found that both focal adhesion dynamics and cell contraction respond rapidly to step changes in force and stiffness, with contraction rate response occurring on a timescale of less than one second. The technique we describe here provides a useful platform for investigating how dynamic mechanical inputs are interpreted by cells during mechanosensing.

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Ventral F-actin waves are coupled to a novel integrin-mediated adhesion complex.

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At the leading lamellipodium of migrating cells, protrusion of an Arp2/3-nucleated actin network is coupled to formation of integrin-based adhesions, suggesting that Arp2/3-mediated actin polymerization and integrin-dependent adhesion may be mechanistically linked. Arp2/3 also mediates actin polymerization in structures distinct from the lamellipodia, in "ventral F-actin waves" that propagate as spots and wavefronts along the ventral plasma membrane. We tested the hypothesis that, similar to Arp2/3 mediated actin polymerization in the lamellipodia, ventral F-actin waves were also coupled to integrin adhesion. We used two-color Total Internal Reflection Fluorescence Microscopy to examine fluorescently tagged F-tractin, an F-actin reporter, coexpressed with fluorescently tagged alpha V integrin. We found that integrins localize to ventral F-actin waves downstream of actin polymerization in several mammalian cell lines. These "adhesive F-actin waves" require a cycle of integrin engagement and disengagement to the ECM for their formation and propagation, and exhibit morphometry and a hierarchical assembly and disassembly mechanism distinct from other integrin-containing structures. Following Arp2/3-mediated actin polymerization, zyxin and VASP are co-recruited to adhesive F-actin waves, followed by paxillin and vinculin, and finally talin and integrin. Adhesive F-actin waves thus represent a previously uncharacterized integrin-based adhesion complex associated with Arp2/3-mediated actin polymerization. The spatiotemporal coordination of actin polymerization and integrin adhesion is important for adhesive cell migration, and our findings support the notion of an inherent mechanistic coupling between Arp2/3-mediated actin polymerization at the plasma membrane and integrin-based adhesion.

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Tension is insufficient to drive focal adhesion maturation in the absence of a dorsal stress fiber template.

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Myosin-II mediated focal adhesion maturation is crucial to modulate physical and biochemical signaling between cells and the extracellular matrix (ECM). Since myosin drives both dorsal stress fiber assembly and enhanced tension at adhesions simultaneously, the extent to which focal adhesion maturation is driven by tension or changes in actin architecture is unknown. Here, we distinguish between the roles of tension and lamellar actin architecture by selectively inhibiting dorsal stress fiber assembly, either through formin inhibition or reduced expression of alpha-actinin 1. We find that force transmission to adhesions can occur in the absence of prominent dorsal stress fibers linking adhesion plaques to lamellar actin. Force-mediated cues are sufficient to stabilize nascent adhesions to the ECM over a wide range of forces and facilitate adhesion dynamics. In the absence of dorsal stress fibers, however, focal adhesions exhibit a decreased length and lifetime relative to those in wildtype cells. In addition, force-mediated adhesion signaling is not sufficient to facilitate classic hallmarks of focal adhesion maturation including the accumulation of phosphorylated paxillin and focal adhesion kinase and the formation of fibrillar adhesions required for fibronectin remodeling. For these compositional and morphological signatures of adhesion maturation, the assembly of a dorsal stress fiber at the focal adhesion plaque must occur. Thus, myosin-mediated mechanotransduction pathways are insufficient to drive essential aspects of focal adhesion maturation in the absence of a dorsal stress fiber template.

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A novel method for the enrichment of structurally intact cytoskeleton and focal adhesions.

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The core mammalian cytoskeleton consists of actin microfilaments, focal adhesions, microtubules, and intermediate filaments, which are constructed through the assembly of free subunits of the cytoskeletal components into highly organized arrays. The biochemical changes of these proteins during assembly are incompletely understood, as the presence of both free and assembled subunits within the cell complicates analysis. Here we present a method for selectively separating soluble cytosolic proteins from adherent cells, which permits sensitive biochemical (including Western blot and mass spectrometric) and imaging analyses of the insoluble cytoskeleton and associated proteins in their native conformations.
The enrichment method entails 1) in situ permeabilization of the plasma membrane with low detergent concentration, 2) partial digestion of nucleic acids, and 3) fixation for immunofluorescence or solubilization for Western blotting or mass spectrometry. Integrity of the HeLa cell cytoskeleton was indicated by comparable rhodamine-phalloidin labeling and vimentin immunostaining in intact and enriched cells. Efficiency of extraction was indicated by complete removal of immunoreactive GAPDH. Staining for vinculin in intact cells yielded diffuse staining with punctae indicative of focal adhesions, whereas enriched cells exhibited staining solely in focal adhesions. Similarly, immunostaining for beta-catenin in intact cells resulted in staining of both cell-cell contacts and cytoplasm, whereas in enriched cells, only the cell-cell contacts were evident. Comparison of intact and cytoskeleton-enriched cells by Western blotting also indicated that GAPDH was completely extracted, whereas vimentin was entirely present in the remaining

adherent fraction. In summary, our methods will facilitate the characterization of mechanisms for regulated partitioning of cytoskeleton-associated proteins between the soluble cytosol and insoluble cytoskeleton.

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The Focal Adhesion: A Regulator of Vascular Stiffness?

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Despite the fact that arterial stiffening with age is now known to be an independent and early predictor of cardiovascular disease, relatively little is known about the contributions of different tissue components to vascular stiffness. The extracellular matrix (ECM) has traditionally been regarded as the main determinant of vascular tissue stiffness, but a recent study (Qiu et al. Circ Res. 2010) implicates the actomyosin-based stiffness of vascular smooth muscle cells as a significant contributor. Here, we investigated the role of differentiated vascular smooth muscle cells in determining tissue stiffness. We measured the stiffness of vascular tissue strips in vitro and observed that stiffness increases unexpectedly when tissue strips are treated with PP2, a small molecule inhibitor of the master focal adhesion (FA) tyrosine kinase Src. This result suggests that FAs, which mechanically link the ECM to the internal actin CSK, may regulate vascular tissue stiffness. To further confirm FA involvement, we examined tyrosine phosphorylation as a biochemical signature of FA signaling and turnover. Phosphotyrosine screening of vascular tissue homogenates via Western blots reveals roles for the FA proteins Src, FAK, and CAS in vasoconstrictor-induced signaling. To directly confirm the role of FAs in the regulation of stiffness, we used optical tweezers to measure stiffness in A7r5 smooth muscle cells by applying piconewton forces localized directly to FAs via cell-bound RGD-coated microbeads. We found that A7r5 stiffness is modulated by FAs and by A7r5 activation state. Stimulation with the vasoconstrictor lysophosphatidic acid (LPA) increases stiffness in a PP2-attenuated manner. Cell immunofluorescence imaging suggests that A7r5 stiffness is related to FA size as well as FA stiffness, since FA size increases in a PP2-attenuated manner with LPA stimulation. Our findings indicate that the FA is a significant component of vascular stiffness. Support: NIH HL86655, HL8003, HL31704.

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Imaging the Cell-Basement Membrane Interface during Anchor Cell Invasion in C. elegans.

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Basement membranes are dense, sheet-like forms of extracellular matrix that underlie all epithelia and endothelia and function in part as natural barriers to cell migration. Despite the impediment posed to migrating cells, specialized cells transmigrate or invade through basement membranes during development, immune surveillance and cancer. Because the complex environments where most cell invasions occur are difficult to experimentally access, researchers have predominately used *in vitro* systems to model cell invasion. These studies have provided considerable mechanistic insight into cell invasion, identifying actin-based membrane structures called invadosomes, which localize to sites of matrix degradation at the ventral surface of invasive cells. It has been postulated that invadosomes are critical to cell invasion through basement membrane, however, whether these structures exist in invading cells *in vivo* and how they would be used to breach a native basement membrane is unclear.

Anchor cell invasion into the vulval epithelium in *C. elegans* provides a simple *in vivo* model of cell invasion through basement membrane that uniquely combines predictability, ease of

visualization and tractable genetic analysis. We have used 3D time-lapse microscopy to focus on the cell-basement membrane interface over the time-course of anchor cell invasion. Using this accessible model, we have identified dynamic actin-based membrane protrusions that mediate the initial stages of basement membrane penetration by the anchor cell. These structures share striking similarities with invadosomes– the invasive membrane structures found in many cell types when cultured *in vitro* or *ex vivo*. The predictability of the event, inherent to the worm's stereotyped development, allows for rigorous quantitative analysis of the dynamics (*i.e.,* numbers, sizes, lifetimes) of these structures and their relationship to the initial breach of the basement membrane. While these structures closely resemble invadosomes that have been described *in vitro*, we have also observed important differences. For example, we find that typically one or two holes are generated in the basement membrane, as opposed to numerous holes being degraded in the matrix. This work provides some of the first clear evidence of invadosome-like structures being used during basement membrane transmigration *in vivo*. Moreover, the powerful genetic tools of *C. elegans* are allowing us to rapidly screen for genes required for invadosome formation and that modulate invadosome dynamics and activity.

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How To Make a Pretzel: the Molecular Machinery That Orchestrates Synaptic Maturation.

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During the formation of neuromuscular junctions NMJs), postsynaptic acetylcholine receptors AChRs) initially cluster in plaque-shaped aggregates that are subsequently transformed into a complex, pretzel-shaped topology. The machinery that orchestrates this transformation remains to be unraveled. We are using cultured myotubes, in which the plaque-to-pretzel transition occurs aneurally, to elucidate its mechanism. Unexpectedly, we found that muscles utilize podosomes, dynamic actin-based organelles, to remodel postsynaptic clusters. Synaptic podosomes influence organization of AChRs by degrading the underlying extracellular matrix (ECM), which is essential for the stability of AChR at the cell surface. As a next step, we are focusing on two proteins, LL5beta (LL5b) and alpha-dystrobrevin-1 (aDB1), which we showed to be components of podosomes(Proszynski, PNAS, 2009) and implicated in AChR organization Grady, JCB, 2003; Kishi, JCB, 2005). We are now using proteomic methods to seek their binding partners. We have identified Flii, Amotl2 and Asef2, all of which organize actin and regulate Rho GTPases, as LL5b interactors. All three interacting proteins are localized to the podosomes and RNAi experiments demonstrated that Amotl2 is essential for podosome formation in Src3T3 cells. We are currently characterizing the roles of these novel podosome components, as well as aDB1 interactors, in AChR clustering using RNA interference.

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TGF-β-induced invadosomes in human trabecular meshwork cells.

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Primary open-angle glaucoma (POAG) is a leading cause of blindness due to chronic degeneration of retinal ganglion cells. It is associated with elevated intraocular levels of TGF-β2, aberrant extracellular matrix (ECM) deposition and increased outflow resistance in the ocular trabecular meshwork (TM). Cell-matrix interactions have a decisive role in TM maintenance and it has been suggested that TGF-β-induced MMP-inhibition may drive ECM deposition in POAG. Invadopodia and podosomes (invadosomes) are distinct sites of cell-matrix interaction and

localized matrix-metalloprotease (MMP) activity. Here, we report on the effects of TGF-β2 on invadosomes in human trabecular meshwork cells. Human TM (HTM) cells were derived from donor tissue and pretreated with vehicle or TGF-β2 (2ng/ml) for 2d. Invadosomes were studied in ECM degradation assays, protein expression and MMP-2 activity were assessed by western blot and zymography and ECM protein transcription was detected by RT-qPCR. HTM cells spontaneously formed podosomes and invadopodia as detected by colocalization of Grb2 or Nck1 to sites of gelatinolysis. Pretreatment with TGF-β2 enhanced invadosomal proteolysis and zymographic MMP-2 activity as well as MMP-2, TIMP-2 and PAI-1 levels in HTM cell culture supernatants. Rho-kinase inhibition blocked the effects of TGF-β2. Concomitant transcription of fibronectin and collagens-1, -4 and -6 was increased by TGF-β2. In contrast to a current hypothesis, our data suggest that TGF-β2 induces an active ECM remodelling process in TM cells, characterized by concurrent increases in localized ECM digestion and ECM expression. Invadosomal cell adhesion and signaling may have a role in POAG pathophysiology.

Bioengineering of Cell-Matrix Interactions

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Molecular dissection and reconstruction of laminin-111 using synthetic peptides conjugated chitosan.

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Laminins, a major component of basement membrane and consisting of alpha, beta, and gamma chains, exert multiple biological functions through interactions with cell surface receptors and with other matrix molecules. Five alpha chains (alpha1- alpha5), three beta chains (beta1-beta3), and three gamma chains (gamma1-gamma3) have so far been identified and they comprise at least 19 different laminin isoforms (laminin-111 to laminin-523). Each laminin isoforms are tissue- and/or developmental stage-specifically expressed and play their own laminin isoform-specific functions. Laminins-111, consisting of laminin alpha1, beta1, and gamma1 chain, plays a critical role during embryonic development and in maintaining the many biological functions of basement membrane. We previously identified about 60 active sequences from laminin-111 through the peptide screening method using over 673 peptides covering the entire sequence of laminin-111. Further, some biological active peptides from laminin-111 had been used to develop the artificial cell sheet by conjugated on chitosan matrix and found that cell-peptide-chitosan sheet accelerate the wound healing. Here, we conjugated 60 cell adhesive peptides from laminin-111 on chitosan matrix and assessed their biological functions to analyze the peptide specific functions. Twenty-six cell adhesive peptide-conjugated chitosan matrices promoted human dermal fibroblasts (HDFs) attachment and twelve peptide-chitosan matrices showed cell spreading. HDF attachment to nine peptide-chitosan matrices was inhibited by heparin and to twelve peptide-chitosan matrices was inhibited by EDTA. Additionally, HDF attachment to several peptide-chitosan matrices was inhibited by anti-beta1 integrin antibodies. Further, seventeen peptide-chitosan matrices promoted neurite outgrowth with PC12 cells. These results suggest that peptide-chitosan matrices exhibited different activities depending on peptide specific manner. We could categorize these active peptide-chitosan matrices into eight groups depend on their biological activities. Next, we conjugated five HDF active peptides, which showed the strongest HDF adhesive activity in the each group, on a chitosan matrix to mimic the multifunction of laminin-111. The mixed peptides-chitosan matrix significantly promoted cell attachment and cell spreading as laminin-111. The peptide-chitosan matrices interact with different cell surface receptors in a peptide dependent manner.

These cell adhesive peptide-chitosan matrices could use to analyze the cellular functions mediated by specific cell surface receptors and have a potential to use as a biomedical material.

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High Cell Aspect Ratio Alters Stem Cell Traction Stresses and Lineage.

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Degenerative diseases, such as muscular dystrophies, often cause abnormal stiffening as a result of fibrosis. Adult mesenchymal stem cells (MSCs) "feel" this stiffness and differentiate in response to it; thus aberrant stiffness could misdirect MSCs into the wrong lineage. Conversely, MSCs have been shown to respond to changes in area and shape in a myosin-dependent mechanism: cells respond to adipogenic and osteogenic media when spread cell area changes from 10^3 to 10^4 μm^2, or when cultured as circles versus rectangles, and polygons, respectively. When both cues are present in a disparate fashion, e.g. highly elongated cells similar to muscle despite the presence of an abnormally stiff microenvironment, we hypothesized that a myosin contraction-dependent balance could induce a subset of the MSC population to differentiate in to a muscle-like phenotype despite residing in a dystrophic-like stiffness. Using microcontact printing to regulate MSC morphology, we patterned fibronectin with polydimethylsiloxane molds in shapes of varying aspect ratios but common area on polyacrylamide substrates of known stiffness. Traction force microscopy (TFM) was used to monitor substrate strain energy from cell-generated normal and tangential forces. Cells on patterns were also fluorescently stained for focal adhesion kinase and vinculin to overlay with traction force measurements and separately with muscle-specific myosin heavy chain (mMHC) to identify early muscle differentiation. MSCs spread to the stamped patterns after two days of culture and localized their focal adhesion in a stiffness and shape-dependent manner. While strain energy scaled with stiffness, it decreased as a function of cell elongation with isotropic cell patterns producing the highest contractile energy in contrast to our hypothesis. mMHC also was expressed in a stiffness and elongation dependent manner. On muscle-like stiffness of 11 kiloPascals (kPa), cells with only minimal elongation, i.e. 1:1 and 3:1 patterns, expressed mMHC most strongly. In contrast on osteogenic-like matrices of 34 kPa, highest MHC expression corresponded to the most elongated patterns. These shape- and stiffness-dependent lineage changes with muscle markers correlated to contractility-based observations suggest that muscle induction may be possible in non-permissive stiffer environments. These results are suggestive of a subpopulation of MSCs that is more responsive to an elongated shape than pathogenically stiff matrix, and could prove to be beneficial for regenerative therapies for fibrotic muscle diseases. Efforts to test this newly identified subpopulation of MSCs in the *mdx* mouse model of muscular dystrophy are ongoing.

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In Vivo Application of Dynamic Hyaluronic Acid Matrix For Myocardial Infarction Therapy.

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Tissue-specific elasticity arises from developmental changes that occur in the environment over time, e.g. ~10-fold myocardial stiffening from E3 to E10 in the chick embryo. Recently, we have shown that pre-cardiac mesodermal cells plated on top of a thiolated hyaluronic acid (HA) matrix engineered to mimic this time-dependent stiffening improves cardiomyocyte maturation compared to cells on static compliant matrices. Here we determined cell-matrix interactions using *in vitro* encapsulation assays and *in vivo* injections. Improved pre-cardiac and embryonic

stem cell (ESC) distribution and viability was observed when cells were encapsulated and bound to immobilized, thiolated fibronectin conjugated to the HA matrix. Though not toxic to cells, we also assessed HA's local and systemic biocompatibility. Prior to assembly, HA was injected subcutaneously into Sprague-Dawley rats and samples were removed over a post-injection time course and subject to histological, immunological, and mechanical analysis. Histological analysis showed minimal infiltration of host cells and capsule formation around the matrix. Hematological analysis showed no significant systemic immune response was elicited in pre- vs. post-injection animals for all time points. Most importantly, atomic force microscopy (AFM) analysis showed dynamically increasing hydrogel stiffness over time similar to that previously found *in vitro*. Finally before cell-matrix co-injection into infarcted tissue, we sought to determine the appropriate time of injection into the stiffened infarct; thus post-infarct remodeling in a total occlusion rat model was monitored using histology and AFM. Minimal infarct remodeling occurred 48 hours-post occlusion, but sufficiently elevated stiffness was observed alongside wall thinning and collagen deposition two weeks post-infarct (monitored out to two months), which suggests two separate approaches with combined HA matrix and ESC or pre-cardiac cell therapy: injection before and after wall thinning to encourage either the prevention of thinning or restoration of myocardial contraction. These *in vitro* data indicate that the combination of cells and developmentally appropriate matrix stiffness may significantly improve cell differentiation while *in vivo* data indicate the injectable feasibility of the HA matrix and an optimal injection time to restore function and prevent myocardial remodeling.

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Fibrillar collagen is equivalent to stiff matrix in driving marrow stromal cell differentiation into matrix-deficient, myofibroblastic-like phenotype.

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Scars tend to be stiffer than normal tissue, which has prompted the use of stiff matrices as models of scars, but scars are also rich in fibrillar collagen-I. Here, we introduce a soft matrix embedded with distinctly fibrillar collagen type I, and show that this is sufficient to drive bone marrow derived stromal cells (MSCs) into a contractile, myofibroblastic-like phenotype – 'myo-MSCs'. These cells have been reported to minimize scarring in a unique wound healing response, exemplified by their application to myocardial infarcts [1]. Transcriptome analysis in response to matrix rigidity points to an upregulation of genes that participate in the cellular contractile machinery, notably α-smooth muscle actin, but a decreased expression of matrix protein genes for collagens type I and VI. MSCs cultured on the embedded-fiber, soft matrix exhibit many similarities to cells on rigid substrates. Phosphorylation at serine-1943 of non-muscle myosin IIA, which deactivates stress fiber assembly, is decreased on both fibrosis-like and rigid substrates but almost twice higher on a soft substrate. Interestingly, nano-indentation measurements of these 'myo-MSCs' hint that, unlike myofibroblasts, they do not become hyper-contractile. This supports the notion that MSC engraftment into wounded tissues suppresses fibrosis, highlighting the promise of these cells in restoring normal tissue function.

[1] Berry, et al. Am J Physiol Heart Circ Physiol 290:H2196-H2203, 2006.

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Investigating the role of vinculin in mechanosensitive stem cell differentiation.

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Human mesenchymal stem cell (hMSC) proliferation, migration, and differentiation have all been linked to extracellular matrix stiffness, but despite serious scientific inquiry, a consensus on the signaling pathways that are necessary and sufficient for this mechanosensitive ability has yet to be reached. An analysis of kinase binding site accessibility revealed MAPK1 to be both prevalent and inaccessible in many focal adhesion/mechanosensing candidate proteins. One binding partner for MAPK1, vinculin, is activated upon binding to talin in a force-sensitive manner. A Cysteine Shotgun/Western Blot was used to confirm the unfolding of vinculin in response to changes in substrate stiffness. RNA interference was used to knock down vinculin, resulting in an 80% decrease in stiffness-induced MyoD, a muscle transcription factor; control cultures that induce Runx2 expression, an osteoblast transcription factor, were insensitive to vinculin knockdown. Vinculin knockdown was not observed to interfere with focal adhesion assembly, alter adhesive properties, or diminish cell traction force generation, indicating that its deletion only adversely affected MAPK1 signaling. In addition, vinculin domains were also selectively deleted and added back into knocked-down cells to assess which domains of the protein were sufficient for stiffness-induced differentiation. Together, these data provide some of the first in situ evidence that force-sensitive focal adhesion proteins can activate stem cell differentiation signals.

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Elastomeric Substrate Panel for Investigation of Cell Adhesion, Proliferation, and Morphology.

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The rigidity of the microenvironment provides important mechanical cues in directing cellular processes, including cell adhesion, spreading, migration, cytoskeletal organization, growth, differentiation, apoptosis, and tissue morphogenesis. Such cellular behavior can result from a complex combination of inputs (cell shape, mechanical forces, chemical cues, etc.), but an elastic matrix assay may facilitate isolating a component of these mechanical cues from chemical signals. In our studies, we have sought to develop a convenient platform of substrates of varying elasticity to investigate changes in cellular behavior caused by mechanically distinct surfaces. Our platform utilized a chemically stable polymer base, polydimethylsiloxane (PDMS), cast and cross-linked within 24-well polystyrene plates for ease of use in the cell culture laboratory. The PDMS was formulated into a range of flexible substrates, which were characterized via parallel plate rheometry. Rheological data demonstrated formulation elastic moduli in the range of 3 kPa to 3 MPa that were stable and consistent over an extended time in storage and across multiple batches of prepared material. PDMS surfaces were first coated with fibronectin to allow for cell adhesion, and then seeded at low density with fibroblast or smooth muscle cell types (primary and immortalized cell lines). The initial extent of cell attachment and spreading were quantified utilizing an acid phosphatase assay and fluorescent imaging, respectively. Cell proliferation and growth distribution were also analyzed following multiple days (~96 hours) in culture. Although the amount of initial (<24 hour) cell adhesion appeared to be comparable between PDMS substrates, average area per cell was observed to decrease with decreasing elasticity/stiffness, and histograms of cell circularity (or roundness) displayed variations in the distribution of cell shape in a surface elasticity-dependent manner. Stiffness-dependent trends in growth were also observed, suggesting that these PDMS substrates may

be successfully utilized in the analysis of mechanical contributions to cellular behavior. A thoroughly characterized elastic matrix panel has the potential to greatly facilitate and increase the reproducibility and throughput of a variety of biomechanical investigations.

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Three-Dimensional Hyaluronic Acid-Based Hydrogel Systems for Mechanistic Studies of Prostate Cancer.

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We developed a cell culture system using three-dimensional (3D) hyaluronic acid (HA)-based hydrogels optimized to grow bone metastatic prostate cancer (PCa) cells. The choice of HA is consistent with the prevalence of HA within the bone marrow microenvironment, providing a typical environment for bone metastatic PCa cells. Correctly simulating the native environment provides more physiologically relevant mechanistic data about the biology of PCa bone metastasis. The biocompatible cross-linking process used allows PCa cell lines to be directly encapsulated within the HA hydrogel where their growth is limited only by time and space. Cells representing distinct stages of metastasis were encapsulated, where patterns of cell growth were found to differ depending on the aggressiveness of the PCa cells. Aggressive PCa cell lines invaded the hydrogel displaying clear filopodia, while less-aggressive PCa cells did not similarly form these invasive processes. Because the HA hydrogel can interact with HA's two receptors (cluster of differentiation 44 (CD44) and receptor for hyaluronan-mediated motility (RHAMM), we studied the impact of 3D culture in the HA hydrogel on localization of the HA receptors and on the activation of pathways downstream of these receptors. A particular focus was on the downstream pathways able to promote invasion and migration of PCa cells during metastasis. We will show the patterns of activation of the Rho GTPases and their downstream effectors in cells grown in 3D hydrogels compared to cells grown conventionally in tissue culture plates. This work highlights the importance of using physiologically relevant culture conditions to simulate a native process *in vivo*.

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Distinct responses to cyclic strain by fibroblasts confined to square versus rectangular fibronectin micro-patterns on elastomer membranes.

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Fibroblasts sense mechanical cues arising from the extracellular matrix via integrin receptors, which transmit forces to the cytoskeleton and transduce them into downstream chemical signals. As we have shown previously, when fibroblasts grown on fibronectin-coated elastomer substrates are subjected to cyclic strain, RhoA is activated, actin stress fibers increase, and the cells contract. This response is more efficient if cells are pre-stressed, i.e. already develop some cytoskeletal tension. Cytoskeletal pre-stress is known to be related to cell shape. On a fibronectin square of limited size, cells form stress fibers along the edges and in the diagonals, and maximal force is localized to focal adhesions at corners. We hypothesized that by manipulating cell shape and hence the arrangement of cell-ECM adhesions, different levels of pre-stress might be generated within cells, which should change their response to externally applied cyclic strain. To this aim, we micro-contact printed fibronectin squares and rectangles of the same area (2000 μm^2) but with different aspect ratios (1:1, 1:2 and 1:4) onto elastomer

membranes. When mouse embryo fibroblasts were plated on the fibronectin patches and allowed to spread, the majority assumed the shape of the patch. We then applied 90 cycles of 10% cyclic strain over 30 min with a custom-made device, fixed the cells, and monitored the changes in cell shape, cell-matrix adhesion points and actin cytoskeleton by anti-vinculin and phalloidin staining. For cells on fibronectin squares, we observed a 40% decrease in total cell area (indicating contraction), a 2-fold increase in the area and a 4-fold increase in integrated intensity of stress fibers after cyclic strain. In about half of the cells on fibronectin squares, cyclic strain induced their polarization along one of the diagonals. Contrary to our expectation, for cells on rectangles (1:4) we could not measure significant differences in the same parameters after cyclic strain, although a redistribution of stress fibers was visible by eye. In summary, our results indicate that cells forced into square (non-polarized) or rectangular (polarized) shape, respectively, react to externally applied cyclic strain with distinct changes in their actin cytoskeleton. However, the rules and mechanisms remain to be determined.

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Mapping mechanical stresses reveals their role in morphogenesis of three-dimensional epithelial tissues.

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Mechanical forces regulate a variety of cellular and morphogenetic processes and drive the changes in shape which sculpt tissues and organs. Deregulation of the mechanical environment can contribute to malignant transformation and progression. Quantitative information about the mechanical forces imparted and experienced by cells and tissues in a physiologically relevant context is important to understand the physical basis of development and disease. Although early development and organogenesis are largely epithelial phenomena and epithelial tissues are the sites of numerous diseases, efforts in mechanobiology have been focused on measuring the forces generated by single cells. Here, we used three-dimensional (3D) traction force microscopy to measure the mechanical stresses exerted by epithelial tissues of arbitrary geometry, embedded within native collagen matrices. Quiescent epithelial tissues were found to exhibit emergent mechanical behavior, different from the individual behavior of their constituent cells. The traction present at the cell-matrix interface was found to be non-uniformly distributed across the epithelium. The mechanical profile of the tissues was dictated by the epithelial geometry, with certain geometrical features consistently exhibiting high levels of traction. Intriguingly, contraction of the epithelial tissue could cause compressive stresses to arise in the microenvironment. The use of native collagen matrices enabled us to recapitulate physiologically relevant tissue-mediated changes in the local material properties. We found that these non-uniformities have a profound effect on the mechanical profile of the tissues. The method presented here allows us to directly measure the mechanical stresses generated by 3D epithelial tissues, define the parameters which govern epithelial force generation and subsequently fabricate tissues with precisely tuned mechanical profiles. The spatially resolved measurement and controlled application of mechanical stress are powerful tools for elucidating the mechanical regulation of epithelial morphogenesis and disease.

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Substratum Compliance and Latrunculin B Regulate Matricellular Gene and Protein Expression in Human Trabecular Meshwork Cells.

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Objective: To determine how substrate compliance influences the matricellular proteins SPARC (serine protein, acidic, and rich in cysteine) and myocilin (Myoc) when human trabecular

meshwork cells are treated with Latrunculin B (Lat B). Lat B disassembles actin cytoskeleton and is currently in clinical trials to reduce intraocular pressure for the treatment of glaucoma.
Methods: Human trabecular meshwork (HTM) cells from three different donors were cultured on hydrogels that are similar to glaucomatous HTM (75 kPa) and on tissue culture plastic (TCP – gigaPascal compliance). Cells were exposed to 2 µM Lat B or vehicle alone (DMSO) for thirty minutes. Seven hours after recovery from Lat B, RT-PCR was done to measure changes in gene expression of SPARC and Myoc. Immunofluorescence was used to compare changes in mRNA to changes in protein expression.
Results: SPARC and Myoc mRNA expression were dramatically increased on the 75 kPa hydrogels compared to TCP. Even on the cells from the least responsive donor, SPARC was increased 2.7 fold and MYOC increased 11.9 fold. Treatment with Lat B significantly decreased the expression of both SPARC and Myoc on the hydrogels. In contrast, cells grown on TCP produced similar amounts of SPARC and Myoc mRNAs under control and treatment conditions. The changes in immunofluorescence of these two matricellular proteins paralleled changes measured by mRNA.
Conclusions: Substratum compliance directly impacts matricellular gene and protein expression and dramatically alters the effects of Lat B exposure. Our results illustrate that the use of stiff TCP may yield incorrect results when employed for *in vitro* studies of HTM cells.

1596

Optimization of cell adhesion, cell spreading and extracellular matrix assembly on mixtures of immobilized fibronectin domains.

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Our research was motivated by the challenge of designing for multiple biological functionalities on synthetic scaffolds. We investigated the influence of varying mixture compositions of a fibronectin and cell binding domains, III_{1-2} and III_{9-10} respectively, on cell adhesion, cell spreading and the amount of fibronectin in the extracellular matrix (ECM) on activated polyurethane surfaces. We used enzyme-linked immunosorbent assays (ELISAs) to detect III_{1-2} and III_{9-10} on the surface and identified a design space defined by the maximum and minimum limits of protein loading. We then obtained a coverage design from the design space; that is, a set of III_{1-2} and III_{9-10} mixture compositions that uniformly covered the design space. NIH-3T3 fibroblasts were cultured on surfaces treated with the coverage design. Cell adhesion and spreading was measured through fluorescence microscopy and the amount of fibronectin in the ECM was determined through immunoblotting. Cell adhesion, spreading and ECM fibronectin were dependent on the ratio of III_{9-10} to III_{1-2}. Models fit to the data were significant ($p<0.05$) and showed maximum values for cell adhesion and ECM fibronectin when III_{1-2} and III_{9-10} were equal. The results obtained from this demonstrate a strategy for the instructive design of multiple biological functions on synthetic scaffolds using different protein domains.

1597

Characterization of Schwann Cells in Self-Assembled Sheets from Thermoresponsive Substrates.

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Schwann cells are the vital glial cells in the development and regeneration of the peripheral nervous system (PNS). Recently, Schwann cell transplantation has emerged as one of many attractive candidates in treating demyelinating diseases resulting from injuries in the central nervous system (CNS) and PNS. Schwann cells for transplantation are usually injected as cell

suspensions or seeded on scaffolds composed of ECM proteins or biodegradable polymers. In these approaches, the adherence junctions between Schwann cells present in vivo are not readily replicated as Schwann cells in suspensions or in scaffolds dispersed as individual cells. Here we describe a procedure to grow large amounts of Schwann cells in a sheet architecture that can be either transplanted or injected, and try to answer some fundamental questions regarding cell viability, proliferation, cell cycle, adhesion, and migration of Schwann cells in the cell sheet. The Schwann cell sheet was successfully generated through coating culture surfaces with layer-by-layer self-assembly of temperature responsive polymer (poly-N-isopropylacrylamide) (PNIPAAM). Further characterization of Schwann cell sheet showed that Schwann cells in sheet were highly viable and maintained lower proliferation rate than individual Schwann cells. The protein level of a cyclin-dependent kinase inhibitor, p27, is increased in Schwann cell sheet. Expression of cadherins and alignment with axon-like nanofibers during adhesion and migration by Schwann cells in cell sheets were not significantly different from these by individual Schwann cells. We conclude that Schwann cell sheet engineering presents a promising method for cell-based nerve injury therapy as well as a model to study the role of interactions between Schwann cells in Schwann cell behavior, i.e. proliferation.

1598

Biomechanical and Biochemical Regulation of Muscle Stem Cell Expansion in Bioengineered Niches.

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Adult muscle stem cells (MuSCs) are a relatively rare cell type and their stem cell properties are rapidly lost once plated in culture limiting their clinical utility. We hypothesized that the long term culture of MuSCs on surfaces mimicking key features of the in vivo microenvironment would be able to increase their viability, promote division, and maintain their in vivo function. To this end, we employed a novel bioengineered hydrogel culture platform in conjunction with a newly developed automated image analysis computer algorithm and assayed the in vitro and in vivo behavior of MuSCs cultured on hydrogels with different biomechanical and biochemical microenvironmental cues. We found that culture upon soft hydrogel that mimics muscle elasticity greatly enhances MuSC viability and prevents their differentiation compared to rigid plastic. Further, transplantation studies revealed that MuSCs exposed to a soft hydrogel surface with mechanical properties similar to native skeletal muscle self renew and retain similar regenerative potential to freshly isolated MuSCs. Though maintenance of self-renewal potential was achieved using this culture paradigm, MuSCs demonstrated a somewhat limited proliferative capacity. As such, recent efforts focused on identifying biochemical cues that promote MuSC proliferation and expansion in the context of our biomimetic culture platform. Here we report that pharmacological manipulation of key signaling pathways in conjunction with culture upon a biomimetic hydrogel substrate can support MuSC expansion in culture. Our results establish parameters for the long term culture and self renewal of MuSCs in vitro that maintain MuSC function in vivo; an essential step to translate MuSC biology to the clinic for treatment of human muscle degenerative disorders.

1599

Quantitatively tuning cell motility, mechanics, and matrix remodeling by graded genetic activation of RhoA.

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The small GTPase RhoA is a well-known activator of actin polymerization and stress fiber formation and has been shown to regulate a number of cell behaviors, including cell migration, contraction, and extracellular matrix (ECM) remodeling. How these behaviors vary quantitatively with the level of RhoA activation, however, is largely unknown since most direct manipulations of mechanotransductive signaling in living cells have focused only on turning specific proteins "on" or "off". Thus, exploring the effects of more measured changes in RhoA activation will advance our quantitative understanding of mechanotransductive signaling and could reveal important nonlinear relationships (such as an optimal activity level for migration). Towards this goal, we have developed a genetic strategy for modulating the activity of RhoA and its downstream effectors by placing mutant genes under a tetracycline-repressible promoter. The expression level of the mutant proteins can be varied in a graded and dynamic fashion by simply changing the concentration of tetracycline in the cell culture media. Here we have introduced the genetic constructs into human glioblastoma cells to investigate the quantitative dependence of cell motility and mechanics on RhoA signaling in the context of glioblastoma multiforme, a very invasive and lethal brain cancer. We show that graded increases in RhoA activation translate to graded increases in stress fiber assembly, cellular stiffness, and traction force generation. We also show that by temporally varying RhoA activity in cells cultured on collagen hydrogels, we can dynamically control complex cell behaviors, including cell migration, invasion, and ECM remodeling. Thus, in addition to providing quantitative relationships between mechanotransductive signal activation, cellular mechanical properties, and dynamic cell behaviors, this genetic strategy could be used to manipulate cellular mechanobiology both as an experimental tool and as a means of directly controlling cell behavior in bioengineering applications.

1600

Elucidating the mechanobiology of malignant brain tumors using brain matrix-mimetic hyaluronic acid hydrogels.

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Glioblastoma multiforme (GBM) is a malignant brain tumor characterized by diffuse infiltration of single cells into the brain parenchyma, which is a process that relies in part on aberrant biochemical and biophysical interactions between tumor cells and the brain extracellular matrix (ECM). A major obstacle to understanding ECM regulation of GBM invasion is the absence of model matrix systems that recapitulate the distinct composition and physical structure of brain ECM while allowing independent control of adhesive ligand density, mechanics, and microstructure. To address this need, we synthesized brain-mimetic ECMs based on hyaluronic acid (HA) with a range of stiffnesses that encompasses normal and tumorigenic brain tissue and functionalized these materials with short Arg-Gly-Asp (RGD) peptides to facilitate cell adhesion. Scanning electron micrographs of the hydrogels revealed a dense, sheet-like microstructure with apparent nanoscale porosity similar to brain extracellular space. On flat hydrogel substrates, glioma cell spreading area and actin stress fiber assembly increased strongly with increasing density of RGD peptide. Increasing HA stiffness under constant RGD density produced similar trends and increased the speed of random motility. In a three-dimensional (3D)

spheroid paradigm, glioma cells invaded HA hydrogels with morphological patterns distinct from those observed on flat surfaces or in 3D collagen-based ECMs but highly reminiscent of those seen in brain slices. This brain-mimetic model system will be used to investigate the mechanisms underlying ECM mechanobiological regulation of brain tumor progression.

1601

Vascular Endothelial Cell Inflammatory Pathways are Dynamically Regulated by Basal and Apical Biophysical Cues.

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Over one third of all deaths in the United States were attributable to cardiovascular disease (CVD) in 2006 and the total direct and indirect cost of CVD is estimated at $503.2 billion in 2010. Human vascular endothelial cells (VECs), involved in the onset and progression of CVD, are exposed to biophysical cues on their apical (shear flow) and basal (compliance and nanoscale topography) surfaces. Previous research from our laboratories and others has demonstrated intrinsic biophysical cues of the extracellular matrix profoundly modulate a host of fundamental VEC behaviors. Importantly, the attributes of the biophysical environment of VECs are known to be altered in CVD. Shear flow also regulates VEC behavior, with unidirectional flow inducing an anti-inflammatory cell phenotype and multidirectional flow leading to a pro-inflammatory profile. Despite the fundamental importance of biophysical cues, a significant knowledge gap remains in our understanding of how these biophysical cues are integrated when provided simultaneously to both apical and basal surfaces. Furthermore, how these cues modulate VEC responsiveness to inflammatory mediators remains unknown. Yorkie-homologues YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) are both mediated by biophysical cues and serve as a pair of transducers linking cytoplasmic signaling events to transcriptional regulation in the nucleus. Importantly, they have recently been implicated as nuclear relays of mechanical signals exerted by substratum rigidity. We hypothesize that the biophysical attributes of substratum topography and local stiffness will influence VEC behavior, gene expression, and inflammatory response in an interdependent manner in the presence of either apical unidirectional or multidirectional shear flow. We also hypothesize that YAP/TAZ are critical mediators of the VEC response to both apical and basal biophysical cues. Exciting data demonstrate a dynamic interaction between multiple biophysical cues (shear flow, physiologically relevant substratum topography and compliance) that ultimately result in modulation of endothelial cell behaviors. In addition, data demonstrate that substratum compliance and nanoscale topography significantly modify YAP/TAZ expression. Patterned substrates with 200 nm wide ridges and grooves (400 nm pitch) were found to down-regulate YAP by 1.7 fold and TAZ by 3.2 fold compared to planar surfaces. Similarly, downstream proteins that are either regulated by or influence the YAP/TAZ pathway are also modulated by biophysical cues including ICAM-1, CTGF, KLF-2, and KLF-4 (participants in VEC inflammatory response). Hydrogels with stiffer (75 kPa) compliance values were found to significantly up-regulate ICAM-1 expression in HAECs when treated with TNFa in contrast to 700 nm wide ridges which were found to down-regulate expression. Overall, these data demonstrate that multiple biophysical cues regulate VEC behavior and expression. The results of these studies contribute to our understanding of biophysical cues and vascular biology, have relevance to the design of improved vascular prosthetics and may identify new therapeutic targets for the treatment of CVDs.

1602

A fibrous poly-L-lactic acid substrate promotes mesenchymal phenotype in human epithelial breast cancer cells.

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It has become increasingly clear that the tumor microenvironment is a key component in the regulation of cancer progression. However, the effect of physical, acellular cues on cellular phenotype remains poorly understood. The stromal tissue surrounding most epithelial cancers are largely comprised of fibrous extracellular matrix such as type I collagen. Here we developed an in vitro model system to test the effect of the fibrous environment alone, independent of ECM composition, on cell morphology and behavior. We grew cells directly on an acellular fibrous material made up of electrospun poly-L-lactic acid (PLLA), and then examined the effect of the fibrous environment upon growth rate, cell morphology, substrate adhesion, cytoskeletal arrangement, and epithelial to mesenchymal transition markers. We compared the growth of the phenotypically normal breast epithelial cell line MCF10a and the breast cancer cell line MDA-MB-231 on three different substrates: glass coverslips, coverslips coated with a thin film of PLLA or coverslips upon which fibers of PLLA had been electrospun. The role of fibers in promoting a more mesenchymal phenotype was apparent in both cell lines and generally exaggerated in the more metastatic MDA-MB-231. These results suggest that the physical structure of fibers alone, regardless of their ECM content, can promote the mesenchymal phenotype observed in metastasizing cancer cells.

1603

Soluble eggshell membrane stimulate dermal fibroblasts to give young ECM environment in vitro.

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Deterioration of dermal extracellular matrix (ECM), mainly constituting fibrous collagens and proteoglycans containing carbohydrate, with aging is due to decreased ECM protein turn over. With increasing age, collagen synthesis reduces and matrix metalloproteinases activity levels are elevated in naturally aged human skin. Avian eggshell membrane (ESM) has long been reported to utilize for recoveries from burns and wound, which may activate dermal regeneration. ESM is similar to basal lamina, therefore we hypothesized ESM may contribute to cell adhesion and facilitate ECM molecule synthesis of young dermal tissue. We found that water-soluble alkaline-digested form of eggshell membrane (ASESM) can be used as a material to provide extracellular matrix (ECM) environment for human dermal fibroblast cells (HDF) in vitro. Avian eggshell membrane (ESM) has fibrous-meshwork structure and has long been utilized as a Chinese medicine for recoveries from burns injuries and wound in Asian countries. Therefore ESM is expected to be providing an excellent natural material for biomedical use but those applications were hampered by the insolubility of ESM proteins. In this study a novel 2-methacryloyloxyethyl phosphorylcholine polymer (PMBN) biointerface selectively capture the ASESM proteins were used. The surface shows fibrous structure under atomic force microscope (AFM) and adhesion of HDF to ASESM was ASESM-dose dependent. Quantitative mRNA analysis showed expression of type III collagen, MMP2, and decorin mRNAs was high at

more than two-fold when HDF was faced to lower dose ASESM-PMBN scaffold, which ECM molecules were produced in young dermis and at the begining of would healing. Particle exclusion assay using fixed erythrocytes visualized secreted water-binding molecules around the cells. Thus HDF seems to provide ECM environment on newly designed PMBN-ASESM biointerface and future application of ASESM-PMBN system for biomedical use will be of great interest.

1604

Development of a Method to Non-invasive Ultra-thin Cell Surface Modification of Hepatocytes with PEG-lipid Derivatives.

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Introduction: The use of primary hepatocytes for cell transplantation or tissue engineering is considered as a promising regenerative approaches for liver diseases. In addition, primary hepatocytes have been used in culture study for offering an important research tool to study liver-specific functions and metabolism. However, hepatocytes are known to poorly engraft in vivo and to lose their functions within several days under culture condition. Since these problems are likely initiated by the inappropriate interactions between the cellular surface and surrounding extracellular situations, a conceivable strategy to overcome these problems would be to modify the hepatocyte cellular surface. In the present study, we created polymers in which PEG was conjugated with 3 different types of phospholipids and investigated their effectiveness to modify surfaces of primary hepatocytes in vitro. Furthermore, we attempted this surface modification procedure on fabricated hepatocyte sheets.
Mehtods: The surface of murine primary hepatocytes was modified using poly(ethylene glycol)-phospholipids conjugate bearing FITC (FITC-PEG-lipid) in suspension. Hepatocyte function was assessed in vitro by examining cell viability, plating efficiency, protein production, metabolizing activity, hepatocyte-specific gene expressions, and cytochrome P450 induction. The engraftment of the PEG-lipid modified cells was studied following transplantation to both the liver or alternate ectopic sites.
Results: Among the types of phospholipids analyzed in our study, 1,2-dimyristoil -sn-glycerol-3-phosphatidylethanolamine (DMPE) was found to be uniformly anchored to the hepatocyte cell membrane (>99% of hepatocytes). Cell surface modification using FITC-PEG-DMPE did not result in any loss of in vitro functional parameters nor affect the engraftment potential in vivo of modified cells. This modification was also successfully performed on fabricated hepatocyte sheets as wells as dispersed hepatocytes.
Conclusions: The present study an efficient and simple surface modification approach to primary hepatpocytes using FITC-PEG-lipid. The replacement of FITC with functional proteins has the potential to move hepatocyte-based cell therapy another step forward as viable therapeutic application.

Rab GTPases

1605

Guanine nucleotide exchange factor is required for the intracellular transport of proglutelins to protein storage vacuole in rice endosperm.

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Rice glutelins are synthesized on the endoplasmic reticulum as a precursor, which is then transported via the Golgi to protein storage vacuole (PSV) where it is processed to smaller subunits. The glup6 mutant, which contains abnormally large amounts of proglutelin, contains a defective guanine nucleotide exchange factor (GEF), a protein which activates Rab GTPases, key regulators of membrane trafficking. A microscopic study was undertaken to determine the role of GEF in the intracellular transport of proglutelin. Immunofluorescence microscopy of several independent glup6 lines demonstrated the appearance of a novel structure, paramural body (PMB), so named for their location in the paramural space between the cell wall and the plasma membrane. Electron microscopy of glup6 endosperm revealed the presence of proglutelin –containing electron-dense granules within the cell wall in young cells; these granules disappeared at later stages accompanied by increases in the size and number of PMBs. Therefore, the loss of GEF function disrupts proglutelin transport from Golgi to PSV, resulting in secretion of these proteins to the extracellular space, which ultimately leads to the formation of PMBs. Further microscopic analysis showed that PMB contained marker proteins of the prevacuolar compartment and the Golgi apparatus as well as layers of ß-glucan, which were continuous with the cell wall. Overall, GEF functions in the formation of the endomembrane system in rice endosperm and participates in intracellular transport of proglutelin to PSV.

1606

The relationship between Rab5a GTPase and guanine nucleotide exchange factor in the intracellular transport of glutelin in rice seed.

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Glutelins, major storage proteins in rice seeds, are initially synthesized on the endoplasmic reticulum as a 57-kDa precursor and then transported via the Golgi apparatus to the protein storage vacuole (PSV) where it is proteolytically processed and stored. Two mutants, *glup4* and *glup6*, have been identified, which contain defects in the small GTPase Rab5a and guanidine exchange factor (GEF) VPS9a, respectively. Both *glup4-rab5a* and *glup6-gef* mutants over-accumulate proglutelin and contain a novel structure, paramural bodies (PMBs), in developing endosperm cells. As rice endosperm expresses several Rab5a and GEF homologues, the relationship between GLUP4-Rab5a and GLUP6-GEF in the intracellular trafficking of glutelin was studied. Immunoprecipitation studies showed that Glup6-GEF and Glup4-Rab5a proteins interacted with each other, suggesting that the Glup6-GEF activates Glup4-Rab5a. Results from additional studies suggest that other Rab5a and GEF homologues may also participate in proglutelin transport, although to a much lesser extent. The ratio of proglutelins to glutelin subunits in mature seeds of the double recessive type of *glup4-rab5a* and *glup6-gef* mutants was significantly higher than seen in each single mutant, indicating that the mutations were

additive. Moreover, microscopic analysis of the double recessive *glup4-rab5a* - *glup6-gef* mutant showed a more severe PMB phenotype than that seen for each individual mutation. These results suggest that although GLUP6-GEF and GLUP4-Rab5a are the dominant GEF and Rab5a partners responsible for intracellular transport of proglutelins to the PSV, other Rab5a and GEF homologues may also play a role, albeit to less prominent extent.

1607

Activation of Rab5 via Diverse Routes of Epidermal Growth Factor Receptor Endocytosis.

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The process of endocytosis is a complex pattern of membrane vesicle trafficking, which permits the continuous flow of membrane between cell surface and various intracellular compartments, via fusion and fission events, with Rab5 mediating the fusion events of early endosomes. Since it has been well established that varying concentrations of EGF during the endocytosis of EGF Receptor (EGFR) can elicit uptake via numerous endocytic routes, we examined which of these endocytic pathways is/are responsible for activation of Rab5.
We have found that with respect to the quantity of EGF administered, Rab5 is activated in a concentration dependent manner. It was also observed that if cells are stimulated with low quantities of EGF [2ng/ml], temporal activation of Rab5 peaks at about 2.5 minutes and diminishes within 10 minutes. However, if cells are stimulated with high concentration of EGF [>100ng/ml], the temporal activation of Rab5 is both heightened as well as prolonged, where even 10 minutes after stimulation, relative activity of Rab5 is much higher than at the maximum point during stimulation with low concentration of EGF. Furthermore, by utilizing specific inhibitors as well as siRNA against key regulators of endocytosis and varying the concentration of EGF stimulation we have provided evidence for the existence of at least three distinct pathways of Rab5-dependent internalization of growth factor receptors.

1608

CLN5 is necessary for Rab7 recruitment and activation.

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Neuronal ceroid lipofuscinoses (NCL) constitute a family of neurogenetic lysosomal storage disorders which show the highest prevalence, reaching 1 in 12 500 in American and European populations. These diseases are characterized by an accumulation of autofluorescent lipopigments in the cells of affected patients. The accumulation of lipofuscin in neuronal cells leads to a progressive loss of motor and psychological skills and eventually death of affected individuals. The Finnish Late Infantile variant of NCL (vLINCL) is caused by mutations in ceroid lipofuscinosis neuronal protein-5 (CLN5), the biological function of which has yet to be determined. Here we show that CLN5 is in a complex with the small GTPase Rab7 located at the late-endosome. Using a membrane topology assay we show that CLN5 is a transmembrane protein with its N-terminal extremity located in the cytosol. We also demonstrate by co-immunoprecipitation that HA-CLN5 can interact with wild-type RFP-Rab7 and with both the dominant active, Rab7Q67L, and the dominant negative, Rab7T22N, mutants suggesting that CLN5 is not an effector of Rab7. We demonstrate using a GTP loading experiment with Guanosine 5'-triphosphate [g] 4-azidoanilide-2',3'-biotin-long chain-hydrazone (GTPγAA) that knockdown of CLN5 expression leads to a decrease of RFP-Rab7 activation. The use of a GST linked Rab7-interacting lysosomal protein (RILP) allowed us to show a decrease in the ability of Rab7 to recruit this effector following CLN5 suppression. CLN5 deletion in HeLa cells also lead

to a deficiency in the recruitment to endosomes of the retromer subunit VPS26. Taken together these data suggest a role for CLN5 at the late-endosome/lysosome via the activation of Rab7.

1609

Centriolin regulates the Rab11-dependent endosomal-recycling pathway through exocyst anchoring at the centrosome.

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Centriolin, a component of the mother centriole, localizes to the cytokinetic midbody and is required for cytokinesis. During cytokinesis, the vesicle-tethering complex known as the exocyst is anchored at the midbody through an interaction with the exocyst subunit Sec15 and centriolin. This anchoring regulates vesicle fusion at the cytokinetic bridge. Exocyst subunits were also found to localize at the centrosome, but it is unclear by what mechanism or for what purpose. Here we have examined a role for centriolin in anchoring the exocyst at the centrosome by first defining whether Sec15 is a bona fide centrosome protein. Centrosomes were isolated after the microtubule and actin cytoskeleton were disassembled. Surprisingly, Sec15 and its binding partner, the GTPase Rab11, were both isolated on centrosomes with centriolin. Furthermore, in cells incubated with centriolin siRNAs the exocyst no longer localized to the centrosome. By confocal microscopy and membrane fractionation, centriolin colocalized with the Rab11- and Sec15-decorated recycling endosome. These results suggest that centriolin, Rab11, and Sec15 are centrosome and the pericentrosomal localized recycling endosome components. In addition, electron microscopy confirmed the intimate association of the recycling endosome with the centrosome. Based on these findings, we examined whether centriolin is required for recycling endosome function. Cells incubated with centriolin siRNAs created a disorganized recycling endosome compartment and caused abnormalities in transferrin recycling. In conclusion, centriolin plays a role in regulating the recycling endosome compartment by anchoring the recycling endosome-associated-exocyst complex at the centrosome.

1610

Rab11-Family Interacting Proteins Regulate Distinct Steps in Transferrin Recycling.

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The Rab11-Family Interacting Proteins (Rab11-FIPs) are critical for efficient recycling of proteins to and from the cell surface. The purpose of the current study was to determine whether Rab11-FIPs (FIP1A, FIP1B, FIP1C, FIP2, FIP3, and FIP5) participate in spatially and temporally distinct steps of the recycling pathway. We transfected HeLa cells with fluorescently labeled Rab11-FIPs, conducted live cell studies, and processed the resulting images using deconvolution microscopy. We evaluated compartment size, distribution, movement, and overlap between Rab11-FIPs as well as fluorescent-Transferrin entry into endosomal compartments labeled with Rab11-FIPs. Our results revealed differences in endosomal tubule size, distribution, and movement following expression of GFP-Rab11-FIPs in live HeLa cells. Rab11-FIP1A, Rab11-FIP2, and Rab11-FIP5 each maintained a wide distribution while labeling shorter tubular compartments (1-2 microns in length) that displayed mobility throughout the entire cell with speeds on the order of 1micron per second. Conversely, Rab11-FIP1B, Rab11-FIP1C, and Rab11-FIP3 each occupied more centrally located compartments with a tighter distribution in the perinuclear region with evidence of longer (3-6 microns in length) tubules emanating from this region. Co-expression studies of Rab11-FIPs revealed overlapping signals on tubular endosomal structures and while some members such as FIP1A induced more

elaborate tubulation of other FIP compartments, FIP3 promoted an accumulation of these proteins in the perinuclear region suggesting these proteins have different effects on components of the recycling system. In addition we learned that some FIPs which appear to occupy similar regions of the cells and display similar types of movement, as in the case with FIP1A and FIP2, do not necessarily overlap, indicating a potential difference in functional roles in recycling. Finally, we observed temporal differences in the overlap of fluorescent Transferrin signal with Rab11-FIP compartments. Transferrin overlap with FIP1A, FIP2, and FIP5 occurs the earliest following loading (5-15 minutes) with Transferrin, whereas Transferrin overlap with FIP1B, FIP1C, and FIP3 does not occur until later (20-30 minutes) suggesting these proteins are involved in temporally different steps of the recycling process. In summary, our current data indicate that the Rab11-FIPs occupy different parts of the recycling network and that the participation of these proteins in the recycling process occurs at spatially and temporally distinct steps.

1611

Rab22 controls biogenesis of signaling endosomes in NGF signal transduction.

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It’s mostly unknown how growth factors and receptors are sorted into signaling endosomes necessary for signal transduction processes. Here we demonstrate that Rab22 is the only endosomal Rab that promotes the sorting of NGF and activated receptor pTrkA into signaling endosomes to sustain the signal and induce neurite outgrowth/differentiation in PC12 cells. NGF binding induces the endocytosis of pTrkA into Rab22-containing endosomes. Knockdown of Rab22 via shRNA blocks NGF-induced pTrkA endocytosis into the endosomes, gene expression (VGF) and neurite outgrowth. Overexpression of human Rab22 can rescue the inhibitory effects of the Rab22 shRNA, suggesting a specific Rab22 function in NGF signal transduction rather than off-target effects. Furthermore, the Rab22 effector, Rabex-5, is necessary for NGF-induced neurite outgrowth and gene expression, as evidenced by the inhibitory effect of shRNA-mediated knockdown of Rabex-5. Disruption of the Rab22-Rabex-5 interaction via overexpression of the Rab22-binding domain of Rabex-5 in the cell also blocks NGF-induced neurite outgrowth, suggesting a critical role of Rab22-Rabex-5 interaction in the biogenesis of NGF signaling endosomes to sustain the signal for neurite outgrowth. The data provide the first evidence for an early endosomal Rab GTPase as a positive regulator of NGF signal transduction and cell differentiation.

1612

The small GTPase Rab27B regulates invasive tumor growth and metastasis through extracellular HSP90α.

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Background:
Vesicle exocytosis and exosome release, controlled by small GTPases such as Rab27B, deliver critical pro-invasive growth regulators into the tumor environment. The biological role and expression status of Rab27B in breast cancer was unknown.
Methods:
Rab27B was studied in estrogen receptor (ER)-positive human breast cancer cell lines (MCF-7, T47D, ZR75.1) using GFP-fusion constructs, including wild type Rab3D, Rab27A, Rab27B and Rab27B point mutants defective in GTP/GDP-binding or geranylgeranylation, and transient siRNA targeting. In cell culture, cell-cycle progression was evaluated by flow cytometry, Western blotting and measurement of cell proliferation rates, invasion was assessed using

Matrigel and native collagen type I substrates. Orthotopic tumor growth, local invasion and metastasis were analyzed in mouse xenograft and chick chorioallantois membrane models. Mass spectrometry was performed to identify Rab27B-secreted pro-invasive growth regulators. In clinical breast cancer, Rab3D, Rab27A and Rab27B mRNA levels were analyzed by quantitative real time-polymerase chain reaction (RT-PCR) and Rab27B protein level was evaluated by immunohistochemistry.

Results:

Rab27B-upregulation promoted G1/S phase cell cycle transition and increased proliferation, F-actin reorganization and invasion in cell culture, and invasive tumor growth and haemorrhagic ascites in a xenograft mouse model . Proteomic analysis of purified Rab27B-secretory vesicles and the secretome of exogenous Rab27B-expressing breast cancer cells identified heat shock protein (HSP)90α as key pro-invasive growth regulator. HSP90α secretion occurred in a Rab27B-dependent manner and was required for matrix metalloproteinase(MMP)-2 activation. All Rab27B-mediated functional responses were GTP- and geranylgeranyl-dependent. Endogenous Rab27B mRNA and protein, but not Rab3D and Rab27A mRNA, associated with lymph node metastasis (P=0.0002) and differentiation grade (P=0.0014) in ER-positive human breast tumors.

Conclusions:

Cancer cells communicate with the environment through delivery of surface proteins, release of soluble factors (growth factors and cytokines) and sophisticated nanovehicles (exosomes) for establishment of invasive tumor growth. This communication occurs in part through constitutive exocytosis, regulated exocytosis or release of intraluminal vesicles and is modulated by small Rab GTPases, the master regulators of vesicle traffic. We studied Rab GTPases implicated in regulated exocytosis and demonstrated a unique role for Rab27B in invasive tumor growth. Emerging evidence indicates that various exocytic routes are implemented by cancer cells to relay crucial information for fostering growth, migration and matrix degradation.

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Rab33a stimulates vesicular trafficking and promotes axon outgrowth.

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Axon outgrowth is accompanied by the transport of newly synthesized membranes from the cell body to the neurite tip. Although the small GTP-binding proteins of the Rab family play a key role in membrane trafficking, the molecules involved in membrane transport for axon outgrowth are largely unknown. Previously, we reported a protrein singar1 which suppresses formation of multiple axons in cultured rat hippocampal neurons. Here, we identified rab33a as a singar1-interacting protein. Expression of rab33a became up-regulated during axon outgrowth of cultured rat hippocampal neurons. Rab33a showed relatively high accumulation in the axonal shaft and growth cone, where it colocalized with synaptophysin. Synaptophisin is well known as a major protein localized to synaptic vesicles, but also preferentially localized in the developing axons before synapse formation. Live cell imaging of EGFP-rab33a showed that it is dynamically transported along the neurite shaft together with synaptophysin. Overexpression of rab33a in cultured neurons stimulated trafficking of synaptophysin positive vesicles and induced multiple axon formation. On the other hand, reduction of the expression of rab33a by RNAi inhibited the trafficking of synaptophysin positive vesicles and axon outgrowth. We propose that rab33a promotes axon outgrowth by regulating the trafficking of synaptophysin positive vesicles in neurons.

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Rab GTPase-MyosinVb Complex Regulates Exocytosis of Discoidal/Fusiform Vesicles in Bladder Umbrella Cells.

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Umbrella cells, the outermost cell layer of the bladder uroepithelium form a selective barrier and experience large variation in hydrostatic pressure and mechanical stress. To neutralize such changes, the umbrella cell modulates its apical surface area by regulated exocytosis of sub apically localized discoidal/fusiform-shaped vesicles (DFV) in response to filling, but little is understood about the molecular machinery that modulates this process. Furthermore, the GTPases that regulate these trafficking events are poorly characterized. We previously localized the small GTPase Rab11a on DFV and showed it regulates DFV exocytosis independent of its well-described role in endocytosis and recycling. Here, we showed that Rab11a-effector MyosinVb was associated with DFV, and the expression of dominant negative (DN) mutant form of MyosinVb inhibited stretch-induced increase in apical cell surface area. Furthermore, the expression of DN-MyosinVb blocked the release of human growth hormone, a secretory protein known to package into DFV. The expression of MyosinVb mutants lacking affinity to Rab8a, Rab10 or Rab11a suggested a role of Rab8a in stretch-induced changes. We observed that Rab8 was expressed in umbrella cells and was partially associated with Rab11a and DFV. Our results indicate that DFV exocytosis depends on the function of the Rab8-Rab11a-MyosinVb complex.

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Distinct Roles of Two Rab GAPs, AS160/Tbc1d4 and Tbc1d1, in Intracellular GLUT4 Trafficking Analyzed by Single Molecule Imaging of GLUT4 Behavior.

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Insulin facilitates translocation of GLUT4, the insulin-responsive glucose transporter, from intracellular storage compartments to the plasma membrane. Insulin signaling regulating these processes is mediated by AS160/Tbc1d4, a TBC1 domain family Rab GTPase-activating protein (GAP), which itself appears to be regulated chiefly by Akt-dependent phosphorylation. Another TBC1 domain family member Rab GAP, Tbc1d1, which is highly homologues with AS160/Tbc1d4 but is abundantly expressed in skeletal muscle cells, has been suggested to control GLUT4 translocation in responses to both insulin and exercise in skeletal muscles. However, due to the technical limitations of conventional approaches, how these proteins regulate intracellular GLUT4 behavior and how their actions differ among each other remains ambiguous. We recently developed a novel method for directly quantifying intracellular GLUT4 movement based on single molecule imaging of GLUT4 behavior (Mol. Biol. Cell 2010) and successfully determined insulin-responsive GLUT4 trafficking systems. Specifically, “static GLUT4 retention” and its “insulin-dependent liberation” are critical properties of these systems, and the former is generated by orchestrated actions of sortilin, a Vps10p family sorting receptor upregulated upon cellular differentiation, with retromer-dependent retrograde trafficking and golgin-97. We herein analyzed the actions of the two Rab GAPs on GLUT4 trafficking systems. In undifferentiated 3T3L1 fibroblasts expressing neither sortilin nor AS160/Tbc1d4 endogenously and therefore incapable of insulin-responsive GLUT4 trafficking, exogenous expression of these two proteins successfully reconstituted the insulin-responsive liberation of static GLUT4. In contrast, fibroblasts expressing both sortilin and Tbc1d1 exhibited no GLUT4 liberation in response to insulin. Interestingly, triple expression of sortilin, AS160/Tbc1d4 and

Tbc1d1 resulted in complete loss of insulin-responsive GLUT4 liberation, indicating that the actions of Tbc1d1 dominate over AS160/Tbc1d4 function. These data suggest that the two analogous Rab GAPs, AS160/Tbc1d4 and Tbc1d1, each have distinct roles in GLUT4 trafficking systems and that the molecular basis of insulin-responsive liberation in muscle cells expressing abundant amounts of Tbc1d1 are more complex than in fat cells expressing mainly AS160/Tbc1d4 alone.

Post-Golgi Trafficking

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Super Hypersensitivity to Hygromycin B (s-hhy) Mutants Converge at the Trans-Golgi and Have a Role in Tor1p Localization to the Vacuole.

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The vacuole in *Saccharomyces cerevisiae* serves as a useful and functional model for the mammalian lysosome. In a genome wide screen for mutants with severe growth hypersensitivity to hygromycin B, our lab identified 14 *HHY* genes. Each of the *hhy* mutants is defective in vacuolar trafficking and/or function (Banuelos et al., 2010). Each of the *hhy* mutants is also sensitive to rapamycin and caffeine suggesting a compromised target of rapamycin (TOR) kinase pathway. Our current research divides the *hhy* mutants into two distinct groups based on quantitative growth analyses in the presence of hygromycin B. 1) a super affected group inhibited at any concentration of hygromycin (s-*hhy's*) and 2) a dose-dependent group, which is more affected as the concentration of hygromycin B increases (d-*hhy's*). The s-*HHY* genes include *ARF1, CHC1, DRS2, SAC1, VPS1, VPS34, VPS45, VPS52,* and *VPS54*. Evaluation of s-*HHY* gene products reveals localization or function at the trans-Golgi to be a common factor. Interestingly, two of the s-*HHY* gene products, Vps52 and Vps54, are components of the GARP complex involved in protein trafficking between the late Golgi and the vacuole, while the deletion strain of a third component of the GARP complex, *vps53Δ*, is unaffected by hygromycin treatment. To assess Tor1 kinase localization, we utilized a strain expressing endogenously tagged Tor1-GFP and assayed localization to the vacuolar membrane in each of the s-*hhy* mutants using confocal microscopy. In wild-type cells, Tor1-GFP co-localizes with the vacuolar membrane marker FM4-64 while several deletion strains including *vps52Δ* and *vps54Δ* fail to localize Tor1-GFP to the vacuolar membrane when treated with hygromycin B. Additionally, s-*hhy* mutants are unable to recover growth after a 4-hour treatment with hygromycin B, similar to EG_0 mutants, which fail to exit from G_0 after treatment with rapamycin.

Based on our data, we propose a model in which the s-*HHY* gene products converge at a hygromycin B hypersensitive microdomain at the trans-Golgi that is involved in post-Golgi vesicular trafficking including recruitment and subsequent transport of Tor1p to the vacuolar membrane. We also propose that Tor1 kinase localization at the vacuole is essential for its cell cycle regulatory function.

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Cargos Specify the Site and Identity of Adaptor Recruitment to the Golgi and Recycling Endosomes.

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About thirty percent of the human proteome moves through the secretory pathway on its way toward secretion from the cell or localization to specific membrane bound organelles. Movement between organelles is marked by the recruitment to nascent carriers by members of the Arf/Sar family of GTPases of specific protein adaptors that mark sites of export, assist in the sorting of transmembrane protein cargos, and help direct movement to the next destination in the endomembrane system; e.g., COPII regulates export from the ER, COPI from the cis-Golgi and AP-1, GGA1-3, and Mint3 are each thought to mark the TGN and help move cargos to endosomes and the plasma membrane. We developed methods to examine specificity and initial sites of adaptor recruitment of Arf-dependent adaptors in a cargo-dependent fashion, with a focus on the Golgi. These methods faithfully recapitulate in cells the specificities determined using in vitro protein binding assays. We find that the same adaptor can be specifically recruited to different organelles by different cargos. In addition, different cargos can recruit a common adaptor to the same organelle but with different consequences to the cargo's export. These results are discussed with respect to the generation of models for molecular mechanisms of membrane traffic and the dangers in extrapolating from one cargo-adaptor pairing to others.

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Loss of adaptor protein AP-3 perturbs the regulated secretory pathway *in vivo.*

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The regulated secretion of many neural peptides and peptide hormones depends on their sorting to large dense-core vesicles (LDCVs) capable of regulated exocytosis. LDCVs form at the trans-Golgi network, but the mechanism for protein sorting to this regulated secretory pathway (RSP) is poorly understood. We have recently described a role for the heterotetrameric adaptor protein AP-3 in targeting proteins to the RSP of both *Drosophila* S2 cells and mammalian neuroendocrine PC12 cells. In both cell types, loss of AP-3 dysregulates exocytosis, resulting in decreased regulated secretion and increased constitutive secretion of LDCV proteins. We are currently assessing the role of AP-3 in sorting to the RSP *in vivo*. Using AP-3 mutant *mocha* mice, we have observed marked reductions in the levels of the major LDCV-resident adrenal proteins Secretogranin II and Chromogranin A, suggesting a defect in RSP formation *in vivo*. To directly assess the regulation of secretion, we have used optical imaging by total internal reflection fluorescence (TIRF) microscopy of *mocha* adrenal chromaffin cells. After lentiviral transduction with neuropeptide Y (NPY)-pHluorin, exocytosis was monitored before and after stimulation. Consistent with our previous findings, we observed a significant increase in constitutive secretion of NPY-pHluorin in cells lacking AP-3, but surprisingly, we found no defect in regulated secretion. To extend these findings to another neuroendocrine tissue and assess secretion of an endogenous peptide hormone, we isolated pancreatic islets from wild type and *mocha* mice, measuring basal and regulated insulin secretion. As in the chromaffin cells, we observed a significant increase in unregulated insulin secretion from *mocha* islets, but no change in regulated secretion. We conclude that loss of AP-3 *in vivo* is sufficient to dysregulate the RSP in multiple neuroendocrine tissues, although the differences observed between cell lines and primary *mocha*-derived cells suggest partial compensation for the loss of

AP-3 *in vivo*. Future work will be aimed at elucidating changes to the molecular composition of LDCVs after loss of AP-3 which result in dysregulated secretion.

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Clathrin and adaptor-dependent trafficking of Atg27.

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Clathrin is involved in the formation of transport vesicles (CCVs) during endocytosis and protein sorting at the trans-Golgi network (TGN)/endosome. It is recruited to membranes by adaptors that recognize sorting motifs on cargo proteins collected into CCVs and/or regulate CCV formation. Proteomic analysis found that purified CCVs are enriched for Atg27, a type-I membrane protein involved in autophagy. Previous studies have shown Atg27 localizes to the pre-autophagosomal structure (PAS), the Golgi, and Atg9-containing compartments. The possibility that clathrin is involved in the trafficking of Atg27 is consistent with previous findings from our lab showing that clathrin mutants display autophagic phenotypes.

In this study, we further investigate the trafficking of Atg27. Atg27 localized to the TGN/endosome and the vacuolar membrane, in addition to autophagy-related structures like the PAS. Atg27 did not accumulate at the plasma membrane upon treatment with Latrunculin-A, which blocks endocytosis, suggesting it does not cycle through the cell surface along its trafficking itinerary.

A class E *vps* mutant that affects multivesicular body function and CPY sorting to the vacuole did not prevent Atg27 from localizing to the vacuolar membrane, suggesting Atg27 does not follow the vacuolar protein sorting (Vps) pathway to the vacuole. Instead, Atg27's vacuolar membrane localization was dependent on the AP-3 pathway.

We looked for potential sorting signals in Atg27's cytosolic C-terminus. The last four amino acids of Atg27 (YSAV) resemble a classic tyrosine sorting motif (YXXΦ) recognized by the μ chains of AP adaptors. Mutation of the YSAV abrogated Atg27's ability to traffic to the vacuolar membrane, consistent with this functioning as an AP-3 dependent sorting signal. Also, YSAV mutants were still recruited to the PAS and endosomal compartments, although Atg27's co-localization with TGN markers was more prominent, consistent with a defect in export from the TGN.

Studies in yeast have indicated that the AP-3 pathway is clathrin-independent. Inclusion of Atg27 in CCVs suggests that, in addition to AP-3 sorting to the vacuole, Atg27 also undergoes clathrin-dependent trafficking. Interestingly, deletion of *CHC1* resulted in Atg27 localization to many small puncta/vesicles and transport to the vacuolar membrane was impaired. Similar results were found for endosomal epsins (*ent3Δ*/*ent5Δ*), but not *gga1Δ*/*gga2Δ* or AP-1/*apm1Δ*. This suggests an interplay between the AP-3 and clathrin-dependent trafficking pathways, which has not been reported to date in yeast.

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Interaction of PAM with μ1A subunit of AP-1 affects trafficking in AtT-20 cells.

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Peptidylglycine α-amidating monooxygenase (PAM-1), a transmembrane protein, is essential in the biosynthesis of many neuropeptides. The trafficking of PAM-1 in AtT-20 mouse corticotrope tumor cell lines revealed essential roles for its luminal catalytic domains and for its heavily phosphorylated, unstructured cytosolic domain (CD). Upon exit from the *trans*-Golgi network (TGN), PAM-1 is directed to immature secretory granules (ISGs), which must undergo a maturation process before their release becomes secretagogue-responsive. Once in the plasma

membrane after exocytosis, PAM-1 is internalized, entering the endocytic pathway. After passage through multivesicular bodies, PAM-1 can be degraded in lysosomes or sent back to the regulated secretory pathway via the TGN. Both the entry of PAM-1 into SG and its endocytic trafficking are affected by phosphorylation of Ser^{949} in its CD. The adaptor protein 1 complex (AP-1) is involved in cargo recognition for proteins moving between the TGN and endosomes in clathrin-coated vesicles. AP-1 has also been found in patches on ISG membranes, where it removes cargo proteins, facilitating ISG maturation. Although the CD of PAM-1 lacks a canonical AP-1 binding site, a yeast-two-hybrid screen showed that it interacts with the µ1A subunit of AP-1. No interaction with µ2, µ3A, µ3B or µ4 was observed. Alanine scan mutagenesis identified a 10 amino acid region of the PAM-1 CD as the µ1A-binding site. Mutation of Ser^{949} revealed a role for this residue in the interaction. Immunofluorescence studies demonstrated co-localization of AP-1 and PAM-1 in the TGN and SGs. PAM-1 AtT-20 cells were infected with lentiviruses encoding five shRNAs specific for µ1A. Based on Western blot analysis for µ1A, a stable line in which expression of µ1A was reduced by 70% was selected. Cleavage of PAM-1 in SGs produces soluble PHM (sPHM), whose secretion can be quantified. Secretion of mature SGs was stimulated with $BaCl_2$. Secretion of sPHM in PAM-1/µ1A knockdown cells was 45% less responsive to $BaCl_2$ than in PAM-1 cells. The endocytic trafficking of PAM-1 in PAM-1/µ1A knockdown cells was quantified using surface biotinylation. Endocytosed PAM-1 was more stable in PAM-1/µ1A knockdown cells than in PAM-1 cells. The biosynthetic and endocytic trafficking of PAM-1 mutants unable to interact with µ1A resembles PAM-1 trafficking in µ1A knockdown cells. The interaction of PAM-1 with AP-1 is essential for PAM trafficking in the secretory and endocytic pathways.

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Dimerization of a model raft-independent protein facilitates apical targeting through transient clustering at the trans-Golgi network.

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Defective intracellular delivery of proteins to the apical surface of renal epithelial cells can disrupt polarity, compromise organ function and lead to chronic kidney disease. Proteins sorted to the apical plasma membrane in polarized epithelial cells can be categorized in two classes based on their differential association with cholesterol and glycolipid-enriched membrane microdomains commonly termed “lipid rafts”. Our laboratory and others have previously demonstrated that newly synthesized lipid raft-associated and lipid raft-independent proteins take different routes to the apical surface. Interestingly, these cargoes appear to be segregated into distinct subdomains even within the *trans*-Golgi network (TGN). Self-association or “clustering” is known to be important for the segregation of raft-associated apical proteins but whether oligomerization also contributes to raft-independent sorting is unclear. The apical sorting determinant in p75, a non-raft associated protein that is expressed in bladder and embryonic kidney, resides within its O-glycosylated stalk, and galectin-mediated crosslinking has been proposed as a sorting mechanism for this protein. Additionally, dimerization motifs within the transmembrane region of p75 could contribute to the formation of oligomers. We used fluorescence correlation spectroscopy (FCS) and photon counting histogram (PCH) analyses to measure the spatial and temporal dynamics of p75 trafficking in live cells. As predicted, p75 exists as a dimer at the plasma membrane in the absence of ligand, as previously reported. However, PCH analysis revealed that p75 forms higher-order oligomers within the *trans*- TGN and this oligomerization is abrogated upon mutation of a conserved transmembrane cysteine residue (C257A). In addition, clustering of p75 in the TGN appears to be dynamic, based on the

range of complex sizes observed and because only the dimer form was detected at the plasma membrane. Finally, the C257A mutation increased the levels of p75 at the basolateral membrane in polarized cells, suggesting that dimerization plays a role in apical targeting.

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STK16, a Novel MAL2 Binding Partner, Regulates Basolateral Secretion in Polarized Hepatic Cells.

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Most classes of newly synthesized hepatic apical proteins take an indirect pathway to the cell surface. They are delivered from the TGN to the basolateral domain, selectively internalized then transcytosed to the apical surface. MAL2 has been implicated in regulating at least two steps in this pathway. Because vesicle formation and delivery are driven by complex machineries, we predict that MAL2 exists in large multi-protein complexes. To identify MAL2 interactors, we performed a split-ubiquitin yeast two hybrid screen using human MAL2 as bait. From a human liver cDNA library, serine-threonine kinase 16 (STK16) was identified. This lipid-anchored kinase is enriched in liver and shown to regulate mammary gland development implicating it as a likely candidate for regulating polarized protein trafficking. In rat liver, MAL2 and STK16 both distributed to the same membrane fractions after differential centrifugation suggesting they interact. This was confirmed by co-immunoprecipitations in rat liver and polarized, hepatic WIF-B cells. When overexpressed in WIF-B cells, STK16 localized mainly to the Golgi with a lesser amount at the basolateral membrane. However, kinase-dead STK16 (E202A) localized to bright puncta in the cell periphery. To determine whether E202A expression disrupted the Golgi, we double labeled with several Golgi markers. Surprisingly, we found that the Golgi remained intact. Treatment with Brefeldin A did not affect E202A, implying they are in post-Golgi vesicles. Additionally, a total TGN block at 20°C followed by a 37°C chase led to redistribution of E202A from the TGN back to the bright puncta, confirming that E202A is in a post-Golgi vesicle. Interestingly, when E202A-expressing cells were labeled for the secretory proteins, albumin and haptoglobin, no staining was detected. Immunoblotting revealed that all albumin forms were decreased to the same extent in E202A-expressing cells indicating that the protein was being properly processed. To account for decreased albumin staining, we reasoned that albumin and haptoglobin were either hypersecreted (preventing Golgi-accumulation) or were misrouted to the lysosome for degradation. Immunoblotting of media from E202A-expressing cells revealed that albumin and haptoglobin were not being hypersecreted. In contrast, in E202A-expressing cells treated with ammonium chloride to deacidify lysosomes, intracellular levels of albumin were increased. Thus, we predict that in E202A-expressing cells, the secretory proteins are being diverted from the TGN to lysosomes for degradation. We are actively examining how MAL2 is involved in this process.

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Basolateral sorting of VSVG and LDLR involves the sub-apical Rab11 compartment.

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Polarized epithelial cells maintain a distinct apical (AP) and basolateral (BL) plasma membrane (PM) protein composition through sorting events in exocytic and endocytic pathways. After exiting the trans-Golgi network (TGN), proteins en route to the cell surface can traverse distinct endosomal compartments. So far, the rab11 sub-apical recycling endosomes have been

identified as an intermediary only for the basolateral sorting of E-cadherin. Here we analyzed by three dimensional live cell imaging the post-TGN routes of three classical basolateral model proteins (VSVG, LDLR and TfR) in MDCK cells stably expressing either TGN38-CFP or Rab11-CFP. After microinjection of plasmids encoding VSVG-mCherry, LDLR-GFP, and TfR-GFP, the expressed proteins were accumulated at the TGN by 20º block and then released at 37°C. VSVG-mCherry and endocytosis defective LDLR-GFP was detectable in the rab11 compartment within 15-30 min, reaching a maximun at about 40 min and then gradually decreased until almost disappearing, while the basolateral cell surface localization increased. TfR reached the basolateral surface without being detectable in this compartment. Membrane permeable peptides carrying competitive BL sorting signals for VSVG and LDLR block trafficking of the corresponding cargo towards both the rab11 endosomes and the basolateral surface. Strikingly, VSVG and LDLR colocalize in the same transport vesicles. These results show for the first time the involvement of the rab11 sub-apical compartment in biosynthetic basolateral sorting of VSVG and LDLR, but not TfR. Experiments with competing peptides indicate that trafficking from TGN to the rab11 compartment is sorting signal mediated and involves different recognition elements, which nevertheless seems to segregate them into the same transport carriers (Financed by CONICYT grant#PFB12/2007, grant#AT24100203 and FONDECYT grant#1110849).

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Rho GTPases are involved in regulating transport of influenza A virus hemagglutinin and neuraminidase in host cell.

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Influenza A virus, a highly infectious respiratory pathogen, is the major cause of annual epidemics and occasional pandemics. Influenza A virus assembles at the plasma membrane of infected host cells, releases by budding, so that possesses a lipid membrane derived from the host cell. This envelope contains two important transmembrane glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Both HA and NA play a critical role throughout the virus life cycle. Because HA and NA are responsible for binding to and release from host cell receptors, they are crucial in determining host specificity. On the other hand, influenza A virus uses the machineries of host cells to synthesize and transport HA and NA. However, little is known about what host cellular factors that are involved in the regulation of intracellular and cell surface transport of HA and NA. Additionally, the mechanism by which viral HA and NA usurps the components of the host trafficking machineries to undergo exocytic transport remains to be further determined. In this study, we have investigated whether Rho family GTPases are involved in regulating the trafficking of HA and NA in the host cell. Using expression of constitutively active or inactive mutants of GTPases, we found that RhoA, Rac1 and Cdc42 exhibited different functions in regulating the intracellular and cell surface transport of HA and NA. Furthermore, using shRNAs to disrupt the expression of these Rho family members, we observed that some but not all Rho family members are required for intracellular and cell surface transport of HA and NA, and thereby affect virus life cycle. Together, our results reveal that members of Rho family are involved in the transport of HA and NA in host cell and thereby impacts virus replication.

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Myosin V transports secretory vesicles via a Rab GTPase cascade and direct contact with the exocyst complex.

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Vesicle transport requires four steps: vesicle formation, movement, tethering and fusion. In yeast, two Rab GTPases, Ypt31/32 are required for post-Golgi vesicle formation. A third Rab GTPase, Sec4, and the exocyst act in the subsequent tethering and fusion of these vesicles. Vesicle production is coupled to transport via direct interaction between Ypt31/32 and the yeast myosin V, Myo2. Here we show that Myo2 interacts directly with Ypt31/32, Sec4, and the exocyst subunit Sec15. Disruption of the interaction between Myo2 and Sec15, or Myo2 and the Rab GTPases, results in severe growth defects and the accumulation of secretory vesicles. Furthermore, mutants that are defective in the interaction of Myo2 with Sec15 result in the mislocalization of Sec15. We identified the Sec15-binding region on Myo2, and found that it is on the opposite face from the Rab-binding site. This suggests that Myo2, Sec4 and Sec15 may form a ternary complex. In addition, we identified residues on Sec15 required for interaction with Myo2. Moreover, we identified a suppressor mutation in this region of Sec15, which rescues the growth defect of the Myo2 mutants that have a defect in binding to Sec15. The finding that Myo2 interacts with Sec15 uncovers a new role for the exocyst as an adaptor for molecular motors, and implies a similar role for other structurally related tethering complexes. Moreover, these studies predict that for many pathways, molecular motors attach to cargoes prior to their formation, and remain attached until fusion at the target membrane.

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The Role of the Exocyst in Ciliogenesis.

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ADPKD is characterized by cyst formation that destroys the kidney. Primary cilia, found on many cell types, including renal tubule cells, participate in flow sensing. Cilia disruption has been linked to ADPKD pathogenesis. Mutations in PKD1 and PKD2, encoding polycystins-1 and-2, cause ADPKD. Polycystins are thought to function in cilia, but it is not well understood how these and other proteins are targeted to cilia. We previously showed that the exocyst, a highly conserved eight-protein trafficking complex, localizes to primary cilia of renal tubule cells and is required for ciliogenesis. We also showed that when a crucial exocyst component, Sec10, is knocked down, the results are ADPKD cellular phenotypes including: loss of flow-generated calcium increases; hyperproliferation; and abnormal activation of the mitogen activated protein kinase (MAPK) pathway. In vivo, Sec10 knockdown in zebrafish phenocopied many aspects of polycystin-2 knockdown, including: curly tail up; left-right patterning defects; glomerular expansion; and MAPK activation. Sec10 morphants also had disorganized cilia. Importantly, we demonstrated a synergistic genetic interaction between zebrafish sec10 and pkd2, in that co-injection of small amounts of sec10 and pkd2 morpholinos, which individually had no effect, together resulted in a severe phenotype. We went on to show that Sec10 biochemically interacts with polycystin-2, IFT88, and IFT20, a ciliary protein known to be trafficked on vesicles. We next wanted to answer the question of how the exocyst first localizes

to primary cilia so that it can participate in ciliary trafficking. Multiple Rho and Rab GTPases regulate the exocyst, and Cdc42, which we previously showed to be involved in cystogenesis, is a candidate to regulate the exocyst at the primary cilium. We now show Cdc42 biochemically interacting with Sec10, and co-localizing with Sec10 at the primary cilium. Dominant negative Cdc42 expression, shRNA-mediated knockdown of Cdc42, and shRNA-mediated knockdown of Tuba, a Cdc42 GEF, all prevent ciliogenesis in Madin-Darby canine kidney cells. Furthermore, exocyst Sec8 and polycystin-2 no longer localize to primary cilia, or the ciliary region, following Cdc42 and Tuba knockdown. We also show Sec10 directly interacting with the Par complex member Par6, which, itself, directly interacts with Cdc42. Finally, we show Cdc42 knockdown resulting in MAPK activation. Several groups have shown that the exocyst interacts with Rab8, the mammalian homologue of yeast Sec4. Sec4/Rab8, a protein found on the surface of vesicles, interacts with Sec10 and Sec15 for vesicle targeting and docking at plasma membranes. Taken together, these data support a model whereby Cdc42 localizes the exocyst to primary cilia, the exocyst is stabilized by binding to Par6, and the exocyst then targets and docks Rab8-positive vesicles carrying ciliary proteins.

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The Oculo-Cerebro-Renal Syndrome of Lowe as a Ciliopathy: Role of Ocrl1 in Primary Cilia Assembly.

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Lowe syndrome is a devastating, X-linked genetic disease characterized by the presence of congenital cataracts, profound learning disabilities and renal dysfunction. Unfortunately, children affected with Lowe Syndrome often die early of health complications including renal failure. Although this syndrome was first described in the early fifties and the affected gene, OCRL1, was identified more than 17 years ago, the mechanism by which Ocrl1 defects lead to Lowe syndrome's symptoms remains unknown.

Here we show that Lowe syndrome display some of the the characteristics of a ciliopathy. Specifically, we found that patients' cells have defects in the assembly of primary cilia and this phenotype was reproduced by knock-down of Ocrl1 in cells in culture. Importantly, this defect could be rescued by re-introduction of WT Ocrl1 in both, patient's and Ocrl1 knock-down cells. In addition, a zebrafish animal model of Lowe syndrome exhibited cilia defects and multiple morphological and anatomical abnormalities typically seen in ciliopathies. Mechanistically, we show that Ocrl1 is involved in protein trafficking to the primary cilia in a Rab8-, Appl1- and IPIP27/Ses-dependent manner via the secretory and endocytic pathways.

Taking into consideration the relevance of the signaling pathways hosted by the primary cilium, our results suggest hitherto unrecognized mechanisms by which Ocrl1 deficiency may contribute to the phenotypic characteristics of Lowe syndrome. This conceptual change in our understanding of the disease etiology may provide an alternative avenue for the development of therapies.

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Relaxin improves cystic kidney disease in rats and alters transcription of ciliary trafficking genes.

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Human autosomal dominant polycystic kidney disease (ADPKD) is characterized by renal epithelial cell proliferation and renal cyst formation, ultimately destroying kidney structure and function. Underlying the pathogenic mechanisms are changes in signaling pathways that affect cellular behavior predominately in the kidney, liver and vasculature. The hormone relaxin is essential for renal vasodilation during pregnancy, regulation of nitric oxide production and several signaling pathways, cAMP production, and collagen formation and degradation. The same pathways and processes are aberrant in ADPKD. Heterozygous Han:SPRD rats manifest ADPKD similar to human ADPKD. We treated male Cy/+ and +/+ Han:SPRD rats with relaxin or vehicle control. The effects on renal structure and function included decreased cyst size and decreased fibrosis. Analyses of renal mRNA profiles from treated and untreated animals revealed expression changes in genes encoding signaling and trafficking proteins. Notable changes were detected in Ras, Rab7, Rab8, and ArfGEF1, in addition to intraflagellar transport protein 80, exocyst complex component 8 and RIMS1. The findings concur with our previous studies demonstrating the importance of GTPase-mediated trafficking in the proper delivery of polycystin-1 to cilia. The data suggest relaxin mediated alterations in GTPase and exocytic protein expression may contribute to improved renal structure and function in the Han:SPRD rat model of ADPKD following relaxin treatment.

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Novel role of PICK1-ICA69 complex in insulin granule biogenesis.

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Diabetes is a group of metabolic diseases characterized by hyperglycemia that result from defects in insulin secretion and/or action. However, the cellular mechanisms and molecular components responsible for the biogenesis of insulin granules are not fully understood and remain an area of intense investigation. PICK1 (Protein Interacting C Kinase 1) is a membrane-associated protein which contains PDZ (PSD-95/Dlg/ZO-1) domain and BAR (Bin/ Amphiphysin/ Rvs) domain. It is widely expressed with high levels in brain, testis and also in pancreas. ICA69 (Islet Cell Autoantigen 69kD), another BAR domain-containing protein, forms tight heteromeric BAR complex with PICK1. As the PDZ domain binds to membrane proteins and the BAR domain binds to liposome, PICK1-ICA69 complex is able to couple several kinds of membrane proteins to the vesicles and regulate their trafficking. We found that that PICK1-ICA69 was also associated with insulin granules and played important roles in insulin granule biogenesis. Deficiency of PICK1 in mice leads to abnormal insulin level and glucose intolerance. To further understand how PICK1 and ICA69 regulate insulin granule trafficking, we have investigated the molecular mechanism underlying PICK1 and ICA69 mediated insulin granule biogenesis and our results have revealed an interesting link between PICK1-ICA69 and insulin granule formation. (Supported by Research Grant Council of Hong Kong).

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Adiponectin is released via a unique regulated exocytosis pathway upon insulin stimulation.

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Adiponectin, a key regulator of insulin sensitivity at the whole body level, is released from adipocytes at high rate into circulation. Reduced serum level of adiponectin and down-regulation of its mRNA expression are strongly associated with insulin resistance, early onset of Type 2 diabetes and obesity. Despite the importance of this adipokine in metabolism and energy homeostasis, the mechanism of its secretion from adipocytes remains poorly understood. Here, we investigated the subcellular localization of adiponectin, and its secretion regulation in differentiated 3T3-L1 and primary mouse adipocytes by using biochemical methods and fluorescence microscopic imaging. Adiponectin was localized in vesicular compartments, with no apparent overlap with p115, GM130 or syntaxin 6. Adiponectin-containing vesicles were enriched in two distinct pools: one at the plasma membrane and the other co-fractionating with ER membranes. We established a stable 3T3-L1 cell line expressing adiponectin-Venus, and confirmed that the adiponectin-venus vesicles completely overlap with endogenous adiponectin vesicles. When viewed under a TIRF microscope, a subset of adiponectin-venus vesicles were readily detected within the evanescent field in close proximity to plasma membrane, and could be released in response to insulin. The N-terminal signal peptide was required to direct the delivery of adiponectin into proper vesicles. Moreover, overexpression of adiponectin-venus carrying an ER retention motif (KDEL) impeded the detection of tubule-vesicular structure, indicating that the adiponectin-containing vesicles in the TIRF zone are post-ER and post-Golgi. Immuno-electron microscopy showed that adiponectin-venus proteins were present in vesicular structures in the vicinity of the plasma membrane. Finally, insulin-stimulated adiponectin release appeared to be from a pre-exiting pool of vesicles, and was not dependent on de novo protein synthesis, as adiponectin mRNA level remained unchanged over a 6-hour period of insulin treatment, and inhibition of protein synthesis had no effect on adiponectin release. Taken together, our results show that adiponectin is stored in a unique subcellular compartment, and a subset of adiponectin is released through a distinctive regulated exocytosis pathway.

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Regulation of cAMP-dependent Weibel-Palade body exocytosis from endothelial cells.

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Vascular endothelial cells provide a dynamic interface between circulating blood and underlying tissues that is critically involved in maintaining vascular integrity and homeostasis. Rapid recruitment of bio-active components from intracellular storage pools has been shown to contribute to the critical role of endothelial cells in maintaining vascular homeostasis. A significant number of haemostatic components and inflammatory mediators originate from endothelial cell-specific, cigar-shaped organelles called Weibel-Palade bodies (WPBs). WPBs function as storage vesicles for von Willebrand factor (VWF), a multimeric adhesive glycoprotein crucial for platelet plug formation, the leukocyte receptor P-selectin and a number of bioactive compounds that include the chemoattractants IL-8 and eotaxin-3. WPBs release their content following stimulation with agonists increasing intracellular Ca^{2+}, like thrombin, or agonists increasing intracellular levels of cAMP, such as epinephrine.

The physiological importance of this cAMP mediated pathway is illustrated by the rise in VWF levels in patients with von Willebrand's disease and mild hemophilia A following administration

of the vasopressin analogue desmopressin (DDAVP) or epinephrine. We have previously shown that this cAMP mediated WPB release is partly dependent on protein kinase A and involves the activation of the small GTPase RalA.During PKA regulated exocytosis a subset of the WPBs is able to escape secretion by perinuclear clustering at the microtubule organizing centre (MTOC), which involves retrograde transport of vesicles mediated by the dynein-dynactin complex
Here, we explored whether guanine nucleotide exchange factor Epac1 (exchange protein activated by cAMP 1) and its substrate, the small GTPase Rap1, are involved in cAMP-dependent regulation of WPB exocytosis. Epinephrine stimulation of endothelial cells leads to activation of the small GTPase Rap1 in a PKA-independent fashion. siRNA-mediated knockdown of Epac1 completely abolishes epinephrine-induced activation of Rap1 and decreases epinephrine-induced WPB exocytosis. We subsequently addressed whether Rap1 is crucial for WPB release using siRNA specific for Rap1. Downregulation of Rap1 expression abolished epinephrine but not thrombin-induced release of WPBs. Prevention of Rap1-activation through over-expression of Rap1GAP also effectively inhibits epinephrine-induced WPB exocytosis. Taken together, these data show that the Epac1-Rap1 pathway regulates WPB exocytosis by endothelial cells in response to cAMP-raising agonists.

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TLR-dependent regulation of MHC molecule transport is orchestrated by MARCH E3 ligase-dependent ubiquitination in antigen presenting cells.

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MARCH ubiquitin ligases have been recently identified on the basis of their homology to immunosuppressive viral factors. In mammalian cells, some MARCHs target immunorelevant receptors, promoting downregulation of specific antigen presentation molecules. Out of a family of 10 related ligases, we have identified MARCH I as the mediator of MHCII downregulation in human dendritic cells. MARCH VIII also targets MHCII and CD86, while MARCH IX acts on MHC I. MARCHs expression in human DCs is differentially regulated in response to specific environmental signals, such as TLR ligation or IL-10. The alteration of the correct expression pattern of these ligases could constitute for tumor cells a strategy to escape for immunosurveillance. To understand their function, we characterized the transcriptional regulation of MARCH E3 ligases in response to different conditions and its effect on the export of neosynthesized MHCI molecules through the secretory pathway, in the attempt to explore their function in the global coordination between pathogen stimulation and antigen presentation up-regulation of DC function.

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A zebrafish mutation in an exocytic machinery component reveals a requirement for membrane trafficking during chondrocyte cytokinesis.

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Vesicular trafficking is a fundamental cellular process and is central to maintaining organization and function along the secretory pathway. In addition to this role during interphase, however, membrane transport is also critical for completing cytokinesis, the terminal stage of mitosis. The mechanisms associated with membrane targeting to the midbody during cytokinesis remain poorly understood. Using a forward genetics approach in zebrafish to identify genes required for craniofacial development, we have identified a mutation in a novel component required for cytokinesis in chondrocytes. We show that mutant chondrocytes initially deposit cartilage extracellular matrix (ECM) proteins normally but then undergo apoptosis at later stages,

disrupting craniofacial morphogenesis. Histological and immunohistochemical analyses reveal that mutant chondrocytes appear to be stalled in cytokinesis; these cells form actin-rich cleavage furrow but fail to complete abscission. Using immunofluorescence and transmission electron microscopy, we show that mutant chondrocytes accumulate vesicular structures similar in morphology to transport vesicles during both cytokinesis and interphase. Unexpectedly, we discovered that although these cells fail to secrete N-glycosylated cargo, secretion of type II collagen is unaffected. Our results suggest that the exocytic machinery is not a homogenous conduit. Instead, we propose a model in which specific components of exocytic machinery differentially regulate ECM protein secretion. Our study further suggests that matrix secretion and membrane trafficking during cytokinesis share common regulatory mechanisms.

Endosomes, Lysosomes, and Lysosome-related Organelles

1634

To enter or not to enter: differentiating machinery from cargo in the MVB pathway.

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The Multivesicular body (MVB) pathway is the major pathway for the degradation of integral membrane proteins under normal cellular conditions. It is also important for the termination of cellular signals by sequestering the signaling membrane proteins away from the cytosol. MVBs are mature endosomes that contain internalized vesicles derived from the limiting membrane by an invagination process. This process is catalyzed and directed away from the cytosol by cytosolic protein complexes called ESCRTs (Endosomal Sorting Complexes Required for Transport). They recognize ubiquitinated (Ub) integral membrane proteins on the endosomal membrane and package them into the intralumenal vesicles. These internalized cargo proteins/lipids are ultimately delivered to the vacuole/lysosome for degradation upon fusion between endosomal and vacuolar/lysosomal limiting membranes. This function of ESCRTs is conserved from yeasts to humans. ESCRTs themselves predominantly stay outside MVBs by getting recycled off the endosome into the cytosol to be used for subsequent rounds of cargo sorting and vesicle formation. This process of ESCRT-recycling is not clear although we know that it requires the AAA ATPase Vps4 and that Vps4 directly binds to the final ESCRT complex, ESCRT-III. It is particularly intriguing as to how the early ESCRTs (ESCRT-0, -I and –II) are recycled back into the cytosol without any known direct interaction with Vps4. In this study, we explored a lattice/strong interaction-mediated recycling model in Saccharomyces cerevisiae wherein the ESCRTs get released off the endosome by virtue of their strong interactions with each other. To test this model, we constructed a chimeric ESCRT-like protein that binds ESCRT-III and endosomal membrane with GFP as a reporter. The ESCRT-III interacting domain is the MIT domain of Vps4 and the endosomal membrane interacting domain is the FYVE domain of the mammalian protein EEA1 that binds phosphatidyl inositol 3-phosphate on endosomes. Like ESCRTs, this chimeric protein gets recycled back into the cytosol. But when its interaction strength with ESCRT-III is weakened, the ESCRT-like protein gets packaged into MVB as a cargo. This observation agrees with recent evidence for ESCRT-I being partially packaged into the MVB. We are currently trying to directly test this interaction-strength-model for the recycling of ESCRT-I and ESCRT-I–like chimeras. Thus this study attempts to explain how ESCRTs manage to stay away from the vacuole to repeatedly function at the endosome. From the cargo point of view, it is noteworthy that we show for the first time that cytosolic cargo can be packaged into MVBs in a Ub-independent fashion.

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Mucolipin-1 induces fusion of lysosomes with the plasma membrane.

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Mucolipins represent a newly described family of cation channels that mediate release of calcium from the lumen of endosomes and lysosomes. It is well established that efflux of luminal calcium is required for endosomal fusion and plays a critical role in the maintenance of luminal pH and homeostasis. In humans, mutations in Mucolipin-1 (MCOLN1) cause Mucolipidosis type IV (MLIV), a lysosomal storage disorder characterized by severe and debilitating neurological and ophthalmologic abnormalities. In agreement with the significant role of calcium efflux in membrane trafficking, we have previously described that fusion between autophagosomes and lysosomes is impaired in fibroblasts from MLIV patients and this leads to defective autophagy and neuronal death. Here we describe a new and novel function for MCOLN1 in inducing lysosomal exocytosis. Expression of MCOLN1 induces fusion of lysosomes with the plasma membrane as revealed by secretion of active lysosomes hydrolases into the medium and by the accumulation of LAMP-1 at the cell surface. Depletion of ALG-2, a penta-EH-hand calcium-binding protein that interacts with MCOLN1 in a calcium-dependent manner, dramatically reduced the secretion of lysosomal enzymes mediated by MCOLN1. Lysosomal exocytosis was also significantly decreased by depletion of Rab7, while absence of PLEKHM1, a negative regulator of Rab7, increased the release. Importantly, no effect was observed upon depletion of VAMP-7, SNAP-23 or syntaxin-4. These results strongly suggest that the fusion machinery involved in this process is different from the one described in lysosomal-mediated membrane repair. Finally, we propose that MCOLN1-mediated lysosomal exocytosis may play a critical role in the clearance of accumulated undegraded material in lysosomal storage diseases.

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Localization and functional requirement of yeast Na^+/H^+ exchanger, Nhx1p, in the endocytic and protein recycling pathway.

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Acidification of the lumen of intracellular organelles is important for post-transcriptional processing, endosomal maturation, receptor recycling, and vesicle trafficking, being regulated by an intricate balance between H^+ influx through vacuolar-type H^+-ATPase and efflux through ion channels and transporters, such as the Na^+/H^+ exchanger (NHE). The eukaryotic NHE family comprises two major subgroups, one residing in the plasma membrane and the other in intracellular organelles. While mammalian intracellular NHE isoforms are localized to various organelles, including the mid-trans-Golgi compartments, early and late endosomes, and recycling endosomes, Nhx1p, the sole NHE in yeast, has been reported to be localized predominantly to the late endosomal/prevacuolar compartment. Here, using live cell imaging, we demonstrated that Nhx1p is localized to the trans-Golgi compartments, late endosomes, and recycling endosomes, similar to mammalian intracellular NHE isoforms. Loss of Nhx1p led to accumulation of components of the retromer complex and late endosomal/prevacuolar compartments, but not trans-Golgi compartments, in aberrant prevacuolar compartments. Importantly, Nhx1p was also required for recycling of the plasma membrane v-SNARE Snc1p. These observations suggest that Nhx1p plays an important role in regulation of the luminal pH of various intracellular organelles, and that this regulation is critical for the protein recycling pathway as well as the biosynthetic and endocytic pathway.

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Saccharomyces cerevisiae ENV7 gene encodes a novel vacuolar membrane protein-kinase and is a homolog of human ser/thr kinase STK16.

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Yeast vacuoles are analogous to mammalian lysosomes and serve as a seminal model system in understanding lysosomal trafficking, biogenesis and function. A genome wide screen for genes involved at endosome and vacuole interface function by our laboratory has identified several novel *ENV* genes. Here we report on multifaceted characterization of the novel *ENV7* gene product. Confocal microscopic studies confirm localization of both endogenously and ectopically expressed GFP tagged Env7p to vacuolar membranes. Sub-cellular fractionation revealed co-fractionation of HA-tagged Env7p with vacuolar CPY, and biochemical manipulations established that membrane association is resistant to salt and carbonate treatments. Bioinformatic analyses suggest no signal peptide or membrane-spanning domains, but identify two highly conserved regions: an N-terminal string of cysteines that may serve as putative palmitoylation sites for membrane anchoring, and a ser/thr kinase domain. By employing site-directed mutagenesis, we show that triple cysteine residues at N-terminus of the protein are essential for its proper membrane association and stabilization. Using *in vitro* kinase assays, we report that Env7p is a novel protein-kinase capable of mediating both autophosphorylation and phosphorylation of exogenous substrates. Bayesian phylogenetic analyses revealed that the *ENV7* gene is well conserved across a wide array of eukaryotes including humans yet is absent in prokaryotes – an evolutionary pattern consistent with endomembrane proteins. The analyses strongly support Env7p as a homolog of human STK16, a palmitoylated and myristoylated ser/thr kinase localized to the endomembrane system with no known function. Site-directed mutagenesis revealed that glutamic acid at position 269 of Env7p is critical for its kinase activity, as it has been reported for its human homolog. E269A substitution resulted in a kinase-dead allele. Interestingly, E269A mutant is unstable *in vivo*, but could be significantly stabilized by chemical as well as genetic interrruption of the proteasomal system. Based on these findings, we conclude that Env7p is a novel and conserved vacuolar membrane protein-kinase whose membrane association and phosphorylation are essential for its *in vivo* stability. We also propose that misfolded or mislocalized Env7p is a substrate for proteasomal degradation.

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The Family of V- Domain Proteins as Endosomal Ubiquitin Sorting Receptors.

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Ubiquitin is used as a lysosomal sorting signal for post-Golgi integral membrane proteins. Ubiquitin induces both internalization and subsequent sorting into endosomal intralumenal vesicles that accumulate in late endosomes/multivesicular bodies. Entry into the MVB pathway is guided by the ESCRTs (Endosomal Sorting Complex Required for Transport) as well as critical ESCRT-associated proteins such as Bro1 and the AAA-ATPase Vps4. ESCRT-0, -I and –II all have ubiquitin binding domains, making at least some of these components excellent candidates for ubiquitin-sorting receptors that could recognize, gather, and sort ubiquitinated cargo proteins into MVB intralumenal vesicles. We present data showing that Bro1p may work as Ub-sorting receptor that works at endosomes in parallel with ESCRT-0. The Bro1p family includes several proteins such as Alix, HD-PTP, and RIM20, which function in a variety of cellular processes such as sorting of cargoes into MVB vesicles, viral budding from cells, cytokinesis, and many of which associate with the ESCRT machinery. These proteins comprise

conserved ESCRT-III targeting Bro1-domain at the N-terminus followed by a middle V-domain. We find that by targeting an engineered Bro1 protein tethered to a deubiquitinating enzyme dramatically inhibits the sorting of ubiquitinated cargo into the MVB pathway, consistent with its role early in the process of sorting. Like other endosomal Ub-sorting receptors such as GGAs, Hrs/STAM (ESCRT-0), and Tom1, Bro1 also binds clathrin via its central V-domain. Mutations that abolish the interaction of Bro1 with clathrin also abolish its ability to affect MVB sorting. We also have discovered that the V-domain of Bro1 binds to ubiquitin. In addition, crystallography, NMR, modeling predictions, and mutagenesis studies show that all V-domains bind Ub, although via distinct binding modes. Together our results indicate that Ub interaction with Bro1 and other family members may provide a variety of functions, and in the case of Bro1, provides a means by which Bro1 acts as an Ub-sorting receptor.

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The Mon1-Ccz1 Complex Promotes Endosome Maturation in *Saccharomyces cerevisiae.*

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Many proteins destined for the lysosome are trafficked through the endocytic pathway. The directionality of transport is regulated by a rab cascade involving Rab5 and Rab7. Rab5 is found on early endosomes and mediates fusion of incoming endocytic vesicles. As an early endosome matures into a late endosome Rab5 is lost and Rab7 is acquired. Acquisition of Rab7 allows for fusion of a late endosome with a lysosome. This transition from Rab5 to Rab7 is regulated by SAND1/Mon1 in *C. elegans* and in mammalian cells. SAND1/Mon1 stimulates this transition by displacing RABX-5, the guanine nucleotide exchange factor (GEF) for Rab5. In the absence of RABX-5, Rab5 is no longer active. The yeast homolog, Mon1, in complex with Ccz1, has recently been shown to be the GEF for Ypt7, the Rab7 homolog. In *Saccharomyces cerevisiae*, Ypt7 regulates fusion with the vacuole, the yeast lysosome. The aim of this work is to understand the role of the Mon1-Ccz1 complex in the yeast endocytic pathway. Using light microscopy we show that deletion of *MON1* leads to a punctate accumulation of the Rab5 homolog, Vps21, as well as its GEF, Vps9. Analysis of *mon1Δ* cells by electron microscopy shows an accumulation of enlarged endosomes, consistent with extended Vps21 activity. However, Vps9 is not displaced following overexpression of either Mon1 alone or both Mon1 and Ccz1. This indicates that Mon1/Ccz1 has a role in down-regulating Vps21 activity but perhaps not through displacement of Vps9, as seen in other systems. Endosomal maturation also involves the sorting of transmembrane cargo, such as carboxy peptidase S (CPS), into inward budding intralumenal vesicles. This requires the action of the Endosomal Sorting Complexes required for Transport (ESCRTs). Upon fusion with the vacuole the intralumenal vesicles are released into the hydrolytic interior of the vacuole lumen. Cells lacking Mon1 are still able to sort GFP-CPS to the vacuole lumen, and electron microscopy shows that these cells contain normal intralumenal vesicles. Taken together these data indicate that Mon1 is not required for ESCRT function. We conclude that the Mon1-Ccz1 complex contributes to endosome maturation by promoting termination of early endosome fusion and this occurs after sorting of cargo by ESCRTs.

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ESCRT-II links RNA localization to endosomal trafficking and translational control.

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Spatial and temporal control of mRNA expression is essential for establishing cell polarity and proper embryonic development. Endosomes are linked to subcellular mRNA localization and translational control, yet little is known about the mechanism of RNA localization to endosomes. Using Xenopus egg extracts we found that the endosomal-sorting complex ESCRT-II bound to

hundreds of mRNAs, including many mRNAs previously identified to localize asymmetrically in oocytes as well as many mRNAs that regulate cell cycle progression. Purified, recombinant ESCRT-II bound directly to single-stranded RNA in vitro. In addition to RNA, ESCRT-II bound to membranes containing PI3P phospholipids, the main phospholipid of endosomes. Interestingly, ESCRT-II simultaneously bound directly to RNA and membranes in vitro and was sufficient to tether RNA to specific phospholipids. Furthermore, immunodepletion of ESCRT-II abolished mRNA localization to endosomes, demonstrating that ESCRT-II is both necessary and sufficient for mRNA localization to endosomes. Finally, ESCRT-II functioned as a translational regulator in vitro and in vivo, and immunodepletion of ESCRT-II from Xenopus egg extracts resulted in multiple defects in cell cycle progression resulting from disregulation of translational control. In sum, our data suggest that ESCRT-II-mediated mRNA localization to endosomes is likely to be a key step in the spatial and temporal control of mRNA expression in all cells.

1642

Investigating the therapeutic potential of HDAC Inhibitors for the treatment of Niemann-Pick type C1 Disease.

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Although some progress has been made in recent years, suitable treatment options for reversal of Niemann-Pick type C1 disease are lacking. Recently, we have shown that treating fibroblasts from NPC1 patients with histone deacetylase inhibitors (HDACi) at sub-micromolar concentrations corrects the NPC1 phenotype (Pipalia et.al. PNAS 108: 5620). This effect was observed in two different NPC1 mutant fibroblasts, but not in NPC2 cells. Although the two NPC1 mutant cells were from different patients, both had one common mutation at I1061T (GM03123 with compound heterozygous and GM18453 with homozygous mutation). These results raised an important question: is the efficacy of HDACi is mutant specific? Hence, we are studying the effect of these HDACi on multiple additional NPC1 human fibroblasts from the patients carrying different type of mutations. Initial results on cells from four patients indicate that two HDACi's (LBH589 and SAHA) used in this study correct the NPC1 phenotype with varying efficacy. We are in a process of screening ~50 additional NPC1 mutations found in patients. This work may help in providing targeted therapy to patients.

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Cellular and Organismal Consequences of Defects in NPC1 Membrane Function.

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Niemann Pick Type C (NPC) disease is a cholesterol transport disorder induced by defects in the NPC1 and NPC2 genes. Focusing on mutations in NPC1, which are responsible for 95% of human cases, we have identified a defect in the dynamic membrane tubulation of late endosomes and lysosomes (LE/Ls). This loss of tubulation correlates strongly with the accumulation of cholesterol and other lipids in LE/Ls and a loss of cholesterol sensing in ER membranes. To determine the basis of this loss of tubulation, we performed MS/MS analysis of purified NPC1 membranes and identified novel tubulation factors. STARD9 was identified as a multi-pass transmembrane protein containing an N-terminal kinesin motor domain. STARD9 is expressed prominently in Purkinje neurons, which are the most vulnerable neuronal cell type in NPC disease. STARD9 was found to be present in wild-type NPC1 membranes but absent from mutant NPC1 membranes. ShRNA analysis of STARD9 revealed a loss of tubulation of NPC1-containing membranes and evidence of cholesterol accumulation. Cholesterol sensing in the ER was also affected by STARD9 depletion. Live-cell imaging of tagged STARD9 constructs

reveals evidence of microtubule binding, strengthening the case for STARD9 to be involved in membrane tubulation. To evaluate zebrafish as a model system to study NPC disease, we have also prepared morpholinos directed against the 5'UTR of zebrafish NPC1. Morpholino injection affects brain development and induces accumulation of cholesterol in multiple brain areas. We observe a significance reduction in the size of the developing cerebellum and the number of Purkinje neurons in the morphant fish. These areas of the brain also display evidence of cell death. Interestingly, cholesterol accumulation is not restricted to Purkinje neurons, suggesting that something other than cholesterol accumulation itself is defective. These studies provide additional details of NPC1 membrane tubulation and suggest that zebrafish will be a powerful model system to study the etiology of NPC disease.

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A Novel Phosphoinositide Pathway Controls EGF Receptor Signaling, Lysosomal Trafficking and Degradation.

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The epidermal growth factor receptor (EGFR) plays pivotal roles in cell proliferation and differentiation. The modulation of EGFR endosomal trafficking and degradation is essential for proper signaling. Here, we show that type Igamma phosphatidylinositol phosphate kinase i5 (PIPKIγi5), an enzyme that synthesizes phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) on the endosomes, controls EGFR lysosomal trafficking and degradation. Loss of PIPKIγi5 via siRNA results in a block of EGFR trafficking to the lysosome, resulting in endosomal retention of activated EGFR. This sorting failure resulted in enhanced and prolonged EGFR downstream signaling. However, loss of PIPKIγi5 did not impact the sorting and degradation of other receptors indicating that overall lysosome function was not impaired.

PIPKIγi5 controls EGFR trafficking by regulating sorting nexins (SNX) 5 and 6, both phosphoinositide effectors and interaction partners of PIPKIγi5. Loss of either SNX also blocked EGFR lysosomal trafficking and degradation similar to that of the PIPKI(i5 knockdown. PIPKIγi5 kinase activity regulates the interaction of SNX5/6 with Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs), a key component of the ESCRT-0 complex that plays important role in EGFR trafficking into MVB. That indicates a role of PIPKIγi5 and SNX5/6 in EGFR MVB sorting. These finding reveal a novel pathway where PIPKIγi5, $PI4,5P_2$, and SNX5/6 form a novel phosphoinositide signaling nexus that controls EGFR signaling by modulating lysosomal trafficking. These results challenge the dogma that PI3P and $PI3,5P_2$ are the only phosphoinositides required for endosomal trafficking and lysosomal degradation.

1645

Endosomal Accumulation of the Activated EGFR Induces Apoptosis.

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Endocytosis both positively and negatively regulates cell surface receptor signaling by temporally and spatially controlling interactions with downstream effectors. Considerable evidence indicates the endocytic pathway controls the biochemistry of receptor:effector communication, however, there are less data indicating endocytosis regulates cell physiology. MDA-MB-468 cells are an excellent model for studying the endocytic regulation of EGFR signaling. In MDA-MB-468 cells, liganded cell surface EGFRs promote cell growth whereas intracellular, liganded EGFRs induce apoptosis. In contrast to most cells, in MDA-MB-468 cells

there is no appreciable decrease in EGFR levels following EGF stimulation [Hyatt and Ceresa (2008) Exp. Cell Res., 314(18):3415-25]. In this study, we report that in MDA-MB-468 cells defective endocytic trafficking causes the phosphorylated EGFR to accumulate in the early endosome up to 24 hours after ligand stimulation. These receptors are on the limiting membrane of the endosome and oriented with the carboxyl terminus in the cytoplasm and the amino terminus in the lumen of the endosome. To determine whether this perturbation in EGFR trafficking is sufficient to cause apoptosis, we used biochemical and pharmacological strategies to disrupt EGFR endocytic trafficking in cells that do not normally undergo EGF-dependent apoptosis. When HeLa cells are manipulated such that active EGF:EGFRs accumulate on the limiting membrane of endosomes, receptor phosphorylation is sustained and the cells undergo apoptosis. However, when cells were manipulated to accumulate the EGF:EGFR complex in the intraluminal vesicles of the late endosome, phosphorylation of the receptor was not sustained nor did the cells undergo apoptosis. The dose-dependent induction of apoptosis indicates that it is a function of the liganded receptor. These data demonstrate that the EGFR-mediated apoptosis is initiated from the limiting membrane of the endosome.

1646

The role of Arl8b and Rab7 GTPases in lysosome tabulation.

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Lysosomes are essential for the catabolism of endocytic and biosynthetic cargo, phagosome maturation and autophagy. Therefore, we are interested in understanding the mechanisms that establish, maintain and/or change the properties and function of these organelles. In resting macrophages, lysosomes are predominantly small, globular organelles, appearing punctate under the fluorescence microscope. In contrast, activation of macrophages with phorbol esters or LPS was previously shown to convert lysosomes into tubular lysosomes (TLs). TLs are suggested to have a role in delivering V-ATPases to phagosomes for acidification and to help retain fluid-phase endocytic uptake in macrophages. However, very little else is known about how TLs form and function. To address this lack of knowledge, we investigated the molecular requirements for LPS-stimulated tubulation of lysosomes in RAW and J774 macrophages. We show that TLs are very dynamic relative to punctate lysosomes; not only did TLs fuse, fission, branch, and displayed bidirectional movement, they also moved at 0.35 µm/sec, which was significantly faster than 0.15 µm/sec for punctate lysosomes. Consistent with this, TL formation required microtubules and the kinesin and dynein motor proteins. The lysosomal Rab7 and Arl8b GTPases are regulators of motor proteins. Thus, we investigated if these GTPases were important for TL biogenesis. Not only did Rab7 and Arl8b GTPases colocalize to TLs, but importantly, expression of dominant negative mutants or siRNA against Rab7 and Arl8b potently blocked TL formation. Moreover, Rab7 anchors dynein and kinesin to membranes via the adaptor proteins RILP and FYCO1, respectively. Indeed, overexpression of RILP and FYCO1, which respectively hyperactivates dynein and kinesin, hindered TL formation. This suggests that dynein and kinesin activities must be balanced for TL biogenesis. Taken together, our data supports a model whereby Rab7 and Arl8b modulate dynein and kinesin motor proteins to transform punctate lysosomes into TLs. In turn, TLs are significantly more dynamic than punctate lysosomes.

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Arf-like GTPase, Arl8b interacts with HOPS complex and regulates lysosomal trafficking and antigen presentation.

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Lysosomes are membrane-bound organelles present in eukaryotes whose catabolic function is essential for maintaining general cellular homeostasis. They receive and degrade macromolecules from the endocytic, autophagic and phagocytic membrane trafficking pathways. A specialized function of lysosomes in antigen presenting cells is to serve as the key cellular compartment where antigen presenting molecules including CD1 and MHC class II intersect with, and bind microbial antigens for presentation to T cells. Thus, uncovering the host endocytic machinery that regulates delivery of antigen presenting molecules to lysosomes is critical to understanding host defense. To identify molecular mediators of CD1 trafficking to and from the lysosomes, we assembled a library of short hairpin RNAs targeting members of the endocytic regulatory protein families and used it to screen for loss of CD1 antigen presenting function. Here, using a lysosome-dependent immunological screen of a trafficking short hairpin RNA library, we identified the Arf-like GTPase, Arl8b as a critical regulator of cargo delivery to lysosomes. In addition to endocytic cargo, delivery of phagocytosed cargo to lysosomes was also impaired in Arl8b-depleted macrophages. To gain insight into the mechanism of Arl8b action, we conducted GST pulldown assays using purified GST-Arl8b and identified HOmotypic fusion and vacuole Protein Sorting (HOPS) complex members as effectors of Arl8b. HOPS complex is a six subunit complex conserved from yeast to humans and is considered to be the critical tethering complex that mediates the fusion of late-endosomes and lysosomes. A direct interaction of GTP-bound Arl8b with Vps41 subunit of the HOPS complex was observed using yeast-two-hybrid and purified proteins. Moreover, overexpression of Arl8b recruited Vps41 and other HOPS subunits to LAMP1 positive lysosomes. On the contrary, knockdown of Arl8b led to complete loss of HOPS complex subunits from lysosomes suggesting that like other small GTPases, Arl8b is critical for membrane association of its downstream effector proteins. Similar to Arl8b knockdown, depletion of HOPS complex subunits also led to delay in trafficking of cargo including CD1d to lysosomes. Together, these results define Arl8b and HOPS complex as key regulators of lysosomal cellular and immunological functions.

1648

Role of Arf-like GTPase Arl8b in Regulating Lysosome/Lytic Granule Motility and Exocytosis.

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A key mechanism that immune cells including Natural killer (NK) cells and cytotoxic T-lymphocytes employ to kill infected or diseased target cells is through regulated exocytosis of lytic granule contents towards the target cell membrane. Although the mechanism by which lytic granules kill the target cells is well-understood, the preceding molecular steps including their movement on microtubules and polarization towards the immunological synapse (IS) is less clear. Previous studies of a small GTPase of the Arl family, Arl8b, had shown that its expression increases motility of lysosomes on microtubule tracks while its knockdown leads to clustering of

lysosomes at the microtubule organizing center (MTOC). Based on these observations, we hypothesized that Arl8b regulates motility and exocytosis of lysosome and lysosome-related organelles required to mediate various physiological events including target-cell killing and plasma membrane (PM) repair. We found endogenous Arl8b co-localized with perforin and granzyme containing lytic granules in both ex vivo NK cells and immortalized NK cell line, YT-Indy. Moreover, in a comparison of cell mediated cytotoxicity as measured using standard Cr(51) release assay, Arl8b-deficient NK cells clearly demonstrated a significant loss of cytolytic activity against 721.221 target cells compared with control cells. To gain insight into the mechanism of Arl8b action, we assessed which step during lytic granule exocytosis is impaired upon Arl8b knockdown. It was previously reported that the microtubule organizing centre (MTOC) translocates towards the IS as it matures. This reorientation is essential for the movement and polarization of the granules to the site of release. We examined the role of Arl8b in these processes using Arl8b-deficient cells. Interestingly, compared to the control knockdown, in NK cells lacking Arl8b, lytic granules and MTOC failed to polarize at the IS and remained almost at the opposite pole to that of the IS. Since Arl8b was first described to be a lysosomal small GTPase, we also analyzed its role in regulating the motility and exocytosis of conventional lysosomes during the process of PM repair. Similar to our findings with lytic granules, conventional lysosomes also showed significantly reduced exocytosis upon treatment with ionomycin, an ionophore commonly used to induce PM repair response. Our findings suggests Arl8b to be a critical factor required for motility and exocytosis of lysosomes and lysosomes-related organelles.

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Glucose starvation regulates clathrin adaptors at the trans-Golgi Network and endosomes.

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Glucose is a rich source of energy and the raw material for biomass increase. Many eukaryotic cells remodel their physiology in the presence and absence of glucose. The yeast Saccharomyces cerevisiae undergoes changes in transcription, translation, metabolism and cell polarity in response to glucose availability. Upon glucose starvation, translation initiation and cell polarity are immediately inhibited and then gradually recover. We demonstrate that like cell polarity and translation, traffic at the trans-Golgi network (TGN) and endosomes is regulated by glucose via an unknown mechanism that depends on Protein Kinase A (PKA). Upon glucose withdrawal, clathrin adaptors exhibit a biphasic change in localization: they initially delocalize from the membrane within minutes and later partially recover onto membranes. These changes are co-incident with changes in posttranslational modifications of adaptors. The clathrin adaptors Ent5 and Gga2 exhibit glucose dependent phosphorylation and dephosphorylation respectively. Ras and Gpr1 signaling pathways, which converge on PKA, are required for changes in adaptor localization and changes in posttranslational modifications. Acute inhibition of PKA demonstrates that inhibition of PKA prior to glucose withdrawal prevents several adaptor responses to starvation including changes in localization and phosphorylation. These findings suggest that membrane traffic at the TGN and endosomes is remodeled by glucose starvation and that PKA activity prior to glucose starvation primes this response.

1650

Cortactin regulates Golgi size by controlling retrograde trafficking from late endosomes.

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Dynamic flow of membrane between cellular compartments is critical for maintenance of organelle size and function. While the actin cytoskeleton is known to regulate Golgi homeostasis and morphology, the mechanism is unclear. Here, we show that knockdown (KD) of the branched actin regulator cortactin leads to a dramatic reduction in Golgi size, dependent on binding the Arp2/3 actin-nucleating complex. Surprisingly, aside from Golgi volume changes, there was little effect of cortactin transient or stable KD on Golgi stack ultrastructure, anterograde trafficking of the constitutive cargo VSV-G, or Golgi assembly from ER membranes. However, 2D and 3D image analysis by electron microscopy revealed a notable expansion of prelysosomal organelles, suggesting an exit block from late endosomes. Inhibition of late endosomal trafficking by treatment of cells with Rab7 siRNA or the endosomal inhibitor chloroquine led to a similar compact Golgi morphology to that found in cortactin-KD cells. These data indicate that vesicular trafficking from late endosomes, regulated by cortactin-decorated branched actin networks, is a major mechanism for maintenance of Golgi homeostasis.

1651

Antagonistic control of lysosomal fusion by Rab14 and the Lyst-related protein LvsB.

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The function of the protein Lyst, defective in patients with the Chediak-Higashi Syndrome, is not known at the molecular level. We have shown that the Dictyostelium ortholog of Lyst, LvsB is a cytosolic protein that associates with lysosomes and postlysosomes. We found that LvsB acts as an antagonist to the activity of DdRab14, a protein that promotes homotypic fusion among lysosomes. DdRab14 is normally found on lysosomes and leaves those lysosomes as they mature into postlysosomes. On the other hand, LvsB binds to those lysosomes that are about to mature into postlysosomes. Interestingly, in the absence of LvsB, DdRab14 is found to colocalize with postlysosomal markers. Importantly, activation of DdRab14 phenocopies the loss of LvsB causing abnormal heterotypic fusion between lysosomes and postlysosomes and the great enlargement of those compartments. In contrast, inactivation of DdRab14 suppresses the phenotype of LvsB null cells restoring their lysosomal size and segregation from postlysosomes. This suppression effect is specific for LvsB function since DdRab14 inactivation does not suppress the phenotype of vacuolin B mutants. Our data suggest a model whereby LvsB binds to late lysosomes and promotes, by an unknown mechanism, the inactivation of DdRab14. This inactivation allows the lysosomes to mature into postlysosomes for eventual secretion. We propose that human Lyst may act in a similar way to promote the maturation of lysosome-related organelles.

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AP-1B and Arf6 cooperate in the maintenance of polarized membranes in epithelial cells.

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Apical-basolateral cell polarity is fundamental for the organization of epithelial tissues. It is well established that the epithelial cell-specific clathrin adaptor complex AP-1B is important for

basolateral sorting of transmembrane receptors from recycling endosomes to the basolateral plasma membrane. Here we show that the small GTPase Arf6 regulates AP-1B function. We found that interfering with Arf6 functions by overexpression of mutant Arf6 alleles or depleting Arf6 expression with shRNAs led to specific apical missorting of AP-1B-dependent cargos. Furthermore, we found that Arf6 could precipitate AP-1B in vitro, and that Arf6 and AP-1B partially colocalized in recycling endosomes. We hypothesize that Arf6 specifically recruits AP-1B onto recycling endosomes to initiate the generation of basolaterally-targeted vesicles. However, a functional interaction between Arf6 and AP-1B may not be restricted to recycling endosomes. Indeed, we also found specific colocalization of AP-1B, but not AP-1A, with Arf6 in membrane ruffles of epithelial cells. Because Arf6 is known to be involved in Rac-induced ruffling and cell migration[1-4], we are now investigating whether AP-1B has a functional role in these processes. So far, preliminary data indicate that exogenous expression of µ1B/AP-1B in µ1B-negative LLC-PK1 cells decreases the migration speed in a wound-healing assay. This was not because of a difference in the actin organization in the leading edges of the migrating cells, suggesting that the cytoskeleton may not be the cause of this speed change. In conclusion, Arf6 and AP-1B may cooperate in diverse cellular processes at multiple intracellular locations.

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1653

Trafficking of Late Endosomes and Secretion of Exosomes in Polarized Epithelial Cells are Regulated by Cholesterol and SNARE Proteins.

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Late endosomes are important hubs for sorting proteins and lipids to lysosomes for degradation or recycling to the plasma membrane. Exocytosis of late endosomes results in the release of intraluminal vesicles or exosomes into the extracellular space. Exosomes are a novel mode of cell-independent communication and function in antigen presentation, immune responses, and stem cell and tumor biology. In polarized epithelia, apical and basolateral endocytic routes converge in late endosomes and polarized secretion of exosomes may participate in the establishment and maintenance of apico-basal polarity and in the epithelial-mesenchymal transition. However, little is known about late endosome trafficking or the machinery involved in exosome release in epithelial cells. Here, we investigated these phenomena in polarized Madin-Darby canine kidney (MDCK) and retinal pigment epithelial (RPE) cells. Live imaging using spinning disk confocal microscopy showed that late endosomal membrane proteins LAMP2, CD63 and Niemann-Pick C1 (NPC1) are present in tubules and vesicles that undergo rapid, bidirectional movement over long distances. In cells treated with U18666A or the retinal lipofuscin fluorophore A2E, both of which cause cholesterol accumulation in late endocytic compartments, there is a significant inhibition of tubulovesicular trafficking from late endosomes and lysosomes. Immunoprecipitation experiments showed that in RPE cells with A2E or excess cholesterol, the v-SNARE VAMP7 is sequestered with Vti1b on late endosomal/lysosomal membranes. This prevents VAMP7 from interacting with the t-SNARE syntaxin 4 at the plasma membrane and inhibits exocytosis of late endosomes. Exosomes released apically have different protein compositions than those released at the basolateral surface. Cholesterol loading and A2E also inhibit the release of exosomes. Accumulation of lipofuscin and A2E are hallmarks of age-related macular degeneration (AMD), the most common cause of vision loss in older adults. Histopathological analyses of eyes with AMD show cholesterol deposits in the RPE

basement membrane and allelic variants in cholesterol modulating proteins have been implicated in AMD. At the cellular level, cholesterol homeostasis and late endosome/lysosome function have emerged as hotspots in the pathogenesis of several diseases of aging such as atherosclerosis, Alzheimer's disease and AMD. Our data show that in the RPE, lipofuscin and cholesterol reorganize late endosomal-lysosomal membranes and interfere with the molecular machinery required for organelle motility and function. Over time, disturbed cholesterol homeostasis in the RPE can cause late endosomal traffic jams, impair lysosome function and lead to oxidative stress, inflammation and chronic RPE cell damage.

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Rac and Rab GTPases Dual Effector Nischarin Facilitates Survival of Intracellular Bacteria.

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Intracellular pathogenic bacteria, like Salmonella typhimurium, survive inside host cells by blocking the fusion of bacteria-containing endosomal vacuoles with lysosomes, thereby preventing degradation of the engulfed bacteria. The transport and fusion of membrane-bound compartments in a cell is regulated by small GTPases including Rac and members of the Rab GTPase family, and their effector proteins. However, the role of these components for survival of intracellular pathogens is poorly understood. Here, we identify Nischarin as a novel dual effector that can interact with members of Rac and Rab GTPase (Rab4, Rab14 and Rab9) families at different endosomal compartments. Sequential interaction of Nischarin with these GTPases, regulates the fate of Salmonella-containing vacuoles (SCV) produced after phagocytosis of the pathogen and prevent fusion of SCV with lysosomes.

1655

Syndecans regulate the biogenesis of exosomes: a non-cell autonomous function in signaling.

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Objectives:

Syndecans, heparan sulfate proteoglycans, are essential for fine-regulating signaling events between cells. The extracellular heparan sulfate chains of these membrane proteins attract several signaling molecules, such as morphogens, to cell surfaces, but the mechanisms of their regulatory functions remain largely unknown.

We have found that, through the PDZ protein syntenin, a well-known syndecan cytoplasmic adaptor, syndecans are connected to Alix, an auxiliary component of the ESCRT machinery, implicated in vesicular budding, and marker of small signaling vesicles released by cells known as ‘exosomes’.

Here, we explore whether the syndecan-syntenin-Alix connection might regulate the biogenesis of exosomes, impacting on the cellular release of morphogen signals.

Results:
Syndecan-1 is released in exosomes and co-fractionates, together with syntenin and Alix, with exosomal marker proteins such as CD63. While syntenin over-expression results, depending on Alix, in an increased release of exosomes, the depletion of syntenin (or Alix) by RNAi treatment blocks the exosomal accumulation of syndecan and marker proteins. Strikingly, syndecan RNAi markedly suppresses exosome production.
Syntenin has an effect on a subset of exosomes: while CD63 and syntenin largely reside in the same exosome population, as shown by immuno-fractionation and by fluorescence cross-correlation spectroscopy, flotillin-1 co-purifies mainly with CD63-negative vesicles and its release is not affected by syntenin.
Syntenin-exosomes are of endosomal origin and depend on endosomal trafficking, as shown by RNAi targeting Rab7, a small GTPase regulating late endocytic membrane traffic. Electron microscopy analysis of syntenin-depleted cells shows that MVBs are largely devoid of intraluminal vesicles, indicating that syntenin supports the intraluminal budding and then production of exosomes.
The molecular machinery responsible for the biogenesis of syntenin-exosomes is ESCRT-dependent, as depletion by RNAi of several components of the ESCRT complexes affects the formation of these exosomes. The presence of heparan sulfate chains on syndecans is necessary for producing exosomes, and the release of exosomes, impaired in HS-depleted cells, can be rescued by anti-syndecan antibody, suggesting that ligand-initiated syndecan oligomerization might be the driving force for the production of syntenin-exosomes.
Syndecan-syntenin exosomes contain HS-dependent signaling cargo, such as p-FGFR, and can transfer FGF-responsiveness to recipient cells, hinting at the functional relevance of these exosomes.
Conclusions:
The results obtained show that interfering with syndecan-syntenin-Alix reduces exosome production by affecting the biogenesis of MVBs and that syndecan-syntenin-Alix complexes control the composition of a specific population of exosomes, potentially transferring specific information between cells. The molecular connection of syndecan with exosomes might thus support a novel role for proteoglycans in vesicular trafficking and trans-cellular signaling, supporting the release of growth factors and signaling components into the extracellular space. The establishment of a direct link between syndecans and exosomes, as a novel mechanism of morphogen spreading, may help explain the role of HSPG in the diffusion of morphogenetic signals, in particular how lipid-modified morphogens travel long-range. Syndecan-syntenin exosomes might also participate in the biology of HS-associated pathological processes, including neurodegenerative and oncogenic diseases.

1656

Characterization of the Vac14 multimer and its role in regulating phosphatidylinositol-3,5-bisphosphate.

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Phosphatidylinositol-3,5-bisphosphate (PtdIns(3,5)P2) is an important regulator of endolysosome function. For example, PtdIns(3,5)P2 deficiency causes a striking enlargement of the yeast vacuole and swelling of endolysosomes in higher eukaryotes. Moreover, PtdIns(3,5)P2 defects is now linked to neuropathologies in humans. To understand PtdIns(3,5)P2 action, it is essential to define the mechanisms underlying the synthesis of PtdIns(3,5)P2. In yeast, PtdIns(3,5)P2 is synthesized by the Fab1 PtdIns(3)P 5-phosphatase Fab1 and degraded by the antagonistic Fig4 phosphatase. Interestingly, Fab1 and Fig4 form a common protein complex, along with Vac14. Although Vac14 binds and regulates both Fab1

and Fig4, we do not understand how Vac14 does this. Yeast two-hybrid assays, co-immunoprecipitation and in vitro protein interaction assays with recombinant Vac14 all indicate that Vac14 self-interacts – thus Vac14 likely is a core scaffold for Fab1 and Fig4. However, it is not known if what the multimeric state of Vac14 is and if its multimerization is necessary for interaction and regulation of Fab1 and/or Fig4. Therefore, to solve the importance of Vac14 multimerization, we set out to identify the multimeric state of recombinant Vac14 and the domain responsible for Vac14 self-interaction. Size-exclusion chromatography and ultracentrifugation with glycerol gradients were used to identify the molecular weight of the recombinant Vac14 multimer. The combined results of these two techniques suggest that the Vac14 multimer is non-globular and exists as a homodimer and/or homotrimer. Moreover, using sequential Vac14 truncations and co-immunoprecipitation from yeast lysates, our data suggests that residues 550-880 in the C-terminus of Vac14 are necessary for Vac14 self-interaction. We are now using analytical ultracentrifugation to obtain a precise multimeric state of Vac14 and developing point mutants in conserved residues of the C-terminus to better characterize Vac14 multimerization. Ultimately, these point mutants will be used in functional assays.

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The Endosomal Rab Cascade is Dependent on ESCRT Activity.

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Maturation of early endosomes into late endosomes is marked by replacement of Rab5 with Rab7 at endosomal membranes, making them competent for fusion with lysosomes (vacuoles in yeast). Maturation also involves the budding of intralumenal vesicles (ILVs), leading to the multivesicular body (MVB) morphology of late endosomes. The switch in fusion properties driven by Rab5-Rab7 conversion coincides with the activity of the endosomal sorting complexes required for transport (ESCRTs), which mediate the budding of ILVs. However, it is unclear if Rab conversion and ESCRT activity are mechanistically coordinated. To address this issue, we examine how loss of ESCRT activity leads to formation of enlarged endosomal structures known as class E compartments. We show in yeast that the loss of ESCRT function disables Rab conversion to result in the chronic localization of Rab5 and its guanine nucleotide exchange factor at class E compartments, leading to the accumulation of membrane destined for the vacuole. More than twice the amount of membrane at an average MVB is found in a single class E compartment cisterna, and restoring ESCRT function causes a burst in MVB formation. Moreover, class E compartment biogenesis requires Rab5 activity, and dominant-active Rab5 expression can drive class E compartment morphology without ESCRT disruption. However, neither Rab5 activity nor Rab5-Rab7 conversion are required for ESCRT-mediated ILV budding. We conclude that Rab conversion at endosomes occurs downstream of ILV budding and requires ESCRT function.

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Intralumenal Vesicle Formation at Multivesicular Endosomes requires Ubiquitinated Cargo.

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Degradation of post-Golgi membrane proteins is regulated by their ubiquitination, which serves as a signal for their internalization and subsequent delivery into lysosomes. Ubiquinated membrane proteins travel to endosomes where the Endosomal Sorting Complex Required for Transport (ESCRT) machinery incorporates them into intralumenal vesicles (ILVs), which bud into the endosomal lumen to form multivesicular bodies (MVBs). ILVs are then transported to

the lysosome / vacuole for degradation. How cargo itself coordinates the activity of the ESCRTs and whether it can regulate the formation of its own ILV carrier are unknown. By fusing a deubiquitnating (DUb) enzyme to ESCRT-0 we have created an environment where all cargo is deubiquinated at the endosome. Under these conditions we show ILV formation is completely ablated. ILV formation was restored when cells expressed cargo fused translationally to a single ubiquitin, which was resistant to ESCRT-DUb activity. These data indicate that vesicle formation is fostered by a cascade of interactions between cargo and the Ub-binding domains within the ESCRT apparatus, helping to organize the sorting machinery into a productive pathway. Finding that ubiquitinated cargo was critical for ILV formation led us to re-examine the integral membrane protein Sna3, which has been reported to follow the MVB pathway in a ubiquitin-independent manner. We have further characterized Sna3 and show it acts as an adaptor protein for the methionine transporter Mup1. This role of Sna3 is induced in conditions distinct from the previously described Mup1 adaptor, Art1, which specifically induces internalization in response to substrate. Furthermore, we have clarified the role of ubiquitin in Sna3 internalization and show that ubiquination plays a crucial role in Sna3 trafficking to the vacuole. Specifically, Sna3 undergoes ubiquitin-dependent MVB sorting either by becoming ubiquitinated itself or associating with other ubiquitinated membrane protein substrates.

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Spongiform neurodegeneration-linked E3 ligase, Mgrn1, regulates endosome-to-lysosome trafficking in neurons.

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A null mutation in the gene encoding the novel protein Mahogunin RING finger 1 (Mgrn1) causes prion disease-like spongiform neurodegeneration in mice. Moreover, depletion of Mgrn1 by the pathogenic forms of prion protein has been implicated in human prion disease pathogenesis. Despite the critical importance of Mgrn1 in maintaining neuronal survival, the mechanism and sites of Mgrn1 action remain mostly undefined and the pathogenic mechanism by which loss of Mgrn1 function causes spongiform neurodegeneration is unknown. We have recently shown that Mgrn1 regulates endosome-to-lysosome trafficking by ubiquitinating TSG101 and that siRNA-mediated depletion of Mgrn1 in HeLa cells results in enlarged endosomes and lysosomes. Western blot analysis reveals that endogenous Mgrn1 protein is expressed in multiple brain regions, including cerebral cortex. Immunofluorescence confocal microscopic studies show that endogenous Mgrn1 protein is localized in axons, dendrites, and synapses of mouse cortical neurons, suggesting that Mgrn1 may play a functional role in neuronal and synaptic physiology. We found that a pool of endogenous Mgrn1 protein is associated with early endosomes in cortical neurons. Furthermore, consistent with our findings in Mgrn1-depleted HeLa cells, primary cortical neurons from Mgrn1 null mutant mice exhibit swollen endosomes and lysosomes. Our results support a role for Mgrn1 in regulation of neuronal endosome-to-lysosome trafficking and have important implications for understanding and combating spongiform neurodegeneration.

1660

ALIX interacts with a YPX_3L motif of Protease-Activated Receptor 1 and mediates MVB/Lysosomal sorting through an ESCRT-III-dependent pathway independent of ubiquitination.

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The sorting of signaling receptors to lysosomes is an essential regulatory process. During degradation, receptors are modified with ubiquitin and sorted by ESCRT-0, -I, -II and -III complexes into intralumenal vesicles (ILVs) of multivesicular bodies (MVBs). However, it remains unclear whether a single universal mechanism mediates MVB sorting of all receptors. We previously showed that protease-activated receptor-1 (PAR1), a G protein-coupled receptor for thrombin, is internalized after activation and sorted to lysosomes independent of ubiquitination and the ubiquitin-binding ESCRTs, Hrs and Tsg101. Here, we now report that PAR1 sorts to ILVs of MVBs through an ESCRT-III-dependent pathway independent of ubiquitination. We further demonstrate that ALIX, a CHMP4/ESCRT-III interacting protein, mediates PAR1/ESCRT-III interaction. ALIX binds directly to a YPX_nL motif localized within the PAR1 second intracellular loop via its central V-domain and directs the lysosomal degradation of PAR1. This study reveals a novel MVB/lysosomal sorting pathway for signaling receptors that bypasses the requirement for ubiquitination and ubiquitin-binding ESCRTs.

Receptors, Transporters, and Channels

1661

Measuring receptor dimerization using proximity biotinylation.

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G-protein couple receptors (GPCRs) represent the largest class of signaling receptors and play an essential role in virtually all cellular processes. In recent years, there is increasing evidence that homo- and hetero-dimerization of activated GPCRs is a means to control the specificity and increase the diversity of signaling events. Several methods have been developed to determine if specific GPCRs would homo-dimerize or hetero-dimerize. In particular, resonance energy transfer techniques have been used to show receptor dimerization. A drawback of such strategies is that dimerization is measured not only on the cell surface, but also for receptors inside the cell. Furthermore, because of the nature of energy transfer, signal is only attained when two receptors are in close proximity and transient dimers that associate and dissociate quickly would yield lower energy transfer than stable dimers, thus hindering the interpretation of data. To address these limitations, we have developed a new strategy for measuring receptor dimerization based on enzyme-based proximity biotinylation. The bacterial biotin ligase BirA catalyzes biotinylation on a lysine residue within a specific motif encoded in an acceptor peptide (AP) fused to the GPCR of interest. In the presence of biotin, receptors conjugated to BirA will biotinylate receptors conjugated to AP when the receptors dimerize. Importantly, biotinylated receptors would remain biotinylated even after receptor dissociation. As a test case, we have focused on three chemokine receptors, CXCR4, CCR2, and CCR5. Here we use CXCR4-BirA as ‘donor’ and CXCR4-AP, CCR2-AP, and CCR5-AP as ‘acceptors’. Using proximity biotinylation, we have shown that CXCR4 homo-dimerizes and hetero-dimerizes with CCR2 and CCR5. Future work will focus on characterizing the dependence of receptor expression levels

on the extent and kinetics of receptor dimerization as well as whether and how agonists/antagonists for CXCR4, CCR2, and CCR5 alter receptor dimerization.

1662

Single molecule study of Thrombospondin receptors in endothelial cell plasma membrane.

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Thrombospondin-1 (TSP-1) is a potent anti-angiogenic factor downregulated in many tumors. CD36 and integrin β-1 are two of its key receptors, mediating its anti-angiogenic activity by initiating signaling cascades that inhibit endothelial cell migration and promote apoptosis. Receptor clustering on the plasma membrane is thought to be important for initiating these signaling cascades. However, little is known about the mechanisms that contribute to CD36 and integrin β-1 clustering and how they lead to downstream signals. In this study, we used quantitative single molecule live cell imaging to measure the dynamics of CD36 in primary human microvascular endothelial cells (HMVECs). To monitor CD36 motion at the single molecule level, we labeled CD36 with primary fab fragments followed by secondary fab fragments conjugated to dyLight 549 fluorophores, and recorded 100ms time-lapse image sequences using total internal reflection fluorescence microscopy (TIR-FM). We also used fixed-cell TIR-FM imaging to study the spatial organization of CD36 and integrin β-1 on the cell surface. Using the two approaches, we compared receptor dynamics and spatial organization between unstimulated cells and cells exposed to TSP-1 or 3TSR, a small subdomain of TSP1 which primarily binds to CD36 and integrin β-1. We found that treatments with either TSP-1 or 3TSR, at doses that lead to HMVEC apoptosis, result in a significant increase in CD36 clustering and mobility and in a differential spatial organization of integrin β-1. Based on our data we propose that the 3TSR domain of TSP-1 results in a change in CD36 dynamics, potentially mediated in part by integrin β-1, that facilitates CD36 clustering and signaling.

1663

Dectin-1, an Innate Immune Receptor, is Endocytosed upon Ligand Binding.

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Dectin-1 is a receptor expressed in macrophages and dendritic cells that plays a key role in anti-fungal immunity. It recognizes ß-glucans, polymers of glucose found in the fungal cell wall. Upon ligand binding, Dectin-1 triggers signaling cascades that lead to phagocytosis of fungal pathogens and the release of pro-inflammatory mediators. Interestingly, soluble ß-glucans themselves have been found to have immunomodulatory effects, suggesting Dectin-1 can bind and internalize soluble ß-glucans to trigger an immune response. The goal of our research is to develop tools to visualize Dectin-1 endocytosis and to characterize the endocytic pathway responsible for ß-glucan internalization.

We stably expressed Dectin-1 in RAW 264.7 murine macrophage cells. Surface Dectin-1 was labeled with antibodies and allowed to internalize into the cells in the presence of the soluble ß-glucans phospho-curdlan and laminarin. Immunofluorescence staining revealed surface Dectin-1 localized to multiple punctate structures inside the cell only upon stimulation with these ligands. This uptake was dependent on the GTPase dynamin, as the intracellular puncta were not observed in cells transiently expressing a dominant-negative dynamin mutant. In addition, total internal reflection fluorescence microscopy showed that at early timepoints, Dectin-1

puncta greatly co-localized with GFP-tagged clathrin. Further dual-fluorescence confocal analysis found Dectin-1 localized to Rab5+ early endosomes by 15 minutes of ligand stimulation, and to recycling endosomes and lysosomes by 30 minutes. We have also observed the recruitment of GFP-tagged Spleen Tyrosine Kinase (Syk), a key player in Dectin-1 signaling, to Dectin-1 puncta.

To quantify Dectin-1 uptake, cell surface biotinylation was performed prior to treatment of the cells with soluble ß-glucans for various times. After the timepoints, non-internalized receptors were stripped of the biotin label with a reducing agent. Subsequent streptavidin pull-down of internalized biotinylated proteins and immunoblotting confirmed that the rate of Dectin-1 uptake increased dramatically upon binding the ß-glucans. Upon treatment with dextran, a sugar that is not a Dectin-1 ligand, the rate of uptake remained only at a constitutive level. By performing the same assay in the presence of pharmacological inhibitors of endocytosis, we determined that ligand-stimulated Dectin-1 endocytosis is also a clathrin- and actin-dependent process.

We conclude that Dectin-1 is internalized rapidly upon binding of soluble ß-glucan ligands, and that several molecular players and events involved in its endocytosis have been identified. Future studies will examine the role of endocytic trafficking in the regulation of Dectin-1 downstream signaling and vice versa.

1664

Activation of the Innate Immune Receptor Dectin-1 by Clustering.

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Dectin-1 is a pattern-recognition receptor that plays a key role in anti-fungal immunity. It specifically recognizes beta-glucans, polymers of glucose found on the fungal cell wall. Upon ligand binding, dectin-1 triggers signaling cascades that lead to various cellular responses, including phagocytosis and the release of pro-inflammatory mediators, which all culminate in the generation of an effective immune response and eradication of the pathogen. The molecular mechanisms underlying dectin-1 activation and signal transduction are not fully understood. The aim of this study is to elucidate such mechanisms. We propose that upon binding of fungal particles, dectin-1 receptors cluster and assemble into multimeric complexes. In these clusters, dectin-1 becomes activated and promotes the recruitment and activation of Syk (spleen tyrosine kinase), which in turn triggers subsequent signaling cascades. To test our hypothesis, we stably expressed human dectin-1 in RAW 264.7 cells. Various levels of dectin-1 clustering were induced using antibody cross-linking or beta-glucans of different sizes. The corresponding activation of dectin-1 was detected by determining the activation of several downstream effectors (Src, Syk, NF-kB). We demonstrate that antibody cross-linking and larger ligands are able to induce more Syk phosphorylation than smaller ligands. To quantify the level of dectin-1 clustering, single molecule analysis was employed. Antibody cross-linking of dectin-1 formed clusters of approximately 50 dectin-1 molecules in nanodomains of 500 nm, which were identified as the nucleation site for intracellular signaling. Additionally, the phosphorylation and recruitment of Syk to regions of the plasma membrane rich in dectin-1 clusters was observed using confocal microscopy. Together, our results suggest that receptor clustering is the mechanism by which dectin-1 is activated.

1665

Investigation of the presence of a Basigin-mediated lactate metabolon in the brain.

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It is generally accepted that plasma membrane-associated expression of monocarboxylate transporters (MCTs) 1, and 4 requires an association with Basigin (CD147), whereas MCT2 does not. In the neural retina, Basigin gene products associate with MCT1 and MCT4 to form a lactate metabolon between Müller glial cells and photoreceptor neurons. The Basigin gene products hold the two halves of the metabolon together and the MCTs transport lactate via facilitated diffusion. It is thought that the metabolon transfers lactate from the highly glycolytic Müller cells to photoreceptor neurons to fuel rapid ATP synthesis in those neurons. In contrast, MCT2 likely plays a minor role in metabolite transfer in the neural retina. Therefore, the purpose of this study was to determine if a similar metabolon exists between neurons and glial cells within other parts of the nervous system as well. The authors believe that such a metabolon could account for the astrocyte-neuron coupling mechanism proposed to exist in the brain. Paraffin-embedded sections of mouse olfactory bulb and midbrain were subjected to immunohistochemical analyses using antibodies specific for Basigin, MCT1, MCT2, and MCT4. In the olfactory bulb, MCT2 is the predominant MCT and is expressed in the granule cell layer, whereas MCT1 and MCT4 are restricted to blood vessel endothelial cells. Basigin expression in the olfactory bulb was observed in the mitral cells and on blood vessel endothelial cells. In the midbrain, MCT1, MCT4, and Basigin are again expressed on blood vessel endothelial cells, but Basigin and MCT4 are also expressed on cell bodies. MCT2 expression was observed on cell bodies as well. The data suggest that a lactate metabolon like that observed in the neural retina does not exist in the regions of the brain tested. It is likely that while neurons within the midbrain and olfactory bulb use lactate as a metabolite based on MCT expression patterns in those regions, rapid delivery from glial cells to neurons is not required like it is in the neural retina.

1666

MCSF Controls Phagocytosis in Macrophages by Stimulating Cytoskeletal Rearrangements.

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Phagocytic engulfment of particles requires the engagement of multiple phagocytic receptors. The principle macrophage growth factor, macrophage colony stimulating factor (MCSF or CSF-1) stimulates phagocytosis within seconds of addition. This enhancement is independent of changes in receptor expression. We demonstrate that the mechanism for this enhancement results from cytoskeleton rearrangements, ruffling and local exploration by extensions of plasma membrane. Drugs that interfere with actin function as well as fixation with paraformaldahyde inhibit the binding of particles. Fluorescence Recovery after Photobleaching demonstrate that net rate of receptor diffusion increased upon MCSF administration and the immobile fraction decreases. These results suggest that perturbations in the local actin cytoskeleton by MCSF allow increased receptor mobility.

1667

Characterization of the Basigin binding domain of Basigin-2 and L1cam.

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The Basigin gene codes for two proteins in the neural retina by splice variation. Basigin and Basigin-2, members of the immunoglobulin superfamily (IgSF) are identical in amino acid

sequence, with the exception of an additional Ig domain in the extracellular portion of the Basigin-2 molecule (Ochrietor *et al*., 2003, *IOVS*). The Basigin-2-specific loop is highly conserved throughout evolution, suggesting it has a specific, conserved role, most likely as a binding domain in the retina. Basigin-2 also contains significant amino acid sequence identity (50%) in its amino terminal Ig loop with an extracellular domain of L1cam, an IgSF neural cell adhesion molecule that is a known binding partner for Basigin in the brain (Heller *et al*., 2003, *J Neurochem*). Therefore, the purpose of this study was to determine whether the regions of significant amino acid sequence identity in both Basigin-2 and L1cam serve a common function as binding domains for Basigin. The cDNA sequences for the potential Basigin binding domains of Basigin-2 and L1cam were each cloned into the pET102 bacterial expression vector (Invitrogen Corporation) and recombinant proteins were generated and purified. ELISAs were then performed to assay the ability of each recombinant protein to bind endogenous mouse Basigin. The data indicate that the conserved regions within Basigin-2 and L1cam do indeed bind to Basigin. In addition, it was determined that the binding affinities of each protein for Basigin were similar, in the □M range, which is consistent with Ig cell adhesive binding affinities. The data suggest that Basigin-2 and L1cam share a common motif: the Basigin-binding domain. Future studies will investigate exactly which amino acids within the motif are involved in the interaction.

1668

Characterization of the interactions between the cell adhesion molecules Basigin and Embigin and monocarboxylate transporters in neural tissues.

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Basigin and Embigin are classified within the same subset of the Immunoglobulin (Ig) superfamily. Both glycoproteins have been implicated in MCT expression at the plasma membrane. The two molecules diverge, however in that Basigin works with more than one MCT (1, 3, and 4) while Embigin is only thought to interact with MCT2. It is known that Basigin and MCT1 must associate for membrane-associated expression in many tissues. Indeed, the MCT1 glycoprotein is not expressed at the plasma membrane in the Basigin null mouse retina. The association utilizes hydrophobic interactions via the transmembrane domain of Basigin. Due to the striking similarities between Basigin and Embigin, including 50% amino acid sequence identity in their transmembrane domains, the purpose of this study was to determine whether Embigin interacts with MCT2 in a similar manner to that of Basigin and MCT1. To answer this question, recombinant versions of the Basigin and Embigin transmembrane domains were generated for ELISA binding assays using endogenous mouse MCT2. The data indicate that neither Embigin nor Basigin transmembrane domains interact with MCT2. This finding suggests that Basigin does not interact with MCT2 due to differences in amino acid sequences of MCT1 and MCT2. The data also raise questions about the validity of naming Embigin as the accessory molecule for MCT2 expression. If it is an accessory protein then it must use a domain other than the transmembrane region. Future studies by this laboratory will examine the other domains of the Embigin glycoprotein to determine if the extracellular or intracellular domains, rather than the transmembrane domain, associate with MCT2.

1669

Enrichment of inositol 1,4,5-trisphosphate receptors and chromogranins in secretory granules of pancreatic β- and α- cells.

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Secretory granules are signature organelles of all secretory cells, and contain not only the largest amounts of calcium in the cell but also function as the major IP_3-dependent intracellular Ca^{2+} store of secretory cells (reviewed Yoo, S.H., *FASEB J.* (2010) 24, 653). Typically, secretory granules contain high concentrations of all three IP3R isoforms and of calcium storage proteins chromogranins and secretogranins. Chromogranins A and B, and secretogranin II are three major members of the granin protein family, and are high-capacity, low-affinity Ca^{2+} storage proteins. Pancreas contains several types of secretory cells such as acinar cells, β- and α- cells along with large numbers of secretory granules in each cell. Yet, there is very little information on the distribution of the IP_3Rs and chromogranins in these cells. Therefore, we have here investigated the distribution and the relative concentrations of IP_3R isoforms and of chromogranin B and secretogranin II in subcellular organelles of rat pancreatic β- and α- cells using immunogold electron microscopy. The IP_3Rs were shown to localize in secretory granules, the endoplasmic reticulum (ER), and the nucleus of rat pancreatic β- and α- cells, and the IP_3R concentrations in secretory granules were shown to be ~2-fold higher than those of the ER or the nucleus. Generally agreeing with pancreatic acinar cells, chromogranin B and secretogranin II were also shown to localize in secretory granules, the ER, and the nucleus of pancreatic β- and α- cells, and their concentrations were also ~2-fold higher than those of the ER or the nucleus. Taken together, these results suggest that secretory granules of pancreatic β- and α- cells also function as the major IP_3-sensitive Ca^{2+} store of these cells as is the case with other secretory cells.

1672

Elevated Levels of Saturated Fatty Acids Disrupt Calcium Homeostasis and Affect Atrial Fibrillation Dynamics in the Ovine Heart.

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Introduction: There is increasing evidence linking obesity and atrial fibrillation (AF). It has been suggested that an excess of myocardial fat can induce ectopic activity and may underlie the arrhythmogenicity associated with an elevated risk for AF. Fat cells (adipocytes) are known to secrete bioactive factors that can modulate cardiac function. The implications of these bioactive factors on myocyte electromechanical function are largely uninvestigated. A major component of these biofactors are free fatty acids (FFA). These FFAs are composed of both saturated Fas (palmitic acid (PA) and stearic acid (SA)) and several unsaturated FAs. We hypothesized that saturated fatty acids detrimentally modulate cardiac electromechanical function. Methods: We have used a combination of optical as well as the whole-cell patch-clamp techniques to examine the effects of saturated and unsaturated fatty acids on the electromechanical function of left atrial ovine myocytes and atrial fibrillation dynamics. Our FFA concentrations were chosen to reflect the elevated levels found in obese sheep (Veiga-Lopez et al, 2010). Results: First, we tested the effects of chronic (24 hr) exposure of left atrial myocytes to 10 μM SA. Treated cells demonstrated an abbreviation of action potential duration at 80% repolarization (APD80) (as a % of control: 63.56 ± 8.39, $p \leq 0.006$), and reduced the density of peak L-type calcium currents (ICa-L) by approximately 50% without significantly affecting voltage-dependent activation or

inactivation. Similarly, chronic exposure to 10 μM PA exposure abbreviated APD80 values (as a % of control 63.68 ± 4.35, p≤0.004) and significantly reduced ICa-L current density. Additionally, 10 μM PA induced a depolarizing shift in voltage- dependent activation of the L-type calcium channel and altered time-dependent inactivation resulting in increased persistent ICa-L. Acute (15 min) exposure of up to 50 μM PA did not significantly affect ICa-L. Furthermore, FA treated myocytes demonstrated reduced calcium transient amplitudes (0.35 ± 0.05 vs. 0.18 ± 0.04 ΔF/Fo, p≤0.007; CTL vs. PA) and the half widths (292.3 ± 19.8 vs. 225.8 ± 11.5 msec, p≤0.007; CTL vs. PA). Importantly, chronic exposure of atrial myocytes to 10 μM oleic acid, a monounsaturated FFA, had no discernable effects on ICa-L. Finally, in optical mapping experiments we examined the effects of 20 μM PA on AF dynamics.PA was perfused into the coronary circulation of isolated ovine hearts. Average APD80 values were abbreviated by 20% (p=0.07). However, dominant frequency maps demonstrated a marked increase in the frequency of fibrillatory activity (17 Hz vs 7.9 Hz; p<0.05; CTL vs PA). Significant recovery was observed after washout. Conclusions: The electrophysiological properties of the left atrium in the whole heart and in isolated cells exposed to elevated levels of saturated, but not monounsaturated FFAs, demonstrate electromechanical alterations consistent with conditions favoring AF. These results may provide insight into the mechanism linking AF with excess adiposity seen in obesity.

1673

Membrane transport activity and dynamics of the ABC transporter, P-glycoprotein.

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ATP binding cassette (ABC) transporters regulate the lipid asymmetry of the plasma membrane by exporting a wide variety of lipids to the outer leaflet. P-glycoprotein (PGP) is an ABC transporter responsible for the efflux of a large number of hydrophobic small molecules, such as drugs and fluorescent dyes. The interactions of PGP with its local environment on the plasma membrane are studied here using confocal and total internal reflection fluorescence microscopy (TIRFM). A fusion protein of PGP with enhanced green fluorescent protein (EGFP) and several rhodamine-derivative and cyanine fluorescent dyes make the transporter and its substrates visible. Here we use fluorescence recovery after photobleaching (FRAP) techniques to assess bulk protein mobility on the membrane. To complement this technique, TIRFM is used to track single PGP molecules and PGP clusters across the surface of the plasma membrane. Using these microscopic methods, the localization and dynamics of PGP on the membrane in the inactive, active, and inhibited states are determined and a preliminary mechanistic model is proposed.

1674

Developmental tradeoffs: endocytosis leads to the down-regulation of ABC-efflux in the sea urchin small micromere lineage.

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Embryos use ATP-binding cassette (ABC) transporters to establish gradients of morphogens and to reduce intracellular accumulation of toxins. We used confocal microscopy to assess ABC-transporter activity in micromeres of sea urchin embryos. Micromeres give rise to the small micromeres, which are essential for the production of germline. As the micromeres divided to produce small micromeres they accumulated 2.2 times more of the ABC-transporter substrate calcein-AM, than other cells in the embryo. Reduced ABC-transporter activity persists in small micromeres, which accumulate an array of ABC-transporter substrates including, CellTrace

RedOrange, BoDipy-verapamil and BoDipy-vinblastine. The reduction in transport activity is mediated by a pulse of endocytosis that occurs 20-60 min after the appearance of the micromeres. Down regulation of efflux is prevented by phenylarsine oxide (PAO), an inhibitor of endocytosis. Micromeres of early 16-cell embryos treated with PAO have a 15% reduction of efflux activity, whereas controls have a decline of 36%. In contrast, 50 min after the start of the 16-cell stage, PAO treatment results in a 31% decrease in efflux activity, roughly equivalent to that measured in controls. Tetramethylrhodamine dextran and cholera toxin B were used to measure endocytosis of apical micromere membrane. During the down regulation of transport, measurements of rhodamine positive endosomes indicated that micromeres retrieve an average of 0.8% of their cellular volume, while the macromeres retrieve only 0.2 %. The removal of raft-associated ganglioside peaks at 6% of cell volume in the micromeres and 2.7% in the macromeres. These results suggest the loss of transport is mediated by selective retrieval of either ABC-transporters or the lipid rafts with which they are associated. The down-regulation of efflux in small micromeres represents a paradox; critical germline precursor cells accumulate more toxins. We hypothesize that a loss of transport may be required for the detection of developmental signaling molecules. If cells in embryos down-regulate ABC-transporter activity to receive signals during development, then this may generate specific windows of germline susceptibility.

1675

A Role for TRPM7's Control of Magnesium Homeostasis in Conferring Resistance to Cell Stress.

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TRPM7, a member of the transient receptor potential melastatin-like (TRPM) ion channel subfamily, is a unique bifunctional protein with ion channel and kinase activities. This Ca^{2+} and Mg^{2+} permeant ion channel is ubiquitously expressed and has been linked to various physiological roles, including magnesium homeostasis, melanopore formation, cell proliferation and early development. Recent studies have demonstrated that depletion of the TRPM7 protein in neurons reduces cell death caused by oxygen-glucose deprivation, however, the mechanism(s) involved remains unclear. We've been investigating how TRPM7 contributes to the demise of cells using HEK-293 cells and Swiss-3T3 fibroblasts as model systems. Our research has revealed that overexpression of TRPM7 in HEK-293 cells increases production of reactive oxygen species (ROS) and the activation of the stress-activated protein kinases (SAPKs), p38 MAP kinase and c-Jun N-terminal kinase (JNK). Conversely, knockdown of TRPM7 in HEK-293 as well as Swiss 3T3 fibroblasts, lessened cell death caused by a wide range of apoptotic stimuli. Inhibition of TRPM7 channel activity using novel TRPM7 channel blockers that we have identified was similarly protective. Consistent with a role of the channel in apoptosis, knockdown of TRPM7 in Swiss 3T3 fibroblasts reduced apoptotic signaling in response to cell stress, as assessed by cleavage of PARP and caspase-3. In addition, TRPM7-knockdown fibroblasts have lower levels of ROS than in control cells, both in the absence of cell stress as well as in response to apoptotic stimuli. Surprisingly, re-expression of the magnesium transporter SLC41A2 was able to restore the concentration of ROS in TRPM7 knockdown cells as well as their sensitivity to apoptotic stimuli. Together these findings underscore a critical role for the TRPM7 channel and its control of magnesium homeostasis in the cellular response to cell stress.

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Acid production in Cystic Fibrosis airway cells measured by flow cytometry.

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Airway pH and bicarbonate transport across CFTR are changed in CF and affect airway epithelial function. Intracellular pH in airway cells is generally measured using wide-field fluorescent microscopy on whole cells with a pH reporter dye. Here we measure intracellular airway cell pHc using flow cytometry and calculate acid production using cell buffer capacity. We used two airway cell lines, one containing the CFTR (CFBE41o- bronchial cells expressing recombinant wt-CFTR), and one without the CFTR (JME CF nasal surface cells). We then compare flow cytometry measurements to both confocal and wide-field microscopy values. Flow cytometry was performed on resuspended (scraped) cells preloaded with the pH reporter dye 5 uM SNARF-1 in HEPES-buffered, nominally bicarbonate-free Ringers and calibration solutions. Confocal microscopy experiments were performed on adherent cells preloaded with 5 uM SNARF-5. Wide-field microscopy was performed on cells on glass preloaded with 5 uM BCECF. All techniques were calibrated using the high K+/ nigericin method and a 2-5 point pH calibration. Flow cytometry data fit a calibration curve derived from 4 to 5 datasets, each dataset containing 30,000 cells set to a unique pH ranging from 6 to 8. Using flow cytometry, CFBE41o-wt cells in Ringers had median pH values of 7.26 +/- 0.01 (n = 3). JME cell pH was 7.41 +/- 0.07 (n = 3). In both cell types, intracellular pH was comparable to wide-field microscopy values. Confocal measurements were variable, possibly due to variable dye localization to intracellular organelles, which were resolved by this technique. When 500 uM amiloride and 10 uM ZnCl2 were added to the cells to prevent acid extrusion, pHc decreased. In CFBE41o-wt cells, pH fell to 6.93 +/- 0.06 (n = 3), corresponding to an initial acid production of 860 +/- 160 fmols / cell hour based on the cell buffer capacity (separately determined). JME pH fell to 7.09 +/- 0.08 (n = 3), corresponding to an initial acid production of 410 +/- 60 fmols / cell hour. By microscopy, we measured an initial acid production of 1620 +/- 730 fmols / cell hour (n = 6) in CFBE41o- cells and 1360 +/- 620 fmols / cell hour (n = 5) in JME cells. We conclude (1) that CFBE41o- and JME airway cells produce substantial, but not significantly different levels of basal acid, and (2) that airway cell pH can effectively be measured by flow cytometry of scraped adherent cells. We suggest that this technique would allow for functional pH analyses on small subpopulations of cells. For comparison, confocal microscopy appears appropriate for measuring subcellular pH compartments. Funded by NIH/NHLBI HL86323, CF Foundation (FISCHE10G0), and the Beverly M. Folgers Foundation.

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Regulation of the expression and cellular localization of aquaporin-3, 9 and 10 in human skin epidermis keratinocytes.

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Skin epidermis is a multi-layered epithelium which undergoes continuous renewal by proliferation and differentiation of keratinocytes. Aquaporins (AQP) are a family of 13 water channels and glycerol transporters in humans. Aquaporin-3 (AQP3) is expressed in skin epidermis keratinocytes in humans and rodents. Aquaporin-9 (AQP9) was also found in mouse skin epidermis. Our objectives were to investigate the expression and regulation of AQP during differentiation in normal human keratinocytes (NHK) and human epidermis. Results: by RT-PCR, AQP3 and aquaporin-10 (AQP10) mRNA and protein were detected in NHK in culture. After 72h calcium-induced differentiation of NHK (with 1.5 mM Ca2+), AQP3 expression was

preserved but AQP10 was replaced by AQP9. In human skin, using immunofluorescence with specific anti- AQP3, AQP9 and AQP10 antibodies on human skin sections, the expression of AQP3 in plasma membranes was confirmed in basal and suprabasal keratinocytes, while AQP10 was localized in keratinocyte intracellular compartments. AQP9 was found only in the stratum granulosum, the epidermis layer prior to terminal differentiation of keratinocytes into corneocytes. In contrast to skin epidermis, AQP3 in proliferative NHK was localized in the endoplasmic reticulum, but AQP3 was targeted to plasma membranes after calcium-induced differentiation of NHK. Interestingly, this behavior was recapitulated in MDCK cells transfected with AQP3-GFP. In these cells, AQP3 trafficking to the plasma-membrane was rapidly reversed by: - lowering extracellular [Ca2+] from 1.5mM to 0.15 mM - the PLCß inhibitor U73343 (2 µM) – the PKC inhibitor Rho320432 (10µM). In conclusion, we show that: - AQP expression is regulated by differentiation in human epidermis keratinocytes: AQP10 is expressed only in proliferative keratinocytes, AQP3 is found in proliferative and differentiated keratinocytes and AQP9 is expressed only in terminally differentiated keratinocytes. Results in MDCK cells suggest that the calcium-sensitive PLC-PKC pathway is directly involved in AQP3 trafficking from the intracellular compartment to plasma membranes during calcium-induced differentiation of keratinocytes.

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The Mitochondrial TIM23 Protein Transport Complex Undergoes Conformational Dynamics Coupled to the Energized State of the Inner Membrane.

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The TIM23 complex of the mitochondrial inner membrane (IM) is a multi-component assembly that mediates the translocation of matrix-targeted precursor proteins as well as the integration of membrane proteins. This complex is energetically coupled to both an ATPase motor and the electrochemical proton potential ($\Delta\tilde{\mu}_{H+}$) across the IM. The central subunit of this complex, Tim23, forms a voltage-gated channel and contains a large soluble domain in the intermembrane space that functions as a substrate receptor. The central objective of this study is to investigate the coupling between the energized state of the membrane and conformational dynamics of the Tim23 channel subunit. To this end, we utilize a newly-developed a fluorescence-based experimental strategy that allows us to explore the structure and function of mitochondrial membrane proteins. In this approach, Tim23 is synthesized with fluorescent probes at specific sites by conducting cell-free translation reactions in the presence of aminoacyl-tRNA analogs that allow for the incorporation of non-natural (probe-bearing) amino acids. Labeled Tim23 is then post-translationally imported into the IM of isolated mitochondria where it properly assembles with native subunits to form TIM23 complexes, allowing for high-resolution fluorescence mapping of the fully operational channel by both steady-state and time-resolved fluorescence measurements [Alder et al. (2008) *Cell* 134: 439-450]. By monitoring the spectral characteristics of environment sensitive probes at key sites within Tim23, we can examine the conformational dynamics of specific regions in response to changes in the energized state of the membrane as they occur in real time. Our results show that the Tim23 subunit undergoes major structural rearrangements in response to changes in the $\Delta\tilde{\mu}_{H+}$, not only within the membrane-bound channel domain but also within the soluble receptor region. Moreover, the kinetics of these conformational changes indicate that they are tightly coupled with changes in the energized state of the membrane. Hence, we conclude that ion gradients drive conformational changes in key sites of Tim23 that are coupled to its function in driving polypeptide translocation and integration. This work provides novel insights into how proteins

within energy-conserving membranes can utilize the $\Delta\tilde{\mu}_{H+}$ to perform work. Moreover, it provides a system for analyzing: (i) how the different components of the $\Delta\tilde{\mu}_{H+}$, the membrane potential and the proton gradient, may differentially drive conformational changes, and (ii) how voltage-gated proteins may sense changes in membrane potential.

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Expression of the Genes Encoding Multidrug Resistance-Associated Proteins (MRPs) and Distribution of MRP1-4 Proteins in Human Skin Cells and Epidermis.

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The nine human multidrug resistance–associated proteins (MRP1-9), which possess an ATP-binding consensus motif, are involved in cellular detoxification processes. Their physiological roles in the human epidermis are unknown. The aim of this study was to characterize the expression, location and functionality of these proteins in normal proliferating human epidermis keratinocytes (NHEK) and melanocytes (NHEM) and in normal human skin samples under various conditions. Judging from reverse transcription-polymerase chain reaction experiments, the genes encoding MRP1, MRP4, MRP5 and MRP7 are similarly expressed in NHEK and NHEM. In contrast, MRP2 gene expression is mainly observed in NHEM and the MRP3 gene is exclusively expressed in NHEK. The expressed proteins were visualized by immunofluorescence. MRP1-4 proteins were found in the intracellular compartment of NHEM and NHEK cell lines. MRP2 was immunodetected around the nuclei of NHEM cells. MRP4 was restricted to the dendrites. Unexpectedly, we found that MRP2 forms a barrier-like structure in the *stratum granulosum* region of the epidermis of normal human skin. We demonstrate that MRPs are functional in NHEK and NHEM cell lines and show that MRP1 and MRP2 are implicated in the sensitivity of both cell lines to doxorubicin, probably by sequestering doxorubicin within the cell. We conclude that these transporters might help protect epidermis cells against xenobiotics.

Mitochondria, Chloroplasts, and Peroxisomes

1680

ER tubules mark sites of mitochondrial division.

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Mitochondrial structure and distribution are regulated by division and fusion events. Mitochondrial division is regulated by Dnm1/Drp1, a dynamin-related protein that forms helices around mitochondria to mediate fission. Little is known about what determines sites of mitochondrial fission within the mitochondrial network. Given that ER and mitochondria exhibit tightly coupled dynamics and have extensive contacts, we tested whether ER plays a role in mitochondrial division. We show that mitochondrial division occurs at positions where ER tubules contact mitochondria and mediate constriction prior to Drp1 recruitment. These data demonstrate that ER tubules play an active role in defining the position of mitochondrial division sites.

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The function of the Mmm1 protein in *Neurospora crassa*.

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Both mitochondria and the endoplasmic reticulum (ER) are essential sites of phospholipid biosynthesis. The recently discovered ER-Mitochondria Encounter Structure (ERMES) complex which comprises the Mdm10, Mdm12, Mmm1 and Mmm2 proteins is thought to function in the exchange of phospholipids between the two organelles. In yeast, the absence of any ERMES complex member causes several phenotypes including the alteration of mitochondrial steady-state lipid ratios, enlarged mitochondria, impaired mitochondrial protein assembly, as well as defects in mitochondrial motility, inheritance and DNA stability. In *Neurospora crassa* we have previously shown that strains lacking Mmm1 contain enlarged mitochondria and are deficient in the assembly of mitochondrial β-barrel proteins. We now demonstrate that mitochondria isolated from *N. crassa* strains lacking Mmm1 do not exhibit obvious alterations in lipid ratios. We also show that HA tagged Mmm1 fractionates with both gradient purified mitochondria and the post-mitochondrial pellet. We demonstrate that Mmm1 forms a dimer with an unknown protein via disulfide bonding. In a cysteine-less mutant, inability to dimerize has no effect on the localization of the protein, but Tom40 is less efficiently assembled into the mature TOM complex. The import and assembly of other outer membrane proteins (Tom22 and Porin) remains unaffected. These results suggest that Mmm1 is directly involved in the late assembly of Tom40 and may not be involved in mitochondrial lipid homeostasis in *N. crassa*. Focusing on a highly conserved region of Mmm1, we constructed another mutant *N. crassa* strain. This mutant is impaired in the assembly of the β-barrel proteins Tom40 and porin, whereas Tom22 assembly remains unaffected. Taken together our results suggest that Mmm1 plays a direct role in mitochondrial protein assembly in *N. crassa* and different domains of the protein appear to be involved in the assembly of specific mitochondrial proteins.

1682

Live-cell imaging of the association of STAT6-GFP with mitochondria.

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STAT6 has been implicated in regulating energy metabolism in cell types such as the hepatocyte. However, all prior discussion of the mechanisms involved have centered on the genomic/transcriptional effects of STAT6. We carried out a survey of all seven STAT proteins (STAT1, 2, 3, 4, 5a, 5b and 6) for their possible association with mitochondria in human Hep3B hepatocytes by first washing out bulk soluble STAT proteins using a low concentration digitonin-sucrose buffer followed by paraformaldehyde fixation and immunofluoresence using respective anti-STAT pAbs. The possible co-localization was evaluated quantitatively using Manders' and Pearson's metrics with mitochondria marked using a mAb to F1-ATPase. STAT6 was observed to be strongly associated with mitochondria; this immunofluoresence was inhibited by STAT6-blocking peptide and reduced by transfecting Hep3B cells with STAT6 siRNA. Following transient transfections of an expression vector, STAT6-GFP, but not N1-GFP, was observed to colocalize with MitoTracker- and TMRE-positive mitochondria in live cells and with F1-ATPase mAb assayed by immunofluoresence after fixation. In contrast, using the same techniques we were unable to detect any association of STAT3-GFP or STAT5a-GFP with mitochondria based on GFP fluoresence. Immunogold EM confirmed the association of STAT6 within the mitochondrial matrix in human pulmonary arterial endothelial and smooth muscle cells. The present data showing the association of STAT6-GFP with mitochondria in live cells represents

the first visible evidence of the association of a STAT protein family member with mitochondria. Supported by NIH grants R01HL087176 and F31HL107013.

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Human MIEF1 Recruits Drp1 to Mitochondrial Outer Membranes and Promotes Mitochondrial Fusion Rather than Fission.

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BACKGROUND: Mitochondrial morphology is controlled by two opposing processes: fusion and fission. Drp1 (dynamin-related protein 1) and hFis1 are two key players of mitochondrial fission, but how Drp1 is recruited to mitochondria and how Drp1-mediated mitochondrial fission is regulated in mammals is poorly understood.
METHODS: This work used a number of cell biological techniques, eg cell culture, generation of expression constructs, mutational analysis, subcellular fractionation, immunoblotting, double and triple immunofluorescence stainings, confocal microscopy, transmission electron microscopy, RNAi silencing, in vivo protein cross-linking, co-immunoprecipitation and cell fusion assays.
RESULTS: We have identified the vertebrate-specific protein MIEF1 (mitochondrial elongation factor 1; independently identified as MiD51), which is anchored to the outer mitochondrial membrane. Elevated MIEF1 levels induce extensive mitochondrial fusion, whereas depletion of MIEF1 causes mitochondrial fragmentation. MIEF1 interacts with and recruits Drp1 to mitochondria in a manner independent of hFis1, Mff (mitochondrial fission factor) and Mfn2 (mitofusin 2), but inhibits Drp1 activity, thus executing a negative effect on mitochondrial fission. MIEF1 also interacts with hFis1 and elevated hFis1 levels partially reverse the MIEF1-induced fusion phenotype. In addition to inhibiting Drp1, MIEF1 also actively promotes fusion, but in a manner distinct from mitofusins.
CONCLUSIONS: Our findings uncover a novel mechanism, which controls the mitochondrial fusion–fission machinery in vertebrates. As MIEF1 is vertebrate-specific, these data also reveal important differences between yeast and vertebrates in the regulation of mitochondrial dynamics.

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Fast oscillations of NAD(P)H in the salivary glands epithelium of live rats imaged by intravital two-photon microscopy.

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Several endogenous small molecules have been described to emit upon two-photon excitation. Among them is the reduced form of nicotinamide adenine dinucleotide (NADH) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) [1]. NAD(P)H molecules are present both in the cytosol and mitochondria and provide the optical contrast to reveal the architecture of various organs. Here we focus on imaging NAD(P)H endogenous fluorescence in the salivary glands (SGs) of live rats by using intravital two-photon microscopy, and show how details of the structure of acini and ducts can be easily resolved. Furthermore, we show that in resting conditions NAD(P)H fluorescence propagates across the salivary epithelium in waves that exhibit a characteristic frequency. By using different experimental approaches we show that these waves are not the result of motion artifacts. Interestingly, the waves seem to be originated from specific foci, most likely the nerve terminals. Stimulation of the β2 adrenergic receptor by

its agonist isoproterenol (ISO), leads to a sharp increase in endogenous fluorescence, whereas the muscarinic agonist Carbachol (Carb) has no effect on the oscillating fluorescence. Moreover, when SGs were labeled with cationic dyes, such as TMRM and Rhodamin123, which are sensitive to the mitochondrial membrane potential, we observe waves that overlaps those revealed by exciting NAD(P)H emission. This finding suggests that the oscillations of the NAD(P)H pool may be linked to mitochondrial metabolism. Although further experiments are required, we speculate that: 1) NAD(P)H oscillations and propagation may be originated from the neurotransmitters originated from nerve terminals, 2) the waves may be used as an indicator of SGs functionality ,raising the possibility of using two-photon stimulated endogenous emissions as a diagnostic tool, and 3) the increase of NAD(P)H upon agonist stimulation may be associated with the regulation of stimulated protein secretion.

1685

The role of a dual specificity phosphatase on alternative oxidase production in *Neurospora crassa.*

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Various inhibitors and mutations result in blockage of the standard electron transport chain. In many organisms, including *Neurospora crassa*, this leads to the expression of alternative oxidase (AOX). AOX is a single protein enzyme that carries electrons from ubiquinol to molecular oxygen. Single gene knockout (KO) strains, available from the Fungal Genetic Stock Centre, were screened for their ability to grow in the presence of Antimycin A, an inhibitor of the standard electron transport chain. KO strains that were unable to grow were selected for further investigation. One KO was found to affect a gene encoding a protein with homology to *Saccharomyces cerevisiae* Yvh1p, a dual specificity phosphatase. The protein contains a HCX_5R phosphatase domain in the N-terminal region and a redox sensing zinc-binding domain in the C-terminal region. The KO has a slow growth phenotypye and under inducing conditions, a low level of AOX. We have shown that the levels of the phosphatase are similar in cultures grown under both non-inducing conditions and conditions that induce AOX. Cell fractionation experiments have revealed that the majority of the protein localizes to the nuclear and microsomal fractions and not to the cytosolic or mitochondrial fractions. Mutational analysis of the two domains has shown that both the phosphatase and zinc binding domains need to be functional to rescue AOX induction.

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Cell-free Reconstitution and Spectral Analysis of the ADP/ATP Carrier within Proteoliposomes.

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The ADP/ATP carrier (AAC) of the mitochondrial inner membrane (IM) transports ADP into the mitochondrial matrix and exports ATP into the cytosol after its synthesis; hence, it is central to cellular energy balance. The AAC contains six transmembrane helices and three matrix-facing loops and represents the only member of the mitochondrial carrier family for which crystal structures are available. Previous work suggests that cardiolipin, the signature phospholipid of mitochondria in eukaryotic cells, is critical for the assembly and function of the AAC. The objective of this work is to investigate the conformational dynamics and lipid interactions of key regions (matrix loops) of the AAC reconstituted into model membrane systems using a fluorescence-based approach. Membrane proteins are innately difficult to analyze because they are not amenable to solution-based studies. We therefore use AAC reconstituted into proteoliposomes for spectroscopic analyses. To synthesize proteoliposomes containing AAC,

we have adapted a novel but well-characterized cell-free translation system whereby polypeptides are integrated directly into preformed liposomes of desired lipid composition. In agreement with previous work with other membrane proteins, AAC is properly integrated as judged by carbonate extraction and proteolysis. Further, we provide evidence that insertion occurs co-translationally by monitoring the interaction of ribosome-bound integration intermediates with liposomes. To synthesize full length AAC with the fluorescent probe NBD at specific sites, we program translation reactions with mRNA encoding AAC bearing unique nonsense (UAG) codons and include suppressor tRNA analogs that allow for the incorporation of non-natural (probe-bearing) amino acids. This has provided evidence for secondary structural elements with membrane interacting surfaces within the matrix loops. In support of this work, synthetic peptides corresponding to matrix helices were used to investigate the innate propensity of these regions to interact with the bilayer. These peptides have been shown to interact with model membranes in a cardiolipin dependent manner by solvent effects, collisional quenching, and resonance energy transfer. In conclusion, we have developed a novel experimental method for the synthesis of proteoliposomes containing AAC site-specifically labeled with fluorescent probes. This technique allows us to monitor structural dynamics of key locations on AAC in relation to the bilayer. Taken together, this work shows that matrix helices of the AAC interact dynamically with the bilayer in a manner dependent on lipid content and conformational state.

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Assembly and Activity of Respiratory Complex II in Reconstituted Membrane Systems.

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Mitochondrial respiratory complex II (succinate:quinone oxidoreductase) is comprised of a soluble catalytic dimer (Sdh1p and Sdh2p in *Saccharomyces cerevisiae*; SdhA and SdhB in mammals) and a membrane-bound heterodimer (Sdh3p and Sdh4p in *S. cerevisiae*; SdhC and SdhD in mammals). The membrane bound subunits coordinate a heme, making complex II a *b*-type cytochrome. As the only membrane-bound complex of the tricarboxylic acid cycle, this complex oxidizes succinate to fumarate and passes the electrons to quinone as part of the electron transport chain of the inner mitochondrial membrane. The primary objective of this work is to investigate the role of the phospholipid environment in: a) the assembly of the membrane subunits, and b) the enzymatic activity of the holoenzyme. Membrane proteins and complexes are traditionally refractory to standard biochemical and biophysical analyses. In order to overcome this technical challenge we have employed the following technically innovative approaches: a) detergent-free proteoliposome synthesis for studying dimerization of membrane-bound Sdh3p/C and Sdh4p/D subunits, and b) reconstitution of active complex II from native membranes into nanolipoprotein particles (nanodiscs) to study isolated function in a lipid bilayer environment. In relation to membrane subunit assembly within proteoliposomes, we have unexpectedly observed that Sdh3p/C form homodimers in a manner that is promoted by heme and also by cardiolipin, the signature phospholipid of mitochondria in eukaryotes. In addition, the Sdh3p/C homodimer is disrupted by Sdh4p/D, suggesting heterodimer formation that can then lead to the assembly of the functional complex. But, interestingly, Sdh4p/D homologs Tim18 and YLR164w appear to interact even more strongly with Sdh3/C, judged by their disruption of the homodimer. Taken together, these results suggest dynamic interaction among complex II membrane subunits and their homologs. By reconstituting the holocomplex into nanodiscs of different lipid composition, we analyzed the effects of phospholipid environment on catalytic activity and complex stability as well. We observed a strong effect of acyl chain length on activity and that maximal activity requires the native-like lipid composition that contains cardiolipin. In conclusion, complex II membrane protein subunit assembly involves multiple

potentially-interacting proteins whose interactions are mediated by lipids and cofactors. Further, complex II catalysis is greatly affected by lipid properties and the presence of cardiolipin.

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MARCH5, a mitochondrial E3 ligase, degrades mitochondrial targeted HBx viral oncoprotein.

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The hepatitis B virus X protein (HBx), oncogenic protein, plays an important role in HBV replication and development of hepatocellular carcinoma.HBx localizes to the mitochondrial outer membrane, which induces mitochondrial dysfunction, oxidative stress and cell death. MARCH5, a mitochondrial E3 ubiquitin ligase localizes to the mitochondrial outer membrane. We here showed that MARCH5 binds HBx and promotes its degradation whereas the MARCH5H43W mutant lacking ubiquitin ligase activity did not, indicating that MARCH5 serves as a protein quality controller on viral oncoprotein. Accordingly, overexpression of MARCH5 alleviated the HBx-induced ROS generation. In addition, HBx-mediated transactivation activity on the COX-2 promoter was weakened in the presence of MARCH5. Thus, the data indicate that MARCH5 contributes to protecting the cells from HBx-mediated cytotoxic effects.

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Reconstitution of the Minimal Mitochondrial Fission Machinery in Yeast and Humans.

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Mitochondrial fission regulates organelle copy number and mitochondrial function in metabolism, development and apoptosis. The dynamin-related GTPases Dnm1 and Drp1 are required for mitochondrial fission in yeast and human, respectively. Dnm1/Drp1 assembles into spirals that encircle and divide mitochondria. In yeast, a tail-anchored protein called Fis1 and an adaptor protein called Mdv1 recruit Dnm1 to the membrane, where Mdv1 co-assembles with Dnm1 and stimulates Dnm1 GTPase activity. Whether Fis1 participates in Dnm1 assembly or post-assembly membrane scission events is unknown. Although Fis1 is conserved in humans (hFis1), an Mdv1 homolog is absent. Instead, humans have at least two different membrane anchored proteins (hFis1 and hMff) whose roles in fission are poorly defined.

Using yeast cells expressing only Dnm1 and a mitochondrial-tethered form of Mdv1, we demonstrated that Fis1 is dispensable for Dnm1 recruitment, assembly, and mitochondrial membrane scission in vivo. Importantly, the absence of Fis1 did not affect distribution of fission complexes on the mitochondrial membrane. Thus, Fis1 acts to direct essential fission components to the correct cellular membrane but does not determine the site of complex assembly. Similar tethering studies reveal that hDrp1 and hMff (but not hFis1) are sufficient to reconstitute mitochondrial fission in yeast. We postulate that the hMff protein combines the hDrp1 GTPase recruitment and effector functions that are carried out separately by Fis1 and Mdv1 in yeast. To test this model, we are using biochemical methods to determine how the cytoplasmic domain of hMff affects the assembly and GTPase activities of hDrp1. Our findings suggest that the conserved role(s) of Fis1 in yeast and human is yet to be identified.

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The Caf4p Adaptor is a Component of Active Mitochondrial Fission Complexes.

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Mitochondrial fission in eukaryotes is mediated by protein complexes that assemble around the cytoplasmic surface of mitochondrial tubules. In budding yeast, fission requires the tail-anchored protein Fis1 and the dynamin-related GTPase Dnm1. Mdv1 and Caf4 are adaptor proteins that interact with both Fis1 and Dnm1 and have redundant functions in Dnm1 recruitment to mitochondria (Griffin et al., 2005). Although Mdv1 is required after Dnm1-recruitment for mitochondrial fission, a role for Caf4p in fission has not been established.
Using time-lapse imaging studies, we established that GFP-Caf4 is present at mitochondrial fission sites and is able to rescue mitochondrial fission defects when Mdv1 is absent. In addition, Caf4 and Mdv1 co-localize in a subset of fission complexes *in vivo*, which also carry out mitochondrial fission. When over expressed 2.5 fold relative to endogenous levels, only Caf4 causes dominant negative fission defects. Experiments are underway to determine whether Caf4 expressed from the genomic MDV1 promoter is sufficient to support wild-type levels of fission *in vivo*. (supported by NIH-RO1-GM053466)

Nuclear Bodies and Dynamics

1691

Discovery of NOL11, a Metazoan-Specific SSU Processome Protein That Is Required For Pre-rRNA Transcription And Processing.

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Ribosome biogenesis is among the most fundamental of cellular processes. Pre-rRNA transcription and processing occur in the subcellular compartment called the nucleolus. The majority of the hundreds of nucleolar proteins involved in ribosome biogenesis were discovered in yeast. While most of these proteins and their functions are conserved to humans, it is becoming clear that important differences exist in how ribosome biogenesis is regulated between yeast and humans. In particular, some essential proteins are not conserved between these two organisms, raising the possibility that they are replaced by functional analogs. Our objective was to identify non-conserved interaction partners for the human ribosome biogenesis protein, hUTP4/Cirhin, since a mutation in the C-terminus of hUTP4/Cirhin is reported to cause North American Indian childhood cirrhosis (NAIC). By screening a yeast two-hybrid cDNA library and through affinity purification followed by mass spectrometry, we identified an uncharacterized nucleolar protein, NOL11, as an interaction partner for hUTP4/Cirhin. Bioinformatic analysis revealed that NOL11 is conserved throughout metazoans, but is not found in any other phylogenetic groups. Co-immunoprecipitation experiments show that NOL11 is in the human ribosomal small subunit (SSU) processome. Using siRNAs to knock-down NOL11 revealed that NOL11 is involved in the cleavage steps that are required to generate the mature 18S rRNA, and further suggested that NOL11 may be required for optimal pre-rRNA transcription. Directed one-by-one yeast two-hybrid analysis shows a complex interaction network between NOL11, other SSU processome subcomplex members, and the RNA polymerase I transcription machinery supporting a role for NOL11 in both pre-rRNA processing and transcription. Finally, yeast two-hybrid analysis using truncated forms of hUTP4/Cirhin shows that NOL11 interacts with the C-terminus of hUTP4/Cirhin, allowing us to propose that NOL11 is the functional analog

of the yeast protein, Utp8. Further study of NOL11 and its interaction with hUTP4/Cirhin may shed light on the etiology of North American Indian childhood cirrhosis.

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Spatio-temporal organization of transcription factories within living cells.

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Recent studies have shown that transcriptional activity in the nucleus is organized in distinct sites called transcription factories (TFs), where genes either loop out or are co-clustered to form active chromatin hubs. In addition, our studies are beginning to reveal that gene-active chromosomes share physical proximity within the 3D architecture of the cell nucleus. However the spatio-temporal organization of TFs and its functional implications are unclear. Using high-resolution live-cell fluorescence imaging and spectroscopy, we analyze the dynamic organization of TFs. For this we labeled TFs using fluorescent UTPs which co-localize with active RNA Pol-II antibody in a transcription dependent manner. Dual color labeling methods revealed that TFs are specialized foci. Further, the local chromatin structure, mapped using fluorescence anisotropy measurements, in vicinity of TFs showed a transcription dependent compaction state. Interestingly TFs exhibited a dynamic behavior with runs, pauses and steps. This dynamic organization of TFs was dependent on ATP, lamin B1, histone acetylation levels and cytoplasmic to nuclear anchorage. Importantly during runs, TFs are mobile within the inter-chromosome territories. The spatio-temporal organization of TFs that we observe may provide possible mechanisms to alter gene expression programs upon integration of physico-chemical signals to the nucleus.

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Digitor, a Novel Zinc-finger Protein Interacts with the Nuclear Spindle Matrix Protein Skeletor as well as with the Dynein Light Chain Ddlc1 in *Drosophila.*

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In *Drosophila* we have used yeast two-hybrid interaction assays to identify a novel zinc-finger protein (CG14962) that we have named, Digitor, which interacts with the COOH-terminal of the spindle matrix protein Skeletor. Skeletor localizes to interband regions of polytene chromosomes during interphase but redistributes during mitosis to form a spindle matrix complex together with Chromator, Megator, and EAST that is distinct from the microtubule-based spindle apparatus. The interaction between Skeletor and Digitor was subsequently confirmed by pull-down assays. Digitor is a 44 kD protein that contains four NH_2-terminal zinc-

finger domains in addition to six TQT motifs in the COOH-terminal domain. Since the TQT motif has previously been identified in dynein light chain binding proteins we made His- and MBP-tagged versions of Digitor and the *Drosophila* dynein complex light chain Ddlc1 (LC8), respectively. Pull-down assays performed with these constructs strongly supported the existence of a direct physical interaction between Digitor and Ddlc1. Furthermore, transient expression studies in S2 cells revealed that GFP-tagged Digitor localizes to the nucleus. Interestingly, it has previously been demonstrated that the spindle matrix protein Megator homolog Tpr in mammalian cells associates with the dynein complex during mitosis (Nakano et al., J. Biol. Chem. 285:10841, 2010). Experiments are in progress to determine whether Skeletor, Digitor, and Ddlc1 all are present in the same complex and to how the interactions between these proteins are coordinated during the cell cycle. Supported by NSF grant MCB0817107.

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The "PcG body" established by correlative light electron microscopy.

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Polycomb group (PcG) proteins are a set of conserved, essential regulatory factors, that through the assembly on key regulatory DNA elements, repress the transcription of their target genes. Proteins of the Polycomb repressive complex 1 (PRC1) are found to be diffusely distributed in nuclei of cells from various species. However, they can be also localized in intensely fluorescent foci, whether imaged using GFP fusions to proteins of PRC1 complex, or by conventional immunofluorescence microscopy. Such foci are termed PcG bodies, and are believed to be situated in the nuclear interchromatin compartment. However, an ultrastructural description of the PcG body has not been reported to date.

To establish the ultrastructure of PcG bodies in human U-2 OS cells stably expressing recombinant polycomb BMI1-GFP protein, we used correlative light electron microscopy (CLEM) implemented with high-pressure freezing, cryosubstitution and on-section labeling of BMI1 protein with immunogold. CLEM approach allowed us to clearly identify fluorescent PcG bodies, not as distinct nuclear bodies, but as nuclear domains enriched in separated heterochromatin fascicles. Importantly, high-pressure freezing and cryosubstitution allowed for a high and clear-cut imunogold BMI1 labeling of heterochromatin structures throughout the nucleus. The density of immunogold labeled BMI1 in the heterochromatin fascicles corresponding to fluorescent "PcG bodies" did not differ from the density of labeling of heterochromatin fascicles outside of the "PcG bodies."

Further, we focused on compaction aspect of the chromatin accumulated into PcG foci and the behaviour of the foci in hyperosmotic environment. Our preliminary data show that with increasing hypertonic load and incubation time the PcG foci reduce their size gradually up to total disappearing and when the cells are washed, the PcG foci appear again. The treated cell reacts within its physiological realms and BMI1 protein undergoes the changes in post-translational modifications.

1695

Characterization of the function of a novel nuclear protein, JC1V2, an interaction partner of Dictyostelium discoideum SUN-1.

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Dictyostelium SUN-1 is the homolog of mammalian Sun proteins. Sun proteins are inner nuclear membrane proteins and are essential components of the LINC complex which connects the nucleus and the cytoskeleton. For the Dictyostelium protein we reported that it connects the centrosome to chromatin and ensures genome stability. However the role of SUN-1 in general is still poorly understood. Here we introduce a novel nuclear protein as an interacting partner of SUN-1, JC1V2, which has a role in gene regulation, aggregation and chemotaxis of D. discoideum. The N-terminus of SUN-1 is important for this interaction. JC1V2 protein is divided in to two domains. The N-terminal domain is highly basic, containing segments of low compositional complexity and C-terminal has a DUF926 domain (Domain of Unknown Function #926 in the NCBI Conserved Domain Database). During interphase JC1V2 is localised in the nucleus in a punctuated pattern, during mitosis it redistributes to the cytosol. Upon ectopic expression of JC1V2 the cells show a severe growth defect, a delay in stream formation, aggregation and chemotaxis. Overexpression of the N-terminus only also led to a growth defect, however streaming and aggregation were enhanced when compared to wildtype cells. This was paralleled by enhanced chemotactic speed and increased motility. To better understand the function of JC1V2, we performed gene expression profiling using microarray studies, which revealed up-regulation of genes involved in stress response whereas genes involved in translation were found to be down-regulated. In conclusion Dictyostelium JC1V2, a SUN-1 interacting protein, is involved in gene regulation, growth and aggregation.

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Son is Important for Cell Cycle Progression.

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The organization of pre-mRNA processing factors into nuclear speckles is important for coordinating pre-mRNA transcription and splicing in mammalian cells. Pre-mRNA processing factors disassemble from these nuclear compartments at the onset of mitosis and assemble into mitotic interchromatin granules clusters (MIGs) in the cytoplasm until they enter nuclei in telophase. A large repeat-rich splicing factor called Son is required for proper organization of pre-mRNA processing factors in nuclear speckles during interphase. Because Son is also required for mitotic progression through metaphase, we analyzed localization of Son in human cells during mitosis. We found Son in MIGs, consistent with the possibility that Son may seed MIG assembly at metaphase to promote normal cell cycle progression. Our recent results revealed that Son is required to maintain the organization and integrity of the mitotic spindle. Son-depleted cells exhibited disorganized spindles with longer interpolar distances than controls. Exon array analysis points toward changes in mRNA levels and alternative splicing of transcripts encoding mitotic regulators as a key mechanism for mitotic defects in Son-depleted cells. Our study also provides a comprehensive view of human transcription and splicing targets for Son in fundamental cellular pathways such as integrin-mediated cell adhesion, cell cycle regulation, cholesterol biosynthesis, apoptosis, and epigenetic regulation of gene expression.

1697

A novel role for β-dystroglycan as a nuclear scaffolding protein required for nuclear domains organization and function.

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β-dystroglycan is a widely expressed transmembrane protein belonging to the dystrophin-associated protein complex. β-dystroglycan anchors α-dystroglycan to the cell membrane via its the N-terminus domain, and binds to dystrophin via its C-terminus domain, providing by this way a tight link between the extracellular matrix and the intracellular cytoskeleton. Nuclear localization of β-dystroglycan has been revealed recently; however, its functional relevance remains unknown. The nucleus of eukaryotic cells is a highly dynamic organelle organized into different specialized domains that perform specific functions, including replication, transcription, RNA processing, and ribosome biogenesis. Therefore, to make an inference concerning the nuclear role of β-dystroglycan, we analyzed its potential association with different nuclear compartment markers, by immunofluorescence and immunoprecipitation assays. We found that β-dystroglycan associates with protein markers from nuclear envelope (lamin A/C, -B1, and emerin),splicing speckles (SC35), Cajal bodies (p80 coilin), and nucleoli (Nopp140). Interestingly, β-Dystroglycan knock-down resulted in mislocalization and decreasing levels of lamin B1, emerin, nopp140 and coilin. Altogether, our data revealed that β-dystroglycan works as nuclear scaffolding protein required for nuclear domains organization and function.

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Quantification of Water and Ions in the Nucleus by Cryo-Correlative Light and Scanning Transmission Electron Microscopy.

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The cell nucleus contains the highest concentration of macromolecules in the cell. Thus, in mammalian cells, mean concentration of macromolecules varies from 65 to 180 mg/ml for whole nuclei and from 75 to 200 mg/ml for interphase chromatin and nucleolus respectively as measured by using different approaches such as chemical assays, interference microscopy or quantitative Scanning and Transmission Electron Microscopy (STEM). Such high concentrations induce macromolecular crowding which predicts that a small variation in the water content could produce strong variations in the activity of numerous macromolecules. Data dealing with water content and ions concentration in hydrated nuclear compartments are unknown. This is limiting for a detailed knowledge of the cell nucleus function because water is recognized as a crucial player in the organization of proteins and nucleic acids.
The objective of our study was to quantify water and ions concentration in the main nuclear domains at nanoscale both within control and stressed cells.
For this, we developed a new correlative light and electron microscopy imaging approach combining observation of the same cryo-ultrathin section first by fluorescence microscopy (localization of GFP in HeLa cell-line expressing H2B-GFP) and then by cryo-STEM. As fluorescence and STEM images were perfectly aligned, nuclear compartments containing chromatin were clearly identified in order to perform simultaneous water quantification and elementary analysis (N, P, K^+, Cl- , Mg^{2+} and S) by STEM with a resolution of 20 nm and expressing data in mmol/L.

We found that nucleoplasm contained more water than clumps of interphase condensed chromatin and than chromatin of mitotic chromosomes (75.7 %, 64.8 % and 63.2% respectively). In the nucleolus, fibrillar centers contained more water than other nucleolar components (dense fibrillar and granular components) (82.9% and 68.8% respectively). Concentration of ions ranged from 128 to 462 mmol/L for K^+, from 23 to 85 mmol/L for Cl- and from 13 to 55 mmol/L for Mg^{2+}. Interestingly, a nucleolar stress induced by actinomycin D increased water content in all the nuclear compartments.
In conclusion, this study simultaneously quantified water and elements in the main nuclear components for the first time. We found that water content is lower than previously estimated in control cells and that it increased after a nucleolar stress.

1699

Coilin implicated in U snRNA 3' end processing: nucleic acid interaction studies point to additional functions of the Cajal body marker protein.

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Coilin is a nuclear protein known primarily as the marker for Cajal bodies, subnuclear domains present in cells with high transcriptional demands and important in the biogenesis of small nuclear ribonucleoproteins (snRNPs), the primary component of the pre-mRNA splicesome. However, the majority of coilin is nucleoplasmic and the function of this fraction has yet to be fully elucidated. In HeLa cells, Cajal bodies have been found to associate with both U1 and U2 gene loci that yield the RNA component of U1 and U2 snRNPs, respectively. The primary transcripts for both U1 and U2 snRNA, products of RNA polymerase II, have been shown to be longer than the mature transcript that is incorporated into the snRNP and must undergo 3' end processing. Both the RNA polymerase II C-terminal domain and the Integrator complex are involved in the processing of U2 snRNA. We hypothesize that coilin can bind nucleic acid and plays a role in the processing of these primary U snRNA transcripts as an additional function to its known role in Cajal body formation. In this study, we seek to characterize the nucleic acid interaction properties of coilin and determine in what capacity it may be involved in snRNA 3' end processing. Using electroelution, we purify bacterially expressed coilin to homogeneity. We then incubate purified coilin with DNA, RNA, and double-stranded RNA. Following incubations, we analyze DNA/dsRNA binding and global RNA degradation by agarose gel electrophoresis and specific RNA degradation by qRT-PCR. We have also targeted coilin for knockdown with siRNA in HeLa cells, and analyzed the effect of this knockdown on U snRNA processing by qRT-PCR. Here, we show purified coilin can bind double-stranded DNA and double-stranded RNA in vitro. Surprisingly, purified coilin degrades HeLa RNA in a concentration-dependent manner, and this activity seems to arise from the N terminus as purified coilin C-terminal fragments have relatively diminished RNase activity. Upon incubation with a U2 snRNA primary transcript in vitro, coilin displays RNase specificity to the CU region, which is downstream of the mature 3' terminus. In HeLa cells with 70-80% coilin mRNA knockdown, we show a significant increase in the level of 3' pre-processed U1 and U2 snRNA by qRT-PCR analysis. Taken together these results support additional functions of coilin, including U snRNA processing, and suggest a more direct role for Cajal bodies in the biogenesis of snRNPs.

1700

Coilin Levels Modulate Cell Cycle Progression and Gamma H2AX Levels in Etoposide Treated U2OS Cells.

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Background: Cajal bodies (CB) are subnuclear domains that participate in small nuclear ribonucleoprotein (snRNP) biogenesis. Coilin is the CB marker protein, but its localization changes in response to various types of cellular stress. Cisplatin induced DNA damage causes nucleolar accumulation of coilin whereas UV-C induced DNA damage fragments CBs and facilitates the interaction of coilin with proteosomal proteins. The main objective of this study was to investigate the role of coilin in the DNA damage response, with specific emphasis on the relationship between coilin and the formation of gamma H2AX, a biomarker for certain types DNA damage.
Results: siRNA mediated depletion of coilin correlated with significantly high levels of gamma H2AX in etoposide treated U2OS cells. We have investigated the role of coilin in the induction of gamma H2AX and found that coilin directly interacts with RNF8, which plays a major role in ubiquitination of gamma H2AX. Further, we have observed that transiently transfected coilin inhibits the nucleolar localization of endogenous coilin in etoposide treated U2OS cells. Modulation of coilin levels in etoposide treated cells is associated with alterations in cell proliferation, with the depletion of coilin levels causing an increased cell proliferation rate and the increase in coilin levels causing a decrease in cell proliferation rates compared to their respective controls. This observation indicates that the coilin has a direct or indirect role in cell cycle check points and may influence the stalling of cells at a specific phase of the cell cycle. To test this, we conducted FACS analysis on etoposide treated U2OS cells transiently transfected with coilin. We found that coilin overexpression increases the percent of cells in S and G2/M and reduces the number of cells in G1 compared to controls.
Conclusion: Coilin reduction increases gamma H2AX levels in etoposide treated U2OS cells. This increase in gamma H2AX levels may be attributed to the reduced activity of RNF8, which we have found to interact with coilin. In U2OS cells treated with etoposide, alteration of coilin levels, either increased or decreased, is associated with changes in proliferation rates. FACS analysis demonstrates that the over expression of coilin arrest cells in the S and G2/M phases of cell cycle in etoposide treated U2OS cells. Collectively, these data further indicate a role for coilin and CBs in the DNA damage response.

1701

Investigation of Functional Diversity within the Ribosomal Protein L22e family in Drosophila melanogaster: Evidence for SUMOylation of RpL22.

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Duplicated ribosomal proteins are found throughout eukaryotic genomes, at times encoding highly similar or identical proteins. Whether these paralogues are functionally redundant or provide a unique cellular role, not limited to translation, remains largely unknown and unexplored. Using *Drosophila* as a model, we have focused on the two members of the eukaryotic-specific ribosomal protein L22e family, RpL22 (ancestral) and RpL22-like (duplicated). Within the reproductive system, RpL22 is ubiquitously expressed, but RpL22-like expression is confined to the testis. Using paralogue-specific C-terminal peptide-derived polyclonal antibodies, Western analysis of fly tissues shows immunodetection for RpL22 at its expected molecular weight (MW) of 33kD as well as a predominant higher MW product at ~50kD, suggestive of post-translational modification. Within the testis, RpL22-like is

predominantly detected at its predicted MW and is a component of ribosomes (Kearse *et al.*, 2011). In this report, we investigate the possible SUMOlyation of RpL22. Computational probing predicts a SUMOylation consensus motif, localized at different sites within the fly-specific N-terminal extension of both family members. Results from tissue culture (S2 cells)-based experiments replicate the electrophoretic pattern when FLAG-tagged RpL22, but not a K39R mutation within the proposed SUMOylation site, is co-expressed with HA-SUMO. These results confirm the *in silico* prediction and demonstrate that RpL22 can be SUMOylated, likely with two SUMO moieties. Interestingly, Western analysis shows that SUMOylated RpL22 is more predominant in adult testis than in S2 cells. Western analysis of RpL23a-FLAG-affinity purified complexes from S2 cells shows that SUMOlyated RpL22 is only a minor component of complexes containing RpL23a or RpL9. Furthermore, whole-mount testis immunohistochemistry reveals distinct nucleoplasmic, but not nucleolar localization within the male germline. These data suggest that within the male germline, RpL22 and RpL22-like have distinct roles. Current efforts are devoted toward elucidating the role of RpL22e paralogues in the male germline using an *in vivo* tissue-specific RNAi approach.

1702

Cells Exhibit a Threshold Dependant Nuclear Reorganization Response to Shear Stress.

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Endothelial cells respond to shear stress by altering gene and protein expression. The mechanisms by which these cells respond to shear stress have not been fully elucidated. Since the probability of gene expression correlates to the position of the genes in the nucleus, we examined reorganization of the nucleus of cells under shear stress by tracking fluorescent intranuclear markers. By studying large scale nuclear response under physiological shear stress conditions, we are able to show that there is a shear-stress threshold dependence to the nuclear response. At high levels of shear stress we observed a shift from random reorganization to a directed reorganization. Additionally, we stiffened the nuclei of cells by overexpressing a mutant nuclear structural protein associated with aging called progerin and considered the effects of chemical stimulation with vascular endothelial growth factor (VEGF). Progerin stiffened nuclei show little response to shear, potentially from altered mechanotransductive response. Stimulation of cells with VEGF also shows increased intranuclear movement in HUVECs. Although not gene specific, these large scale organizational responses across the nucleus could correspond to substantial changes in gene expression. HUVECs and HeLa cells both showed a threshold response to shear stress. At high shear stress cells show a directed reorganization in response to shear stress. The expression of progerin more than doubles the continuum stiffness of the nucleus as shown by micropipette aspiration. However, in progerin expressing cells, there is increased intranuclear movement in cells under shear stress and altered nuclear response. Here, we show that the nucleus responds to extracellular shear stress with both increasing levels of shear stress and with time under shear. Thus, in addition to force-sensitive elements in the plasma membrane, extracellular adhesions and cytoskeleton, the nucleus itself may be a mechanosensitive element of the cell. The introduction of progerin results in an interruption of these responses that could be either from stiffening of the nucleoskeleton, from damage to other underlying nuclear structures or from altered nucleoskeletal-cytoskeletal connections. These nucleus-wide mechanical studies show that there is a materials response of the nucleus to shear stress in addition to an active biological response; by changing the material mechanical properties of the nucleus the biological activities of the nucleus are also altered.

1703

Characterization of the Enlargement of Speckles of SF2/ASF due to Loss of Function of Smu1 in the Mammalian Temperature-Sensitive Mutant.

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A temperature-sensitive (ts) CHO-K1 mutant cell line, tsTM18, exhibits chromosomal instability with decreased DNA synthesis at the nonpermissive temperature, 39°C. An amino acid substitution in Smu1 underlying the ts phenotypes of tsTM18 cells was identified previously. We also found a ts defect in splicing of the *unc52/perlecan* gene. In the present study, we have generated cell lines expressing Smu1 tagged with green fluorescent protein (GFP) to study the dynamics of Smu1 in living cells. The hybrids complement deficiencies in tsTM18 cells and allow them to grow normally at 39°C. GFP-tagged Smu1 is found in speckles in many discrete nucleoplasmic sites, and most of these also contained SF2/ASF. SF2/ASF is a member of the serine/arginine (SR)-rich splicing group of factors that are necessary for spliceosome assembly and can influence alternative splicing. SF2/ASF is also involved in the integrity of genome maintenance. In tsTM18 cells cultured at 39°C, the Smu1 ts defect appears to alter SF2/ASF localization, suggesting a physiological association of Smu1 with SF2/ASF. The significant decrease of Smu1 may lead the enlargement of speckles of SF2/ASF. These data show the importance of Smu1 as a regulator of splicing and genome maintenance.

1704

Visualization of Eukaryotic DNA Mismatch Repair Reveals Distinct Recognition and Repair Associated Intermediates.

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DNA Mismatch Repair (MMR) increases the fidelity of DNA replication by eliminating mispaired bases resulting from replication errors. MMR proteins are conserved from bacteria to humans and people with defective mismatch repair develop cancers at much higher rates due to an increased accumulation of mutations. In eukaryotes, Mispaired bases are recognized by two partially redundant heterodimer complexes, Msh2-Msh6 or Msh2-Msh3. After the mismatch recognition factors bind a mispaired base, accessory factors including the Mlh1-Pms1 complex are recruited, ultimately targeting repair to the daughter DNA strand. We used high-resolution microscopy to visualize functional fluorescent-tagged versions of the Msh2-Msh6 and Mlh1-Pms1 MMR complexes in living S. cerevisiae cells. Msh2-Msh6 forms foci in S-phase that colocalize with nuclear foci composed of the DNA replication machinery, often called replication factories. This localization was independent of mispair recognition. The association of Msh2-Msh6 with replication factories is completely dependent on the interaction of Msh6 with the DNA polymerase clamp PCNA (Pol30). Replication factory-association accounts for 10 to 15% of MMR in wild-type cells but is essential for MMR in the absence of the exonuclease Exo1. Mlh1-Pms1 forms nuclear foci that are dependent on mispair recognition by Msh2-Msh6 or Msh2-Msh3. Surprisingly, these foci rarely colocalize with Msh2-Msh6 foci. Mlh1-Pms1 foci increase in frequency in response to increased levels of mispaired bases. These foci also increase in response to the disruption of processes downstream of mispair recognition, indicating an accumulation of foci that cannot be efficiently processed. Based on these data, we conclude that Mlh1-Pms1 foci are sites of active mismatch repair; this is the first time mismatch repair intermediates have been observed in eukaryotes. These results suggest that (I) mispair recognition involves either the concentration of Msh2-Msh6 at replication factories or a second distinct pathway that depends on both Msh2-Msh6 and Exo1, and that (II) accumulation of Mlh1-Pms1 triggered by Msh2-Msh6-dependent mispair recognition defines sites of active MMR.

1705

Involvement of p32, fibrillarin, and Nop52 in pre-90S particle separation during human ribosome biogenesis.

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Ribosome biogenesis, which takes place in the nucleolus, is fundamental cellular metabolism to produce ribosomes. It is coordinated by cell growth and proliferation, and its aberrant regulation causes some human diseases and cancer. Ribosome biogenesis starts with the transcription of a large ribosomal RNA precursor (47S pre-rRNA in human cells), which soon combines with numerous factors to form 90S preribosome (pre-90S). This pre-90S undergoes further processing and modification of pre-rRNAs, and association with ribosomal proteins before splitting into pre-40S and pre-60S particles. Despite that this splitting step is one of the most important processes to produce small and large ribosome subunits in ribosome biogenesis, its molecular mechanism remains to be determined even in yeast cells that have been analyzed extensively.
In this report, we present evidence that p32, fibrillarin (FBL; a human homolog of yeast Nop1p), and Nop52 (a human homolog of yeast Rrp1p) involves in this splitting step. We first isolated and analyzed the nuclear p32-associated protein complex by mass-based proteomic approaches, and showed that p32 associated with 31 pre-rRNA processing factors including not only an early pre-rRNA processing factor FBL but also a late processing factor Nop52. Both FBL and Nop52 interacted directly with p32. Using immunofluorescence microscopy, we next showed that p32 co-localized with FBL or Nop52 in the nucleolus and Cajal bodies. p32 was also present in the pre-ribosomal fractions prepared by cell fractionation or separated by ultracentrifugation of the nuclear extract. In addition, we showed that p32 associated with pre-rRNAs including 47S/45S and 32S pre-rRNAs. We then found that knockdown of p32 with a siRNA slowed the early processing from 47S/45S pre-rRNAs to 18S rRNA and 32S pre-rRNA. Furthermore, we found that Nop52 competes with FBL for binding to p32 probably in the nucleolus. Given the fact that FBL and Nop52 were associated with preribosome particles distinctly different from each other, we suggest that p32 is a new rRNA maturation factor involved in the remodeling from pre-90S particles to pre-40S and pre-60S particles that requires the exchange of FBL for Nop52.

RNA Localization and Transport

1706

The hnRNP protein CBF-A, a novel transacting factor for mammalian mRNA transport and localization.

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Upon export certain mRNAs are assembled into granules and transported to subcellular compartments for localized translation through mechanisms that require interplay of cis-acting elements and transacting factors. The A2RE or RNA trafficking sequence (RTS) in the 3' untranslated region (UTR) of the myelin basic protein (MBP) mRNA is recognized by the A/B type hnRNP proteins hnRNP A2 and CBF-A. In oligodendrocytes RTS recognition by CBF-A is

necessary for transport and localization of MBP mRNA to the myelin compartment. CBF-A has a conserved role in RTS-mediated transport and localization of Arc, CaMKIIα and BDNF mRNAs at neuronal synapses. In the nucleus of brain cells CBF-A is excluded from dense chromatin and localizes to nascent pre-mRNPs within the perichromatin region. Since CBF-A does not bind to transcripts without RTSs, co-transcriptional RTS-recognition by CBF-A de facto provides a sorting mechanism for transport-competent mRNAs at an early stage in RNP biogenesis. Recently we discovered that the role of CBF-A is conserved in spermatogenic cells where CBF-A is involved in transport and localization of protamine 2 mRNA. Here we show that CBF-A binds to the conserved RTS in the protamine 2 mRNA 3' UTR and in round spermatids, both CBF-A splice variants p37 and p42 associate with protamine 2 mRNA and accompany the transcript to chromatoid body. The p42 isoform appears in protamine 2 mRNA-containing polysomes and interacts with the 5' mRNA cap structure. We conclude that in elongating spermatids when a distinct protamine 2 mRNP emerges in the cytoplasm to engage the translation machinery, p42 remains associated with the transcript, facilitating targeting to the translation machinery.

1707

Cotranslational transport of ABP140 mRNA to the distal pole of S. cerevisiae.

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In budding yeast, several mRNAs are selectively transported into the daughter cell in an actin-dependent manner. This directional transport involves a specialized myosin system, the SHE machinery. With ABP140 mRNA, we now describe the first mRNA that is transported in the opposite direction and localizes to the distal pole of the mother cell, independent of the SHE machinery. Distal pole localization is not observed in mutants devoid of actin cables and can be disrupted by latrunculin A. Furthermore, expression of Abp140p is required for localization of its mRNA; more specifically, the N-terminal actin-binding domain of Abp140p is necessary for distal pole localization of the mRNA. By replacing the N-terminal localization motif, ABP140 mRNA can be retargeted to different subcellular structures. In addition, accumulation of the mRNA at the distal pole can be prevented by inhibition of protein translation. We therefore propose a model of translational coupling, in which ABP140 mRNA is tethered to actin cables via its nascent protein product and is transported to the distal pole, possibly by actin retrograde flow.

1708

Endoplasmic Reticulum (ER) Resident Protein-Encoding mRNAs Undergo Translation-Independent Localization to Subdomains of the ER Membrane.

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mRNAs encoding secretory and integral membrane proteins are localized to the ER membrane via the SRP pathway, a translation- and topogenic signal-dependent mechanism for segregating the synthesis of topogenic signal-encoding proteins to the ER, the gateway to the secretory pathway. In a recent report, we demonstrated that topogenic signal-encoding mRNAs sequence display cohort-specific patterns of enrichment on the ER as well as distinct modes of attachment to the ER membrane (1). Resident endomembrane protein-encoding mRNAs are the most highly enriched class of mRNA on the ER membrane; secretory pathway cargo (secretory and membrane protein)-encoding mRNAs, in contrast, display diverse subcellular distributions, ranging from cytosol-enriched to highly ER-enriched. These subcellular distribution patterns were mirrored in distinct modes of mRNA association with the ER membrane, where resident endomembrane protein-encoding mRNAs undergo direct, ribosome-independent binding interactions with the ER and secretory cargo protein-encoding mRNA are bound to the ER via

their functional association with membrane-bound ribosomes. These findings demonstrate that topogenic signal-encoding mRNAs undergo hierarchical, cohort-specific modes of localization to the ER. In current studies, we extend these findings in studies of the subcellular localization of newly synthesized mRNAs. By tracking the subcellular distributions of metabolically labeled, newly synthesized mRNA as a function of time after nuclear export and in the presence or absence of the protein synthesis inhibitor cycloheximide, we demonstrate that secretory cargo protein-encoding mRNAs rapidly accumulate in the cytosol when translation is inhibited whereas resident endomembrane protein-encoding mRNAs localize to the ER in a translation-independent manner. Furthermore, in cell fractionation studies, we report that cargo- and resident endomembrane protein encoding-mRNAs bind to distinct subdomains of the ER. Using Brij 35 in combination with buffer conditions that release ribosomes from the ER, secretory cargo-encoding mRNAs (β-2-microglobulin) were efficiently released from the ER membrane whereas endomembrane protein-encoding mRNAs (GRP94) were retained in a Brij 35-resistant ER membrane domain. We speculate that translation- and ribosome-independent mRNA localization to the ER represents a previously unexplored mechanism for establishing the intrinsic self-organization capacity of the endomembrane system.

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1) Chen et al. (2011) Mol. Bio. Cell (14):2646-58.

1709

Genome-scale survey of subcellular mRNA translation reveals compartment-specific regulation of cellular protein synthesis.

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In eukaryotic cells, protein synthesis is primarily catalyzed by two populations of ribosomes, cytosolic and endoplasmic reticulum (ER)-bound, which catalyze the synthesis of distinct cohorts of proteins, cytosolic and nucleoplasmic in the case of cytosolic ribosomes and secretory and integral membrane, in the case of ER-bound ribosomes. The signal recognition particle (SRP) pathway, which directs the co-translational recruitment of topogenic signal-encoding mRNAs to the ER, is an established mechanism for regulating mRNA partitioning between the cytosol and ER. Recent genomic studies of subcellular mRNA partitioning have demonstrated that cytosolic protein-encoding mRNAs are also represented and in some cases enriched on the ER, suggesting both a broad role for the ER in cellular protein synthesis and the existence of additional, SRP-independent mechanisms trafficking mRNAs to the ER. We have investigated the genomic and biochemical patterns of mRNA partitioning to the ER and report that topogenic signal-encoding mRNAs are partitioned to the ER in a hierarchical, cohort-specific manner where mRNAs encoding resident proteins of the endomembrane system displayed high ER enrichment and mRNAs encoding secretory pathway cargo were represented on free and ER-bound ribosomes, though biased to the ER. In agreement with previous studies, mRNAs encoding cytosolic and nucleoplasmic proteins were represented on the ER, with small cohorts displaying substantial ER enrichment. To assess the consequences of bi-compartmental mRNA partitioning on mRNA translation, we performed a genome-wide interrogation of subcellular mRNA translation profiles by ribosome footprinting. Cytosolic and ER-bound polyribosomes were treated with nuclease and the ribosome-protected mRNA fragments subjected to deep sequencing. By this technique, the position of ribosomes on each mRNA, in either compartment, can be determined at single nucleotide resolution. These studies demonstrate that the cytosol and ER target specific subsets of biologically related mRNAs for translation. Additionally, the biochemical properties of translation are divergent, with ER-bound ribosomes displaying greater processivity and more efficient termination, as compared to cytosolic ribosomes. On the basis of these data, we speculate that mRNA localization to the ER

serves as a novel mechanism for the post-transcriptional regulation of gene expression, and operates on a genome-wide scale to regulate steady-state cellular protein levels. (Supported by NIH GM077382, C.V.N.)

1710

The human enhancer of mRNA decapping, Hedls, regulates the decapping enzyme, Dcp2, protein levels and activity.

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mRNA decay is a key step in the regulation of gene expression. A critical step in several mRNA decay pathways is decapping. A major decapping complex consists of the decapping enzyme Dcp2 as well as several decapping enhancers. An outstanding question is what role does each of these enhancers play in regulating the activity of Dcp2. We have identified a role for the metazoan specific decapping enhancer Hedls (also called Ge-1/Edc4) in the regulation of Dcp2 protein stability and activity. We observed that knockdown of Hedls results in the accumulation of deadenylated mRNA using a beta globin reporter containing the AU-rich element from the 3'UTR of granulocyte-macrophage colony-stimulating factor mRNA. This suggests that Hedls is required for the efficient 5'-3' decay of the message following deadenylation. We also observed that endogenous Dcp2 levels were reduced dramatically upon Hedls depletion. In addition exogenously expressed Dcp2 protein is highly unstable but can be stabilized by Hedls co-expression or by deletion of or five point mutations within the C-terminal Hedls interaction domain of Dcp2. Thus Hedls promotes Dcp2 activity at least in part by interacting with and preventing the activity of a C-terminal Dcp2 instability domain. These results provide novel insights into the regulation of mRNA decay mediated by the Dcp2 decapping complex.

1711

A subset of axonally transported RNAs associates with Myosin Va in ribonucleoprotein complexes.

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Sorting of specific messenger RNA to particular cytoplasmic locations and regulated translation has been demonstrated to occur in multiple systems. While mature axons have been shown to contain specific ribosomal and messenger RNAs, their origin and roles are still unclear. RNA trafficking by means of kinesins and dyneins moving along microtubules has been clearly established in several cell types, including neurons. Since the highly processive myosin Va (MVa) has been described as a component of ribonucleoprotein particles (RNPs) in the rat nervous system, we characterized myosin Va-associated RNPs in rat adult brain and spinal root axons. Using fluorescence microscopy, we show that myosin Va colocalizes with RNA and with RNA binding proteins in discrete structures present in spinal cord root axons. We also show that the axonal structures known as periaxoplasmic ribosomal plaques are enriched in RNPs, some of which are equipped with a dual kinesin and myosin Va molecular motor complex and MVa mRNA. Immunoprecipitation of myosin Va-associated messenger RNAs and microarray analysis demonstrated that myosin Va associates with a specific subpopulation of neuronal mRNAs both in brain and ventral/dorsal root axons. In conclusion, our data suggest that myosin Va is involved in mRNA trafficking within axons.

1712

Schwann cells as local supply of axonal RNA in regenerating mammalian.

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The existence and extent of axonal protein synthesis has been a contentious issue for decades, but evidence supporting it has steadily accumulated. In turn, this raises the question of whether axonal mRNAs are transcribed in the cell body, glia, or both? Here we show that proximal segments of transected sciatic nerves, in which newly synthesized RNA was labelled in the absence of neuronal cell bodies in vitro, accumulate labelled RNA in axons. We show that myosin-Va function is required for this cell-to-cell transfer, as segments from null mutant mice have no detectable labelled axonal RNA. We also demonstrate that the integrity of the actin cytoskeleton is necessary for RNA transference from glia to axon, since latrunculin impairs such transference in a dose dependent way. Our results demonstrate cell-to-cell transfer of RNA (including neurofilament mRNA and ribosomal RNA) and suggest that its mechanism may be similar to the mechanism of transfer of melanosomes, which also is disrupted in myosin-Va-deficient mice. The enhancement of cell-to-cell transfer by injury suggests that interventions following injury or degeneration, particularly gene therapy, may be accomplished by applying them to nearby glial cells (or implanted stem cells) at the site of injury to promote regeneration.

1713

Expression of a Putative miR, Lb-miR-1 in Leishmania braziliensis.

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MicroRNAs have been described in most organisms from worms to human and more recently in protozoans. MicroRNAs are a group of small RNAs that regulate gene expression post-transcriptionally in a complex process of binding to mRNA in a perfect complement or almost perfect complement that cleave mRNAs or inhibit their translation. Gene expression in *Leishmania* is not well understood, however, it is known to be post-transcriptionally regulated. Argonaute-like and Dicer-like protein, the machinery needed for the processing of miRNAs have been shown to exist computationally in *Leishmania braziliensis.* Our hypothesis is that microRNAs may regulate gene expression in *L. braziliensis*. Here we show our results of computational data analysis showing potential MicroRNAs in *L. braziliensis.* We also show that Lb-miR-1 is expressed in *L. braziliensis* promastigotes.

Epigenetics and Chromatin Remodeling

1714

Generation of a Novel Chromatin E-map in a Mammalian Cell Line.

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Quantitative epistasis mapping (E-MAP) is a powerful method for identifying synergistic or antagonistic functional relationships between genes. E-MAPs have been successfully generated in two yeast species, *E. coli*, and *Drosophila* S2 cells, but until now, this approach has not been

utilized in mammalian cell lines. In this presentation, we report a method for generating an E-MAP using GFP labeled mouse fibroblast cell lines and a highly efficient transient knock-down (KD) approach with esiRNA (endoribonuclease-prepared siRNA).

Interrogation of pair-wise combinations of approximately 160 genes focused on chromatin regulation and epigenetics was carried out post-transfection using an Acumen® eX3 plate laser scanning image cytometer. This approach allowed us to rapidly and accurately enumerate whole wells for cell proliferation, at multiple time points, without further sample manipulation. Using this data, a mammalian E-MAP based on more than 12,000 unique combinations of double KDs was generated using hierarchical clustering algorithms. As expected, functionally related genes exhibited similar phenotypes and members of known multi-protein complexes were clustered together. Two complexes, Ccr4-Not and Paf1, were also seen to be located near each other in a common sub-tree and in addition, we observed buffering interactions between the complexes. This synergistic mode of action has also been debated in the literature. Further screening studies analyzing cell cycle and apoptosis with additional components for these complexes will provide further information about the interactions of these complexes.

This presentation demonstrates the power and utility of generating E-MAPs in mammalian cells and is applicable to many cell types and genes of interest or gene and drug combinations.

1715

Centromere maintenance is dynamic throughout the cell cycle.

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The centromere is a defining feature of chromosomes. On mitotic chromosomes, it determines the primary constriction where it templates kinetochore assembly, thus linking chromosomes to the mitotic spindle. It is thus surprising that centromeres are specified epigenetically in most model organisms. The epigenetic mark is widely considered to be the substitution of histone H3 for the variant CENP-A in centromere nucleosomes.

Epigenetic marks are dynamic throughout the cell cycle, changing with transcriptional activation for instance. CENP-A was thought to be an exception since maintaining centromere identity is critical for survival. This hypothesis is supported by evidence that during replication, CENP-A from the mother centromere is evenly distributed among the daughter centromeres, and that newly synthesized CENP-A is only loaded after mitotic exit. Furthermore, FRAP experiments have shown that CENP-A does not turn over at centromeres. This suggested that once incorporated into the centromere, CENP-A would remain stable for many cell cycles.

We hypothesized that specific mechanisms regulate the stability of centromere identity throughout the cell cycle. Using a novel high-resolution live cell microscopy assay, we find that centromere levels of CENP-A are dynamic throughput interphase in HeLa cells. Loading of new CENP-A to centromeres persists throughout G1, repopulating centromere CENP-A levels to that seen pre-replication with first-order kinetics (time-constant ~2.5h). Continued analysis of CENP-A levels in control cells revealed that centromeric CENP-A levels increase to greater than pre-replication levels, and that the excess CENP-A is lost during S/G2. Taken together these results suggest that CENP-A levels at centromeres is dynamic throughout the cell cycle.

Depletion of CENP-A or its chaperone HJURP simply abolished new CENP-A loading, but did not affect pre-assembled centromeric CENP-A. Thus these data support recent findings that HJURP is required for the physical assembly of CENP-A nucleosomes. On the contrary, depletion of the licensing factor KNL-2 lead to a loss of centromeric CENP-A, suggesting that

KNL-2, which is recruited to the centromere in early G1 and remains bound until S-phase, is important for the maintenance of centromere identity. Co-immunoprecipitation of KNL-2 and subsequent mass spectrometry identified MgcRacGAP, which has been previously reported to play a role in centromere function. We find that MgcRacGAP localizes transiently to centromeres at the end of G1 and is required to stabilize newly incorporated CENP-A at the centromere. We propose a model where KNL-2 acts as the epigenetic mark for the centromere during G1 via three mechanisms. First, KNL-2 is required to protect preexisting CENP-A from removal during G1. Second, it targets the CENP-A loading machinery to replenish CENP-A levels. Finally KNL-2 recruits MgcRacGAP at the end of G1 to protect newly incorporated CENP-A from removal in G2. In sum, centromere epigenetic identity is dynamic and this may represent a mechanism for error correction of the epigenetic mark.

1716

CENP-C recruits M18BP1 to centromeres to promote CENP-A chromatin assembly.

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Eukaryotic chromosomes segregate by attaching to microtubules of the mitotic spindle through a chromosomal microtubule-binding site called the kinetochore. Kinetochores assemble on a specialized chromosomal locus termed the centromere, characterized by the replacement of histone H3 in centromeric nucleosomes with the essential histone H3 variant centromere protein A (CENP-A). Understanding how CENP-A chromatin is assembled and maintained is central to understanding chromosome segregation mechanisms. CENP-A nucleosome assembly requires the Mis18 complex and the CENP-A chaperone HJURP. These factors localize to centromeres in telophase/G1, when new CENP-A chromatin is assembled. The mechanisms that control their targeting are unknown. Here we identify a mechanism for recruiting the Mis18 complex protein M18BP1 to centromeres. We show that depletion of CENP-C prevented M18BP1 targeting to metaphase centromeres and inhibited CENP-A chromatin assembly. We find that M18BP1 directly bound CENP-C through conserved domains in the CENP-C protein. Thus, CENP-C provides a link between existing CENP-A chromatin and the proteins required for new CENP-A nucleosome assembly.

1717

Function of HP1 proteins as a component in kinetochore formation and its relation with chromosome instability.

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HP1 Family of proteins are involved in the formation and maintenance of chromatin higher order structure. In mammals there are known three isotypes (HP1α, HP1β and HP1γ). Recently, it has been proposed that HP1 may play an important roll in inner centromere establishment, generated by its interaction with HMis12 complex, (HMis12C) which is relevant in kinetochore formation and microtubule recognition which ensure correct chromosomal segregation. However, alterations in chromatin structure or loss in H3K9 methylation lead to a reduction of the protein presence and changes of HP1 proteins localization to heterochromatin followed by chromosome instability. It has not been studied if this is mediated by loss of recruitment of HMis12C to the kinetochore and which is it relation with chromosomal instability generation. Thus, the aim of this study is to determine if alteration of HP1 proteins is capable of reducing HMis12C recruitment to the kinetochore. We elaborated transfected of constructions of HP1-GFP for each isotype in HCT116 cells and performed time-lapse to observe localization along

cell cycle by confocal microscopy; in addition, we treated cells with TSA 1uM to analyze changes in HP1 localization. We used ChIP assay in satellite alpha and satellite 2 to determine presence of HP1, and HMis12 in HCT116 transfected cells with HP1-GFP and with Jmjd2b to observe the effect of the loss of H3K9me3 to HMis12C incorporation. We found that each isotype present a different localization at interphase, this localization is highly dynamic in mitosis where HP1α is removed and HP1β is enriched at the chromosomes centromere. Treatment with TSA generates a 70% relocalization of HP1 proteins to pericentromeric chromatin and an increase of chromosome instability. Jmjd2b over-expression reduces HP1 presence at chromatin and also reduces HMis12 in mitosis. These results support another function of HP1 as a kinetochore partner leading incorporation of HMis12 during cell division.
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1718

USP22 Mediates Puripotency, Differentiation and Sox2 Transcriptional Repression.

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USP22 is a ubiquitin hydrolase that we and others have recently shown cleaves mono-ubiquitin moieties from histones H2A and H2B. We have reported that USP22 is a stable component of the hSAGA transcription co-activator complex. (We have also shown that USP22 is required for cell cycle progression in differentiated cells, with USP22 depletion causing G1 arrest). A number of lines of evidence suggest a potential role for USP22 in stem cell function. USP22 was included in an 11-gene polycomb/cancer stem cell signature identified through a translational genomics analysis of metastases and primary tumors from both human patients and a mouse model of prostate cancer. The histone H2B deubiquitnase activity of nonstop, the drosophila ortholog to USP22, is required for early glial development and migration in flies. USP22 increases as mouse embryonic stem cells (mESCs) differentiate, either spontaneously through embryoid body formation, or forcibly through treatment with retinoic acid. We report that USP22 is important for mediating mammalian differentiation programs through transcriptional regulation. USP22 depleted mESCs exhibit a defect in differentiating upon embryoid body formation, as measured by alkaline phosphatase expression. This correlates with retention of a more stem-like morphology in these cells. Furthermore, after embryoid-body induced differentiation, USP22-depleted mESCs show decreased expression of markers for all three germ layers. These cells show no change in their cell-cycle profile or BrdU incorporation. Before induction of differentiation, USP22-depleted mouse and human ESCs display increased expression of the Sox2 transcription factor. Sox2 is a core transcription factor involved in the maintenance of pluripotency and one of four transcription factors needed to induce pluripotency in somatic cells. This coincides with increased RNA Polymerase II recruitment across the Sox2 locus and enhancer region, as well as a loss of histone H2B. Interestingly, ubiquitylated H2B levels increase across the Sox2 locus and enhancer region upon USP22 depletion, which is consistent with a decrease in the ability to remove ubiquitin from H2B when USP22 is depleted. Following Sox2 induction, the cells show increased transcription of Sox2 target genes including Nanog and TDGF1. These data suggest a model in which USP22 is important in maintaining steady-state Sox2 levels in ESCs and upon depletion of USP22, ESCs are defective in differentiation, potentially due to an overabundance of Sox2. These findings propose a role for the SAGA deubiquitylase activity in maintaining pluripotency and executing differentiation programs. The SAGA complex is traditionally though to act as a co-activator complex and we report a novel co-repressor function of the SAGA member USP22.

1719

Epigenetic status of cardiac master genes in native adipose derived stem cells.

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Adipose derived stem cells (ADSCs) are mesenchymal stem cells that can be easly isolated from lipoaspirates. They show multilineage differentiative capacities, including the cardiac phenotype. Therefore they represent a promise for regenerative medicine (Zuk 2001), for the treatment of those diseases affecting organs with extremely low potential of repair and regeneration, such as the heart. The knowledge of the molecular mechanisms that control development and differentiation - such as epigenetic DNA promoter methylation at CpG sites and histone modifications inducing chromatin remodelling and changes in DNA accessibility (Bernstein 2007) - will help to understand their potential in this regard.
Aim of this study was to evaluate the epigenetic status of cardiac specific transcription factors, such as GATA-4, MEF-2C and Nkx2.5 (Brand 2003) in undifferentiated human ADSCs. The analysis of DNA promoter methylation, using Methylation Specific PCR (MSP), revealed the unmethylated status of the promoters of the above cardiac master genes. This finding suggested that ADSCs are not prevented to differentiate towards the cardiac phenotype by DNA methylation, as otherwise reported for myogenic genes (Sørensen 2010).
Chromatin immunoprecipitation (ChIP) showed association of a well known transcriptional repressive mark [trimethylation of Lys27 in H3 (H3K27me3)] with GATA-4 and Nkx2.5 promoters. On the other hand, an enrichment in acetylation of histone 3 (acH3), hallmark of transcriptional potential, was detected for MEF-2C promoter. These results are in agreement with those of the gene expression analysis, showing the presence of only MEF-2C transcript in total mRNA extracted from undifferentiated human ADSCs of three different donors.
In conclusion our results suggest that the epigenetic signature of ADSCs represents a level for intervention to address their differentiation towards the cardiac lineage.

1720

Regulation of Gene Expression on Mouse PAD4 in vivo.

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Peptidylarginine deiminase (PAD) is a Ca^{2+}-dependent enzyme that catalysis the conversion of protein arginine residues to citrulline, and classified five different types of human PADs encoded by the genes PAD1-PAD4 and PAD6 in those characters. Human PAD4 (hPAD4) is localized in the cell nucleus, and has been shown to target multiple arginine sites in histons H3 and H4 including those sites methylated by coactivator-associated arginine methyltransferase and protein arginine methyltransferase1. It is thought that hPAD4 participates in transcriptional regulation of gene expression by antagonizing protein arginine methylation by methltransfelase. The expression of hPAD4 has been reported in human granulocytes/monocytes, synovial membranes, and some tumor cell lines such as MCF-7, but tissue-specific expression in vivo have not been studied in detail.
In this study, we investigated PAD4 gene expression and it's regulation in various tissues of mouse. Mouse PAD4 (mPAD4) is highly expressed in the estrogen responsive tissues, such as vagina, uterus and pituitary. It is speculated that mPAD4 expression may be controlled by estrogen in vivo. To further analysis, we use the ovary ectomized (OvX) mouse in order to exclude the endogenous estrogen. In the OvX mouse, the mPAD4 expression in vagina and

uterus were down regulated both at the mRNA and protein levels. Moreover, we stimulated the OvX mouse by injection of 17-beta-estradiol (E_2), the expression of mPAD4 mRNA and protein were up regulated in those tissues. These results suggested that the expression of mPAD4 is regulated by estrogen in the mouse tissues in vivo.
To investigate whether mPAD4 participates in transcriptional regulation as histone demethyltransferase, we examined the cellar localization of mPAD4 in vagina, at first. As a result of immunohistochemistry, mPAD4 was expressed in the epithelium of vagina. Furthermore, mPAD4 was existed in the cytoplasm of proliferating layer neighboring basement membrane, on the other hand, in the nucleus of differentiated layer. And then, we are examining whether mPAD4 is functional in vagina by using anti histone citrullination antibody.
Now, we attempt to analyze immunologically cellular localization of mPAD4 in the OvX and E_2 injected mouse, in order to study the relationship between proliferation, differentiation, and cellular localization of mPAD4 in vaginal epithelium by the treatment of E_2.

1721

Conservation of the SIR complex in fission yeast.

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In the budding yeast, *Saccharomyces cerevisiae*, heterochromatin contains nucleosomes that are hypoacetylated and are bound by a complex of three proteins, Sir2, Sir3 and Sir4 (Silent information regulator), named the SIR complex. Sir2 is the founding member of a conserved family of NAD-dependent protein deacetylases and creates the hypoacetylated domains of nucleosomes within heterochromatin. Sir3 and Sir4 are histone-binding proteins that appear conserved only in related budding yeasts, not larger eukaryotes. Current models for SIR complex assembly of heterochromatin propose that after initial recruitment to a DNA element, iterative rounds of histone deacetylation and SIR complex recruitment lead to spreading of heterochromatin. Although the role of histone deacetylation by Sir2 has been well established, the precise role of Sir3 and Sir4 in the establishment and spreading of heterochromatin is not fully understood.

Although Sir2 is highly conserved through evolution and is required for heterochromatin assembly in many eukaryotes, Sir3 and Sir4, and the SIR complex, are thought to be present only in budding yeasts. Recent work has suggested that Sir3 arose from a duplication of Orc1, a component of the Origin Recognition Complex (ORC). In budding yeasts that did not duplicate Orc1, Orc1 plays a role in replication and heterochromatin assembly. Orc1, which is highly conserved, therefore may be part of a SIR complex in larger eukaryotes. Work on budding yeast has pioneered the understanding of heterochromatin, and demonstrating that the SIR complex is conserved through evolution will alter current models for how heterochromatin is assembled and regulated in vertebrates.

We have purified Orc1-TAP in the fission yeast, *Schizzosaccharomyces pombe*, and found that in addition to purifying the other five ORC components, it also co-purifies Sir2 and a protein that may be analogous to Sir4. We are currently testing if this complex is distinct from ORC, if it localizes to sites of heterochromatin, and if mutants in the Sir4-like protein have defects in heterochromatin formation. We are also curious how the spSIR complex interacts with the other conserved components of heterochromatin.

1722

C/EBP[beta]-LAP* mediated recruitment of SWI/SNF modulates Ric-8B gene repression during osteoblast differentiation.

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The Ric-8B gene is essential for cell proliferation and is down-regulated during osteoblast differentiation by a mechanism involving C/EBP[beta]-LAP*-mediated repression and SWI/SNF-dependent chromatin remodeling. Interaction of SWI/SNF and C/EBP[beta] has been previously demonstrated as a principal regulatory mechanism in myeloid gene promoters. Here, we address whether C/EBP[beta]-LAP* mediates the recruitment of SWI/SNF during Ric-8B gene repression in osteoblastic cells. C/EBP[beta] silencing in osteoblastic cells was achieved by specific siRNAs or lentiviral-mediated expression of specific shRNAs. Binding of SWI/SNF and C/EBP[beta] to the Ric-8B gene promoter was determined by chromatin immunoprecipitation (ChIP). Changes in gene transcription were evaluated by qPCR. The analyses were also carried out in stable osteoblastic cell lines expressing dominant-negative forms of SWI/SNF subunits. To evaluate Ric-8B gene promoter activity, undifferentiated pre-osteoblastic cells were co-transfected with constructs carrying the Ric-8B promoter fused to a Luciferase reporter gene together with vectors coding for mutated C/EBP[beta] proteins that impair interaction with SWI/SNF. Interaction between C/EBP[beta] and SWI/SNF was evaluated by co-immunoprecipitation. We find that silencing of C/EBP[beta] impairs SWI/SNF binding to the Ric-8B promoter and increases Ric-8B expression levels. This increased Ric-8B transcription is also observed in cell lines expressing dominant negative forms of SWI/SNF. Over-expression of a C/EBP[beta]-LAP* isoform carrying a mutation that impairs interaction with SWI/SNF is unable to repress the Ric-8B promoter activity. Co-immunoprecipitation studies show that C/EBP[beta] and SWI/SNF interact in nuclear extracts from osteoblastic cells. Together, our results support a model where C/EBP[beta]-LAP* recruits SWI/SNF to the Ric-8B promoter leading to Ric-8B gene repression during osteoblast differentiation.
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1723

The Complete Chemical Synthesis of Histones H3 and H4 Containing Epigenetic Modifications and Their Use in Characterizing Arginine Methylated Histone Antibodies.

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Arginine methylation occurs on numerous cytoplasmic and nuclear proteins including histones (H2A, H2AX, H2B, H3 and H4). In the case of histone modification, arginine methylation is known to play role in chromatin's accessibility to the enzymes that control transcription. Arginine methylation is catalyzed by a family of 9 enzymes called protein arginine methyl transferases (PRMTs). All PRMTs catalyze the addition of monomethyl arginine (MMA) onto a nitrogen within the guanidinium side-chain of arginine. Class I PRMTs further catalyze the formation of asymmetric dimethyl arginines (ADMA) where both methyl groups are on the same nitrogen whereas Class II PRMTs catalyze the formation of symmetric dimethyl arginines (SDMA) where each methyl group is on a different nitrogen. Critical to unraveling the role of arginine methylation and other epigenetic modifications (e.g., lysine methylation, acetylation, serine or threonine phosphorylation) are the availability of well characterized antibodies and peptides that

can be used in a variety of applications (western blotting, ICC/IHC, ChIP). One of the issues with antibodies that detect the specific methylation of arginine (MMA, ADMA or SDMA) is the difficulty in their characterization using short synthetic peptides and dot blots, competition ELISAs or similar methods. To better characterize various epigenetic antibodies, we have successfully synthesized numerous epigenetically modified forms of histones H3 and H4 in mg quantities. Peptide synthesis was affected using a combination of standard Fmoc amino acid building blocks, pseudoproline dipeptides to minimize inter-chain aggregation, and differing solvent mixtures to affect difficult couplings, followed by a variety of HPLC methods to isolate the full length peptides. The epigenetically modified histone proteins were characterized by HPLC, nanospray MS, and the sequence confirmed by CID MS/MS. The full length proteins were then used to characterize antibodies manufactured to specific MMA, ADMA or SDMA sites on histones (e.g., H3R8, H4R3 and H4R17) by western immunoblotting and dot blots. Antibodies that had appeared to cross-react to varying degrees with non-target modifications using short peptides were in fact shown to be specific using full length, epigenetically modified H3 and H4 protein. These proteins can also be used for the assembly of artificial nucleosomes and biotinylated forms of these proteins can be used for pull downs and other biochemical experiments. Thus synthetic histones containing defined epigenetic modifications are valuable tools for characterizing the specificity of epigenetic antibodies.

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The SCF^{Slimb} ubiquitin-ligase regulates Cap-H2 levels to control interphase chromosome condensation and spatial organization.

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Chromosome condensation is essential during mitosis, however little is known about how or if interphase chromosome compaction is also modulated. Two conserved complexes (condensins I and II) facilitate chromosome condensation in mitosis. Recent work has revealed that condensin II also functions as an anti-pairing factor, preventing polytene chromosome assembly in non-dividing *Drosophila* nurse cells and inhibits homolog pairing essential for transvection. These interphase functions suggest that condensin is regulated throughout the cell cycle and, at present, it is not clear how interphase condensin is different from its mitotic activities. Since control of protein turnover is a prominent mechanism cells use to regulate cell cycle-dependent events, we examined whether the ubiquitin machinery could play a role in chromosome condensation. By examining a previous RNAi screen of the Cullin-based E3 ubiquitin-ligase family, we found that depletion of the SCF components (Cullin-1 or SkpA) or the F-box protein, Slimb, resulted in a chromosome hyper-condensation phenotype in cultured *Drosophila* S2 cells. Specifically, SCF^{Slimb} RNAi causes a dramatic change in the morphology of chromatin in non-mitotic cells; compacting chromosomes into a cluster of 8-12 spheres per nuclei that we call the ‘gumball’ phenotype and are likely to be individualized chromosome territories. Strikingly, we find that double RNAi of Slimb and either SMC-2, Cap-D3, or Cap-H2 completely rescues the gumball phenotype, suggesting that condensin II activity is negatively regulated by SCF^{Slimb}. Indeed, we also find that the non-SMC subunit, Cap-H2, is a novel Slimb ubiquitin-target, as Cap-H2 possesses a conserved Slimb-binding domain, co-immunoprecipitates with Slimb, and is stabilized upon Slimb depletion. Furthermore, mutation of the Slimb-binding domain stabilizes Cap-H2 and expression of this mutant in S2 cells is sufficient to induce the hyper-condensation gumball phenotype. Here, we also present *in vivo* evidence supporting a Slimb/Cap-H2 genetic interaction in modulating polytene chromosome assembly and pairing. Our results reveal a previously unknown mechanism for regulating condensin that utilizes ubiquitin-mediated

proteolysis of a key regulatory subunit to attenuate chromosome compaction and spatial organization during interphase of the cell cycle. We propose a model where SCF^{Slimb} controls Cap-H2 proteins levels thereby linking mitotic exit and chromosome decondensation to interphase specific compaction and chromosome organization.

Cytokinesis II

1725

Microtubule and actin cytoskeleton rearrangements within intracellular bridge mediates terminal cytokinesis.

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Abscission, the final event during cytokinesis consists of orchestrated events of membrane and cytoskeleton reorganization, yet the exact mechanisms and timing of these events are unknown. Previously, we have shown that recycling endosomes are required for cytokinesis and their trafficking to, and fusion with, the intracellular bridge connecting the two daughter cells is necessary prior to abscission. We have also demonstrated that before endosome fusion, microtubules undergo a dramatic reorganization allowing for localized microtubule clearance from the intracellular bridge. Other labs have shown that the ESCRT complexes are also required for cytokinesis and abscission, but the construction of ESCRT filaments and the actual abscission step, is still a mystery. While recycling endosome and the ESCRT based abscission models are not mutually exclusive, their cohesiveness has not been investigated and remains unknown. Here we investigate the timing of recycling endosome secondary ingression and CHMP-4B, an ESCRT-III member responsible for forming ESCRT filaments, localization in respect to abscission. Our data show that the endosome-induced secondary ingression precedes and is likely required for ESCRT-III-mediated abscission. What remains unclear, is how endosomes induce secondary ingression and ESCRT-III recruitment during abscission. Proteomic analysis of recycling endosome cargo delivered to the intracellular bridge identified several actin cytoskeleton regulators that have not been studied in the context of cell division. We also show that endosome fusion with the furrow plasma membrane is required for the localized disassembly of cortical actin cytoskeleton, resulting in the formation of the secondary ingression, and the recruitment of the ESCRT-III protein complex during the terminal step of cytokinesis.

1726

Cyclin B1* expression and endopolyploidization during flower development in *Phalaenopsis aphrodite* subsp. *Formosana.

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Endopolyploidy has been found in various organs and tissues during plant development and flower development in *Phalaenopsis* orchids. Endopolyploidy is caused by endoreduplication / endomitosis, in which cytokinesis is not completed after one or several rounds of DNA replication and results in more amount of DNA content. Flower is the major attraction in

Phalaenopsis industry. Not only flower color and shape, flower size is also an important issue. In sepal and petal of different floral development in *Phalaenopsis aphrodite* subsp. *formosana*, the proportion of 2C nuclei decreased, the polyploidy proportion of 4C and 8C increased. There were 4C nuclei after one round of endoreduplication and 8C nuclei after twice endoreduplication existed simultaneously. The 8C nuclei had been detected at 1.1 cm sepal and 1.2 cm petal or larger flower bud. In pedicel, polyploidy levels and percentage were higher and more than sepal and petal, 8C nuclei had been showed in pedicel from 0.6 cm flower bud. However, ploidy degree in ovary remains monoploid because of germline stability. These results indicate that different endopolyploidy regulated-mechanisms might be existed in different floral tissues. *Cyclin B1* gene expression was detectable in sepal, petal and pedicel. In sepal and petal, *Cyclin B1* displayed abundantly among 0.5-1.1 cm and 1.4-1.8 cm of floral bud. The peak of *Cyclin B1* gene expression is highly correlated with endopolyploidization during floral development because of similar timing and pattern. To identify the regulatory role of this gene, three constructs were created. The first one contains the full length of *Cyclin B1* (Wild Type, abbreviated as WT). The second one includes two point mutations located on destruction box (Mutation, abbreviated as Mut). The third one excludes 5'-sequence deletion of destruction box (ΔDbox). All constructs were transient expression in the flower buds of diploid *P. aphrodite* by *Agrobacterium*-mediated transformation, respectively. Construct containing empty vector was also transferred as control (MOCK). GUS staining revealed that MOCK, WT, Mut, and ΔDbox gene did transient express. *Cyclin B1* gene expressions were amplified via RT-PCR. All of WT, Mut, and ΔDbox showed abundant exogenous *Cyclin B1* gene expression. All transient over-expression of WT, Mut, and ΔDbox appeared higher and faster endopolyploidy percentage and endoreduplication cell cycle (endocycle) index than MOCK. These results suggested that *Cyclin B1* may involve in endoreduplication and manipulate endopolyploidy in *P. aphrodite* during flowering.

Keywords: cyclin B1, endocycle, endomitosis, endopolyploidy, endopolyploidization, endoreduplication, orchid, Phalaenopsis

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Phosphorylation of NudC by Aurora B regulates cytokinesis.

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Nuclear distribution protein C (NudC) is a highly conserved protein that plays a role in mitosis and cytokinesis. We recently discovered that NudC is phosphorylated by the mitotic kinase Aurora B. Whether NudC phosphorylation by Aurora B plays a role in cytokinesis is not known. We found that NudC and Aurora B associate in the late stages of mitosis, from anaphase to cytokinesis. In vivo labeling showed that NudC is phosphorylated by Aurora B, as evidenced by reduced phosphorylation in the presence of the Aurora B inhibitor ZM447439. Using a series of GST-NudC truncation proteins in IP kinase assays, we mapped a site within the N-terminus of NudC at residue T40 as an Aurora B phosphorylation site. An anti-phospho-T40 (pT40) NudC peptide antibody was generated and used to show that pT40 NudC localizes to the midbody where Aurora B is localized during cytokinesis.

To determine the potential function of pT40 NudC in cytokinesis, we knocked down NudC in HeLa cells and rescued with wild-type NudC or NudC containing a T40A (phospho-defective) or

T40D (phospho-mimetic) mutation. NudC knockdown increased the percentage of cells exhibiting elongated intercellular bridges up to 30 µm in length, demonstrating a lack of cell abscission and failure in cytokinesis. Furthermore, Aurora B was found to be mislocalized along the elongated bridges instead of being concentrated at the midbody. Both wild type and T40A NudC rescued the cytokinesis phenotype and Aurora B localization. In contrast, T40D NudC was unable to rescue the cytokinesis defect and Aurora B remained diffused along the elongated bridges, indicating that a function of pT40 NudC is to prevent the completion of cytokinesis. Our studies suggest that phosphorylation/dephosphorylation of NudC plays a role in cytokinesis regulation, and that dephosphorylation of pT40 NudC is required for timely cell abscission.

1728

F-actin assembly at the cell division ring in fission yeast.

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Cytokinesis in many eukaryotes involves the function of an actomyosin based contractile ring. One of the key steps in the assembly of the actomyosin ring is the polymerization / accumulation of actin filaments at the division site. In recent years, the fission yeast Schizosaccharomyces pombe has emerged as an attractive model for the study of cytokinesis, since it divides using an actomyosin ring. Here we investigate the mechanism of actin filament assembly at the division site in fission yeast using the recently developed lifeact-peptide, as a probe for studying actin dynamics. Although previous work had shown that actin filaments for the actomyosin ring assemble exclusively at the division site, we find that cable-like actin filaments are also assembled away from the division site, in a manner dependent on the formin-related protein Cdc12p. Non-medially assembled actin filaments accumulate at the division site and contribute to the assembly of actomyosin ring in a myosin II and myosin V-dependent manner. Our studies taken together with some studies in animal cells establish that overlapping mechanisms, of de novo actin filament assembly at the division site and of transport of preexisting actin filaments to the division site, may facilitate actomyosin ring assembly in many organisms.

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The condensation of actomyosin network into a contractile ring depends on actin cross-linkers alpha actinin and fimbrin during fission yeast cytokinesis.

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Quantitative studies of the assembly of the contractile ring in the fission yeast S. pombe provides insights of how actin, myosin motors, and cross-linkers self-organize into contractile structures in cells. The fission yeast contractile-ring assembles through the condensation of a broad band of membrane-bound nodes containing myosin-II and formin. In the Search, Capture, Pull, and Release (SCPR) model, formins nucleate actin filaments from nodes and establish transient actomyosin connections among them. Myosin pulls and condenses the band of nodes into a ring. However, the role of actin cross-linkers in ring assembly is unclear. To dissect their

roles we mutated them and modified the concentrations of α-actinin Ain1 and fimbrin Fim1 in vivo. We also extended the SCPR model to account for actin filament flexibility, representing filaments as beads connected with springs and cross-linking as connections among actin filaments. Both Ain1 and Fim1 localize to the division site during node condensation. Ain1 localizes between nodes. Nodes collapse into clumps instead of a ring in *ain1Δ* cells. Surprisingly, in *fim1Δ* cells, condensation is not affected. However, reducing the level of both Ain1 and Fim1 severely affects node condensation, suggesting that they cooperate for ring formation. By titrating Ain1 and Fim1, we find that node condensation rates depend on their concentration, with meshworks forming instead of rings at high concentrations. The Kd of Ain1 with the ring is in the µM range. Using the in vivo parameter values, the model reproduces many of the observed dependencies on cross-linker concentrations, including morphological features such as branched meshworks and clumps. These results indicate that actin cross-linkers are crucial for node condensation during cytokinesis.

1730

Spatial and temporal control of lipids and F-actin in cytokinesis completion.

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Cytokinesis leads to the physical separation of the daughter cells, and is characterized in animal cells by a complex series of cell shape changes and membrane remodelling. Following furrow ingression, daughter cells are connected for most of cytokinesis by an intercellular bridge, which is cut in a final step termed abscission. Cytoskeleton and lipid composition are profoundly remodelled during late cytokinesis steps, but how this occurs and whether it is important for successful cytokinesis remains to be established.
Growing evidence indicate that membrane traffic has a key contribution in the post-furrowing steps of cytokinesis. In order to identify more precisely the transport routes involved and how they function in cytokinesis, we recently conducted a systematic RNAi screen focused on Rab genes, which encode key conserved GTPases that regulate membrane trafficking in eukaryotic cells. We found that Rab35 regulates an endocytic pathway and is involved in the initial stability of the intercellular bridge, after furrow contraction, by controlling the proper localization of SEPTIN2 in human cells. In addition, we noticed that Rab35 depletion led to additional cytokinesis defects characterized by either delayed or inhibited abscission.
We will present the molecular mechanism by which the Rab35 GTPase regulates lipid and cytoskeleton remodelling in terminal cytokinesis steps. We found that Rab35 controls the localization of a phosphatase that regulates the turnover of PI(4,5)P2 in late intercellular bridges. Our results indicate that PI(4,5)P2 hydrolysis is a key requirement for local removal of cortical F-actin and therefore normal cytokinesis abscission. We will also discuss how these findings shed new light on the pleiotropic phenotypes associated with a disease in which this phosphatase is mutated.

1731

The roles of C. elegans MRCK-1 & Rho-Kinase in myosin organization and contractile ring dynamics.

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During cytokinesis, the duplicated and segregated genome is physically compartmentalized into two daughter cells. The contractile ring, a highly dynamic structure, is made of actin, myosin, other actin binding proteins and additional accessory factors. Actomyosin contractility provides

the force to deform the cell equator into a cytokinetic furrow. The myosin holoenzyme of two heavy chains and four light chains can adopt three conformations: closed, open and oligomeric bipolar mini-filaments. Mini-filament assembly has major implications for myosin's processivity, and crosslinking and bundling activities. Conversion among these conformations is thought to be regulated by two phosphorylation events on the regulatory light chain protein, by conserved kinases. We are working to understand how myosin is activated, organized and regulated during cytokinesis. We **hypothesize** that the conserved kinases Rho-kinase and MRCK-1 contribute distinctly to myosin regulation in cytogenesis. We image *C. elegans* zygotes expressing GFP-tagged myosin heavy chain (NMY-2) by real-time confocal microscopy and perform quantitative image analysis with custom bio-informatics tools. We first confirmed published observations that Rho-kinase depletion leads to decreased myosin recruitment, a delay in furrow initiation, slower closure and a significant increase in ring asymmetry. Also, we measured defects in contractile ring cortical organization into patches. *C. elegans* MRCK-1 is thought to indirectly regulate myosin activation, but its mammalian homologue can directly phosphorylate myosin. We show that MRCK-1 depletion does not affect furrow initiation or closure kinetics, nor significantly affects ring closure concentricity. Paradoxically, MRCK-1 depletion increases cortical myosin recruitment, but simultaneous depletion with Rho-kinase decreases equatorial myosin compared to Rho-kinase depletion alone. Furthermore, the double kinase depletion leads to a unique ring asymmetry phenotype with very asymmetric initiation, following a recentering of the ring. Together, our results support the idea that these two kinases have different contributions to myosin organization, thus are non redundant.

1732

Roles of Gef2 in division-site positioning and contractile-ring function in fission yeast cytokinesis.

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Cytokinesis is crucial for integrating genome inheritance and cell functions. In higher eukaryotes, Rho-GEFs and Rho GTPases are key regulators for division-plane specification and contractile-ring formation during cytokinesis. However, how they regulate early steps of cytokinesis remains unknown in fission yeast. Here we show that *S. pombe* Rho-GEF like protein Gef2 and Polo kinase Plo1 coordinate to control the medial cortical localization and function of anillin-related protein Mid1, which specifies the medial division plane by recruiting contractile-ring components. The division-site positioning defects of *gef2 plo1* double mutant can be partially rescued by increased dosages of Mid1. We find that Gef2 modulates Mid1 cortical binding and is required for the cortical localization of Mid1 NH2 terminus by physical interactions. Gef2 cortical localization depends on its last 145 residues. In addition, Gef2 also functions in late cytokinesis involved in contractile-ring stability/anchoring. We propose that the mechanism regulating cytokinesis by Rho-GEF related proteins is conserved in fission yeast: Plo1 dependent phosphorylation triggers Mid1 nuclear exit, and Gef2 facilitates the binding of Mid1 to the medial cortex, subsequently Mid1 induces division-plane specification and contractile-ring assembly.

1733

Supervillin-mediated Myosin II Activation Is Required for Normal Cytokinesis.

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Supervillin (SV), a large member of the villin/gelsolin/flightless family, coordinates motor proteins at membranes during motile processes. Supervillin binds tightly to lipid raft membranes

and is a regulatory hub for cytoskeletal, membrane, and signaling proteins. Many supervillin interactors are involved in cell cycle control, and supervillin knockdown inhibits early cytokinesis. In the absence of supervillin, myosin II heavy chain and phosphorylated myosin light chain are recruited normally to the furrow, but activated myosin II subsequently becomes mis-localized to non-equatorial sites. GFP-tagged full-length supervillin partially rescues the RNAi-mediated defect in cytokinesis, and overexpressed full-length supervillin improves the fidelity of cell division in HeLa cells. By contrast, expression of supervillin lacking the N-terminal 170 amino acids (SV171-1792) increases the numbers of bi- and multi-nucleated cells. We have shown previously that the N-terminal 174 amino acids in supervillin (SV1-174) enhance myosin II contractility, and we have proposed that they do so by cross-bridging the S2 regulatory region in non-muscle myosin II heavy chain with the long (L) isoform of myosin light chain kinase (L-MLCK). Expression of GFP-SV1-174 has no effect on the inherent cell division defect in HeLa cells, even though it is present at the furrow. However, GFP-SV1-174 promotes hyper-activation of myosin II during interphase, leading to the disruption of stress fibers into myosin II "punctae" containing supervillin and L-MLCK. We have used smaller deletions and point mutagenesis to identify sequences within SV1-174 that participate in myosin II activation. Supervillin residues 1-11 and 127-174 are necessary for the formation of myosin II punctae. Pulldowns with GST-tagged constructs indicate that SV1-11 enhances and that SV127-174 is absolutely required for binding to the S2 regulatory region of myosin IIA. In addition, we find that SV23-101 is required for binding to the GST-tagged L-MLCK N-terminus. These results suggest a U-shaped model for the supervillin N-terminus, in which L-MLCK binds to sequences interior to the myosin II-binding sites at each end of SV1-174. In ongoing experiments, we are selectively disrupting individual binding sites in full-length supervillin to examine the molecular basis for the important role of the supervillin N-terminus for myosin II anchorage to the cytokinetic cell furrow. Supported in part by NIH grant GM33048.

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Roles of the Drosophila RZZ complex in membrane traffic and cytokinesis.

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The conserved Rod-Zwilch-Zw10 (RZZ) complex is a well-known kinetochore component required for proper activity of the spindle assembly checkpoint in both Drosophila and mammals. In yeast and mammals, Zw10 participates in a second complex involved in membrane traffic. However the role of the Drosophila RZZ complex in membrane trafficking has never been addressed. We found that Zw10 is enriched at both the Golgi and the ER of Drosophila spermatocytes; Rod concentrates at the Golgi but not at the ER, while Zwilch does not accumulate in any membrane compartment. Mutations in zw10 and rod severely affect Golgi structure, while zwilch is not required for Golgi maintenance. In addition, loss of Zw10 results in frequent failures of spermatocyte cytokinesis, whereas Rod and Zwilch are not required for this process. zw10 mutant spermatocytes assemble regular central spindles and acto-myosin rings, but furrow ingression halts prematurely due to defective plasma membrane addition. Collectively, our results suggest roles for Zw10 in the ER-Golgi traffic and membrane addition during cytokinesis, while Rod appears to mediate proper Golgi function but is dispensable for cytokinesis. Given their different functions and subcellular localizations, Zw10 and Rod might be part of different subcomplexes involved in membrane traffic during Drosophila spermatogenesis.

1735

Activated H-Ras in recycling endosomes regulates endosomal trafficking to the cleavage furrow and is required for efficient cytokinesis.

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We describe here a new set of functions for the Ras small G protein: regulation of endosomal traffic into the cleavage furrow in dividing cells, and a requirement for this process in efficient cytokinesis. Ras small G proteins are molecular switches which drive signaling pathways stimulating transcription and cell proliferation, apoptosis, differentiation and migration. Transcriptional activation leading to cell proliferation is the best studied Ras function, and this cellular function has been considered the principal explanation for the very high incidence of somatic activating mutations in hyperproliferative diseases, most notably cancers. However, Ras genes are clearly pleiotropic, as indicated in part by distinct signaling patterns and cellular and physiological functions of Ras isotypes which share common effector pathways. Much current research is focused on the role of spatial segregation of Ras signaling in specifying these distinct functions. Owing to the requirement of membrane anchorage of Ras for its functions, Ras signaling was originally believed to be restricted to the plasma membrane. However, several Ras isotypes also attach to the membranes of internal organelles such as the Golgi and ER. Recently H-Ras and N-Ras, but not K-Ras, were localized in cells to recycling endosomes (REs), dynamic endosomal compartments engaged in receptor recycling and secretory trafficking pathways. A functional role for Ras at these sites remains to be elucidated. Also recently, REs were shown to traffic centripetally into the cleavage furrow which develops between daughter cells during telophase, and this trafficking is essential for cytokinesis, the physical separation of the daughter cells. Using real-time live cell confocal fluorescence microscopy, we have found that activated H-Ras (expressed as a red fluorescent protein fusion), highly concentrated on recycling endosome membranes, engages in centripetal trafficking patterns as endosomal cargo into the cleavage furrow in cells, beginning in late anaphase and continuing through telophase. Activated H-Ras was also concentrated in the midbody as the daughter cells pulled apart, whereas dominant negative H-Ras was excluded from the midbody. Furthermore, dominant negative H-Ras, although localized to REs, did not traffic efficiently into the cleavage furrow. We found two important Ras effectors, RalGDS and the p110α subunit of PI3 Kinase, but neither c-Raf nor B-Raf, co-localized with activated H-Ras on recycling endosomes in the cleavage furrow, suggesting that activated H-Ras engages in signaling at these sites. Interestingly, dominant negative H-Ras severely delayed abscission, the final separation step in cytokinesis, in synchronized cells compared to control. Conversely, constitutively active H-Ras slightly accelerated the time to abscission. Thus, blockade of H-Ras activity and signaling stalls recycling endosome traffic to the cleavage furrow with a subsequent inhibition of cytokinesis. These data represent two related functions of Ras previously undescribed: regulation of endosome traffic and its downstream requirement for efficient cytokinesis. Thus, H-Ras regulates cell proliferation at multiple stages – transcriptional activation and regulation of cell division. Whereas the former function is driven by signaling relays, the latter is due to H-Ras effects on the trafficking of sub-cellular organelles and their contributions to cell morphology. These results therefore widen the picture of the pleiotropic cellular functions of Ras genes to include multiple processes throughout the cell cycle.

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Super-resolution Analysis of Cytokinetic Ring Formation.

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The formation of the cytokinetic ring has been visualized by many imaging modalities. We previously used TIRF microscopy to visualize the accumulation of Myosin II, Kinesin-6, Diaphanous, and Aurora B with high temporal and spatial resolution. We observed that there is a substantial pool of Kinesin-6 that accumulates on the cortex at the site of cytokinetic ring formation and is not associated with microtubule tips. We hypothesized that this motor protein may be associated with the nascent ring through an interaction between centralspindlin and the scaffold protein anillin in order to concentrate this Rho activator and stimulate faster and more robust cytokinetic ring assembly. In order to test this model, we've been performing more detailed spatial analysis of the distribution of Kinesin-6, microtubules, and the proteins of the cytokinetic ring with super-resolution imaging combined with RNAi of various components of the cytokinetic ring. Additionally, we are trying to dissect the signaling pathways and interactions that allow for the recruitment of centralspindlin to the cytokinetic ring, independent of microtubule binding, by generating some novel mutants that may uncouple these interactions and then observing the effects that these mutants have on the spatial and temporal dynamics of Kinesin-6 and myosin recruitment. We hope that these results will better illuminate the feedback loops and pathways that drive cytokinetic ring formation and allow for assembly to occur even in areas of low microtubule density.

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Role of the Contractile Ring in Cytokinesis.

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In animals and fungi cytokinesis involves constriction of an actomyosin contractile ring, but the constriction mechanisms and the role of the ring are not established. The constriction rate could be determined only by the properties and internal dynamics of the ring itself, as recently suggested by experiments on *C. elegans* embryos [Carvalho et al, Cell (2009)]. Alternatively, the constriction rate could be set by other processes coupled to constriction. In fission yeast constriction is tightly coupled to septation, the growth of new cell wall in the wake of the constricting ring. To isolate ring mechanisms, we combined mathematical modeling of the ring with experiments on fission yeast protoplasts which lack cell wall and adopt a rounded shape. Protoplasts contained ring precursor nodes as in normal cells and assembled functional contractile rings that constrict without septation by sliding along the plasma membrane without cleaving the cell. By manipulating the shape of the protoplasts, we showed that the ring's shape evolution and constriction rate depend on the shape of the protoplast surface, in remarkably close quantitative agreement with model predictions. Therefore, ring constriction dynamics depend on external factors: in protoplasts, the ring tension couples to ring anchors whose sliding response determines the constriction rate. This suggests that in normal yeast cells the function of the ring is to exert tension upon the septum and the septation rate then determines the closure rate. We used the model to examine how ring tension depends on the dynamics of actin and the relatively disordered non-sarcomeric arrangement of ring components suggested by prior experiments. Simulations predicted that the ring generates ~40-80 pN of tension, and comparison with our observations of protoplast ring constriction revealed that the net drag of ring-membrane anchors during constriction is ~20-fold greater than the combined drag of the precursor nodes. Anchoring of actin filament barbed ends maximizes tension, and actin turnover

plays a vital role by remodeling the ring throughout constriction while maintaining organizational correlations that set the tension. In agreement with experiment, the model showed that suppression of actin polymerization slows ring constriction because actin filaments are then shorter and couple to fewer myosins, with consequently reduced tension. Thus, the model articulates a mechanistic relationship between organization, turnover kinetics, ring tension and closure rate.

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Interdependence of ring constriction and septation in fission yeast cytokinesis.

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Cytokinesis is the final stage of the cell cycle where the cell physically divides into two daughters. In animals and fungi, cytokinesis involves constriction of an actomyosin contractile ring coupled to other simultaneous processes. While the ring is thought to generate tension, its role has not been established, and whether the ring or coupled processes drives constriction is unknown. In fission yeast, constriction is tightly coupled to septation: the growth of new cell wall in the wake of the constricting ring. How these processes coordinate to complete cytokinesis is not established: septation could be driven by ring tension, or ring closure could instead be driven by septation. Here we developed a model of cytokinesis in fission yeast that describes the coupling of the tensile contractile ring to the growth of primary septum by Bgs1p and other proteins that synthesize septum material. The model hypothesizes that ring tension influences the local rate of septum growth through the effect of radial ring-generated forces on Bgs1p or other motors. We used our model to calculate the rate of inward growth of the septum and the evolution of the shape of the inner septum boundary during ring closure. Our model results are consistent with experimental observations in wild type and mutant yeast cells, and suggest that while the ring-septum closure rate is independently set by the septation process, the ring tension serves a vital role by regulating septum growth, and thereby suppressing roughness of the septum boundary, and maintaining its circular shape. From the model, we calculated the minimum ring tension required for a given degree of smoothness in the growing septum, consistent with calculations of wild type fission yeast ring tension. Thus, our model suggests that the primary role of the contractile ring in fission yeast cytokinesis is as a tension-producing machine that regulates septum circularity to ensure ordered closure and separation of the daughter cells.

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Forces in fission yeast cytokinesis.

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A paradigm of cytokinesis in animal cells is that the actomyosin contractile ring provides the primary contractile force to divide the cell. In the fission yeast Schizosaccharomyces pombe, cytokinesis also involves a conserved actin-myosin ring, which has been generally assumed to provide the force for cleavage. However, in contrast to animal cells, yeast cells assemble a cell wall septum concomitant with ring contraction and possess large (MPa) internal turgor pressure. Here, we show that the inward force generated by the division apparatus must oppose turgor pressure. Reducing effective turgor pressure leads to an increase in cleavage rate. We estimate that the contractile ring can only provide a tiny fraction (less than 1/1000) of the mechanical stress required to overcome turgor. Indeed, we find that once septation has started, cleavage can continue in the absence of the contractile ring. Instead, we propose that cell wall synthesis provides the primary force for cleavage furrow ingression. We develop a quantitative model in

which the assembly of cell wall fibrils in the growing septum pushes in the plasma membrane for ingression using a rachet-based mechanism. We show that this mechanism may be sufficient to provide the large MPa pressures to work against turgor pressure. This work begins to provide some initial estimates of force production in division of a walled cell.

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In vivo measurements of cell shape reveal the dynamics of early cytokinesis.

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Cytokinesis is the final step of cell division. Failure of cytokinesis can lead either to cell death, or to cancerous transformation. Cytokinesis is achieved by the contractile ring, located at the cell equator, which constricts to split the cell into two daughter cells, dramatically deforming the cell membrane in the process. Most work has been done on the signalling that elicits contractile ring assembly. Investigating membrane curvature and the early mechanics that drive cytokinesis remain experimental challenges because of the difficulty of extracting 3D shape information from microscopy data.

We investigate the early events of cytokinesis by determining the 3D cell shape of living cells by combining high resolution microscopy with automated image analysis. We use the *C. elegans* zygote expressing fluorescently labelled membrane-associated proteins, a powerful system due to the highly stereotypical nature of cell division events in this cell. To visualize and analyze cell shape dynamics, we map deformation, protein distribution, and membrane curvature into 2D via Mercator projection, the method used to visualize the surface of the Earth, for example, in 2D.

We find that furrow ingression starts locally and propagates around the circumference of the division plane at ~65 μm/min. Initially, when the furrow has gentle curvature, it radially ingresses at ~2.4μm/min, which is consistent with measurements made using traditional imaging approaches. During furrow initiation, we observe local changes in the concentration of a PH domain marker for PIP2: it is first depleted from the future furrow site and then from regions flanking the furrow. Finally, we note that the cytokinetic ring is surprisingly non-planar, suggesting that cortical mechanics outside the division plane play a more important role in shaping cytokinesis than previously appreciated.

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Molecular basis for the interactions of the F-BAR protein Hof1 with septin filaments and myosin-II during yeast cell cycle.

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F-BAR domain belongs to a subfamily of the BAR domains that are known to dimerize, bind phospholipids, and cause membrane curvatures. Hof1, a cytokinesis protein in the budding yeast *Saccharomyces cerevisiae*, contains a F-BAR domain at its N-terminus and a SH3 domain at its C-terminus. Hof1 localizes to the mother-bud neck in a septin-dependent manner from S phase to the onset of cytokinesis and contracts with the actomyosin ring (AMR) during cytokinesis. The mechanism underlying the dynamic localization of Hof1 during the cell cycle remains largely unknown. We found that the F-BAR domain of Hof1 co-localizes with the septins throughout the cell cycle and interacts strongly with septin filaments *in vitro*. The F-BAR also interacts with a number of septin subunits *in vivo*, particularly strongly with Cdc10, by bimolecular fluorescence complementation. This result is further supported by the striking observation that GFP-tagged F-BAR fails to associate with the septins in *cdc10* but not in *shs1* deletion cells. Unlike other F-BAR domains, the F-BAR of Hof1 does not bind any phospholipid

with specificity and high affinity. These data suggest that the F-BAR of Hof1 binds to septin filaments in a Cdc10-dependent manner.

The C-terminal half of Hof1 (C-Term) localizes to the mother-bud neck right before septin hourglass splitting and contracts with the AMR during cytokinesis. The C-Term interacts with the tail of Myo1, the sole myosin-II in budding yeast. In the absence of Myo1, the level of C-Term phosphorylation is reduced and the C-Term co-localizes with split septin rings during cytokinesis. These data suggest that Myo1 may provide a scaffold for protein kinases to phosphorylate Hof1 during cytokinesis. Together, our study provides biochemical evidence for the discrete localizations of Hof1 during the cell cycle, which are presumably associated with its role in cytokinesis and beyond.

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Reactive oxygen species increase binucleated cells through perturbation of cytokinesis progression via unresolved DNA bridges in HeLa cells.

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Aneuploidy, an abnormal number of chromosomes which is one of the major characteristics of cancer cells, can arise from tetraploid cells resulting from a failure of the cytokinesis. Reactive oxygen species (ROS) have been implicated in processes as diverse as cancer, inflammation, cardiovascular disease and ageing. However, the role of ROS in cytokinesis progression has not been studied well. In time-lapse analysis, 100 μM H2O2 treatment during mitosis induced early- and late-cytokinesis failure in HeLa cells. In addition, the number of binucleated cells was significantly increased by H2O2 treatment. Treatment with 10 mM N-acetyl-L-cystein (NAC), an anti-oxidant reagent, abrogated cytokinesis failure and binucleated cell formation induced by H2O2, suggesting that ROS is involved in cytokinesis perturbation. Next we examined whether major molecules working in cytokinesis progression such as Anillin and Rho A were affected by treatment with ROS. However, our observation revealed that these proteins did not participate in H2O2 induced cytokinesis failure. One of the possible ways inducing cytokinesis failure is increase of DNA bridges. Immunocytochemistry and confocal microscopy data revealed a significant increase in DNA bridges as well as γ-H2AX expression (80.9%), a maker for DDSBs. Moreover the binucleated cells resulted from the cytokinesis failure by ROS also contained DNA bridges (probably nucleoplasmic bridges; NPBs) between two nuclei. Interestingly, while the DNA bridges induced by etoposide were rapidly resolved during telophase, those induced by H2O2 were mostly maintained during the same period, indicating the perturbation in DNA bridge resolution by H2O2 treatment. Taken together, we propose that ROS increases polyploid cells resulting from cytokinesis failure *via* DNA bridge formation that are not effectively resolved.

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Chlamydia trachomatis Inclusions Induce Asymmetric Cleavage Furrow Formation and Ingression Failure in Host Cells.

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Chlamydia trachomatis, an obligate intracellular bacterium, is the most common cause of bacterial sexually transmitted diseases worldwide. Epidemiological studies have linked *C. trachomatis* infections to increased risks of cervical cancer. Here we show that *C. trachomatis* disrupts host cell division and induces host genome duplication, a phenomenon well linked to

tumorigenesis. Through long-term live cell imaging of *C. trachomatis*-infected cells, we observed the consistent formation of a single unilateral cleavage furrow instead of the normal bilateral furrows that occur during host cell division. The absent furrow and the *C. trachomatis* inclusion always resided on the same side of the cell, when unilateral cleavage furrow was observed. In these instances, host cells would often prematurely exit mitosis, resulting in binucleated cells. We demonstrated with both fluorescent live cell imaging and immunofluorescence that *Chlamydia* inclusions located in the polar region of the host cells did not disrupt the normal bilateral cleavage furrow formation. We recapitulated the unilateral cleavage furrow defect in CHO cells that engulfed 15-micron latex beads, indicating that the *Chlamydia* inclusion acted as a physical barrier to prevent normal cleavage furrow initiation. We also performed an exhaustive study of various signaling proteins involved in furrow formation in *Chlamydia*-infected cells using immunofluorescence. The accumulation of F-actin, myosin II, RhoA, anillin, Ect2, centralspindlin complex, Aurora B, PRC1 and Plk1 were sharply reduced or completely absent at the side of the cell containing *Chlamydia* inclusions. We confirmed that the localization of these signaling proteins was normal in cells containing *Chlamydia* inclusions in the polar region. Since *Chlamydia* is genetically intractable at this time, we prevented bacterial protein synthesis with antibiotics. A significant increase in the number of inclusions located in the polar region of the host cells after antibiotics treatment was observed. Moreover, *Chlamydia* inclusions localized to the cell center more efficiently than similarly-sized latex beads, indicating *Chlamydia* was actively localizing to the host cell equator. We took advantage of the robustness of the equatorial localization and protein displacing capability of the *Chlamydia* inclusions to address a prevalent controversy regarding the role of MTs in cleavage furrow formation. It has been proposed that astral MTs have an inhibitory effect on the contractile activity of the plasma membrane and the absence of astral MTs leads to ectopic furrowing. In our experiments, *Chlamydia* inclusions consistently generated regions on the host plasma membrane receiving minimal astral MT and in such regions, no ectopic furrowing was ever observed, indicating that the lack of astral MTs do not promote furrow formation. This study not only delineates the mechanism by which *Chlamydia* causes cleavage furrow defects and host genome duplication but also illustrates how we can take advantage of this bacterium to address the basic cell biology question of how the cleavage plane is defined.

Mitosis and Meiosis: Focus on Centrosomes, Kinetochores, and Microtubules

1744

Accurate chromosome segregation requires a tunable binding affinity between core kinetochore components and microtubules.

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The ability to stably bind microtubules (MTs) is an obvious requirement for the proteins that make up the kinetochore-MT attachment site. However, the binding strength must be finely tuned to allow for the maintenance of force-generating attachments and for the fluid gain and loss of tubulin subunits at MT plus-ends. The kinetochore-associated NDC80 complex can bind directly to MTs and is proposed to couple chromosome movements to growing and shortening MT plus-ends. The Hec1 protein of the NDC80 complex contains two putative MT-binding motifs: a well-structured calponin homology domain and a poorly-structured amino terminal “tail” domain. Recent studies have indicated that both domains are required to achieve high affinity

MT binding in vitro and for stable kinetochore-MT attachment in vivo. Additionally, the tail domain plays a role in regulation of the attachment strength between kinetochores and MTs through phosphorylation by Aurora kinases. How tail domain phosphorylation contributes to the regulation of kinetochore-MT attachments remains a highly debated topic. To investigate this issue, we generated a series of Hec1 constructs encoding for either truncated tail domains or tail domains containing various phospho-mimetic mutations. The mitotic phenotypes of PtK1 and HeLa cells expressing these mutants were then analyzed. We find that only a very short tail domain (6 amino acids) is required for the formation of stable kinetochore-MT attachments in cells, but a much longer tail is required for proper regulation of kinetochore-MT binding. In addition, we find that kinetochore-MT attachment strength in cells is tunable, as increasing the number of phospho-mimetic mutations within the tail domain decreases kinetochore-MT attachment strength and increases the rate of kinetochore oscillatory movements. To gain molecular insight into these cellular phenotypes, we turned to in vitro single molecule approaches using TIRF microscopy. The diffusion coefficient and residency time of purified GFP-NDC80Bonsai complexes containing Hec1 phospho-mimetic substitutions were measured on the surface of coverslip-attached taxol-stabilized MTs under physiologically-relevant conditions. To determine the dissociation constants for these proteins we developed a TIRF-based assay, in which the intensity of the GFP-NDC80 complex-MT decoration was measured for a wide range of soluble protein concentrations. At saturation, we observe that both the unphosphorylated complex and the mutant forms achieve a high density of binding, which corresponds to about 2 NDC80 complexes per tubulin dimer. Our in vitro data show that even a single phospho-mimetic substitution in the Hec1 tail has a measurable impact on the interaction of NDC80 complexes with MTs: with increasing phospho-mimetic mutations, the dissociation constant and diffusion coefficient increase, while the residency time shortens. These results are consistent with the view that the differential phosphorylation of Hec1, rather than an abrupt switch from the fully phosphorylated to fully unphosphorylated form, regulates the ability of the NDC80 complex to couple kinetochores to MTs, and to produce cohesive chromosomal oscillations.

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Cdk activity couples epigenetic centromere inheritance to cell cycle progression.

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Centromeres are chromosomal loci that drive their segregation during mitosis. Structure and identity of centromeres are maintained epigenetically by nucleosomes containing the histone H3 variant CENP-A. Propagation of CENP-A chromatin is uncoupled from DNA replication and initiates following mitotic exit implicating mitotic events in controlling the propagation of centromeric chromatin. However, the nature of the signal that triggers assembly of CENP-A is unknown.

We now show that mitotic passage itself is dispensable for CENP-A loading and that this process is directly triggered by the loss of cyclin dependent kinase 1 (Cdk1) activity in early G1. Treatment of cells in G2 phase with Cdk inhibitors induces rapid assembly of newly synthesized CENP-A at centromeres. Using pharmacogenetics in chicken DT40 cells we show that direct inhibition of Cdk1 and Cdk2 activities is sufficient for rapid CENP-A assembly at centromeres throughout the cell cycle. Consistent with canonical loading in G1, premature CDK inhibition induces recruitment of the CENP-A assembly factors hMis18 and Mis18BP1/HsKNL2 to centromeres that precedes arrival of CENP-A. G2 phase loading is dependent on these factors as well as on the CENP-A chaperone HJURP. Critically, drug induced centromere targeting of

CENP-A does not require proteolysis, uncoupling CENP-A assembly not only from mitotic events but also from the specific involvement of any APC targets that are degraded upon mitotic exit. We further show that the key CENP-A assembly factor Mis18BP1/HsKNL2 is phosphorylated in a cell cycle dependent manner controlling its centromere localization during mitotic exit.
Our results strongly support a model in which the CENP-A assembly machinery is poised for activation throughout the cell cycle, but kept in an inactive non-centromeric state by Cdk1 and Cdk2 activities during S, G2 and M phases, thus ensuring tight coupling between DNA replication, cell division and subsequent centromere maturation.

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Nucleophosmin/B23 acts as an interphase activator of Aurora-A at the centrosome.

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Aurora-A is a serine/threonine kinase required for centrosome maturation and spindle assembly. In a proteomic screen designed to unravel new Aurora-A substrates, we identified Nucleophosmin/B23 (NPM). NPM is a histone chaperone that is involved in regulation of transcription and translation, in DNA replication, in centrosome duplication and in cytoplasm/nucleus trafficking. We showed that NPM is not only a substrate, but also an activator of Aurora-A. In vitro NPM activates Aurora-A in a way that is different from the activation of Aurora-A by TPX2. Indeed, NPM doesn't trigger autophosphorylation of Aurora-A on T288 and doesn't protect T288 from dephosphorylation by PP1. In vivo, Aurora-A and NPM co-localize at centrosomes during interphase and mitosis. We validated physiological activation of Aurora-A by NPM, since NPM depletion by RNAi leads to the decrease in CDC25B phosphorylation at centrosomes in early prophase. In summary, our work suggests that NPM is a new Aurora-A activator that may act through an original activation mechanism.

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Temporal regulation of Haspin, the histone H3 Threonine 3 kinase, by Polo-like Kinase 1 and Cdk1.

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Haspin is a critical activator of Aurora B, one of the major mitotic kinases that orchestrate cell cycle progression. Phosphorylation of histone H3 at threonine 3 by Haspin (H3T3ph) creates a chromosomal docking site that recruits and ultimately activates Aurora B, leading to reaction cascades on chromosomes that coordinate mitosis. The temporal regulation of Haspin is key for proper cell cycle progression; in its absence, metaphase spindles are shorter, while persistence of its activity after exit from M phase results in a delay in chromosome decondensation and nuclear re-formation. We employed the Xenopus egg extract system to show that Haspin activity is restricted to M phase through the combined action of Cdk1 and Polo-like kinase (Plx1). Upon entry into M phase, Cdk1 phosphorylates the N terminus of Haspin to recruit Plx1. The N terminus of Haspin contains an evolutionally conserved polo-box binding domain, an STP motif, and we show that the prephosphorylation of this motif by Cdk1 promotes binding of purified Plx1-PBD to Haspin. Consistently, a point mutation of the Plx1-docking site abolishes Haspin-dependent phosphorylation of H3T3 in egg extracts. In Plx1-depleted egg extracts, histone H3T3 phosphorylation is greatly impaired and the phospho-dependent mobility shift of Haspin is greatly reduced. Therefore, Plx1 seems to phosphorylate Haspin on multiple sites to

maintain the kinase active up to anaphase. The molecular details of this phospho-dependent activation are still under study. We identified a C-terminal domain within Haspin that possesses inhibitory activity and removal of which renders the kinase constitutively active in a Plx1-independent manner. Plx1 and Cdk1 may control Haspin kinase activity either by inducing a direct conformational change through phosphorylation to relieve this inhibition, or by regulating the binding of an inhibitor in that region. We are currently investigating a number of potential Haspin binding partners as well as further dissecting the structure of Haspin to gain insight into its cell cycle dependent activation.

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Elucidation of the Aurora A activation module that drives centrosome maturation.

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The function of centrosomes as the major sites of spindle microtubule (MT) assembly relies on recruitment of pericentriolar material (PCM), a proteinaceous matrix containing the gamma tubulin ring complexes (γ-TuRC) and other spindle assembly factors. This process, known as centrosome maturation, occurs at the onset of mitosis via incompletely understood mechanisms.

Our recent study revealed that Cep192/Spd-2, a key regulator of centrosome biogenesis and PCM recruitment, is a cofactor of the mitotic serine/threonine kinase, Aurora A (AurA), at centrosomes. Specifically, Cep192 targets AurA to these organelles, where it promotes oligomerization-dependent activation of the kinase characterized by extensive phosphorylation at a critical threonine residue within the activation loop (T-loop) (Joukov et al., 2010, PNAS 107:21022). This process is essential for the recruitment of the PCM components, such as γ-TuRC and pericentrin, and for the function of centrosomes as MT-organizing centers (MTOCs).

Although at least two other cofactors, Bora and TPX2, have previously been implicated in AurA regulation during mitosis, the Cep192-mediated mechanism appears to account for most of the T-loop phosphorylated AurA. We found that Bora, Cep192, and TPX2 form distinct endogenous complexes with AurA and compete with one another for binding to the kinase, thus suggesting that the binding occurs at an overlapping segment on AurA. Cep192 appears to regulate AurA downstream of Bora since Cep192-dependent AurA activation occurs at mitotic entry and coincides with the degradation of Bora.

While elucidating the mechanisms underlying Cep192-dependent AurA activation, we identified CCDC21 (Coiled-Coil Domain Containing 21), a centrosomal protein of unknown function, as an abundant component of the AurA-Cep192 complex. CCDC21 localizes to mitotic centrosomes independently of AurA and it is not required for the targeting of Cep192 and AurA to these organelles. Yet, CCDC21 is essential for AurA activation by centrosomes, as well as for PCM recruitment and centrosome-mediated spindle MT assembly. Based on our data, CCDC21 seems to function by facilitating the formation and/or stability of AurA-Cep192 oligomers.

These observations reveal that PCM recruitment, a prerequisite for centrosomes to act as major spindle MTOCs, relies on a specialized mechanism of potent AurA activation, which involves at least two proteins, Cep192 and CCDC21 (“AurA activation module”).

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Rsf-1, a protein involved in chromatin remodeling, is functionally related to the activation of aurora B kinase.

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Rsf-1/HBxAP interacts with hSNF2H, a ATPase to form a chromatin remodeling complex, RSF (remodeling and spacing factor). Recently, Rsf-1 is suggested to be involved in centromere formation. Here, we show that Rsf-1/HBxAP protein accumulates in mitosis and localizes at the centromeres. Depletion of Rsf-1/HBxAP caused severe defects in chromosomal congression, resulting in accumulation of prometaphase cells. Despite the defects in congression, the analysis of time-laps images showed the acceleration of mitotic progression, which indicates that the mitotic checkpoint is compromised in Rsf-1 deficient cells. In Rsf-1 depleted cells, Aurora B and hBubR1 properly localize to kinetochores. However, the activity of Aurora B kinase was significantly decreased in the absence of Rsfp1/HBxAP or hSNF2h. Depleted cells defect localization of Plk1 at the kinetochore. Our results suggest that Rsf-1/HBxAP depletion decreases Aurora kinase activity by impairing the localization of Plk1 at the kinetochore. This study provides the evidence that Rsf1/HBxAP play an important role in mitosis.

1750

IPL1/Aurora B kinase inhibits bipolar spindle assembly during meiotic prophase.

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The coordination of spindle assembly with chromosome dynamics is a prerequisite for correct chromosome segregation during mitosis and meiosis. Spindle assembly occurs following the separation of side-by-side microtubule organizing centers (MTOC). In budding yeast, the spindle pole body (SPB) functions as the MTOC. In mitotic growth, the single SPB duplicates in G1/S and the duplicated SPBs soon separate to form a bipolar spindle. However, in meiosis, the duplicated SPBs stay side-by-side and their separation is delayed during a protracted prophase in which pairing and recombination between homologous chromosomes prepares them to be segregated at metaphase I. Only as the cell exits prophase do the duplicated SPBs separate to form a spindle. Thus, it appears there is a mechanism that coordinates these two seemingly independent events (chromosome interactions/spindle assembly) so that SPB separation does not precede the completion of homologous chromosomal interactions. Here, we combine genetic and microscopic approaches to demonstrate that Ipl1/Aurora B kinase is essential for inhibiting bipolar spindle assembly during prophase. When this inhibition is removed spindles form prematurely in prophase cells. We examined whether premature spindle formation in prophase leads to chromosome mis-segregation. Our results show chromosomes do not interact with the precocious spindles in prophase but that when cells exit prophase in the presence of pre-formed spindles they exhibit increased rates of chromosomes mis-segregation. Our results suggest that Ipl1/Aurora B kinase plays a critical role in coordinating the timing of bipolar spindle formation with chromosomal events to prevent chromosome mis-segregation in meiosis.

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Mechanisms of acentrosomal spindle assembly during C. elegans oocyte meiosis.

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Although centrosomes serve to organize microtubules in most cell types, oocyte spindles form in their absence. We are investigating acentrosomal spindle assembly using *C. elegans* as model. High resolution imaging of acentrosomal spindle formation revealed that following nuclear envelope breakdown, microtubules form a "cage-like" structure that forms adjacent to remnants of the nuclear envelope. At later stages, arrays form where minus ends are associated at multiple sites before achieving bipolarity. These findings suggest a model where acentrosomal spindle assembly proceeds by: 1) microtubule nucleation within the disassembling nuclear envelope, 2) organization of minus ends at sites away from the chromosomes, and 3) coalescence of these sites into two poles. We are currently testing this model and identifying proteins required for these events.

Through the course of an RNAi screen, we identified proteins required for multiple aspects of acentrosomal spindle assembly. Depletion of either KLP-18 (kinesin-12 family) or MESP-1 (meiotic spindle 1) results in the formation of monopolar oocyte spindles, implicating these proteins in establishing and/or maintaining spindle bipolarity. KLP-18 was previously shown to localize to the poles of oocyte spindles, and we found that MESP-1 colocalizes with KLP-18 throughout the cell cycle. In *klp-18* mutants MESP-1 fails to localize to the poles of the monopolar spindles, and KLP-18 fails to localize to microtubules following *mesp-1 (RNAi)*, suggesting that these proteins are interdependent for their localization and likely collaborate to promote acentrosomal spindle bipolarity. In contrast, depletion of ASPM-1 (homolog of *Drosophila* Abnornal spindle protein) in oocytes results in disorganized structures that either lack or have unfocused poles. ASPM-1 localizes to spindle poles and to regions where minus ends begin to associate during the early stages of acentrosomal spindle formation, and KLP-18 and MESP-1 are not required for this localization. Double depletion of ASPM-1 and either KLP-18 or MESP-1 prevents monopolar spindle formation, and a disorganized structure without a focused pole forms. Therefore, ASPM-1 is required for acentrosomal pole formation, and we propose a model in which ASPM-1 contributes to coalescing minus ends during spindle assembly.

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The *fragile centrioles (fract)* gene is required for the maintenance of centriole integrity during *Drosophila* male meiosis.

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We have isolated and characterized a mutation in a *Drosophila melanogaster* gene we name *fragile centrioles (fract)*; this mutation disrupt male meiosis and causes male sterility. Immunostaining with antibodies against centriolar components such as DSpd2 and Asl showed that premeiotic spermatocytes of *fract* mutants display 2 centrioles at each cell pole. However, meiotic ana-telophase I figures of *fract* mutants often exhibit two regular centrioles at one cell pole but only one at the opposite pole. As a result, a number of secondary spermatocytes assemble bipolar monastral spindles that are unable to mediate proper chromosome segregation. *fract* encodes a 322 aa protein that contains WD repeats; this protein is expressed only in testes, suggesting that *fract* is specifically required for meiotic centriole stability. The EMS-induced *fract*[1] mutant allele we characterized carries a stop codon that truncates the Fract protein into a 298 aa polypeptide. A polyclonal antibody raised against Fract decorates the distal end of male meiotic centrioles. This specific staining pattern is lost in *fract* mutants, where the

antibody decorates the entire centriole, suggesting that the C-terminal region of Fract is crucial for its correct localization. Asl, DspD2, Sas4 and Dplp localize normally to the intact centrioles of *fract* mutants, indicating *fract* is not is not required for recruitment of these major centriole components. Collectively our results strongly suggest that *fract* does not play an essential role in centriole assembly but it is instead required for the maintenance of centriole integrity during male meiosis. We are currently characterizing centriole behavior in living spermatocytes of *fract* mutants and looking for proteins that physically interact with Fract.

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Regulation of kinetochore-microtubule attachment during meiosis I by Mps1 and Ipl1/Aurora-B.

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Kinetochores are large protein complexes which define the site of microtubule attachment to the chromosome. Regulation of kinetochore attachment and orientation on the spindle is essential to insure proper segregation of chromosomes during mitosis and meiosis. The 1st meiotic division, which separates homologous chromosomes, requires attachment of the sister chromatids to the same pole. This monopolar attachment is promoted in budding yeast by the monopolin complex and Ipl1 (=Aurora-B). Recent observations in budding yeast suggest that in meiosis I, there are two periods in which kinetochore-microtubule (kMT) associations are regulated: an early release of attachments in prophase and a period of attachment and orientation in metaphase.
Mps1 is an evolutionarily conserved Serine/Threonine kinase. Mps1 is implicated in different functions linked to chromosome segregation including spindle pole body duplication (Mono-Polar Spindle 1), the spindle checkpoint and promotion of bi-orientation in mitosis. The pleiotropic phenotypes of MPS1 mutations have complicated the studies of the different individual functions/targets of Mps1, and for this reason the precise roles of this kinase remain mysterious. We have circumvented this problem with a separation of function point mutation of MPS1 that is located in domain recently described as specifically implicated in promoting bi-orientation during mitosis. Mutants bearing this allele are perfectly viable during vegetative growth. Surprisingly, the same mutants exhibit extremely high levels of mis-segregation during meiosis I. During the 1st meiotic division, this allele mutant suppresses the monopolar attachment defect of monopolin mutants, much like IPL1 mutants do. Comparative analyses with ipl1 mutants suggest that Ipl1 and Mps1 play similar roles in promoting kMT attachment in meiosis but at different times in the meiotic program.

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Pac-man motility of kinetochores unleashed by laser microsurgery.

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Typically anaphase segregation entails a kinetochore moving poleward as its kinetochore (K-) fiber shortens. In crane-fly spermatocytes, the sex chromosomes – X and Y – are found as univalents during meiosis I, and they exhibit a very atypical segregation mechanism in which each has K-fibers to both poles. X-Y segregation is based on their being led to opposite poles, each connected with a leading fiber (which shortens) to one pole, and a trailing fiber (which elongates) to the other. We used a combination of fluorescent speckle microscopy, liquid crystal

polarized light microscopy and laser microsurgery to show that segregation is based on a traction fiber model in which leading kinetochores function in a polymerization (reverse pac-man) state, whereas trailing kinetochores are in a neutral state that cannot foster poleward movement. To investigate the motile states that the kinetochores are able to support, we used the laser to knock out one of a univalent's two kinetochores, so as to release the remaining intact kinetochore from the resistive influence of its sister's K-fiber. This resulted in impressively more-rapid-than-normal poleward movement of the released kinetochore and attached arms. Fluorescent speckle microscopy of such released kinetochores revealed their motile state. Through analysis of poleward movement of released kinetochores relative to fluorescent speckles within microtubules, we found that our experiment had caused the released kinetochore to convert from reverse pac-man to pac-man motility. This novel result demonstrates that it is indeed possible for a spindle that normally employs traction fiber mechanics to exhibit pac-man motility. This unleashing of kinetochore motility through loss of resistive force is further evidence for the emerging model that kinetochores are subject to tension-sensitive regulation.

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Architecture and kinetochore binding of the yeast monopolin complex.

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In fungi, the monopolin complex prevents certain types of improper kinetochore-microtubule (MT) attachments, both in mitosis and meiosis. In *Saccharomyces cerevisiae*, monopolin binds kinetochores in meiosis I and enforces co-orientation of sister chromatids; while in *Schizosaccharomyces pombe*, the complex inhibits merotelic attachment of individual kinetochores in mitosis and meiosis II. How monopolin accomplishes these tasks, in addition to a seemingly unrelated function in rDNA silencing and recombination control, is not well understood. We have examined the architecture of the conserved core of monopolin from both *S. cerevisiae* (the Csm1/Lrs4 complex) and *S. pombe* (Pcs1/Mde4). The *S. cerevisiae* Csm1/Lrs4 complex adopts a distinctive V-shaped structure, with two globular 'heads' spaced ~10 nm apart. We identified direct physical interactions between the globular head domains of Csm1 and both the MIND/Mis12 complex, a conserved inner-kinetochore subcomplex, and the inner-kinetochore subunit Mif2/CENP-C. Point-mutations in Csm1 that disrupt these interactions in vitro result in a loss of sister chromatid co-orientation in meiosis I, indicating their functional importance. Together, our data support a model in which Csm1/Lrs4 and Pcs1/Mde4 act as bivalent clamps, which can cross-link kinetochores' microtubule-binding sites in order to co-orient sister chromatids in meiosis I (*S. cerevisiae*) or prevent merotelic attachment (*S. pombe*). More recently, we have dissected the protein-protein interactions of the two other *S. cerevisiae* monopolin complex subunits, Mam1 and the casein kinase Hrr25. We have determined the structure of the Mam1/Csm1 complex, and are also working to image the intact *S. cerevisiae* monopolin complex, which we recently expressed and purified.

1757

Replacement of Histone H3 with CENP-A Directs Global Nucleosome Array Condensation and Loosening of Nucleosome Superhelical Termini.

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Centromere protein A (CENP-A) is a histone H3 variant that marks centromere location on the chromosome. The goal of this work was to study the subunit structure and folding dynamics of human CENP-A-containing chromatin. We generated a set of nucleosomal arrays with canonical core histones and another set with CENP-A substituted for H3. Then we coupled folding measurements using analytical ultracentrifugation (AUC) with mass spectrometry-based hydrogen/deuterium exchange (H/DX-MS). The AUC studies measure the bulk behavior of the arrays, while H/DX-MS is used to measure the dynamic behavior of the polypeptide backbone of each histone in the nucleosome core. At the level of quaternary structure and assembly, we find that CENP-A arrays are composed of octameric nucleosomes that assemble in a stepwise mechanism, recapitulating conventional array assembly with canonical histones. At intermediate structural resolution, we find that CENP-A-containing arrays are globally condensed relative to arrays with the canonical histones. At high structural resolution, using H/DX-MS, we find that the DNA superhelical termini within each nucleosome are loosely connected to CENP-A, and we identify the key amino acid substitution that is largely responsible for this behavior. Also the C-terminus of H2A undergoes rapid hydrogen exchange relative to canonical arrays, and does so in a manner that is independent of nucleosomal array folding. These findings have implications for understanding CENP-A-containing nucleosome structure and higher order chromatin folding at the centromere.

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Quantification of CENH3/CENP-A and NDC80 Complexes in Individual *Arabidopsis* Kinetochores During Mitosis and Meiosis.

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The kinetochore protein complex attaches chromosomes to spindle microtubules to facilitate chromosome segregation. Kinetochore size varies greatly between organisms, but the effect of this structural plasticity on protein stoichiometry is not well understood. We replaced the *Arabidopsis thaliana* kinetochore proteins CENH3 and NUF2 (a member of the microtubule binding NDC80 complex) with GFP-tagged variants. This allowed us to measure their amounts in individual mitotic and meiotic kinetochores using quantitative fluorescence microscopy with *Saccharomyces cerevisiae* GFP-tagged proteins as standards. *A. thaliana* kinetochores contained an average of 375 ± 111 CENH3 molecules, and had a much higher CENH3:NDC80 ratio than budding yeast kinetochores. The number of CENH3 molecules in plant mitotic kinetochores varied more than the number of NDC80 complexes. Interphase kinetochores contained fewer CENH3 molecules, further indicating that the level of CENH3 is plastic. Meiotic kinetochores differ from mitotic kinetochores in three respects. First, the NDC80 complex is transiently removed from meiosis I kinetochores during chromosome pairing. Second, fused kinetochores in meiosis I are larger than mitotic kinetochores and have an elongated morphology. Third, the lowest levels of CENH3 and NDC80 complexes are observed in the tetrad stage of meiosis, indicating that kinetochores are significantly smaller before meiocytes return to the mitotic cell cycle.

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KNL-2 acts as a licensing factor for CENP-A loading in G1 through its Myb domain binding to centromeric DNA.

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Centromeres are chromosomal loci that direct kinetochore assembly in mitosis. Unlike genetic features, centromere DNA sequence is not conserved through phylogeny nor is it sufficient for centromere function. Therefore, it is commonly accepted that centromeres are epigenetically defined; a process mediated by the histone H3 variant CENtromere Protein-A (CENP-A). CENP-A loading at centromeres is a replication independent mechanism, occurring from late telophase until early G1. However, the mechanism of centromere recognition by the loading machinery is not well understood. Kinetochore NulL-2 (KNL-2) was identified in *Caenorhabditis elegans* (*C. elegans*) by functional genomics as required for CENP-A loading at centromeres. Sequence analysis reveals a conserved MYB like DNA binding domain within KNL-2. In other well studied MYB proteins, this domain is known to bind DNA with high specificity via the 3rd of three helices. Here we show that the *C. elegans* KNL-2 (CeKNL-2) MYB domain is able to bind genomic DNA in vitro. Furthermore, CeKNL2 localizes to centromeres when ectopically expressed in human cells and in rat kangaroo kidney epithelium (PtK2). Together, these results indicate that the KNL2 Myb domain does not utilize a sequence specific DNA binding mechanism and possibly recognizes a specific structure generated by the presence of CENP-A nucleosomes at centromeres. To test this model, we solved the 3 dimensional structure using Nuclear Magnetic Resonance (NMR). This revealed an expected helix-loop-helix-loop-helix structure. Also, a 1H-15N Heteronuclear Single Quantum Coherence (HSQC) NMR spectra of the MYB domain in presence of centromeric DNA sequence (α-satellite sequence) revealed the amino acids that are potentially interacting with the DNA, and surprisingly they all reside within the first helix. The NMR results were further confirmed by an Electrophoretic Mobility Shift Assay (EMSA), deletion of the first helix of the Myb domain is not able to bind DNA compared to full length (or control deletions). To test whether KNL2 recognizes a specific structure given by CENP-A at centromeres, we are currently testing Myb domain binding to chromatin using Total Internal Reflection Fluorescence (TIRF) microscopy. Preliminary data shows that the CeKNL-2 Myb domain can localize to CENP-A-YFP foci. Moreover, Chromatin ImmunoPrecipitation followed by deep Sequencing (ChIP-Seq) shows an enrichment at centromere loci. Together, those results indicate that KNL-2 recognizes a specific structure given by CENP-A chromatin. Although not conserved at the sequence level, CeKNL-2 MYB domain displays conserved activity in human cells. Thus, the mechanism controlling centromere specification may be ubiquitous in metazoans.

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A test of structural rigidity of CENP-A-containing chromatin as the epigenetic mark of centromere identity.

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The centromere is a specialized chromosomal domain necessary for the correct segregation of eukaryotic chromosomes during cell division. This region is a complex chromosomal structure containing extensive tandem repeated arrays of a DNA sequence element called α-satellite. The centromeres have a distinct type of chromatin in which histone H3 is replaced by a structurally different homologue identified in humans as CENP-A. These conformational differences can be transmitted into histone H3 when the 22 amino acid differences between helix α1 and α2 of CENP-A and histone H3 are substituted into histone H3. Since this short domain is sufficient to

drive histone H3.1 to the centromere when substituted into histone H3, this domain is called the CENP-A targeting domain (CATD). Furthermore the chimeric histone $H3^{CATD}$ can maintain centromere function when CENP-A levels are lowered. For these reasons, CENP-A chromatin is thought to be the major determinant of centromere identity since specific DNA sequences are neither required nor sufficient for centromere identity.
I aim to define the importance of the conformational difference of chromatin assembled with CENP-A compared with chromatin with canonical histone H3 to test the hypothesis that the structural rigidity conferred by CENP-A to centromeric chromatin is the epigenetic mark of centromere identity that templates its own replication.
For this purpose I used gene replacement in diploid human cells to construct a CENP-A Flox/- cell line that have null allele missing exon 3 and 4 (where the CATD domain is) and a conditional allele with two LoxP sites flanking exons 3 and 4. Inactivation of the floxed CENP-A allele is achieved after transient expression of Cre recombinase. I then determine if the chimeric histone H3 with the centromeric targeting domain of CENP-A ($H3^{CATD}$) can maintain centromere functionality and identity when endogenous CENP-A is completely absent.

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CENP-A Exceeds Microtubule Attachment Sites in Centromere Clusters of Both Budding and Fission Yeast.

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The kinetochore stoichiometries in yeast and vertebrate cells were determined using the histone H3 variant CENP-A, Cse4 of budding yeast, as a counting standard. One Cse4-containing nucleosome exists in the centromere of each chromosome, so it is assumed that each anaphase centromere/kinetochore cluster contains 32 Cse4 molecules. We report that anaphase centromere clusters contained ~4× as much Cse4 in *S. cerevisiae* and ~40× as much CENP-A (Cnp1) in *S. pombe* as reported. These results suggest that CENP-A molecules exceed the numbers of kinetochore-microtubule attachment sites on each centromere and that CENP-A is not the sole determinant for specifying kinetochore assembly sites in either yeast. In addition, fission yeast has enough Dam1/DASH complex for ring formation around attached microtubules. This study prompts the need for significant revision of the centromere/kinetochore architectural models.

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HP1-Borealin interaction specifies CPC localization to the centromere.

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During cell division, chromosome segregation is orchestrated by the interaction of spindle microtubules with the centromere. Accurate attachment of spindle microtubules to kinetochore requires the chromosomal passenger of Aurora B kinase complex with borealin, INCENP and survivin. However, the chromatin factors that recruit the CPC to centromeres have remained elusive. Here we show that the C-terminal borealin contains readers for the chromatin shadow domain of heterochromatin protein 1 (HP1£\). This borealin-HP1£\ binding is very specific and it mediates recruitment of the CPC to the centromere and the resulting activation of its kinase subunit Aurora B. Consistently, perturbation of such an borealin- HP1£\ interaction leads to defects in CPC targeting and aberrant Aurora B activity in centromere. Importantly, the

kinetochore microtubule attachment and tension across the kinetochores are attenuated due to the perturbation of borealin targeting. These findings establish a direct link between HP1£\ and CPC plasticity in centromere and its cellular role for Borealin-HP1£\ƒn interaction in orchestrating an accurate cell division. Using an optical sensor reporting kinase in centromere, we are illustrating the temporal dynamics of aurora B/haspin activity during kinetochore maturation and tension development during mitotic chromosome segregation.

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Crosstalk Between the Ndc80 Complex and the Dynein Motor Module at the Kinetochore.

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The Ndc80 complex forms the primary attachment site for microtubules at kinetochores. Microtubules capture kinetochores via transient lateral attachment, which mature to end-on stable load-bearing attachment. A poorly conserved basic 60-100 amino acid tail and a highly conserved globular calponin homology (CH) domain in the N-terminus of the Ndc80 subunit directly contact the microtubule lattice in vitro. Divergent results have been obtained on the importance of the basic tail in vivo; in yeast the tail is dispensable whereas in mammalian cells it is reported to be essential. Phosphorylation of the tail by Aurora B kinase is proposed to regulate the stability of kinetochore microtubule attachment by the Ndc80 complex. Analysis in the C. elegans embryo has also revealed that the formation of load-bearing microtubule attachments by the Ndc80 complex is regulated by the dynein motor module, presumably to facilitate the transition from an initial dynein motor-mediated lateral to end-on attachment. To investigate formation of end-on microtubule attachments by the Ndc80 complex in vivo, we developed a single copy transgene insertion system that enables precise phenotypic characterization of engineered mutants in the early C. elegans embryo. In parallel, we analyzed reconstituted complexes in vitro. Deletion of the tail, which severely reduced microtubule-binding affinity in vitro, or mutation of Aurora B sites on the Ndc80 complex, did not perturb load-bearing attachment formation or cause detectable chromosome missegregation. By contrast, mutations in the CH domain severely inhibited attachment formation and chromosome segregation, indicating that the primary load-bearing attachment activity of the Ndc80 complex resides in the Ndc80 CH domain. Unexpectedly, deletion of the Ndc80 basic tail disrupted the ability of the dynein motor module to regulate the formation of load-bearing attachments. Prior work indicates that the dynein-tethering RZZ (Rod/Zwilch/Zw10) complex (Gassmann et al. Genes Dev. 2008 22:2385-99) transiently inhibited the formation of load-bearing attachments by the Ndc80 complex. Deleting the Ndc80 tail prevented this crosstalk between the dynein motor module and the Ndc80 complex, accelerating the formation of end-on attachments. Taken together, our results reveal that the basic tail of Ndc80 is dispensable for load-bearing attachment formation but serves as the primary target for regulation by the dynein tethering RZZ complex at the kinetochore. We propose that crosstalk between the two major microtubule-binding activities at the kinetochore in metazoans coordinates conversion of initial dynein-dependent lateral microtubule capture into the load-bearing end-on Ndc80 complex-mediated attachments that align and segregate chromosomes.

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Fast removal of TACC3/ch-TOG/clathrin inter-microtubule bridges from kinetochore fibers during mitosis.

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The equal distribution of chromosomes between daughter cells during mitosis by the spindle apparatus is essential to avoid aneuploidy, one of the main hallmarks of cancer. The regulation of spindle microtubule dynamics and kinetochore fiber (K-fiber) stability is key to achieve correct mitosis. A TACC3/ch-TOG/clathrin complex was previously shown to stabilize K-fiber microtubules by acting as an inter-microtubule bridge, thus contributing to the alignment of chromosomes on the metaphase plate.

The use of RNAi to study spindle protein function has disadvantages due to slow depletion over several days, during which the cell can compensate for the missing proteins. Furthermore, distinguishing between the roles of proteins in either spindle assembly or spindle maintenance is difficult. Here, we removed specific spindle proteins from K-fibers by rapamycin-induced rerouting to the mitochondria: a technique known as knock-sideways (KS).

KS of TACC3 resulted in complete removal of the protein within 10 minutes of rapamycin application. Its mitotic partners, ch-TOG and clathrin were also removed from K-fibers; whereas the unrelated spindle proteins, NuMA and HURP were unaffected. Interestingly, KS of clathrin light chain induced the removal of clathrin heavy chain but only partial removal of TACC3 and ch-TOG from spindle fibers. This argues that TACC3, and not clathrin, serves as the microtubule-binding element of the TACC3/ch-TOG/clathrin complex.

Removal of the TACC3/ch-TOG/clathrin complex by KS of TACC3 during metaphase resulted in delayed progression to anaphase. By immunofluorescence, KS of TACC3 resulted in a ~20% decrease in the half-spindle length suggesting that K-fibers had become destabilized. Tension was reduced, as evidenced by a reduction in the inter-kinetochore distance. Moreover, a subset of kinetochores in metaphase-like cells where TACC3 had been rapidly removed still recruited the mitotic checkpoint protein MAD2. Correlative light-electron microscopy was used to study the loss of inter-MT bridges and the MT packing in K-fibers at either 10 or 30 minutes after removal of the complex.

Our results show that the TACC3/ch-TOG/clathrin complex contributes to maintaining the integrity of the mitotic spindle by stabilizing K-fiber microtubules. Using the KS method it was possible to distinguish these functions from roles in early mitosis, such as spindle assembly. As such, rapid methods for removal of spindle proteins show clear advantages over more chronic methods, such as RNAi.

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The Role of Slk19 in Centromere Cohesion.

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To ensure faithful chromosome segregation, sister kinetochores must attach to microtubules emanating from opposite spindle poles to establish chromosome bipolar attachment. Once attached, the microtubules apply a pulling force against the sister kinetochores. This force is counter-acted by the cohesin complex, which holds sister chromatids together until anaphase onset to prevent premature chromosome separation. This conserved complex, thought to be in the form of a ring structure which entraps sister chromatids, is located throughout the

chromosomes and plays an essential role in chromosome segregation. Slk19 is a budding yeast kinetochore protein that has been previously shown to interact with a cohesin subunit Mcd1/Scc1, but its role in chromosome segregation has not been well defined (Zhang et al. 2006. J Cell Sci 119:519-31). Our data indicate that Slk19 could function as a centromeric-specific cohesin. We have observed separated sister centromeres in *slk19*$_{\Delta}$ mutant cells even when the pulling force is absent. Although *slk19*$_{\Delta}$ mutants undergo a relatively normal mitosis, they exhibit an anaphase entry delay after the kinetochore-microtubule interaction is disrupted by nocodazole. This delay is dependent upon Sgo1, a tension checkpoint component, which suggests that Slk19 is required for the reestablishment of bipolar attachment after the disruption of the kinetochore-microtubule interaction. We found evidence of a Slk19-Slk19 physical interaction and interestingly, we observed that overexpression of Slk19 also leads to centromeric cohesion defects. Furthermore, our evidence indicates the presence of a cohesin-cohesin interaction, but this interaction is reduced in *slk19*$_{\Delta}$ mutants. All of these results support the possibility that a single sister chromatid is entrapped within the cohesin ring at the centromeric region and dimerized Slk19 brings the cohesin rings from sister centromeres together.

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Enrichment of the Aurora B Kinase on the Centromeres of Incorrectly Attached Chromosomes.

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Maintenance of genome stability during cell division depends on establishing correct attachments between chromosomes and spindle microtubules. Correct, bi-oriented attachments are stabilized, while incorrect attachments are selectively destabilized. This process relies largely on increased phosphorylation of kinetochore substrates of Aurora B kinase at misaligned versus aligned kinetochores. Current models explain this differential phosphorylation by spatial changes in the position of substrates relative to a constant pool of kinase at the inner centromere. However, these models are based on studies in aneuploid cells. We show that normal diploid cells have a more robust error correction machinery. Aurora B is enriched ~3-fold at misaligned centromeres in these cells. Live-cell imaging studies demonstrate that the Aurora B enrichment at misaligned centromeres is due to a switch-like mechanism based on chromosome attachment status. Indeed, re-enrichment of Aurora B occurs within 1-4 minutes on the centromeres of individual chromosomes that lose initial bipolar attachment in otherwise unperturbed cells or on all the centromeres of cells treated with the microtubule poison, nocodazole. We show that this enrichment substantially increases the dynamic range of Aurora B substrate (Dsn1) phosphorylation at misaligned versus aligned kinetochores. We also show that, in addition to Aurora B, phospho-H2A-T120 levels, but not phospho-H3T3 levels, are enriched at the centromeres of misaligned chromosomes. Further, using cell fusions, we show that the deficiency of Aurora B enrichment in aneuploid cells can be rescued by the cytoplasm of healthy diploid cells. Our findings indicate that in addition to Aurora B regulating kinetochore-microtubule binding, the kinetochore also controls Aurora B recruitment to the inner centromere. We show that this recruitment depends on both activity of Plk1, a kinetochore-localized kinase, and activity of Aurora B itself. Our results suggest a feedback mechanism in which Aurora B both regulates and is regulated by chromosome attachment to the spindle, which amplifies the differential phosphorylation of kinetochore substrates and increases the efficiency of error correction.

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Characterization of centrosome positioning at nuclear envelope breakdown and its potential role in cancer cell chromosome mis-segregation.

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The microtubule-based bipolar mitotic spindle ensures accurate partitioning of chromosomes to two identical daughter cells. Although the mitotic spindle can assemble in the absence of centrosomes, when centrosomes are present, they act as major sites of MT nucleation. In preparation for mitotic spindle assembly, the replicated centrosomes can move to opposing sides of the nucleus before nuclear envelope breakdown (NEB) through a mechanism typically referred to as the prophase pathway of centrosome separation. However, in some cases centrosomes do not completely separate to opposite sides of the nuclear space until after NEB, which occurs through a mechanism known as the prometaphase pathway. Using PtK1 cells with fluorescently labeled centrosomes we found a continuous distribution of inter-centrosome distances at NEB, arguing against the presence of two distinct pathways of centrosome separation. Moreover, we found elevated numbers of anaphase lagging chromosomes in cells that have an inter-centrosome distance at NEB that is less than 75% of the inter-centrosome distance at the end of prometaphase. Additionally, we observed three types of arrangements in cells that fail to complete centrosome separation before NEB. These include a top-bottom, top-side, and a side-side orientation of centrosomes around the nucleus. Interestingly, when centrosomes are positioned in top-bottom configuration (which would entail completion of centrosome separation before NEB), they move to the same focal plane in the center of the nuclear space upon NEB, thus turning into a situation of incomplete centrosome separation. Finally, we tested whether failure of centrosomes to completely separate prior to NEB is a common phenomenon in cancer cells. We found that in a panel of human cancer cell lines 30-70% of early prometaphase cells exhibited incomplete centrosome separation. Such high frequencies suggest that incomplete centrosome separation at NEB may represent an additional mechanism by which cancer cells mis-segregate chromosomes at high rates.

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BubR1 Autophosphorylation is a Signaling Switch at the Kinetochore in Response to Microtubule Capture.

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Whether and how spindle microtubule capture can be translated into signaling at the kinetochore remain unresolved. Here we demonstrate that the kinetochore-associated mitotic kinase BubR1 is able to autophosphorylate itself in vitro and in cultured cells in the presence of its binding partner, a kinetochore motor CENP-E. This autophosphorylation is sensitive to microtubule attachment in cells. Replacing endogenous BubR1 with a nonphosphorylatable BubR1 mutant, but not a phosphomimetic mutant, results in not only metaphase chromosome misalignment, but also a decrease of Aurora B kinase activity. Depletion of CENP-E, the BubR1 kinase activator, also causes decreases of BubR1 autophosphorylation and Aurora B kinase activity. Furthermore, expression of a phosphomimetic BubR1 mutant in CENP-E-depleted cells substantially reduces incidents of polar chromosomes. Thus, CENP-E-dependent BubR1 autophosphorylation serves as a signaling switch at the kinetochore to directly regulate Aurora B kinase activity in response to spindle microtubule capture by CENP-E.

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Three-dimensional high-resolution colocalization of centromere and kinetochore proteins.

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The kinetochore is the primary microtubule-binding site on the chromosome and governs accurate chromosome segregation during cell division. Kinetochores form during mitosis on a specialized chromatin domain called the centromere. The centromere and kinetochore are large multiprotein complexes. Understanding centromere and kinetochore function requires knowing how individual protein subunits of the centromere and kinetochore are spatially arranged and how this organization changes in response to microtubule binding and spindle forces. High-resolution localization of many centromere and kinetochore proteins has provided a map of the linear distribution of proteins along the sister kinetochore axis (Wan et al. 2009) but it has not been possible to measure the three-dimensional organization of centromere and kinetochore subunits or how this organization responds to microtubule based tension.

We present an extension of the high-resolution colocalization technique that allows us to measure three-dimensional distances between diffraction-limited objects to better than 10 nm absolute accuracy using standard widefield fluorescence microscopy. We apply this method to measure the three-dimensional distances between proteins of the centromere and kinetochore in human cells and examine asymmetry about the interkinetochore axis between two paired metaphase chromatids. We compare these distances between individual kinetochores from paired chromatids in untreated cells and cells treated with taxol to examine the differential response of paired kinetochores to microtubule-based tension.

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Tension dependent nucleosome remodeling at the pericentromere in yeast.

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Nucleosome positioning is important for the structural integrity of chromosomes. During metaphase the mitotic spindle exerts physical force on pericentromeric chromatin, and the cell must adjust the pericentromeric chromatin to accommodate the changing tension resulting from microtubule dynamics. Here we examine the effects of spindle-based tension on nucleosome dynamics by measuring the histone turnover of the chromosome arm and the pericentromere during metaphase in the budding yeast *Saccharomyces cerevisiae*. We find that both histones H2B and H4 exhibit greater turnover in the pericentromere during metaphase. Loss of spindle-based tension by treatment with the microtubule depolymerizing drug nocodazole results in reduced histone turnover in the pericentromere. The observed dynamics in the pericentromere under tension are influenced by the chromatin remodeling activities of STH1/NPS1 and ISW2. Sth1p is the ATPase component of the remodel the structure of chromatin (RSC) complex and is known to displace histones from chromatin, whereas Isw2p is known to insert nucleosomes via sliding activity. The balanced displacement and insertion of histones in the pericentromere allows the cell to accommodate spindle-based tension while maintaining proper chromatin packaging during mitosis.

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Defining how HJURP Differentiates CENP-A/H4 from Canonical H3/H4 in the Centromere Chromatin Assembly Pathway.

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Centromeres are the genomic locus required for proper chromosome segregation. Centromere Protein A (CENP-A) is a histone H3 variant present at all active centromeres and is the best candidate to provide the epigenetic mark that specifies centromere location on the chromosome. The CENP-A Targeting Domain (CATD), containing the L1 and α2 helix from the histone fold domain, confers unique dynamic and structural features to CENP-A-containing complexes and is sufficient for centromere maintenance when substituted into H3. HJURP (Scm3 in yeast) is the CENP-A specific chaperone that is required for recruitment of newly expressed CENP-A to centromeres, and its ectopic targeting onto a chromosome arm site directs local CENP-A loading and acquisition of a functional centromere (Barnhart et al., 2011, J. Cell Biol. 194: 229-43). How HJURP recognizes and binds specifically to CENP-A remains unclear despite a recent crystal structure of the ternary HJURP/CENP-A/H4 complex (Hu et al., 2011, Genes Dev. 25:901-6). By reconstituting complex formation with purified components and using the cell-based HJURP chromosome-tethering approach, we have now performed extensive mutagenesis of both CENP-A and H3 and defined the 7 residues within the CATD that are necessary and sufficient for HJURP binding. Our findings are a direct challenge to the proposal that a single CENP-A residue outside of the CATD is the major HJURP recognition element (Hu *et al*., 2011, Genes Dev. 25:901-6), and, indeed, direct tests in our functional biochemical and cell-based assays failed to support the earlier GST pulldowns experiments that originally led to this view. Surprisingly, we found that HJURP binding is insufficient for centromere targeting of CENP-A, suggesting that the dynamic and structural features of the CATD have other essential functions in conferring centromere targeting to CENP-A. In addition, we also found that a mutant version of CENP-A that cannot form a $(CENP\text{-}A/H4)_2$ subnucleosomal heterotetramer robustly assembles into a complex with HJURP but fails to assemble at centromeres, strongly suggesting that two copies of CENP-A are required in a stable centromeric nucleosome. In sum, our findings suggest that the CATD provides HJURP specificity and fulfills structural and dynamic requirements for effective centromeric nucleosome assembly and stability.

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The organization of the pericentromere chromatin spring by cohesin and condensin determined using model convolution.

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The mitotic chromatin spring is composed of cohesin, condensin, and an intramolecular loop of pericentric chromatin. We utilized model convolution methods to compare quantitative computer simulations of pericentric cohesin and condensin to experimentally acquired microscopy images. Condensin lies proximal to the spindle microtubules in line with and spanning the distance between the inner kinetochores whereas cohesin is radially displaced from condensin by ~75 nm and does not span the full interkinetochore distance. Experimental condensin fluorescence is accurately modeled by clustering condensin complexes between the kinetochores whereas cohesin is modeled as a random distribution of single or paired complexes. Condensins aggregate to accomplish the compaction of the primary chromatin axis

forming loops. Cohesin slides along the pericentric chromatin to a spindle distal position where it interconnects and confines inter-chromatid loops generated by condensin. In conjunction, we are developing a mathematical model of the mitotic spindle to deduce the structure of the chromatin spring. Utilizing model convolution we report the sub-structures of cohesin and condensin that organize the pericentromere chromatin.

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Point centromeres contain greater than a single centromere-specific Cse4 (CENP-A) nucleosome.

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Cse4 is the budding yeast homolog of CENP-A, a modified histone H3 that specifies the base of kinetochores in all eukaryotes. Budding yeast is unique in having only one kinetochore microtubule attachment site per centromere. The centromere is specified by CEN DNA, a sequence specific binding complex (CBF3) and a Cse4-containing nucleosome. Here we compare the ratio of kinetochore proximal Cse4-GFP fluorescence at anaphase to several standards including purified EGFP molecules in vitro to generate a calibration curve for the copy number of GFP-fusion proteins. Our results yield on average ~5 Cse4s, ~2.5 inner kinetochore CBF3 complexes and ~20 outer kinetochore Ndc80 complexes per kinetchore. Our calibrated measurements increase ~2.5 fold protein copy numbers at eukaryotic kinetochores based on previous ratio measurements assuming 2 Cse4s per budding yeast kinetochore. All ~5 Cse4s may be associated with the CEN nucleosome, but we show that it is possible that ~3 Cse4s on average could be located within flanking nucleosomes at random sites that differ between chromosomes. Supported by NIGMS 24364(EDS) and 32238(KB).

Spindle Checkpoints

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Resolving the Spindle Assembly Checkpoint.

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Accurate chromosomal segregation is governed by the spindle assembly checkpoint (SAC) which functions to ensure that anaphase onset is delayed until all chromosomes are under tension on the mitotic spindle. The SAC signal is generated by a mitotic checkpoint complex (MCC) formed by interactions amongst Mad2, Mad3/BubR1, Bub3, and CDC20 which together inhibit the E3 ubiquitin-ligase anaphase promoting complex/cyclosome (APC/C). The high affinity between the MCC and APC/C protects cyclin B1 and securin from ubiquitination while the MCC is bound to the APC/C. The pathway of MCC formation and inhibition of the APC/C is understood at the level of a generally excepted working model. However, the pathway of APC/C activation via disruption of the bound MCC is not well established. Using mitotic HeLa extracts, we have examined how the checkpoint protein p31comet contributes to APC/C activation. In HeLa extracts, synergy between UbcH10, a critical E2 of the APC/C, and p31comet is observed and depends on p31comet binding Mad2. To understand this synergy we measured the in vitro activities of p31comet and UbcH10 against anaphase inhibitors. P31comet acts primarily to dissociate the free MCC complex, whereas UbcH10 acts primarily on the MCC-APC/C to disrupt this complex by ubiquitination of Cdc20. Thus the observed synergy is due to two parallel

pathways one where p31 disrupts the free MCC and the other being UbcH10 dissociating the MCC-APC/C complex. Coordinating p31comet binding to Mad2 and UbcH10 binding to the APC/C is a key regulatory step in releasing MCC from the APC/C and subsequently resolving the spindle assembly checkpoint. Indeed, computational simulations show that a kinetochore-regulated dissociation pathway is necessary for tight APC/C inhibition followed by rapid re-activation. These calculated predictions agree well with the observations regarding the long time cells can maintain mitotic arrest and the short time cells take to transition from metaphase to anaphase.

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Quantitative analysis of the spindle assembly checkpoint.

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The spindle assembly checkpoint (SAC) is a surveillance mechanism, which ensures that cells only enter anaphase once all chromosomes have become properly attached to the mitotic spindle. The proteins constituting the SAC network are known and are conserved in eukaryotes. SAC proteins enrich at unattached kinetochores and ultimately lead to the inhibition of the anaphase-promoting complex. However, the complex *in vivo* signaling pathway is still only fragmentarily understood. Mathematical modeling can be a valuable means to explore possibilities for the signaling mechanism, but requires accurate quantitative data. We have determined the relative and absolute abundance of GFP-labeled SAC proteins *in vivo* in fission yeast by quantitative fluorescence microscopy and fluorescence correlation spectroscopy. The SAC proteins differ in their abundance, but all are present in the fission yeast cell at low nanomolar concentration. To further explore the robustness of the signaling mechanism and our mathematical representation of it, we modified SAC protein abundances and compared the outcome on SAC activity. We found strong differences in the ability of the SAC to tolerate changes in the abundance of single SAC proteins, and these results shed light on the signaling mechanism. To further unravel the organization of the SAC network, we determined the hierarchy of SAC protein localization to kinetochores upon mitotic entry and SAC activation. When comparing our results to data obtained in other eukaryotes, evolutionary differences become apparent. Exploring how the conserved SAC signaling mechanism has been shaped in different organisms in adaptation to their specific needs will be an interesting area for future investigation.

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Artificial Checkpoint Activation by Overpression of Checkpoint Complexes in the budding yeast Saccharomyces cerevisiae.

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The spindle checkpoint is an elaborate system that is essential for accurate chromosome segregation. It involves proteins such as Mad1p, Mad2p, Mad3p, Mps1p, Bub1p, and Bub3p, most of which are highly conserved among eukaryotes. The absence of microtubule attachment or the lack of tension at the kinetochore (because of a failure to establish bi-orientation) activates the checkpoint, which arrests cells at the metaphase-to-anaphase transition by targeting the anaphase promoting complex (APC) and its co-activator Cdc20 for inhibition until errors are corrected. Despite numerous studies, we still do not understand how the checkpoint proteins coordinate with each other to inhibit APC-Cdc20 activities in the presence of errors at kinetochores.

To investigate how the checkpoint proteins induce metaphase arrest, we generated and express different checkpoint fusion proteins in the budding yeast Saccharomyces cerevisiae to mimic complexes that might form during checkpoint activation. Unexpectedly, we found that overexpression of Mad2-Mad3 fusion proteins arrest the cells in metaphase in the absence of obvious chromosome and spindle defects. The arrest doesn't require the presence of functional kinetochores or other checkpoint proteins. The effect of the Mad2-Mad3 construct could be due to its ability to bind and inhibit APC-Cdc20, as artificially tethering Mad2 to Cdc20 in cells can also lead to metaphase arrest. The Mad2-Mad3 construct may represent a complex formed during normal checkpoint activation and additional studies on this system will be helpful for understanding the complicated interactions among the checkpoint proteins.

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Par1b regulates mitotic spindle orientation and promotes asymmetric division in epithelial cells.

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Symmetrically dividing columnar epithelial cells align their mitotic spindle parallel to the basal domain in metaphase. However, the molecular mechanisms implicated in mitotic spindle orientation remain poorly understood. Here we show that the serine/threonine kinase Par1b controls mitotic spindle orientation along the x-z axis in columnar and hepatic epithelial cells. Par1b overexpression causes a hepatic-like phenotype in kidney-derived MDCK cells where their mitotic spindles are oriented at a 20°-45° angle rather than parallel to the substratum, and divide asymmetrically relative to the luminal domain. Tilted spindles were also found in β1-integrin disrupted MDCK cells. Interestingly, the expression of dominant-negative Par1b promotes parallel spindles and symmetric divisions in hepatic-derived WIF-B9 cells. We observed that Par1b overexpression causes a defect in the localization of endogenous collagen IV and disrupts the localization of spindle orientation-related proteins dynein, NuMA, and $p150^{glued}$ in the cell cortex during mitosis. Strikingly, extracellular collagen IV rescues the localization of these proteins at the cell cortex and promotes parallel spindles in Par1b-overexpressing and β1-integrin disrupted cells. Taken all together, we suggest that Par1b regulates both mitotic spindle orientation and type of cell division through the β1-intengrin/collagen IV signalling.

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Changes in molecular composition and kinetochore structure in response to different types of attachments.

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Each mitotic chromosome is constituted by two sister chromatids whose correct segregation to the daughter cells is ensured by amphitelic attachment, in which the two sister kinetochores (KTs) are attached to microtubules (MTs) from opposite mitotic spindle poles. Other types of KT attachment can occur in early mitosis and can cause chromosome mis-segregation and aneuploidy if not converted into amphitelic attachments before anaphase onset. These attachments include monotelic (one attached and one unattached sister KT), syntelic (both sister KTs attached to the same spindle pole), and merotelic (a single KT attached to MTs from opposite spindle poles) attachment. A biochemical pathway named the Spindle Assembly Checkpoint (SAC) is responsible for delaying anaphase onset to allow correction of KT mis-attachments. SAC activation is believed to occur due to KT localization of certain SAC proteins and/or lack of tension, but only monotelic attachment has been proven to activate the SAC. To determine if and how other types of KT attachment may activate the SAC, we investigated the

localization of certain SAC proteins, as well as members of the chromosome passenger complex at the KT/centromere of chromosomes with different types of attachment. Furthermore, we measured intra- and inter-KT stretching for chromosomes with different types of attachment, as both intra- and inter-KT stretching have been proposed to play some role in SAC signaling, correction of KT mis-attachments, or both (reviewed in Maresca and Salmon, J. Cell Sci., 2010; Nezi and Musacchio, Curr. Opin. Cell Biol., 2009). Our data suggest that monotelic attachment is the only type of attachment that can induce a SAC response thanks to the accumulation of the SAC protein Mad2 at the KT. Our data also suggest that structural changes of the KT, measured as intra- or inter-KT stretching, do not directly induce a SAC response, although they may contribute to correction of mis-attachment as previously proposed.

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Mps1/Mph1 kinase recruits SAC components to kinetohores through phosphorylation of KNL1/Spc7.

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The spindle assembly checkpoint (SAC) delays anaphase onset in the presence of unattached kinetochores. Bub1 is a well conserved SAC protein and temporarily recruited to kinetochores in mitosis. In addition to SAC function, kinetochore associated Bub1 has a role in proper chromosome segregation in part through recruiting shugoshin proteins to centromeres.The kinetochore localization of Bub1 requires its binding partner Bub3 as well as another SAC protein kinase Mps1/Mph1. But how Mps1/Mph1 regulates the kinetohore localization of Bub1 is unknown. Here, we show that Mph1 promotes the interaction of the Bub1-Bub3 complex with the kinetochore protein KNL1/Spc7 through phosphorylation of Spc7 in fission yeast. Mph1 phosphorylates Spc7 at multiple sites containing conserved MELT repeats in vitro. In cells expressing the non-phosphorylatable spc7-12A mutant, the kinetochore localization of Bub1 and Bub3 are abolished, resulting in impaired chromosome segregation and SAC. Moreover, artificial targeting of Mph1 to kinetochores or expression of the phospho-mimetic mutant spc7-12E causes ectopic localization of Bub1 and Bub3 at kinetohores in interphase. Our results suggest that Spc7 is a hitherto unknown critical target of Mph1 in both chromosome segregation and SAC.

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Loss of function of the Cik1/ Kar3 motor complex results in chromosomes with syntelic attachment that are sensed by the tension checkpoint.

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The attachment of sister kinetochores by microtubules emanating from opposite spindle poles establishes chromosome bipolar attachment, an essential step for sister-chromatid separation. The bipolar attachment generates tension on chromosomes, and lack of tension or defects in kinetochore attachment activate the spindle checkpoint to prevent anaphase onset. Tension checkpoint proteins, Aurora/Ipl1 kinase and centromere-localized Sgo1, are required to prevent anaphase entry when tension is missing, but they are dispensable for the checkpoint response to detached kinetochores. Syntelic attachment occurs when both sister kinetochores are attached by microtubules from the same spindle pole and this attachment is unable to generate tension on chromosomes, but a reliable method to induce syntelic attachments is not available. In budding yeast Saccharomyces cerevisiae, we found that the loss of function of a motor protein complex Cik1/Kar3 leads to syntelic attachments. First, we showed that the expression of the coiled-coil domain in Cik1 from a galactose-inducible promoter disrupts Cik1-Kar3

interaction, therefore the function of Cik1/Kar3 can be inactivated conditionally. With this approach, we demonstrated that inactivation of either the spindle or tension checkpoint enables premature anaphase entry in cells with dysfunctional Cik1/Kar3. In these cells, both sister chromatids move along with one spindle pole, resulting in co-segregation. Moreover, the abolished Kar3-kinetochore interaction in cik1 mutants suggests that the Cik1/Kar3 complex mediates chromosome movement along microtubules, which could facilitate bipolar attachment. Consistent with the role in chromosome bipolar attachment, the association of Cik1/Kar3 with kinetochores occurs when cells are treated with a DNA synthesis inhibitor or microtubule poison, but the association disappears when cells arrest at preanaphase or telophase. In summary, we can induce syntelic attachments by inactivating the Cik1/Kar3 complex and this approach will be very useful to study the checkpoint response to syntelic attachments.

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Both Plk1 and AurB Are Required for Assembly of a Dynein-binding Platform on Kinetochores.

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Cytoplasmic dynein is a prominent component of kinetochores from nuclear envelope breakdown to anaphase onset. During prometaphase, dynein is responsible initiating and regulating linkages to microtubules. At metaphase, dynein is responsible for silencing the spindle assembly checkpoint by removing checkpoint proteins from kinetochores through poleward streaming. Polo-like kinase 1 (Plk1) and Aurora B (AurB) are important for both of these processes, so we investigated if they regulated dynein. Inhibition of Plk1 with BTO-1 or analogue-sensitive Plk1 constructs ablated dynein phosphorylation, reduced recruitment of dynein to kinetochores and induced errors in chromosome alignment during prometaphase. Dynein-binding proteins implicated in recruiting kinetochore dynein were not affected. Based on the antagonistic roles of PP1 phosphatases and AurB homologues in yeast, we assessed the requirement for AurB in dynein recruitment. Inhibition of AurB blocked recruitment of dynein to kinetochores. However, the effects on dynein recruitment were indirect. Dynactin, spindly and ZW10 were each reduced after AurB inhibition, whereas zwint-1, Hec1 and Knl1 were not affected. Because the interaction between zwint-1 and ZW10 has been implicated in recruiting the rod-zw10-zwilch (RZZ) complex to kinetochores, we compared phosphorylation of zwint-1 and ZW10 using in vitro kinase assays. Zwint-1 but not ZW10 was phosphorylated by AurB, and a set of three novel AurB phosphorylation sites was mapped in zwint-1 by MS/MS analysis. A 3A zwint-1 mutant blocked recruitment of the RZZ complex and all RZZ-dependent proteins to kinetochores and induced prometaphase arrest. A 3E zwint-1 mutant overcame the effects of AurB inhibition on kinetochore assembly. However, the 3E mutant interfered with dynein-driven removal of checkpoint proteins at metaphase, inducing metaphase arrest. These results suggest that phosphorylation of zwint-1 by AurB is required for assembly of a dynein-binding platform at kinetochores during prometaphase. However, dephosphorylation of zwint-1 at metaphase defines the boundary between stable and streaming proteins implicated in checkpoint silencing. These studies indicate that Plk1 and AurB play critical roles in regulating kinetochore dynein.

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Dynamic regulation of kinetochore microtubules by changes in Plk1 activity.

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Plk1 (Polo-like kinase 1) is a highly conserved mitotic kinase that regulates multiple processes in cell division. Plk1 localizes to kinetochores and spindle poles but disappears from kinetochores at metaphase. The loss of kinetochore localization as chromosomes align is similar to many mitotic checkpoint signaling proteins, but Plk1 is not required for the checkpoint. Plk1 function at kinetochores and the importance of the localization change are unclear. Here we show that changes in Plk1 activity at kinetochores are critical for regulation of microtubule dynamics. Constitutive targeting of Plk1 to kinetochores suppresses microtubule dynamics and leads to reduction of both intrakinetochore and interkinetochore tension and mitotic arrest. Conversely, prematurely displacing Plk1 from kinetochores slows the initial formation of stable microtubule attachments. Using a FRET-based biosensor, we find that a Plk1 substrate at kinetochores is highly phosphorylated in the absence of microtubules and subsequently dephosphorylated as kinetochore align at metaphase. Our results reveal that kinetochore microtubules are regulated by a dynamic balance between the destabilizing activity of Aurora B and the stabilizing activity of Plk1. Initially, Plk1 activity allows attachments to form even when Aurora B activity at kinetochores is high, and later Plk1 is removed so that microtubules remain dynamic to apply tension and satisfy the spindle checkpoint.

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COMA/CENP-H/I/K kinetochore proteins and Aurora B kinase are required to generate the spindle checkpoint.

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It is currently unclear how kinetochores generate a spindle checkpoint signal and how this is regulated by kinetochore microtubule attachment. Budding yeast cannot generate a spindle checkpoint signal in the presence of unreplicated chromosomes if the Aurora phosphorylation site on Mad3 (Mad3-S337) is mutated, but they can arrest in nocodazole. We have screened for benomyl sensitive mutants in a Mad3(S337A) background to identify proteins required to arrest cells by the putative occupancy branch of the checkpoint. Budding yeast lacking the kinetochore proteins Chl4, Ctf3 and Ctf19 arrest in nocodazole but not if they also contain the Mad3(S337A) mutation. These proteins are members of the COMA complex, which is a large set of conserved kinetochore proteins with unknown functions. The human homologs of the COMA proteins are a recently identified complex containing CENP-N, CENP-H, CENP-I, and CENP-K. We have found that after siRNA knockdown of CENP-N, CENP-H, CENP-I or CENP-K, cells arrest in nocodazole but this arrest is sensitive to low doses of the Aurora B inhibitors ZM447439 or Hesperadin. These cells can arrest in response to taxol. Mad2 binding to kinetochores is lost in cells knocked down of CENP H/I/K. We argue that the COMA/CENP H/I/K proteins have a highly conserved role to generate a spindle checkpoint signal before microtubule attachment and they function in a pathway that is redundant with Aurora B. CENP H/I/K is the first set of proteins implicated in the spindle checkpoint that are not required after microtubule attachment. In addition, it is currently controversial if the Aurora B kinase is required to generate a spindle checkpoint or only to release microtubules from kinetochores. Aurora kinase activity is activated by CENP H/I/K knockdown and cells with completely depolymerized microtubules require Aurora activity to maintain a functioning spindle assembly checkpoint when the CENP H/I/K complex is absent.

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Aurora B kinase activation requires PLK1-mediated survivin phosphorylation.

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During cell division, chromosome segregation is orchestrated by the interaction of spindle microtubules with the centromere. Accurate attachment of spindle microtubules to kinetochore requires the chromosomal passenger of Aurora B kinase complex with borealin, INCENP and survivin. The current working model argues that survivin is responsible for docking Aurora B to the centromere whereas its precise role in Aurora B activation has been unclear. Recently, we showed that Aurora B kinase activation requires survivin priming phosphorylation at Ser20 which is catalyzed by PLK1 (Chu et al., 2011. J. Mol Cell Biol.). Inhibition of PLK1 kinase activity or expression of non-phosphorylatable survivin mutant prevents Aurora B activation and correct spindle microtubule attachment. The PLK1-mediated regulation of Aurora B kinase activity was examined in real-time mitosis using fluorescence resonance energy transfer-based reporter and quantitative analysis of native Aurora B substrate phosphorylation. We reason that the PLK1-mediated priming phosphorylation is critical for orchestrating Aurora B activity in centromere which is essential for accurate chromosome segregation and faithful completion of cytokinesis. Currently, we are delineating the spatiotemporal dynamics of Aurora B activity during kinetochore maturation and spindle microtubule attachment error correction.

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CDK-dependent potentiation of MPS1 kinase activity is essential to the mitotic checkpoint.

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Accurate chromosome segregation relies upon a mitotic checkpoint that monitors kinetochore attachment towards opposite spindle poles before enabling chromosome disjunction. MPS1/TTK is a protein kinase essential to this process, but the mechanisms underlying its regulation during mitosis remain elusive. By mass spectrometry, we mapped several phosphorylation sites onto MPS1 and we show that phosphorylation of one site in the non-catalytic region of MPS1 is required for full kinase activity. Strikingly, this phosphorylation potentiates MPS1 catalytic efficiency without impairing its affinity for the substrates. Using Xenopus egg extracts depleted of endogenous MPS1 and reconstituted with single point mutants, we show that phosphorylation of that site is essential to activate the mitotic checkpoint. This phosphorylation does not regulate the localization of MPS1 to the kinetochore, but is required for the recruitment of MAD2, demonstrating its role at the kinetochore. This phosphorylation is not sufficient to prevent the metaphase-to-anaphase transition in the absence of kinetochore-dependent signaling. Nevertheless, this essential phosphorylation of MPS1 is mediated by CDK1 in the cytoplasm independently of the kinetochore. Altogether, this study uncovers a new step of amplification of the checkpoint signal via a CDK-dependent phosphorylation of MPS1.

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MPS1 kinase activity is essential for C-MAD2 production and assembly of the mitotic checkpoint complex (MCC).

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The spindle assembly checkpoint (SAC) is an evolutionarily conserved mechanism that preserves genomic integrity through regulating proper timing of the metaphase-to-anaphase transition during cell division. Closed MAD2 (C-MAD2), one of the two native conformations adopted by the checkpoint protein MAD2, is a well-recognized component of the "wait anaphase" signal generated in response to SAC activation. However, it has been controversial whether MAD2 is an integral component of the mitotic checkpoint complex (MCC), the suggested SAC effector that potently inhibits the anaphase promoting complex/cyclosome (APC/C). We have recently provided strong evidence demonstrating that C-MAD2 (but not the inactive O-MAD2) is indeed incorporated into the MCC. Intriguingly, selective incorporation of C-MAD2 into the MCC arises not only from the previously characterized CDC20:C-MAD2 interaction, but also from a novel direct interaction between C-MAD2 and BUBR1. The C-MAD2:BUBR1 interaction has been observed both *in vitro* and *in vivo*, and is fundamental for both assembly and inhibitory activity of the MCC (Tipton AR et al., JBC, 286:21173). Here we report that MPS1, a classical SAC kinase, functions through regulating MCC assembly, yet appears to have no role in maintaining the stability of pre-assembled MCC. Inhibition of MPS1 kinase prevents MAD2 incorporation into the MCC, simultaneously impairing both CDC20:MAD2 and BUBR1:MAD2 interactions but having no apparent effect on the BUBR1:CDC20 interaction. Strikingly, the impairment caused by MPS1 inhibition can be rescued by expressing a C-conformation locked MAD2 mutant in mitotic cells. C-MAD2 incorporation into the MCC correlates with MCC:APC/C binding, APC/C inhibition and SAC function. We propose a model whereby MPS1 kinase contributes to the SAC through promoting C-MAD2 production and subsequent MCC assembly.

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Tpr promotes the formation of the Mad1-cMad2 template required to amplify and sustain spindle assembly checkpoint response.

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Translocated promoter region (Tpr) is a highly conserved nuclear pore complex protein involved in nuclear transport. Recently, we and others have uncovered a novel role of human Tpr, and respective orthologs in Drosophila and fungi, in the spindle assembly checkpoint (SAC). This is likely to rely on a functional interaction between Tpr, Mad1, Mad2 and Mps1, however, the precise mechanism remains unknown. By using conformation-specific antibodies here we show that human Tpr promotes the dimerization between Mad1 and the closed conformer of Mad2 (c-Mad2), being rate-limiting in the formation of the Mad1:c-Mad2 template before it reaches the kinetochore. Consistent with this function, Tpr depletion prevented normal Mad1 and c-Mad2 recruitment to nuclear pores, but Mad1 targeting to kinetochores was independent of Tpr. Due to a limited amount (50%) of the Mad1:c-Mad2 template at kinetochores, the conversion of the open-Mad2 (o-Mad2) conformer into new c-Mad2 at the basis of the signal amplification cascade was also compromised. In agreement, Tpr depleted cells exited mitosis faster than normal and were not able to sustain a prolonged SAC response. Importantly, this was independent of the role of Tpr in mRNA transcription/export and rather reflected a role in the stabilization of SAC proteins. Finally, we show that Mps1 spatially regulates Tpr localization

during mitosis, independent of its kinase activity and Mad1:c-Mad2. Overall, our data implicate Tpr in the formation of the Mad1:c-Mad2 template required to amplify and sustain SAC response.

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A Role for the KNL1-PP1 Interaction at the Kinetochore in Coupling Microtubule Attachment to SAC Silencing.

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Accurate chromosome segregation in mitosis is necessary to ensure that each daughter cell gets a full complement of the genome. This is accomplished by a pair of sister kinetochores on each chromosome attaching to microtubules emanating from opposite poles of the spindle (biorientation). The spindle assembly checkpoint (SAC) inhibits anaphase onset until all kinetochores establish bipolar attachment. A network of protein kinases, localized to centromeres and kinetochore, are required for SAC activation, while phosphatase protein phosphatase 1 (PP1) is required to silence the SAC. We have recently shown that the evolutionarily conserved PP1-binding motif at the N-terminal region of the kinetochore protein KNL1 (Spc105 in budding yeast) is essential for silencing the SAC. However, constitutive PP1-Spc105 interaction alone is insufficient to silence the SAC in the absence of microtubules. While constitutive PP1-Spc105 interaction itself has no impact on cell cycle progression or chromosome segregation, targeting one extra copy of PP1 to Spc105 is detrimental. These results indicate that PP1 activity at the kinetochore must be both finely tuned and coupled to kinetochore-microtubule attachment so that the SAC is silenced only when kinetochores accomplish bipolar attachment. To understand the molecular basis for the coupling, we are currently systematically investigating how the phosphorylation level of different kinetochore proteins is affected by kinetochore-microtubule attachment and the PP1-Spc105 interaction. Furthermore, our preliminary data indicate that the N-terminal region of Spc105 plays an important role for cell proliferation other than its function for PP1 recruitment and SAC silencing. Together, our studies will help understand how phosphorylation and dephosphorylation reactions at the kinetochore are spatiotemporally coordinated with microtubule attachment status for proper SAC signaling.

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Structure Function Analysis of the mitotic checkpoint kinetochore protein, Spindly.

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Spindly was shown to recruit dynein to the kinetochore in Drosophila S2 cells and the dynein/dynactin complex in human cells. The dynein/dynactin complex is required for alignment of chromosome during prometaphase and silencing the mitotic checkpoint following the proper attachment of chromosomes by mediating the transport of the checkpoint proteins off the kinetochore. Recruitment of Spindly to the kinetochore is dependent on the RZZ (Roughdeal, Zeste-White10 and Zwilch) complex in Drosophila, C. elegans and human cells. Spindly is localized to kinetochores at prometaphase and once the chromosomes are aligned properly on the metaphase plate, it is translocated to the poles. Little is known about the interactions of Spindly or its mechanism of function. It has been shown that Spindly mediates the mitotic checkpoint signaling by acting as an adaptor between RZZ and the dynein complex but its interacting partners are not yet known. The aim of the study is to characterize the structural and functional domains of Spindly. Spindly consists of two coiled coil domains, which are separated by a conserved spindly box. We have made 18 insertion, 16 truncation, 15 site-directed and 6 phospho Spindly mutants. These mutants were transfected into HeLa cells followed by fluorescence microscopy analysis. The kinetochore localization of Spindly is found to be

dependent on both N and far C-terminal regions, which correspond to the data, published by Barisic et al. Also, we are looking into the potential interacting partners of Spindly using yeast 2-hybrid and pull down assays. We are also interested in how Spindly is recruited to kinetochore by RZZ complex and dynein/dynactin recruitment by Spindly.

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Novel Role for a Mitotic Checkpoint Protein in the Termination of Vacuole Inheritance.

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The initiation and termination of organelle inheritance is coordinated with the cell cycle. In *Saccharomyces cerevisiae*, organelle specific adaptor proteins in conjunction with the myosin V motor, Myo2, play a key role in this coordination. To date, the best characterized example is vacuole inheritance, where the vacuole specific adaptor, Vac17, attaches the vacuole to Myo2. Regulation of the interaction between Vac17 and Myo2 both initiates and terminates vacuole movement.

Early in the cell cycle, Vac17 is phosphorylated by the cyclin dependent kinase, Cdk1, which promotes its interaction with Myo2 and initiates vacuole movement. Once the vacuole arrives in the bud, Vac17 dissociates from Myo2 and vacuole movement is terminated even though Myo2 continues to move other organelles to different places. We sought to determine the mechanisms that signal the vacuole to detach from Myo2. We found that further post-translational modifications of Vac17 regulate its dissociation from Myo2 and terminate vacuole inheritance.

In a random mutagenesis screen of Vac17 we identified two new phosphorylation sites, Ser222 and Thr240, which are required for the regulated dissociation of Vac17 from Myo2. Moreover, in an EMS mutagenesis screen we identified Dma1, an E3 ubiquitin ligase and spindle positioning checkpoint protein, as a novel regulator of the detachment of the vacuole from Myo2. We found that Dma1 binds specifically to phosphorylated Vac17-T240 but not mutant vac17-T240A. Furthermore, we show that Dma1 localizes to the vacuole and colocalizes with the Myo2/Vac17 vacuole transport complex. Expression of the enzymatically inactive mutant, dma1-I329R, results in the constitutive attachment of Vac17 and the vacuole to Myo2. Together, these results suggest that phosphorylation of Vac17 at Thr240 recruits Dma1 to the vacuole transport complex where Dma1 ubiquitinates an as yet unknown substrate and thus signals the dissociation of Vac17 from Myo2. Dma1 is a mitotic checkpoint protein, therefore it is tempting to speculate that Dma1 coordinates the termination of vacuole movement with mitotic exit.

Signaling Receptors (RTKs and GPCRs)

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Thrombin promotes cyclin D1 upregulation and RPE cell proliferation through the activation of Akt.

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The retinal pigment epithelium (RPE) forms the outer blood-retina barrier (BRB) and plays an essential role in the survival of retinal neurons and the maintenance of visual function. Proliferative ocular pathologies are characterized by the epithelial-mesenchymal transition, proliferation and migration of RPE cells, eventually, leading to blindness. Existing evidence

suggests that exposure of RPE cells to serum-contained thrombin upon BRB breakdown may be responsible for the development of these diseases.
The serine protease thrombin stimulates growth factor secretion and mitogenic activity in several cell types, acting on a family of protease-activated receptors (PARs 1-4), coupled to G-proteins Gq, Gi/s and G12/13. We have previously shown that thrombin promotes cyclin D1 expression, the progression of cell cycle, and the proliferation of RPE cells. The present study was aimed to identify the molecular mechanism leading to thrombin-induced cyclin D1 expression in RPE.
Results demonstrate that: 1) thrombin stimulates Ser 473 Akt phosphorylation without affecting Thr 308 basal phosphorylation; 2) thrombin-induced Akt activation indirectly promotes cyclin D1 accumulation through the phosphorylation/inhibition of GSK-3β, which prevents cyclin D1 phosphorylation, nuclear export and degradation; 3) Akt signaling requires the upstream activation of PLC and PI3K. Since pharmacological inhibition of these pathways or the silencing of cyclin D1 expression prevent proliferation, these results contribute potential information for preventing the development of proliferative eye diseases. This work was partially supported by Grants IN228203 (PAPIIT, U.N.A.M) and CB-80398 (CONACyT) to A.M.L.C.

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ARRDC2: a Human Arrestin-Related Protein with a Potential Role in Cargo Protein Trafficking within the Endo-Lysosomal System.

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β-Arrestins are essential adaptors for G-protein coupled receptor (GPCR) trafficking. Recently, arrestin-related proteins have been identified in yeast/fungi that, similar to β-arrestins, recognise cargo proteins and mediate their intracellular trafficking. In mammals, five largely uncharacterized arrestin domain-containing (ARRDC) proteins display a predicted arrestin fold; here we focus on human ARRDC2. Confocal microscopy was used to determine the subcellular localization of ARRDC2. In cultured cells (osteosarcoma; U2OS) transiently transfected fluorescent protein-tagged ARRDC2 localized to punctate vesicular structures throughout the cytoplasm and the plasma membrane (PM). ARRDC2 puncta colocalized with late endosomal/lysosomal markers (Rab7, Lamp1, LysoTracker) but not early/recycling endosomal markers (Rab5, EEA1, transferrin, Rab11). However, overexpression of constitutively active Rab5 or Rab7 mutants (commonly used to block transport through early or late endosomes, respectively) resulted in ARRDC2 accumulation within Rab mutant-positive endosomes. These data suggested that ARRDC2 predominantly localizes to late endosomes/lysosomes, but may also be dynamically distributed throughout the endocytic system. Biochemical studies were next used to investigate ARRDC2 interactions with potential trafficking related partners. ARRDC2 coimmunoprecipitated with several members of the homologous to E6-AP C-terminus (HECT) E3 ligase family. These included WW domain-containing ubiquitin protein ligase-1 (WWP1) which, when coexpressed with ARRDC2 and imaged by confocal microscopy, changed from a diffuse cytoplasmic distribution to a more punctate pattern that colocalized with ARRDC2. ARRDC2 contains two proline-proline-x-tyrosine (PY) motifs that we hypothesized might bind WW domains in the HECT ligases. Accordingly, mutation of both ARRDC2 PY motifs (ARRDC2ΔΔ) abolished the interaction detected with one of the ligases, NEDD4-like ubiquitin protein ligase-1 (NEDL1), and ARRDC2ΔΔ was no longer able to induce WWP1 redistribution. Coimmunoprecipitation was also used to detect basal ubiquitination of ARRDC2; this was unaltered in ARRDC2ΔΔ implying that ARRDC2 ubiquitination is not dependent upon ARRDC2-HECT ligase interaction. Interestingly, ARRDC2 also coimmunoprecipitated with itself and β-arrestin2, suggesting that the purported ability of β-arrestins to oligomerize may also extend to the ARRDC proteins. In conclusion, we have identified ARRDC2 as a human arrestin-related

protein localized to the endo-lysosomal system. The distant homology of ARRDC2 to β-arrestins, ARRDC2 interaction with multiple E3 ubiquitin ligases, and the potential for ARRDC2 to homo-/hetero-oligomerize with itself and β-arrestins, collectively point towards a potential role for ARRDC2 in cargo protein trafficking that merits further investigation.

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Melanin-concentrating hormone receptor signaling is sensititve to both β-Arrestin levels and Caveolae.

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The melanin-concentrating hormone signaling pathway is a candidate in the pharmacological treatment of eating and appetite disorders. G protein-coupled receptors for MCH are expressed in the central nervous system and peripheral tissues such as the pancreas and adipose tissue. Unfortunately, little is known about how these receptors signal and desensitize. Investigations by other groups point to clathrin-mediated endocytosis as a desensitization mechanism for MCHR1, however our initial observations of little to no visible internalization of MCHR1 in BHK570 cells led us to question this conclusion. We began with measurements of VSVg-tagged MCHR1 surface levels following timed MCH treatments by cell-based ELISA. This resulted in barely 15% of MCHR1 internalizing following 30 min agonist exposure; which was undetectable by fluorescence microscopy. We next co-transfected GFP β-arrestin 1 or β-arrestin 2 to see if the potential for MCHR1 internalization existed and indeed we observed up to 40% loss of surface receptor via both methods. Overexpression of GRK2 resulted in a partial recovery while dominant-negative GRK2 had no effect. Therefore MCH signaling is influenced by the levels of both arrestins and GRK2. We have previously identified caveolae as a potential mediator of MCH signaling and our working hypothesis is that these membranes act to enhance MCH signaling in some cell types. We previously reported pharmacological depletion of cholesterol decreases, but does not eliminate ERK1/2 phosphorylation by MCH in CHO cells. In this study we also reaffirm co-localization of MCHR1 in caveolar membranes biochemically by several different extraction methods and begin to explore the effects of its depletion on MCH-signaling. For example, MCH-mediated activation of a leptin promoter is enhanced in cells expressing a RNAi-plasmid targeting Cav-1, which specifically depletes caveolae. While this seems contradictory, it may signify differences in the regulation of MCHR1 by membrane microdomains for short- and long-term signaling events. Future experiments will extend these studies into an adipocyte cell culture model with variable caveolae membrane content depending upon differentiation state.

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G Protein-Coupled Receptor Kinases 2 and 3 Regulate CCR7 Internalization.

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C-C chemokine receptor 7 (CCR7) targets migration of T cells to the lymph nodes. While the two known agonists for CCR7, CCL19 and CCL21, internalize to differing extents, the underlying molecular mechanisms regulating CCR7 internalization and downstream signaling in T cells to control migration, remain largely undefined. G protein coupled receptor kinases (GRKs) can regulate receptor internalization, and their functions are largely dependent upon the relative levels that are expressed in different cells. To characterize the roles of GRKs in the internalization of CCR7 in T cells, we used wild type (WT), GRK2+/- and GRK3-/- primary murine cells. Unlike WT murine T cells that internalized only ~38% of CCR7/CCL19 and failed

to internalize CCR7/CCL21, we found that GKR2+/- T cells internalized ~57% of CCR7 in the presence of either ligand. In contrast, GRK3-/- cells failed to internalize CCR7 independent of ligand. To determine if the levels of GRKs expressed control CCR7 internalization we used HEK293T cells to titrate GRK2-GFP or GRK3-GFP levels and assayed for CCR7 internalization. We found that low levels of GRK2 promoted CCR7 internalization, while higher levels stabilized CCR7 surface expression. Expression of GRK3 promoted CCR7 internalization. We characterized the effect of GRK2 on T cell migration and found that while WT murine T cells migrated to both CCR7 ligands, GRK2+/- cells migrated only in the presence of CCL19 and GRK3-/- cells failed to migrate independent of ligand. Taken together, these results indicate that the agonist-induced internalization and downstream signaling which controls T cell migration via CCR7 is regulated by GRK2 and GRK3.

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Dependence of the mating pathway on components of the Gpr1p-mediated glucose sensing pathway in *Saccharomyces cerevisiae.*

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Haploid strains of *Saccharomyces cerevisiae* express two unique GPCRs, a pheromone receptor (Ste2p or Ste3p) and a glucose sensor (Gpr1p). GPCR-mediated pathways in other organisms have been found to interact with one another either through direct contact between the GPCRs or through interactions between downstream elements. This research focused on identifying the components of the Gpr1p-mediated glucose sensing pathway required for full activation of the pheromone sensing pathways in Mata and Matα *S. cerevisiae*. To achieve this, the ability of strains lacking various components of the Gpr1p-mediated glucose-sensing pathway to undergo growth arrest and shmooing in response to α-factor, mate with wild-type cells, and activate fus1 transcription was determined. Mating efficiency is significantly reduced in Mata cells lacking Gpr1, Gpa2, Plc1, Asc1, Gpb1, and Gpb2, all of which encode components of the Gpr1p-mediated glucose sensing pathway. Preliminary data indicates that Mata strains lacking components of the Ras signaling pathway also had reduced mating efficiency. Preliminary data also indicates that Matα strains lacking Gpa2 and Gpr1 have reduced mating efficiencies compared to wild-type cells. In many of the Mata strains examined, including strains lacking Gpr1 and Gpa2, shmoo formation and growth arrest in response to α-factor were not depressed to the same degree as mating efficiency, narrowing down the possible mechanisms by which glucose-sensing impacts mating efficiency. In addition, a Fus1-lacZ assay was used to demonstrate that deletion of Gpr1 in Mata cells reduces expression of Fus1, and addition of Gpr1 on a plasmid can restore fus1 expression to wild-type or higher levels. Overexpression of Gpr1 in wild-type cells leads to increased expression of Fus1. Together, these data indicate that components of the Gpr1p pathway are required for proper function of the Ste2p and Ste3p pathways

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Heat Shock Analysis of the Interdependency of Pheromone and Glucose Sensing G-Protein Coupled Receptor Pathways in *Saccharomyces cerevisiae.*

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In *Saccharomyces cerevisiae*, the G-protein coupled receptors (GPCR) Gpr1p and Ste2p/Ste3p are known to initiate signaling through two separate pathways, the glucose sensing pathway and pheromone sensing pathway, respectively. One known role of the glucose-sensing pathway is to cause the cell to exhibit an increased sensitivity to heat shock when introduced to high concentrations of glucose after a period of starvation. The pheromone-sensing pathway does

not share this characteristic. However, research in our lab has indicated that disruption of the Gpr1 pathway negatively affects mating in Mat**a** cells. In an attempt to determine if the reciprocal effect might be present, mutants of Matα and Mat**a** cells lacking components of the pheromone pathway were assayed for their sensitivity to heat. The results show that Matα cells exhibit no increased resistance to heat shock when the components of the pheromone-sensing pathway are removed, because the relative survival of cells after heat shock is approximately equal to the relative survival of wild type cells. On the other hand, Mat**a** cells can exhibit between a 5 to 25-fold increase in relative survival or resistance to heat shock when components such as STE2, STE4, STE5, STE7, STE11, STE18, and FUS3 are removed from the pheromone-sensing pathway. These results support the hypothesis that in Mat**a** cells (but not Matα cells) there is a connection between the components of the pheromone and glucose sensing pathways that directly affects the cell's response to heat shock in the presence of glucose. The interaction point between the two pathways is certainly crucial in regard to the heat shock stress response and, thus, is likely to play an important role controlling the many additional functions that each GPCR pathway executes.

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CNP-1 (ARRD-17), a novel substrate of calcineurin, is critical for modulation of egg laying and locomotion in response to food, and lysine sensation in Caenorhabditis elegans.

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CNP-1 (ARRD-17), a novel substrate of calcineurin, is critical for modulation of egg laying and locomotion in response to food, and lysine sensation in *Caenorhabditis elegans.*
Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase involved in calcium signaling pathways. In *Caenorhabditis elegans*, the loss of calcineurin activity, causes pleiotropic defects including hyperadaptation of sensory neurons, hypersensation to thermal difference and hyper-egg-laying when worms are re-fed after starvation. In this study, we report on *arrd-17* as *calcineurin interacting protein-1* (*cnp-1*), which is a novel molecular target of calcineurin. CNP-1 interacts with the catalytic domain of the *C. elegans* calcineurin A subunit, TAX-6, in a yeast two-hybrid assay, and is dephosphorylated by TAX-6 *in vitro*. *cnp-1* is expressed in ASK, ADL, ASH and ASJ sensory neurons as TAX-6. It acts downstream of *tax-6* in regulation of locomotion and egg-laying after starvation, and lysine chemotaxis, which is known to be mediated by ASK neurons. Altogether, our biochemical and genetic evidence indicates that CNP-1 is a direct target of calcineurin and required in stimulated egg-laying and locomotion after starvation, and attracts to lysine, which is modulated by calcineurin. We suggest the phosphorylation status of CNP-1 plays an important role in regulation of re-fed stimulating behaviors after starvation and attraction to amino acid, which provides valuable nutritious information.

Key Words: calcineurin, CNP-1, ARRD-17, ASK

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Constitutive Activity of Orphan G Protein Coupled Receptors.

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Constitutive signaling in the absence of an activating ligand has been reported in over 60 members of the G-protein coupled receptor family (GPCRs). The signaling pathways of orphan GPCRs are difficult to characterize because their native activators are unknown, a limitation that can be partially overcome when the receptors exhibit constitutive activity. Thirty-six Class-A orphan GPCRs were screened for constitutive activity using luciferase coupled reporter vectors. Orphan GPCRs and reporter plasmid pairs were transiently co-transfected into CHO-K1 cells (40,000 cells in 96-well plates) and grown for 24 hours before measuring luciferase expression. Experiments were performed three times in quadruplicate. Receptor constitutive activity was reflected by induction of luciferase expression under the control of either the cAMP response element (CRE) or the Nuclear Factor of Activated T-Cell response element (N-FAT). Gαs pathway activity increased expression under control of the CRE promoter; Gαi pathway activity inhibited forskolin-induced expression under control of the CRE promoter; Gαq/11 pathway activity increased expression under control of the calcium-sensitive NFAT promoter. Most of the orphan receptors (86%; 31 of 36) exhibited some form of constitutive activity: 31% of the receptors more than tripled CRE mediated expression through the Gαs pathway; 64% of the receptors inhibited (by more than 50%) forskolin-induced CRE mediated expression through the Gαi pathway; and two receptors more than doubled N-FAT mediated expression through the Gαq pathway. Seven of the receptors increased signaling through both the Gαs and Gαi pathways (i.e., both increased CRE mediated expression over baseline and decreased forskolin-stimulated CRE mediated expression). Interestingly, 8 receptors increased Gαi signaling while decreasing basal CRE and N-FAT mediated expression. These findings suggest that constitutive activity plays a prominent role in the physiological activities of the majority of orphan GPCRs.

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A Novel Mechanism of Signal Transduction: Oxytocin Receptors Activated by the Ligand Translocate in the Nucleus to Enhance Osteoblast Transcription.

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Oxytocin (OT), acts through a seven-transmembrane G-protein coupled receptor OTR, and exert a direct anabolic effect on bone (Tamma et al., 2009) upregulating the expression of osteoblast markers and transcription factors as Osterix, Schnurri 2 and 3, Atf-4, Osteocalcin and Osteopontin. OT or OTR null mice develop by the time a reduced bone mass ascribable to a low turnover osteoporosis. OTRs are activated by OT signal through Gq/11, but we unexpectedly found after OT stimuli (15-30') OTRs largely relocated in osteoblast nuclei as identified by western blot analysis of cytoplasmic and nuclear extracts. These results were confirmed by immunogold identification of OTR in osteoblasts nucleus with TEM and by confocal immunofluorescence studies. Thus we asked two questions 1) how a GPCR receptor is transported to nuclei and 2) to do what.

Results 1) Within 2-3 min from OT treatment in primary osteoblasts OTRs were colocalized with â-arrestin1/2, thereafter, after â-arrestins detachment, were found colocalized with rab5 in the early endosomal compartment. Eventually the vesicles were taken in charge by transportin-1

and sorted to the nuclei. All these data were obtained by confocal microscopy of OTR-GFP in fixed and in living cells. MALDI-TOF analysis, was performed on nuclear proteins immunoprecipitated with anti-OTR 30' after OT treatment and revealed the presence of four peptides corresponding to OTR intracellular loops, further giving strong evidence of OTR nuclear localization.
Results 2) â-arrestin1/2 and Transportin-1 silencing in osteoblasts not only affected OTR nuclear localization but prevented OT induced up-regulation of several genes as ATF-4,Osterix and osteocalcin. Immunoprecipitation of OTR or the osteoblast transcription factor Runx2, and the transcription co-activator Schnurri-2 from nuclear lysates after OT treatment, demonstrated that the three proteins immunoprecipitated together. Furthermore an OTR/Smad-4 interaction was also found when osteoblasts were simultaneously stimulated with OT and BMP2. A molecular analysis of the receptor protein sequence led us to identify three putative nuclear localization sequence (NLS) in the first and second loop of OTR, and in the C-terminal tail. Surprisingly, however, the deletion of any NLS did not block its nuclear localization, suggesting that OTR translocation could be mediated by OTR-another associated protein as Schnurri-2, or Smad-4.
Conclusion. Some OT anabolic effects on osteoblast activities are mediated by a novel mechanism involving OTR nuclear interaction with the BMP-2/ Runx2 pathway with a positive regulatory effect.

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Single molecule studies reveal insights on the nature of constitutively activated HER2 on living cells.

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HER2 is a highly active kinase that is distinguished from the other HER family members in that no ligand has been identified to directly bind the receptor. Although HER2 can be trans-activated by forming complexes with other receptors, it exhibits constitutive activation when it is over-expressed. This ligand-independent activation plays an important role in driving the growth of HER2 amplified tumors. To gain further mechanistic understanding of the constitutive HER2 activation, we performed single molecule tracking studies of HER2 and its mutants, also further developing new analyses tools. From these studies, we found that activation of HER2 is less regulated by the structural features of its ectodomain than for EGFR as we previously demonstrated. Rather, HER2 activation may be largely related to its density, which in turn is affected by its interaction with membrane compartments. Indeed, we found that the variation in local HER2 densities is associated with HER2 phosphorylation. It appears that some of the membrane compartments modulating HER2 density and activation of the receptor are cholesterol rich regions. Alteration of the cholesterol content of the membrane changed the diffusion behavior and distribution pattern of HER2 and its phosphorylation status. We are now examining how these HER2 density changes might affect tumor cell responsiveness to trastuzumab.

1801

ErbB1 Dimerization is Promoted by Domain Co-confinement and Stabilized by Ligand-binding.

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Ligand-induced signaling by the epidermal growth factor receptor (EGFR/HER1/erbB1) drives cell growth and survival, with roles in normal development and disease pathogenesis. A wealth of structural knowledge supports a model of signal initiation through the formation of back-to-back erbB1 dimers. However, the size and ligand-occupancy of the erbB1 signaling complex remains controversial.

Imaging technologies and biological tools have developed to a point where many fundamental biological questions can now be addressed at the molecular level. In particular, single particle tracking (SPT) using bright and photostable quantum dots (QDs) provides information about protein dynamics with high spatial (~10 nm) and temporal (>30 Hz) resolution. We used two-color QD tracking to directly visualize erbB1 homodimerization and determine the dimer off rate on living cells. Kinetic parameters were extracted using a 3-state Hidden Markov Model to identify transition rates between free, co-confined, and dimerized. We report that erbB1 homodimers composed of two ligand-bound receptors are long-lived and their off rate is independent of kinase activity. By comparison, unliganded erbB1 homodimers have >4-fold faster off rates. These results unequivocally link ligand occupancy to dimer stability. Transient co-confinement of receptors by the actin cytoskeleton is shown to promote repeated encounters, enhancing dimer formation. Mobility decreases when ligand-bound receptors form dimers. Inhibition of erbB1 kinase activity or disruption of the actin cytoskeleton results in faster diffusion of receptor dimers. These results implicate both signal propagation and the cortical cytoskeleton in reduced mobility of signaling-competent erbB1 dimers.

1802

Regulation of EGFR Degradation by Ankyrin105.

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Growth factors can regulate a variety of cellular processes, including cell growth, proliferation, differentiation, survival and migration, by activating receptors on the cell surface. Many of the receptors belong to the receptor tyrosine kinase (RTK) family, such as platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) and its related family members (e.g. ErbB2). Activated RTKs are rapidly endocytosed. Most of them are recycled back to the cell surface and only a small fraction may be diverted to late endosome and lysosome for degradation. Continuously activated RTKs and downstream signaling pathways (PI3K/Akt and Ras/MAPK pathways) are frequently observed in cancer cells. Therefore, understanding the degradation mechanism of receptors is important and may facilitate the development of new prognostic or treatment strategies. Ankyrin105 (Ank105) is the smaller isoform of Ankyrin3, and is localized to late endosomes and lysosomes. Our lab has previously shown that Ank105 can stimulate degradation of PDGFR and thus reduce its signaling. To determine whether Ank105 can induce degradation of multiple RTKs similarly, we have now extended these studies to EGFR and its signaling. We introduced HA-tagged Ank105 into several cell lines that endogenously express EGFR, and carried out EGF stimulation time courses. Cell lysates were immunoblotted with antibodies specific for phosphotyrosine, EGFR

and downstream signaling proteins to test if Ank105 expression affects the degradation kinetics of EGFR. Interestingly, some cell types were susceptible to Ank105 facilitated EGFR degradation whereas others were not. These studies will improve our understanding of how Ank105 affects EGFR degradation.

1803

Mechanisms of EGF receptor internalization.

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Endocytosis is the major regulator of epidermal growth factor receptor (EGFR) signaling. However, the molecular mechanisms of EGFR endocytosis through clathrin coated pits remain to be poorly understood. We have recently demonstrated that clathrin-dependent internalization of activated EGFR is regulated by multiple redundant and partially interdependent mechanisms. Mutations of 21 lysines, that are modified by ubiquitination and acetylation, together with mutations of AP-2 binding motifs yielded the first internalization-defective EGFR mutant, 21KRΔAP2, with functional kinase and normal tyrosine phosphorylation. To define the mechanism of residual internalization of 21KRΔAP2, siRNA analysis was performed in human adenocarcinoma HuTu-80 cells lacking endogenous EGFR. Knock-down of clathrin heavy chain and dynamin strongly inhibited internalization of 21KRΔAP2 indicating that this mutant is internalized via clathrin coated pit pathway. This conclusion is also supported by partial inhibition of the 21KRΔAP2 mutant endocytosis by depletion of FCHo1, FCHo2 or CALM, proteins involved in the general clathrin-dependent endocytic process. As in the case of wild-type EGFR, internalization of the 21KRΔAP2 was strongly inhibited by siRNA knock-down of the Grb2 adaptor protein. Because Grb2 is known to mediate receptor ubiquitination, we first tested whether Grb2 role in 21KRΔAP2 endocytosis is ubiquitination-independent by depleting Grb2-binding protein Tom1L, that has been previously implicated in EGFR internalization by others. However, Tom1L depletion did not affect internalization of wild-type and 21KRΔAP2 mutant EGFRs. Surprisingly, depletion of the E2 ubiquitin-conjugating enzyme UbcH5b/c strongly inhibited internalization of the 21KRΔAP2 mutant, suggesting that the ubiquitination reaction is involved, despite that the mutant is not ubiquitinated. Moreover, knockdown of the E3 ubiquitin ligase c-Cbl also decreased EGF internalization rates in HuTu80 cells. Based on these results we hypothesize that in the absence of lysine residues and AP-2 binding motifs in the EGFR, the receptor is still internalized by means of Cbl/UbcH5 dependent ubiquitination of a protein associated with the receptor.

1804

Determining if spatio-mechanosensitivity of EphA2 signaling stems from physical impedance of endocytosis.

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We recently found that the spatial organization of the receptor tyrosine kinase EphA2 plays a key role in signal transduction in breast epithelial cancer cells. Upon stimulation from its native ligand ephrinA1 bound to a supported lipid membrane, EphA2 is spatially reorganized into large clusters at the junction between the cancer cell and supported membrane. Using the spatial mutation technique, in which nanopatterned structures on the supported membrane substrate impose physical restrictions to movement of EphA2-ephrinA1 clusters, alterations in

downstream signaling events as a function of EphA2 organization were discovered. In particular, recruitment of the disintegrin and metalloprotease 10 (ADAM10) to the clusters is inhibited. ADAM10 has been implicated in the cleavage and subsequent endocytosis of other ephrin ligands, and may play a role in the endocytosis of EphA2-ephrinA1. Here we report that dynamin2, a large GTPase involved in the scission of endocytic vesicles, is recruited to the EphA2-ephrinA1 clusters. Preliminary evidence suggests that clathrin-mediated endocytosis (CME) is involved in the initial endocytosis of EphA2-ephrinA1. Finally, we have also found that the function of ADAM10 is necessary for ephrinA1 endocytosis. The implications of these observations for spatial regulation of EphA2 signaling will be discussed.

1805

Internalization and traffic of the macrophage colony stimulating factor receptor.

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Macrophage colony stimulating factor receptor (MCSFR aka CSF-1R) is essential for normal development and function of macrophages. Additionally, hyper activaiton of the MCSFR is involved in various leukemias. Here, we define the endocytic trafficking and down-modulation pathways of the MCSFR in a bone marrow derived macrophage model. To visualize the MCSFR endocytosis and traffic, we developed Texas Red labeled rhMCSF (TXR-rhMCSF). Additionally, live cell fluorescent microscopy and immunostaining were used to define the transport and degradation of the receptor. Through the use of inhibitors, we show that activated MCSFR complexes are internalized exclusively by clathrin mediated endocytosis. The vesicles containing the MCSFR undergo sequential maturation from nascent clathrin coated vesicles, to Rab5-positive early endosomes, to Rab7-positive late endosomes whereupon the receptor is packaged into multivescular bodies and finally to fuses with lysosomes for degradation.

1806

Honokiol blocks store operated calcium entry in CHO cells expressing the M3 muscarinic receptor.

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Honokiol, a cell-permeable phenolic compound derived from the bark of magnolia trees and present in Asian herbal teas, has a unique array of physiological actions, including the inhibition of multiple autonomic responses. In excitable tissues, honokiol displays antioxidative actions and a depression of glutamate-receptor and calcium mediated signaling. In this study, we determined the effects of honokiol on calcium signaling underlying transmission mediated by human M3 muscarinic receptors expressed in Chinese hamster ovary (CHO) cells. Receptor binding was determined in radiolabelled ligand binding assays using [3H]N-methylscopolamine ([3H]MS); changes in intracellular calcium concentrations were determined using a fura-2 ratiometric imaging protocol; cytotoxicity was determined using a dye reduction assay. Activation of M3 muscarinic receptors with carbamylcholine induced a biphasic increase in [Ca2+]i: an initial, IP3-mediated release of Ca2+ from endoplasmic reticulum stores followed by a sustained phase of steady Ca2+ entry (i.e., store operated calcium entry, SOCE). Honokiol had a potent (EC50 ≈ 5 μM) inhibitory effect on store operated calcium entry (SOCE) that was induced by the activation of the M3 receptors. SOCE was further examined by acute exposure to thapsigarin (2 μM) in a calcium-free medium, followed by reintroduction of external calcium. Again, SOCE was severely depressed by honokiol. This effect of honokiol on SOCE was

specific, rapid and partially reversible, and was seen at concentrations not associated with cytotoxicity, inhibition of IP3 receptor-mediated calcium release, or depletion of ER calcium stores. Moreover, honokiol did not disrupt the ligand binding properties of M3 receptors as reflected in [3H]MS binding affinity, agonist binding affinity, or allosteric receptor interactions. It is likely that an inhibition of SOCE contributes to honokiol disruption of parasympathetic motor functions, as well as many of its beneficial pharmacological properties.

1807

Critical role of farnesoid X receptor (FXR) for hepatocellular carcinoma cell proliferation.

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Farnesoid X receptor (FXR) is a bile acid-activated nuclear receptor mainly expressed in liver. FXR was originally identified as a pivotal factor maintaining bile acid homeostasis, and has been recently shown to be a critical factor required for liver regeneration. The elucidation of the mechanism how FXR controls the proliferation of hepatocellular carcinoma cells is useful to establish the novel types of therapy applicable for liver cancer. Here we show that FXR plays a crucial role in the proliferation of human hepatocellular carcinoma cell line, HepG2. The treatment of HepG2 cells with FXR siRNA elevates the level of p16/INK4a expression resulting in the inhibition of cell proliferation. By contrast, FXR activation in HepG2 cells treated with the synthetic FXR ligand GW4064 reduces p16/INK4a expression and stimulates the cell proliferation. Hepatocyte nuclear factor 4 alpha (HNF4alpha), a nuclear receptor which plays a key role in regulating the expression of metabolic genes in various tissues, is known to be involved in p16/INK4a expression. Since it has been known that knockdown of HNF4alpha stimulates p16/INK4a expression, we have examined whether or not knockdown of FXR or FXR activation alters the HNF4alpha expression. In fact, knockdown of FXR downregulates p16/INK4a expression while FXR activation stimulates it, suggesting that p16/INK4a expression is regulated by FXR in HNF4a-dependent manner. The ectopic expression of the active form of Ras that causes strong activation of extracellular signal-regulated kinase (ERK) leads to the decrease in FXR expression, suggesting that FXR expression is negatively regulated via Ras/ERK pathway. In addition, FXR level is drastically lowered in HepG2 cells treated with hepatocyte growth factor (HGF), whereas it is restored in HGF-stimulated cells pretreated with the MEK inhibitor U0126. These results indicate that HGF downregulates FXR through the activation of ERK. In this study, we have suggested a novel mechanism by which hepatocellular carcinoma cell proliferation is regulated: FXR stimulates cell proliferation by suppressing the p16/INK4a expression whereas Ras/ERK pathway downregulates the FXR expression, leading to the suppressed proliferation of HepG2 cells.

1808

Differential regulation of CIDEA and CIDEC expression by insulin via Akt1/2- and JNK2-dependent pathways in human adipocytes.

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White adipose tissue (WAT) is a key organ for energy homeostasis. Excessive lipid accumulation of WAT in obesity contributes to severe diseases, such as type 2 diabetes, hypertension, cardiovascular and kidney diseases, and several types of cancer. WAT mass is determined by the number and size of adipocytes regulated by cell differentiation, apoptosis, and lipid droplet formation. Both insulin and the cell death-inducing DNA fragmentation factor-α-like effector (CIDE) family play important roles in apoptosis and lipid droplet formation, and are closely related to energy balance and obesity. Previously, we reported that CIDEA and CIDEC

are differentially regulated by insulin and contribute separately to insulin-induced anti-apoptosis and lipid droplet formation in human adipocytes. However, the upstream signals of CIDE proteins remain unclear. Here, we investigated the signaling molecules involved in insulin regulation of CIDEA and CIDEC expression, and analyzed apoptosis and lipid droplet formation. Gene expression analysis was performed by quantitative real-time PCR. Analyses of apoptosis and lipid droplet formation were performed by fluorescence microscopy of adipocytes stained with TUNEL, Nile Red, and/or DAPI. The phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and PI-103 blocked both insulin-induced downregulation of CIDEA and upregulation of CIDEC. The Akt inhibitor API-2 and the c-Jun N-terminal kinase (JNK) inhibitor SP600125 selectively inhibited insulin regulation of CIDEA and CIDEC expression, respectively, whereas the MAPK/ERK kinase inhibitor U0126 and the p38 inhibitor SB203580 did not. Small interfering RNA-mediated depletion of Akt1/2 prevented insulin-induced downregulation of CIDEA and inhibition of apoptosis. Depletion of JNK2, but not JNK1, inhibited insulin-induced upregulation of CIDEC and lipid droplet enlargement. Furthermore, insulin increased both Akt and JNK phosphorylation, which was abrogated by the PI3K inhibitors. These results suggest that insulin regulates CIDEA and CIDEC expression via PI3K, and it regulates expression of each protein via Akt1/2- and JNK2-dependent pathways, respectively, in human adipocytes. The identification of novel signaling pathways mediating insulin regulation of CIDEA and CIDEC will provide additional targets for the development of effective therapeutic strategies to combat obesity and its associated disorders.

Cell Death

1809

The N-end rule pathway counteracts cell death by destroying proapoptotic protein fragments.

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The N-end rule relates the regulation of the in vivo half-life of a protein to the identity of its N-terminal residue. Apoptosis (a specific type of cell death) in a multicellular organism makes possible the selective elimination of supernumerary, damaged or otherwise abnormal cells. Apoptotic death of a cell is mediated by nonprocessive proteases such as caspases that make specific cleavages in a number of cellular proteins. These proteases are normally dormant, but can be activated by inducers of apoptosis. Some caspase/calpain-generated protein fragments are known to be proapoptotic in that they increase the probability of apoptosis. The naturally produced proapoptotic fragments Cys-RIPK1, Cys-TRAF1, Asn-PKCδ, Asp-EPHA4, Asp-BRCA1, Asp-BCL-xL, Tyr-MET, Tyr-HEF1, Trp-ETK, Leu-LIMK1, Ile-Hpk1, Ile-MLH1, Lys-PKCθ, Arg-BID, and Arg-BIM_{EL} bear destabilizing N-terminal residues, i.e., those that can be recognized by the Arg/N-end rule pathway of protein degradation. We found that these proapoptotic fragments are, in fact, short-lived N-end rule substrates. The resulting down-regulation of proapoptotic fragments, through their degradation by the Arg/N-end rule pathway, comprises a new layer of antiapoptotic control. In agreement with this understanding, we show that even a partial ablation of the Arg/N-end rule pathway makes mice and their cells hypersensitive to apoptosis. Together, our findings indicate that the Arg/N-end rule pathway acts to counteract cell death through the degradation of proapoptotic protein fragments, thereby setting specific thresholds that prevent a transient or an otherwise weak apoptotic signal from reaching the point of commitment to apoptosis.

1810

Stress-inducible caspase substrate TRB3 promotes nuclear translocation of procaspase-3.

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Endoplasmic reticulum (ER) stress response normally promotes cell survival by reducing the accumulation of unfolded proteins through global transcriptional control, well known as the unfolded protein response (UPR). However, apoptosis is considered selected when the apoptotic pathway gains ascendancy over the adaptive pathway by overwhelming the ER stress. The mechanism controlling cell fate is not completely understood. Pseudokinase TRB3, one of the human homologs of Drosophila Tribbles, is up-regulated in a variety of cell types under various stress conditions including ER stress. It has recently been suggested that TRB3 functions as an important component of the stress response mechanism. We recently demonstrated that TRB3 was cleaved by caspase-3 (CASP3) at Asp338. To investigate the role of TRB3 cleavage in the apoptotic process, we carried out cell-based analysis using the wild-type and a non-cleavable mutant of TRB3. In this study, we show that the cleavage of TRB3 produced a pro-apoptotic response, and present evidence for a novel anti-apoptotic mechanism involving TRB3-mediated nuclear translocation of procaspase-3 (proCASP3). Overexpression studies revealed that the cleavage of TRB3 promoted CASP3/7 activation and apoptosis. In contrast, the anti-apoptotic effects were found under TRB3 non-cleavable conditions, such as ER stress, and also when the CASP3/7 activation was enhanced by knockdown of endogenous TRB3 expression. Interestingly, nuclear translocation of proCASP3 was observed in cells either overexpressing TRB3 or under tunicamycin-induced ER stress. Although forced cytoplasmic expression of proCASP3 enhanced apoptosis significantly, its nuclear expression did not produce any pro-apoptotic effect, suggesting that nuclear distribution of proCASP3 is not critical for the execution of apoptosis. TRB3 might prevent cytoplasmic activation of CASP3 by promoting proCASP3 entry into the nucleus, and thereby inhibit apoptosis. Taken together, our results suggest that TRB3, through its own cleavage, functions as a molecular switch between the cell survival and apoptosis pathways under stressful conditions.

1811

A Complex Role for PA28γ as a Regulator of Cell Fate Decisions Following Stress.

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PA28γ is a nuclear proteasome activator that facilitates protein degradation by the 20S proteasome in an ATP- and ubiquitin-independent manner. Some targets of PA28γ include key cell cycle regulators such as $p21^{Cip1}$, $p16^{INK4a}$, $p19^{ARF}$, and p53, however PA28γ also has important roles in controlling cell death. PA28γ-deficient cells display increased spontaneous apoptosis in culture, but activate apoptosis signaling to different degrees following various cell stressors. These data suggest that PA28γ has a complex role in regulating survival signals, apoptosis signals, or both. We have investigated the response of PA28γ-deficient Murine Embryonic Fibroblasts (MEF) to a variety of cell stressors and characterized both the viability of the cells as well as the activation of apoptosis signaling. The response to cell stress divides into several categories that are correlated with activation of the pro-survival kinase Akt-1 and the stress response MAP-Kinases p38 and JNK. We conclude that PA28γ, through its role as a substrate-selective activator of proteasomes, controls the ratio of pro-survival to pro-apoptotic

signaling following cellular stress. Since PA28γ is differentially expressed in several cancers, these data may demonstrate one mechanism by which cancer cells can circumvent apoptosis by altering PA28γ expression.

1812

Effect of Extracellular Alzheimer Disease's Hyperphosphorylated Tau on Neuronal Toxicity.

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An inverse relationship has been found between the number of TAU tangles and the number of surviving cells in Alzheimer Disease damaged brain regions. The addition of recombinant pathological TAU protein to cultured neuronal cells leads to neuron degeneration as well. Experimental findings have also shown the transmission of neuropathy induced by injection of pathological TAU-brain extract in the hippocampus and overlying cortex of mice. This spreading event has been postulated in Alzheimer Disease as well. At present, there is no disease described where TAU accumulates and it is not hyperphosphorylated. In this regard, phosphorylation of some specific TAU sites, such as Thr212, Thr231 and Ser262, is sufficient to induce cell death. Indeed, the presence of TAU is required for the toxicity associated to Beta Amyloid. Those neurons that are successful in keeping low levels of hyperphosphorylated TAU are able to survive longer. The objective of this work is to test if the presence of TAU stably transfected to the neurons can protect against substances such as the minimal TAU sequence inducing aggregation, Amyloid-β peptide, and TNFα. We used PC12 and CHO cell cultures stably transfected with TAU. Apoptotic events were determined by the Active Caspase-3 inmunostainning. Initial results showed a neurotoxic effect of TNFα in PC12 cells but not in PC12-TAU cells at 24 hours as the early apoptotic marker Caspase-3 indicated it. We also found a Caspase-3 activation and membrane blebbing induced by phosphorylated TAU. These effects are ameliorated by the overexpression of normal TAU. Similar evidence showed a toxic effect of the TAU heptapeptide in cell culture. This neurotoxic effect is also decreased by the overexpression of normal TAU. Beta-Amyloid toxicity in the presence of this overexpression is currently being elucidated. All together, these results shed light on how the cellular balance regarding TAU phosphorylation determines a neuronal commitment to death in pathological conditions. The avenues for successful treatment for tauopathies may involve down regulation of the phosphorylation and up regulation of the clearance of the abnormal form of TAU.

1813

HeLa cells expressing the nuclear Poly (A) binding protein with expanded alanines undergo cell death through GAPDH nuclear translocation and stabilization of p53.

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The nuclear Poly (A) binding protein (PABPN1) is present ubiquitously in the nuclei of eukaryotic cells, and play an important role in polyadenylation of mRNAs. The wild type protein with a stretch of 10 alanines (PABPN1-A10) is concentrated in nuclear speckles. However, in Oculopharyngeal muscular dystrophy patients, the stretch of alanines is extended to 17 (PABPN1-A17) causing aggregation of the protein within the nucleus. We have previously shown that over expression of the PABPN1-A17 in non muscle HeLa cells leads to increased cell death compared to the wild type protein. Thus, in this study we have investigated the mechanism of cell death following expression of PABPN1 containing expanded alanines (A17 and A40). We showed that over expression of PABPN1 with A17 or A40, leads to increased

nuclear translocation of a glycolytic enzyme, GAPDH, initiating a p53 mediated apoptosis. This event is associated with phosphorylation of p53 at serine 46 in A17 and A40 cells. Once stabilized, p53 is transported to the nucleus leading to up regulation of Puma. Puma then binds to the anti apoptotic protein Bcl2. As Bcl2 is unavailable to form complex with p53, the later is free to activate and oligomerize Bax. We further detected mitochondrial localization of Bax, release of Cytochrome c from the mitochondria and increased levels of activated Caspase 3 in PABPN1-A17 cells. All these findings, therefore, suggest a possible role of GAPDH triggered p53 mediated apoptosis in cells expressing expanded alanine containing PABPN1.

1814

The multidrug resistance-associated transporters offer a differential protection against oxidative stresses induced by hydrogen peroxide or 4-hydroxynonenal in normal human epidermal keratinocytes.

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In normal cells the expression of membrane transporters like the multidrug resistance-associated proteins (MRPs) is often correlated with an increase detoxification capacity. This is due to their transport substrate promiscuity and their ability to segregate inside or to efflux out of the cell the toxic molecules. Recent studies revealed the expression of multiple MRP isoforms in normal human epidermal keratinocytes (NHEK). Because NHEK cells are located closer to the surface of the skin they are subjected to various oxidative stresses. Our aim was to assess whether the MRP tranporters expressed in NHEK cells could offer a protection against oxidative stresses caused by exposure to hydrogen peroxide (H_2O_2) or 4-hydroxynonenal (4-HNE). Primary cultures of purified human normal keratinocytes were treated for various incubation time (2, 12, 24 h) and concentrations of H_2O_2 or 4-HNE. Then, cell viability was determined using the tetrazolium salt 3-(4,5-dimethylthiazol bromide (MTT) assay and the effective concentrations (EC50) at which 50% of the cells died were calculated. Cell death was characterized by flow cytometry and fluorescence microscopy using propidium iodide and annexin-V-FITC as fluorescent markers of necrosis and apoptosis. For each oxidant agent, the involvement of MRPs was evaluated by comparing the effects observed in the absence or presence of a non-toxic concentration of MK571, a specific MRP inhibitor. The MTT-reducing activity of NHEK cells dose-dependently decreased in the culture exposed to either H_2O_2 (0-10 mM) for 2 h or 4-HNE (0-100 µM) for 24 h with EC50 values of 0.60 mM and 10 µM respectively. In the presence of 10 µM of MK571 a significant decrease of the EC50 value to 3 µM for 4-HNE was observed suggesting a role for MRPs in the protection of NHEK cells against 4-HNE toxicity. Moreover, flow cytometry and fluorescence microscopy data indicated that NHEK cells exposed to 0.3 to 3 mM H_2O_2 for 2 h or to 0.3 to 30 µM 4-HNE for 24 h followed a necrotic cell death pattern.

1815

Nox4, a Novel Mediator for TGF-β-Induced Apoptosis in Mouse Podocyte.

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Podocytes are terminally differentiated, actin-rich epithelial cells present on the glomerular basement membrane and critical for glomerular filtration process. Podocyte injury leads to onset of chronic renal diseases characterized by proteinuria. Elevated level of TGF-β is associated with podocyte damage ultimately resulting in apoptosis and detachment. The underlying

mechanism of apoptosis, however, remains unclear in these cells. We investigated pathogenic mediators for proapoptotic effects of TGF-β in cultured mouse immortalized podocytes. Exogenous TGF-β1 selectively up-regulates the expression of only Nox4, but not those of other NADPH oxidase (NOX) family enzymes such as Nox1, Nox2 and Nox3. Consistent with Nox4 upregulation, the total activity of NOX was higher in TGF-β1-treated podocytes. TGF-β1 enhanced reactive oxygen species (ROS) level and elicited apoptosis indicated by the increased the number of apoptotic nuclei and the level of cleaved caspase-3. All these changes by TGF-β1 in podocyte were abolished by SB431542, a potent inhibitor of ALK-5 (Type I TGF-β receptor). Apocynin and DPI, selective inhibitors of NOX, blocked TGF-β1-induced ROS generation and apoptosis. N-acetylcystein as an antioxidant also attenuated the detrimental changes by TGF-β1. In addition, TGF-β1-induced apoptosis was inhibited by SB202190, a p38 MAPK inhibitor. These results suggest that ROS production via Nox4 closely participates in TGF-β-induced apoptosis that might be a crucial mechanism in the development and progression of proteinuric glomerular diseases.

1816

Oxidative Stress Markers and Apoptosis in the Prostate of Diabetic Rats is Diminished by Vitamin C.

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Negative consequences of diabetes on the prostate such as involution are associated with diminished testosterone, insulin deficiency and hyperglycemia. The contributions of oxidative damage, which usually increases with diabetes, are unknown for these alterations. This study evaluated the impact of streptozotocin-induced diabetes on the biomarkers of the antioxidant system of rat ventral prostate, the influence of vitamin C supplementation on these biomarkers and on the balance between cell proliferation and death. Diabetes (D) was induced in Wistar male rats by streptozotocin (5mg/100g b.w., i.p.). Control animals (C) were injected with a vehicle. Vitamin C (150mg/kg b.w./day) supplementation was introduced by gavage in diabetes (D+V) as well as control (C+V) groups. Thirty days after diabetes onset, the rats were killed and the ventral prostates were analyzed using light microscopy, immunocytochemistry and biochemical assays for biomarkers of oxidative stress. In comparison to control groups, the levels of circulating testosterone, proliferating and androgen-receptor positive cells decreased in diabetic groups regardless of vitamin C treatment whereas apoptosis was increased. The levels of superoxide dismutase and glutathione peroxidase did not change, but the levels of catalase and glutathione S-transferase (GST) were increased in diabetic prostate. Vitamin C supplementation normalized GST activity and recovered the apoptotic rates in the prostate. These results suggested that oxidative damage is, at least in part, responsible for the unbalanced cell proliferation and apoptosis observed in the rat prostate after diabetes, and GST is a good indicator of compensatory oxidant defense in the gland at earlier stages of metabolic disease.

1817

Development of apoptotic cell mimics to assess protein binding to curved membranes.

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Cells have intricately shaped membranes that are created and maintained by the interplay between proteins, lipids, and physical forces. These curved membranes function to control protein activity, to sort proteins in space, and are the product of protein-lipid interactions. The shapes vary dramatically in size, from nanometers to microns, and the affinity of a protein for a curved lipid surface are shape dependent. In our work, novel, nanoparticle-templated membranes have been developed and used to characterize protein interactions with spherically shaped lipid surfaces. Two types of nanoparticle-templated membranes are used. 1) Gold nanoparticles (10-100 nm) are coated with a lipid bilayer for bulk assays of protein binding using gel electrophoresis and fluorimetry. 2) Surface studies are done on single areas of curvature by depositing polystyrene nanoparticles (20-100 nm) on a glass surface and forming a conformal bilayer over top. Both types of sensors allow for the curvature and lipid composition to be separately tuned. Our results reveal that C-reactive protein (CRP), a protein involved with binding to LDL and apoptotic cells as a part of the complement immune response, preferentially binds regions of high curvature. The transition of CRP from a soluble pentamer to a modified, membrane bound form has been assessed and this transition occurs on the curved membranes. These results suggest that regions of high curvature may recruit CRP and allow for the transition of CRP to a form that is capable of binding C1q, another protein in the complement immune response, to signal clearance of apoptotic cells.

1818

C-reactive protein binding to apoptotic cellular blebs.

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This project is advancing research in the study of the innate immune response by investigating C-reactive protein's (CRP) interaction with curved membrane surfaces, or blebs, a condition present in apoptotic cells. CRP has been known to bind to apoptotic cells to facilitate clearance, possibly converting between two isoforms – pentameric CRP and modified CRP. CRP binding to apoptotic mimics has been explored. Nanoparticles were deposited onto a glass substrate prior to creation of a supported lipid bilayer, which induces curvature in the bilayer as it forms. Using confocal microscopy, fluidity was observed and diffusion rates characterized for bilayers formed over the nanoparticles (20-100 nm in diameter). CRP binding to this induced curvature has been explored. Furthermore, for our *in vivo* work, we induced apoptosis in Chinese Hamster Ovary (CHO) cells and measured the binding of CRP, using Western blotting and fluorescence microscopy. Using the drug, Valinomycin, apoptosis has been confirmed after one hour of incubation and up to 7 hours of incubation. Blebs have been visualized using Annexin V, which binds phosphotidylserine present on the extracellular side of apoptotic membranes, and apoptosis is additionally confirmed using DAPI to visualize the condensed nucleus. Visualization of the blebs allows us to image the binding of CRP to the curved membrane. Preliminary work has been done to determine if CRP undergoes isoform conversion from pentamer to monomer on the surface of the apoptotic cellular membrane and if membrane bound CRP interacts with C1q in an effort to better understand how apoptotic cells are cleared.

1819

Role of lipids in apoptosis in *Xenopus laevis* eggs.

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The eggs of many species spontaneously die due to apoptosis (Pasquier et al., 2011). The fertilized egg (zygote) switches off this suicide pathway whereas aged unfertilized eggs rapidly undergo apoptosis (Pasquier et al., 2011). Perhaps to prevent the use of damaged eggs in fertilization, the egg will spontaneously undergo apoptosis unless fertilization takes place. This creates problems in storage and use of eggs for in vitro fertilization. Tilly et al. (2002) and Kolesnick et al. (2007) found that apoptosis in mouse eggs can be induced by exogenous addition of ceramide. In effort to better understand the role of ceramide and other lipids in the apoptotic switch in unfertilized eggs, we conducted our studies using the National Institute of Health recognized model organism *Xenopus laevis*.

Spontaneous Apoptosis: One major enzyme of apoptosis is caspase-3 which is activated by limited proteolysis. To define apoptosis in *X. laevis* eggs, we developed an assay for detecting cleaved caspase-3. Detection of cleaved caspase-3 began ~16 hours after incubation (RT) in 100% Modified Barth's Saline (MBS). Micrographs show cells became mottled soon after this time. In addition, preliminary data suggest that ceramide mass increases as *X. laevis* eggs die naturally in storage media. Ceramide was separated by HPLC and quantified by evaporative light scattering mass detection. A time course of these three apoptotic measures during spontaneous egg apoptosis will be compared and presented.

The Role of Ceramide in Apoptosis: To investigate the role of lipids in egg apoptosis, the addition of the sphingolipid C2 ceramide to *X. laevis* eggs was found to induce caspase-3 activation and egg mottling over four hours. Ceramide addition also increased intracellular calcium which was measured using a calcium sensitive Fluo-4 dye. The calcium sensitive dye was injected into *Xenopus* oocytes prior to ceramide addition.

1820

Activation of intracellular tissue transglutaminase 2 by hyperglycemia potentially contributes to endothelial apoptosis in diabetes.

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Elevation of intracellular reactive oxygen species (ROS) and calcium (Ca^{2+}) has been implicated in various hyperglycemia-induced endothelial dysfunctions in diabetes. However, the individual roles of ROS and Ca^{2+} as well as their unified molecular mechanism linked with endothelial dysfunctions in diabetes remains to be clarified. The aim of this study was to explore the role of intracellular ROS and Ca^{2+} in activation of tissue transglutaminase 2 (TG2), and induction of endothelial cell apoptosis in vascular system. We observed that hyperglycemia substantially increased intracellular Ca^{2+}, and increased ROS predominantly via Ca^{2+}, activation of PKC and NADPH oxidase pathway. Intracellular ROS- and Ca^{2+}-dependent increase in TG2 activity, was revealed as a previously unrecognized mediator of endothelial cell apoptosis in diabetes. In addition, elevated TG2 activity was observed in aortic segments of streptozotocin-induced diabetic mice in comparison to normal mice. Prevention of cellular apoptosis by inhibition of intracellular Ca^{2+}, ROS, and knockdown of TG2 by siRNA, indicating the crucial role of intracellular ROS, Ca^{2+} and TG2 in cell death mechanism. These findings identify intracellular ROS, Ca^{2+} and TG2 as potential new therapeutic targets for the prevention and treatment of diabetic vasculopathies.

1821

Mitochondrial dysfunction and Purkinje cell loss in the human spastic ataxia ARSACS.

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Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) (OMIM: 270550) is a childhood-onset neurological disease resulting from mutations in the *SACS* gene encoding sacsin, a massive 4579 amino acid protein of unknown function. Originally identified as a founder disease in Quebec, ARSACS is now recognized worldwide. Prominent features include pyramidal spasticity, peripheral neuropathy and cerebellar ataxia but the underlying pathology and pathophysiological mechanisms are unknown. We have now generated sacsin knock out mice that display age-dependent neurodegeneration of Purkinje cells and modified mitochondrial function. Mitochondrial dysfunction is a common pathophysiological feature of major neurodegenerative diseases including Huntingtin's, Parkinson's, and Alzheimer's. We show that sacsin localizes to mitochondria in neurons and nonneuronal cells and that it interacts and co-localizes with dynamin-related protein 1, which participates in mitochondrial fission. Disruption of sacsin function leads to an overly interconnected and functionally impaired mitochondrial network. Mitochondria accumulate in the soma and proximal dendrites of neurons and there are striking alterations in the organization of dendritic fields and the morphology of dendritic spines that precede neuronal cell death. Our data reveals mitochondrial dysfunction/mislocalization as the likely cellular basis for ARSACS.

1822

Visualization of Bcl-2 family protein interactions enables screening and characterization of Bcl-2 family small molecule inhibitors in live cells.

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Bcl-2 super-family of proteins interact at the mitochondria to regulate apoptosis. Overexpression of pro-survival Bcl-2 family proteins is observed in some cancers and confers resistance to chemotherapeutics. Therefore, small molecule inhibitors (SMI) are being developed to block interactions of pro-survival Bcl-2 proteins with their pro-apoptotic counterparts, thereby causing apoptosis.

Current cell-based assays for screening SMIs of Bcl-2 pro-survival proteins rely on indirect, downstream read-outs of apoptosis such as cytochrome-c release, caspase cleavage or cell viability. These assays are unable to detect Bcl-2 family member specificity and may identify compounds that cause cell death by off-target mechanisms. To address these shortcomings, we set out to develop live-cell assays that would detect the direct interactions of Bcl-2 pro-survival proteins with pro-apoptotic BH3-only proteins. This was accomplished by establishing stable cell lines expressing differentially fluorescently-tagged Bcl-2 super family proteins. These fluorescent fusion proteins were engineered to facilitate quantitative assessment of pro-survival Bcl-2 protein binding-dependent localization of BH3-only proteins at the mitochondria. We showed that disruption of the Bcl-2: BH-3-only protein-protein interaction results in cytoplasmic retargeting of BH3-only proteins and that the sensitivity of these assays can be adjusted by

using BH3-only proteins engineered to have reduced binding affinity for Bcl-2 pro-survival proteins. We observed that the decreased binding affinity of these affinity mutants results in BH3-only displacement from the mitochondria in the presence of lower potency compounds.

In addition to SMI screening, we are currently characterizing the biological mechanisms that regulate interaction between pro-survival and pro-death proteins with these novel assays. Given that these protein-protein interactions are critical to determining cell survival, a better understanding of how they are regulated within the cell will allow for the development of new and more effective strategies for targeting them in a clinical setting.

1823

Silver nanoparticles induce apoptotic cell death in *Candida albicans* through the increase of hydroxyl radical.

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Silver ions are well known to have inhibitory and antimicrobial effects, but the effects of silver nanoparticles on microorganisms and antimicrobial mechanism have not been revealed clearly. In this study, it was found that silver nanoparticles exerted its antifungal effect via apoptosis. *Candida albicans* exposed to silver nanoparticles showed the increased reactive oxygen species (ROS) production, measured by DHR-123 staining and then we investigated that silver nanoparticles induced the accumulation of hydroxyl radical, which is one of the more active oxygen among the reactive oxygen species (ROS) formed, on *C. albicans* as an oxidative stress response to the drug. The apoptotic effects of silver nanoparticles were detected by fluorescence microscope using Annexin V-FITC, TUNEL, DAPI and FITC-VAD-FMK staining further confirmed diagnostic markers of yeast apoptosis including phosphatidylserine externalization, DNA and nuclear fragmentation, and activation of metacaspase. In addition, the reduction of mitochondrial membrane potential through flow cytometry analysis after staining these with $DiOC_6$ or JC-1, and the release of cytochrome *c* from mitochondria were also assured. Furthermore, the effect of thiourea as hydroxyl radical scavenger in *C. albicans* was investigated. The results suggest that silver nanoparticles possess antifungal effects with another mechanism promoting such as apoptosis and we thought hydroxyl radical generated by silver nanoparticles play a significant role in mitochondrial dysfunction.

1824

Evaluating the Neurotoxicity of Manganese Oxide Nanoparticles using Caenorhabditis elegans.

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Nanoparticles are defined as the smallest unit (1-100nm) that can still represent the characteristics and functionality of an entity in terms of properties and transport. They can be applied to electronics, medicine, cosmetics and clothing. Recently, various neurodegenerative diseases have been linked to metal oxide nanoparticle exposure. The exact mechanism of toxicity following exposure to these nanoparticles is unknown. Our interest in metal oxide nanoparticles is based on the fact that experimental studies have shown a positive link between manganese exposure and a Parkinson's disease-like syndrome called manganism. The symptoms of this manganism include tremors, bradykinesia, and involuntary muscle movements. In this study, to assess the neurotoxicity manganese oxide nanoparticles, *C.*

elegans were used as the model organisms. These small nematode worms have been used in several areas of research including developmental biology, neurobiology, oxidative stress, and aging. In this study, *C. elegans* were exposed to manganese oxide nanoparticles (Mn_2O_3), silicon dioxide (SiO_2), titanium dioxide (TiO_2), and manganese chloride (MnCl) as an ionic control. The nanoparticles were introduced to bacterial cultures at concentrations of up to 100µg/ml and compared to untreated controls. Following the growth of treated bacteria on petri dishes, the C. elegans were then applied to the bacterial plates and allowed to grow on feed on bacteria containing particles. After 1 and 2 weeks of feeding, the plates were then washed and worms were collected. The worms were evaluated for viability and the induction of oxidative stress. In summary, increasing nanoparticle concentration resulted in decreased viability and oxidative stress occurring in pharynx and intestine of the worm. These studies suggest *C. elegans* as a potential model system in evaluating a role of metal oxide nanoparticles in neurodegenerative disorders.

1825

Immunoassays for the quantitation of two key autophagy biomarkers p62 and NBR1.

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p62 and NBR1 share similar functions within the autophagy pathway. NBR1 and p62 aid in protein trafficking and degradation. Although the proteins differ in size and sequence, both proteins contain N-terminal Phox domains and Bem1 (PB1) domains, LC3-interacting region (LIR) motifs, and C-terminal ubiquitin-associated (UBA) domains. Although NBR1 and p62 interact and form oligomers, they can also function independently.

Immunoassay kits using a colorimetric 96-well microtiter plate format ELISA have been developed for the detection and quantitation of p62 and NBR1. The sensitivities of the individual kits were determined to be 500 pg/mL and 66 pg/mL, respectively. Both assays demonstrated parallel dose response curves between the recombinant standard and cell lysates from human, mouse and rat origin. Distinct concentrations of recombinant p62 were spiked into three cell lysates, diluted in assay buffer and run in the assay. Similarly, recombinant NBR1 concentrations were spiked into lysis buffer, diluted in assay buffer and run in the assay. The recovery of the spiked p62 was 91, 90 and 95% following a 1:16 dilution. The recovery of the spiked NBR1 was 89, 88 and 106% following a 1:8 dilution. The p62 ELISA intra-assay precision ranged from 3.7 to 3.9% CV and inter-assay precision ranged from 5.2 to 7.0% CV. The NBR1 ELISA intra-assay precision ranged from 4.4 to 7.8% CV and inter-assay precision ranged from 15.6 to 21.8% CV.

The immunoassays were used to measure autophagy flux by detecting changes in p62 and NBR1 levels. This was shown in a time course study in which MDA-MB-231 human breast cancer cells were treated with an autophagy inducing drug withaferin A (WA). Both p62 and NBR1 levels were reduced in response to drug treatment at 6 hour and 12 hour time points. Interestingly, both protein levels increased at the 24 hour time point. p62 ELISA results correlated with western blot (NBR1 not tested). WA treatment of cells correlated with induction of autophagy as confirmed by LC3-II levels in western and puncta formation in immunofluorescence. The accumulation of p62 and NBR1 at the 24 hour time point contrasted with elevated LC3-II possibly indicating the beginning of autophagy resolution. In a second experiment, HeLa cells were treated with Bafilomycin A1 or Rapamycin. After Bafilomycin A1 treatment, both p62 and NBR1 protein levels showed significant increases, 18-fold and 1.8-fold, respectively. In the Rapamycin cell treatment, p62 protein level showed a modest decrease,

25%, whereas no decrease in NBR1 protein level was noted. ELISA results for both p62 and NBR1 correlated with western blotting results.
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1826

An attempt to identify biomarkers in mouse lung exposed to single wall carbon nanotubes.

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With the widespread application of carbon nanotubes (CNTs) in diverse commercial processes, scientists are now concerned about the potential health risk of occupational exposures. In this study, Single wall carbon nanotubes (SWCNTs) induced pulmonary toxicity was investigated by exposing Balb/c mice to aerosolized SWCNT (5µg/g of mice) for 7 consecutive days in nose-only exposure system. The microscopic studies showed that inhaled SWCNTs were homogeneously distributed in the mouse lung. The total number of bronchoalveolar lavage (BAL) polymorphonuclear leukocytes recovered from the SWCNT exposed mice (1.2×10^6 ±0.52) was significantly greater than control mice (5.46×10^5 ±0.78). Rapid development of pulmonary fibrosis in SWCNTs inhaled mice was also confirmed by significant increases in the collagen level. The lactate dehydrogenase (LDH) levels were increased nearly by 2 folds in SWCNT inhaled mice respectively, as compared to control mice. In addition, exposure of SWCNTs to mice showed a significant ($p<0.05$) reduction of antioxidants; glutathione, superoxide dismutase, catalase activity and induction of oxidants; myloperoxidase, oxidative stress lipid peroxidation product compared to control. Apoptotic related proteins such as caspase-3 and -8 activities were also significantly increased in SWCNTs inhaled mice than control. Together, the present study shows that inhaled SWCNTs induce an inflammation, fibrosis, alteration of oxidant and antioxidant levels and induction of apoptotic related proteins in the lung tissues to trigger the cell death.

Polarity in Development

1827

The role of RNA binding proteins in dendritic morphogenesis.

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Neurons have highly asymmetric cellular morphologies and polarized cellular functions that are necessary for establishing neural circuitry and for proper functioning of the nervous sytem. Specialized processes called dendrites are used by neurons for reception of stimuli, while axons function in the transmission of signals. The mechanisms for establishing neuronal polarity are poorly understood, however, it is well established that mechanisms that generate asymmetric protein distributions are essential for establishing such morphological and functional polarities in many different cell types. In neurons, mRNA localization and translational repression are used to change the protein composition of various regions of the cell, allowing for distinct axonal and dendritic morphologies and environments that are equipped for their various cellular tasks. A significant portion of eukaryotic genomes encode for RNA-binding proteins and other components of post-transcriptional regulatory machinery. Moreover, a large number of dendritically localized mRNAs have been identified, suggesting that regulation of localized

mRNAs contributes broadly to neuronal development and function. **Objective:** We performed a candidate RNAi screen to identify genes involved in the development of the highly complex dendritic trees of Class IV dendritic arborization (da) neurons of the *Drosophila* larval peripheral nervous system in order to assess the role of RNA binding proteins in dendrite morphogenesis. **Methods:** We systematically knocked down expression of genes that have been annotated as translation factors and RNA binding proteins within the *Drosophila* genome using RNA interference, activated specifically within Class IV da neurons by the bipartite Gal4/UAS system. **Results:** The screen uncovered substantial roles in dendrite development for genes encoding translational repressors, eukaryotic initiation factors, as well as genes involved in mRNA splicing. We find that the translational repressor Brain tumor (Brat) and the heterogeneous nuclear ribonucleoprotein Squid are expressed within Class IV da neurons and function in the development of higher order dendritic branching within these neurons. We find that *brat* functions cell autonomously in dendrite morphogenesis in Class IV da neurons. Furthermore, we show that *brat* genetically interacts with the translational repressors *nanos* and *pumilio* in order to promote the generation of complex dendritic trees. **Conclusions:** Our screen highlights an important role for post-transcriptional gene regulation in generating morphological cellular asymmetries during Drosophila neurogenesis.

1828

Morphogenesis of the Intestinal Epithelium during Zebrafish Development.

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Proper sorting and targeting of endocytosed proteins is necessary for the maintenance of epithelial cell polarity and apical lumen formation. FIP5, a Rab11 effector protein, is known to be involved in apical-directed transport in polarized cells and is thought to act as a scaffolding protein that recruits and binds effectors of endocytosis. Recently, using 3D culture assays, we have shown that FIP5 is an integral mediator of the formation of the epithelial lumen by cavitation in MDCK cells, with the knock-down of FIP5 resulting in the formation of multiple lumens. Additionally, we demonstrated that FIP5 regulates the formation and scission of endocytic carriers in vivo.

Following these data obtained in cell culture, we propose that FIP5 is also involved in the regulation of polarity maintenance and lumen formation in vivo, and have focused our studies on the zebrafish model. In zebrafish, the cavitation model of lumen formation allows for the lumenal opening of the intestinal mesoderm. Moreover, the proper formation of the intestinal tract within zebrafish is highly regulated, with the polarized integrity of the gut's neighboring epithelial structures, the lateral plate mesoderm (LPM), being required for the proper formation and localization of the intestinal precursor during development. Here, using morpholino antisense oligonucleotides directed against FIP5, we characterize the role of FIP5 in the morphogenesis of the zebrafish intestinal tract, along with the ability of the LPM to effect the asymmetric localization of the gut at early stages of development. We propose that FIP5 mediates the positioning of the intestinal tract, as well as the formation of the gut lumen, during early stages of zebrafish development.

1829

The PKD1 locus encodes an alternative transcript that induces mesenchymal to epithelial transition in COS-1 cells.

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Recent evidence from a genome wide cDNA library construction project suggests that the HmPKD1 locus encodes a multiplicity of transcripts (Kimura et al, Genome Research, 2006). We have been characterizing one of the cDNA's generously supplied by Dr. Sagano (Helix Research Institute, Chiba, Japan). We sequenced the entire cDNA clone and found that the start site of the clone is in intron 40 of the HmPKD1 locus. There is also a unique splice between exons 41 and 43. When transfected into COS-1 cells, an SV40 transformed cell line, transfected cells exhibit cobblestone morphology, develop a measurable trans-epithelial resistance (~100 Ohm-cm^2) and increase E-cadherin expression. Furthermore, when transfected COS-1 cells are plated in 3D collagen matrix, the cells form cysts. Control, non-transfected cells do not form any three dimensional, organized structures. This data demonstrates that an alternative transcript from the Human PKD1 locus induces an epithelial phenotype in a mesenchymal cell line.

1830

Effects of Early Disruption in Polarity on Later Development in the Sea Urchin Embryo.

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Establishment and maintenance of cell polarity has become an increasingly interesting biological question in a diversity of cell types and has been found to play a role in variety of biological functions. Previously, it was thought that the sea urchin embryo remained relatively unpolarized until the first asymmetric division at the 16cell stage of development. However, there is mounting evidence to suggest that polarity is established much earlier. We analyzed roles of the cell polarity regulators, the PAR complex proteins, and how their disruption in early development affects later developmental milestones such as blastula and gastrula formation. We found that PAR6 along with aPKC and CDC42 localize to the apical cortex (free surface) as early as the 2-cell stage of development and this localization is retained through the blastula stage. These proteins are anchored in the cortex by myosin as disruption of myosin light chain kinase activity with ML-7 resulted in cytoplasmic pooling. Additionally, pulse treatments with ML-7 at the 1 or 2-cell stage prevented the embryos from reaching the gastrula stage. This same pulse disrupted PAR6 localization at the fertilized egg and maintained this disruption through the first cleavage division. Interestingly, aPKC inhibition early in development prevented blastula formation but not normal cleavage divisions. aPKC inhibition did not effect micromere formation at the first asymmetric division. These observations suggest that disruptions of the polarity complex in the early embryo can have a significant impact on the ability of the embryo to reach later critical stages in development. This work is supported by NIH GM093978.

1831

Differential segregation of apical domain markers during chick lens placode elongation.

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Vertebrate lens originates from pre-lens ectoderm, a simple cuboidal epithelium that overlies the optic vesicle. After induction by the optic vesicle, the pre-lens ectoderm cells elongate at their

apico-basal axis, becoming columnar and forming a pseudostratified epithelium known as the lens placode.
We have shown previously that actomyosin contractile filaments are homogeneously distributed along the apico-basal cell sides of the chick pre-lens ectoderm (embryonic stage HH11) and become enriched apically at later stages of the lens placode (embryonic stage HH 13) and during lens placode invagination. Therefore, the actomyosin filaments redistribution to the cell apex accompanies the morphological transition from pre-lens ectoderm to lens placode. Here, we investigate the segregation dynamics of apical proteins during the establishment of the placode´s apico-basal polarity.
Beta-catenin is found in lateral membranes of pre-lens ectoderm and is apically concentrated in elongated placodal cells. In contrast, PAR3 is already apically located in pre-lens ectoderm. PAR3 is a scaffold protein that establishes and maintains epithelial cell polarity in a diverse set of models. Similarly, occlusive junctions (detected by ZO-1 immunolocalization) and aPKC are also apically located in pre-lens ectoderm and remain so with placode elongation.
Taken together, these data suggest that the pre-lens ectoderm has an intrinsic polarity reflected by the apical concentration of PAR3, ZO-1 and aPKC. Thereafter, placodal elongation involves further polarization of additional elements such as acto-myosin network and membrane catenins. This latter step of polarization is required for the onset of placode invagination

1832

Localization of LET-99 during asymmetric division in C. elegans embryos.

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Positioning of the mitotic spindle during asymmetric divisions is essential for generating cell diversity during development. In several systems, PAR polarity proteins and Gα signaling control spindle positioning. In the C. elegans 1-cell embryo, the Gα complex components, GPR and LIN-5, are asymmetrically localized at the cortex in a dynamic pattern in response to PAR cues. GPR and LIN-5 generate asymmetric cortical pulling forces that move the spindle, possibly by regulating dynein. LET-99, a DEPDC1 family protein, is a key intermediate in this pathway. LET-99 is asymmetrically localized at the cortex in a lateral posterior band pattern. LET-99 in turn inhibits the cortical association of GPR and is needed for asymmetric GPR localization. Quantitative analysis of LET-99 localization in mutant backgrounds shows that PAR-3 inhibits cortical LET-99 localization at the anterior cortex, while a gradient of PAR-1 inhibits LET-99 at the posterior-most cortex. Additionally, cytoplasmic polarity mediators are not required. PAR-3 colocalizes at the anterior an atypical protein kinase C, PKC-3. PAR-1 is a Ser/Thr kinase and associates with LET-99 in immunoprecipitations from embryo extracts and in pull-down assays. These results lead to the hypothesis that phosphorylation of LET-99 by PAR-1 and PKC-3 prevents cortical accumulation. However, PAR-3 also colocalizes at the anterior with PAR-6 and CDC-42. To determine which of the anterior polarity proteins are required to regulate LET-99, we are examining LET-99 localization in embryos depleted for each of the anterior proteins. In vitro kinase assays will be used to test for phosphorylation of LET-99 by PAR-1 and PKC-3. Additionally, we are testing LET-99 interacting proteins for a role in anchoring LET-99 at the cortex.

1833

LET-99, a novel G protein regulator for asymmetric division.

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Asymmetric divisions that generate cell diversity are required for normal development and stem cell maintenance. Spindle positioning is a key aspect of asymmetric division. The mitotic spindle

must be aligned with the axis of cell polarity in order for cell fate determinants to be differentially segregated to daughter cells to give them different fates. The conserved PAR polarity proteins establish cell polarity, and they also regulate spindle movements via a complex involving Gα subunits, GPR and LIN-5. The Gα/GPR/LIN-5 complex is necessary for the cortical forces that pull on astral microtubules, potentially by recruiting regulators of the microtubule motor dynein. In C. elegans one-cell embryos, GPR and LIN-5 first show an overall anterior enrichment during prophase, when pulling forces center the nuclear-centrosome complex and rotate it on to the polarity axis. GPR/LIN-5 enrichment then switches to the posterior during the spindle displacement movements that produce an unequal division. We previously showed that LET-99 is a key component of this pathway and acts downstream of the PAR proteins during both nuclear centration/rotation and spindle displacement. LET-99 antagonizes G protein signaling by inhibiting the localization of GPR at the cortex. The highest levels of LET-99 are present in a posterior-lateral cortical band, which results in the lowest levels of cortical GPR in this region; nonetheless, LET-99 function is also needed for the overall anterior and posterior enrichment of GPR through the cell cycle. To begin to elucidate the mechanism of LET-99 action, we tested LET-99 for interaction with other components of the pathway. In vitro pull down and yeast two-hybrid assays show that LET-99 can interact with GOA-1 and GPA-16, the two Gα subunits used in this system. Direct binding of LET-99 to Gα could directly interfere with formation of a Gα/GPR/LIN-5 complex; alternatively, LET-99 could regulate an upstream step involving other pathway components such as Gβ or RIC-8. To further elucidate the molecular mechanism of LET-99 action, we are carrying out additional genetic and interaction studies with Gβ and Ric-8.

Tissue Development and Morphogenesis II

1834

Morphophysiological Regulation of the Female Prostate by Progesterone Hormone.

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The objective of this investigation was to evaluate the direct effects of hormonal suppression on the female prostate gland, as well as the replacement of progesterone (P4) associated or not with testosterone (T) and estrogen (E2). For this purpose, surgical castration was performed in female gerbil (*Meriones unguiculatus*) at early puberty (45 days). After adulthood (90 days), some of the castrated animals were sacrificed, forming the control group (CCa), while others were treated with subcutaneous doses (1 mg/kg b.w.) of P4 (PCa), E2 plus P4 (PECa) and T plus P4 (PTCa), during 14 days. For the intact control group (NC) non-castrated adult gerbils were used. The surgical castration trigged a diminished glandular development and a reduction in the secretory capacity, as well as decreased the amount of androgen receptor (AR) positive cells, but increased cell proliferation (PCNA) and the number of estrogen receptor cells (ERα). The replacement of P4 unleashed a hyperplastic and hypertrophic process in the epithelial secretory cells, recovering the pattern of glandular secretion with increased number of AR and ERα-positive cells. When P4 was associated with E2, the glandular cellularity became similar to that observed in NC group, occurring however, an intense epithelial hyperplasia associated to a high proliferative rate of stromal and epithelial cells. In the PTCa group, the epithelial hyperplasia was even more evident, presenting an intense immunostaining for AR and ERα and a normal secretion pattern. Furthermore, important hormonal oscillations were observed in the different treatments, with peaks of P4 in the PCa and PECa groups and a testosterone in the

PTCa animals. Thus, the results show the impact of hormonal imbalance on the prostate morphophysiology, highlighting that the female gland is very dependent of androgen and estrogen, but that the progesterone hormone is also a potential regulator of its physiology. Financial support: FAPESP.

1835

Securin is Essential for Mammary Gland Morphogenesis and Tumor Suppression.

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Background & Objective: Securin, also known as PTTG1 (pituitary tumor transforming gene 1), is an inhibitor of separase, a protease catalyzing the cleavage of sister-chromatid cohesions during mitosis. There is strong evidence for securin as a dynamic player involved in gene regulation, organ development and tumorigenesis. However, these earlier reports combined mutations and knockouts of other proteins such p53, Retinoblastoma and p21 with securin. We want to understand how securin directly affects these processes. Our study focuses on the securin loss-of-function effects on mouse mammary gland development, gene regulation and tumorigenesis.

Methods and Results: Our lab generated a securin knockout ($Sec^{-/-}$) mouse model that was normal with no decreased fecundity. Previous *in vitro* studies indicated that securin enhanced promoter activity of specific genes involved in proliferation (c-Myc, Cyclin D3, FGF-2), inhibition (p21) and extra cellular matrix modifiers (MMP2). Using quantitative real-time PCR (qRT-PCR) we analyzed $Sec^{-/-}$ mouse embryonic fibroblasts to reveal a significant reduction of the mRNA transcripts of matrix metalloproteinase 2 (Mmp2) and p21. Preliminary reports demonstrated that Mmp2 knockout mice had a disturbed mammary gland branch network, with increased lateral branching and decreased invasion of the developing ducts. We have completed developmental time points for 4, 7 and 13 wk old mice and our analysis shows that $Sec^{-/-}$ glands have super-numerated secondary and tertiary lateral branching as well as decreased invasion. Using BrdU incorporation to analyze proliferation, we have revealed that 7 and 13 wk old mice have increased proliferation in the branch network. To eliminate the systemic effect of the knockout, we performed mammary gland transplants and found similar results to the $Sec^{-/-}$, indicating that the alteration of the gland is contained within the stem cell niche and not caused by a hormone-signaling imbalance due to the systemic knockout. Analysis of the glands by qRT-PCR reveals similarly low levels of mRNA transcripts in Mmp2 and p21. These $Sec^{-/-}$ mice also develop tumors exclusively in the breast (17%). Current studies are assessing the ability of MMP2-lentivirus to rescue the gland phenotype.

Conclusion: In gland development, securin is more than a regulator of mitosis; it controls the gene expression of both MmP2 and p21. The misregulation caused by the knockout increases proliferation and uncontrolled branching; a factor that is necessary for tumorigenesis. Therefore, securin is essential for gland architecture and tumor suppression.

1836

HIF-dependent upregulation of Bnip3 promotes apoptosis and cavitation during epithelial morphogenesis.

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Apoptosis is an essential step in cavitation during embryonic epithelial morphogenesis, but the mechanisms are largely unknown. In this study we used embryonic stem cell-differentiated

embryoid bodies (EBs) as a model and found that Bnip3 (Bcl-2/adenovirus E1B nineteen-kDa interacting protein), a BH3-only pro-apoptotic protein, is significantly upregulated during cavitation in a hypoxia-dependent manner. shRNA silencing of Bnip3 inhibits apoptosis of the core cells and delays cavitation. Immunoblot analysis reveals that HIF-2α is selectively increased during EB morphogenesis. Inactivation of HIF-2α but not HIF-1α markedly inhibits Bnip3 elevation and apoptosis. Similarly ablation of HIF-1β, the common β subunit of HIF-1 and -2, completely abolishes Bnip3 upregulation and delays cavitation, whereas overexpression of Bnip3 in HIF-1β-deficient EBs rescues apoptosis and cavitation. These results uncover a mechanism of cavitation through hypoxia-induced apoptosis of the core cells mediated by HIFs and Bnip3.

1837

NF-kappa B Activation Promotes a Phenotype Shift in Fetal Mouse Lung Macrophages.

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Objective: Macrophages are present in multiple embryonal tissues and may play key roles in morphogenesis. We have previously shown that NF-□B activation in fetal lung macrophages inhibits airway morphogenesis and may play a role in neonatal lung disease. The role of NF-□B activation on fetal lung macrophage phenotype remains unknown. We hypothesized that NF-□B signaling in fetal macrophages drives an M1, pro-inflammatory phenotype, which may be detrimental to normal lung morphogenesis.
Methods: Macrophage marker expression was measured by real time PCR and immunostaining. A fetal lung explant model was used to study saccular stage airway branching. In addition to LPS treatment, we tested the effects of NF-□B activation in macrophages using a transgenic mouse model with a macrophage-specific, tet-inducible constitutively active IKKβ mutant (IKFM).
Results: To determine macrophage phenotype in the fetal lung, we measured expression of pro-inflammatory, M1 markers and alternative activation, M2 markers in fetal and adult lung sections. Compared to adult cells, fetal lung macrophages had increased expression of the M2 marker CD206. Isolated macrophages from fetal lung tissue were still able to mount an M1 response to LPS, expressing IL-1β, TNF-α, and the M1 markers CXCL-10 and CCL-3. LPS had no effect on M2 marker expression in isolated cells. In contrast, the T_H2 cytokines IL-4 and IL-13 increased expression of the M2 markers CCL-17, CD206, and Ym1 in isolated macrophages, while decreasing expression of M1 markers. When tested in fetal lung explants, LPS also inhibited expression of Mgl1/2, suggesting a more M1 phenotype. We have previously shown than LPS inhibits airway branching in fetal lung explants. Adding IL-4 or IL-13 prevented the effects of LPS on branching and maintained expression of the M2 marker Mgl1/2. When constitutive NF-□B activation in fetal lung macrophages was tested using doxycycline-treated IKFM macrophages, we again measured increased expression of IL-1β, TNF-α, and CCL-3 and decreased CD206 and Ym1. IL-4 and IL-13 increased CD206 and Ym1 expression in activated IKFM macrophages, but did not decrease expression of CCL-3, IL-1β, and TNF-α.
Conclusions: These results demonstrate that NF-□B activation in fetal lung macrophages stimulates an M1 versus M2 cell phenotype. This shift may play a role in the altered lung morphogenesis seen with exposure to inflammation, as suppression of the M1 phenotype and maintenance of the M2 phenotype with IL-4 or IL-13 can preserve airway branching.

1838

PTEN-dependent upregulation of Bnip3 mediates apoptosis and cavitation during embryonic epithelial morphogenesis.

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Gene targeting experiments have demonstrated that the tumor suppressor phosphatase and tensin homolog (PTEN) is essential for mouse early embryogenesis. To elucidate the role of PTEN in embryonic epithelial morphogenesis and the underlying mechanisms, we employed embryonic stem cell-differentiated embryoid bodies (EBs), which are epithelial cysts consisting of an outer endoderm, an underlying basement membrane, and the epiblast epithelium encompassing a proamniotic-like cavity. We found that PTEN is upregulated during EB morphogenesis in parallel with decreased Akt activation and increased inner cell apoptosis, the latter is essential for EB cavitation. Genetic ablation of PTEN in EBs causes over-activation of Akt and inhibition of apoptosis-dependent cavitation. However, pharmacological inhibition of Akt fails to induce cavitation in PTEN-null EBs. Additionally, overexpression of myristoylated, constitutively active Akt in wild-type EBs only slightly inhibits apoptosis and cavitation, suggesting the existence of an Akt-independent mechanism. Our previous study showed that hypoxia-induced upregulation of Bnip3, a pro-apoptotic BH3-only protein, contributes to EB cavitation. To test whether Bnip3 mediates PTEN-dependent cavitation, we analyzed Bnip3 expression in wild-type and PTEN-null EBs. PTEN inactivation inhibits Bnip3 elevation during EB differentiation and in response to hypoxia. Furthermore, overexpression of Bnip3 restores the apoptosis of core cells in PTEN-null EBs. Lastly, reconstitution of PTEN-null EBs with wild-type PTEN or the G129E mutant, which lacks the lipid phosphatase activity, completely rescues Bnip3 expression and cavitation, whereas expression of the C124S mutant, which lacks both the lipid and protein phosphatase activities, restores Bnip3 expression and apoptosis but not cavitation. These results suggest that PTEN facilitates hypoxia-induced Bnip3 upregulation and apoptosis independently of its phosphatase activity. Yet its protein phosphatase activity is required for the clearance of apoptotic corpse and cavitation.

1839

Genome-wide mapping of Sox6 binding sites reveals coordinated regulation of sarcomere protein gene expression by Sox6 in developing skeletal muscle.

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The molecular mechanisms underlying the terminal differentiation of vertebrate skeletal muscle are not well understood. Our laboratory investigates the role of the transcription factor Sox6 in fiber type (slow-twitch vs. fast-twitch) specification in mammalian skeletal muscle and has previously reported that Sox6 functions as a transcriptional suppressor of the slow fiber gene program during skeletal muscle development. [Objective] The objective of the current study is to identify genome-wide binding sites of Sox6 and uncover the functional role(s) of Sox6 during terminal differentiation of skeletal muscle. [Methods] We conducted ChIP-seq, conditional inactivation of the Sox6 gene in mouse muscle, and reporter gene assays of the newly identified Sox6 binding cis-regulatory sequences using muscle primary cultures. [Results] We report that: (1) A total of 1,066 Sox6 peaks were identified by ChIP-seq using mouse fetal myotubes and were assigned to 867 RefSeq genes. Gene ontology analysis of the Sox6 peak-associated genes revealed that the categories relevant to muscle functions, muscle development, and transcriptional regulation were significantly enriched. The genes encoding slow fiber-specific sarcomere proteins and transcription factors highly expressed in muscle were found to be frequently associated with Sox6 peaks; (2) The majority of the Sox6 peak-associated genes were classified as transcriptionally inactive based on Pol II ChIP-seq data, suggesting that the

main function of Sox6 is transcriptional suppression; (3) This notion was confirmed by the phenotype of Sox6 conditional knockout muscle, which exhibited a significant increase in the expression of slow fiber, cardiac, and embryonic isoform genes (associated with Sox6 peaks), accompanied by a major decrease in the expression of fast fiber-specific genes; and (4) Sox6 binding sites were found in the previously reported slow fiber-specific Tnni1 and Tnnc1 enhancer regions. These Sox6 binding sites stifled the enhancer activity, suggesting that transcriptional levels of muscle fiber type-specific genes may be dictated by the combination of modular cis-regulatory elements, which exert positive or negative influence on transcription. [Conclusions] Our data indicate that Sox6 plays a critical role in fiber type specification of skeletal muscle. The main function of Sox6 is transcriptional suppression of developmentally regulated genes, whose combinatorial expression determines the physiological maturity of skeletal muscle.

1840

Tissue Geometry Regulates the Axis of Cell Division in Epithelial Tissues.

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Control of the orientation of cell division is critical for regulating normal tissue structure and function. Misorientation of the axis of cell division can cause organ malformation and tumorigenesis. Recent studies examining the biophysics of single cells have revealed that cell shape and cell-matrix interactions can act as significant determinants for the orientation of the plane of cell division. Spatial patterns in cell behavior emerge when groups of cells interact, in part through endogenous mechanical stresses transmitted between cells. Here we investigated the influence of these emergent biophysical signals on the axis of cell division within epithelial tissues. Microlithography was used to engineer epithelial sheets of defined geometry. The axis of cell division was recorded within these microscale tissues, and composite maps of division axis were correlated with computational predictions and experimental validations of the patterns of endogenous mechanical stresses. Cell proliferation correlated with the magnitude of mechanical stress, with increased proliferation in regions of high mechanical stress. In contrast, the axis of cell division was largely perpendicular to the gradient of the endogenous stress. Additionally, the axis of cell division was influenced by the global geometry of the tissue and the local motions of neighboring cells. These data implicate emergent mechanical signals that arise from tissue multicellularity as key determinants of the orientation of cell division, and suggest another mechanism through which tissue geometry might control patterns of morphogenesis.

1841

Inhibition of Sp1-Mediated FGF-10 Expression by Sp3 and NF-κB.

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Objective: Microbial products and inflammatory mediators that activate NF-κB can inhibit branching morphogenesis of the fetal lung, and may lead to developmental lung disease. A key mechanism in this process appears to be the NFκB mediated suppression of FGF-10 expression in the fetal lung mesenchyme. The transcriptional mechanisms for this inhibition are unknown. We hypothesize that NF-κB activation interferes with normal transcriptional regulation of FGF-10 expression.

Methods: We measured FGF-10 promoter activity using an FGF-10 luciferase reporter plasmid in CHO cells. NF-κB activation was modulated by either IL-1β addition or overexpression of a constitutively active IKKβ; (cIKKβ). NF-κB was also blocked using a dominant-negative IκB (dnIκB). ChIP was performed on LPS-treated primary fetal mouse lung mesenchymal cells using antibodies against Sp1, Sp3, and the NF-κB subunit RelA.

Results: Both IL-1β and cIKKβ expression inhibited FGF-10 promoter activity. Conversely, inhibiting NF-κB activation by transfecting cells with dnIκB increased FGF-10 promoter activity above basal levels. The effect of NF-κB on the FGF-10 promoter appeared to be indirect, as deletion a predicted NF-κB binding site in the FGF-10. This result suggested that NF-κB might inhibit the function of other transcription factors at the FGF-10 promoter. Following in silico identification of several GC boxes within the FGF-10 promoter, we next tested if Sp family transcription factors regulated FGF-10 transcription. Overexpressing Sp1 increased FGF-10 promoter activity. Sp1 also co-immunoprecipitated with RelA, suggesting physical interaction. The stimulatory effect of Sp1 was inhibited by co-expression with Sp3. Interestingly, only the two longest translational isoforms of Sp3 inhibited Sp1-mediated FGF-10 promoter activity. NF-κB activation may promote the recruitment of Sp3 to the FGF-10 promoter, as ChIP from LPS-treated cells measured increased RelA and Sp3 promoter association. Co-transfection with dnIκB blocked the inhibitory effects of Sp3, suggesting that NF-κB activation and nuclear translocation may be required for Sp3 to block FGF-10 transcription.

Conclusions: These data suggest that inflammatory signaling through NF-κB inhibits FGF-10 expression by altering the normal transcriptional activities of Sp1 and Sp3 at the FGF-10 promoter. NF-κB might therefore use this a novel mechanism to disrupt normal developmental regulation of FGF-10 in the fetal lung mesenchyme.

1842

PI3K/Akt1 plays a pivotal role in retinal angiogenesis.

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Phosphoinositide 3-kinase (PI3K) and Akt, downstream target molecule of PI3K, regulate multiple cellular functions including cell proliferation, cell cycle progression, tumor growth, and angiogenesis. Disruption of Akt1 in mice shows reduced organism size and endothelial dysfunction. Blood vessels comprise endothelial cells, mural cells. During angiogenesis, mural cells are recruited to sprouting endothelial cells and stabilizing newly formed vessels. In this study, we investigated the role of Akt in endothelial cells or in mural cells during retinal angiogenesis. Vascular endothelial growth factor (VEGF)-induced capillary-like tube formation was significantly abolished in Akt1 knock-down human umbilical vein endothelial cells (HUVECs), whereas Akt2 knock-down cells were not affected. Moreover, retinas from WT or Akt1 deficient mice were stained with endothelial cell marker, CD31 and smooth muscle cell marker, SM22a. Interestingly, retinas from Akt1 knock-out mice showed tortuous, irregular and clustering retinal vessels compared to that of WT mice. It represented that loss of Akt1 caused vascular morphological alterations. Given these results, we suggest that Akt1 plays an important role during retinal angiogenesis.

1843

Role of Pinin in Alternative pre-mRNA Splicing during Epithelial Differentiation.

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Pinin (Pnn) is a member of the SR proteins, which comprise a large portion of the non-snRNP spliceosomal proteins. Our previous investigations have revealed that Pnn plays essential roles in the differentiation and maintenance of epithelial cells. To further investigate Pnn's function during epithelial differentiation and to explore the potential underlying mechanism, we now focus on Pnn's role in alternative pre-mRNA splicing. Conditional inactivation of Pnn in multiple types of developing epithelia by utilizing Shh-Cre and Pax6-Cre mouse lines resulted in significant disruption of alternative splicing pattern of Fibroblast growth factor receptor 2 (Fgfr2), leading to the aberrant ratio between IIIb (epithelial-type) and IIIc (mesenchymal-type) isoforms, while not affecting total Fgfr2 transcript level or the alternative splicing of other genes such as Pyruvate kinase isozyme type M2 (Pkm2). Further alternative splicing-specific RT-PCR analyses revealed that other epithelial patterns of pre-mRNA splicing such as Cd44, p120-Catenin (CtnnD1), and enabled homolog (Enah), were also markedly altered in Pnn depleted epithelial cells. It has been demonstrated that epithelial-specific alternative splicing may to a great extent be mediated by Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and 2). Furthermore, ESRPs have been shown to be crucial for the epithelial differentiation and maintenance. Notably, our recent co-immunoprecipitation assays revealed that PNN is in a complex with ESRP1, along with other SR Proteins, SF3b155 and U2AF65. Finally, consistent with our gene inactivation studies in mice, inducible shRNA-mediated knockdown of either PNN or ESRP1 in human corneal epithelial cells led to the similarly decreased levels of epithelial-type FGFR2 isoform, IIIb. Taken together, our study suggests that Pnn is involved in the regulation of epithelial-specific alternative splicing of Fgfr2, Cd44, CtnnD1, and Enah, and Pnn may act through modulating alternative pre-mRNA splicing, possibly through coordination with ESRP proteins, during epithelial cell differentiation. (Supported by NIH R01 EY07883)

1844

Developmentally Regulated Expression of Intracellular Fgf11-13, Hormone-like Fgf15 and Canonical Fgf16, -17, and -20 mRNAs during Odontogenesis.

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Objective: To investigate and compare the cellular expression of nonsecreted Fgf11-14 and secreted Fgf15-18 and -20 mRNAs during tooth formation. Material and methods: mRNA expression was analyzed from the morphological initiation of the mouse mandibular first molar development to the onset of crown calcification using sectional in situ hybridization. Results: We found distinct, differentially regulated expression patterns for the Fgf11-13, -15-17 and -20, in particular in the epithelial-mesenchymal interface whereas Fgf14, and 18 mRNAs were not detected. Fgf11, -15, -16, -17, and -20 were seen in the epithelium whereas Fgf12 and -13 signals were restricted to the mesenchymal tissue component of the tooth. Fgf11 was observed in the putative epithelial signaling areas, the tertiary enamel knots and enamel free areas of the calcifying crown. Fgf15, Fgf17, and -20 were transiently colocalized in the thickened dental epithelium at E11.5. Later Fgf15 and -20 were exclusively expressed in the epithelial enamel knot signaling centers. In contrast, Fgf13 was present in the dental mesenchyme including odontoblasts cell lineage whereas Fgf12 appeared transiently in the preodontoblasts. Conclusions: The expression of the Fgf11-13, -15, -17 and -20 in the epithelial signaling centers and/or epithelial-mesenchymal interfaces at key stages of the tooth formation suggest important functions in odontogenesis

1845

Mitochondrial development of the *in vitro* hepatic organogenesis model with simultaneous cardiac mesoderm differentiation from murine ES and iPS cells.

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Induced pluripotent stem (iPS) cells, resembling embryonic stem (ES) cells in many phenomena, including differentiation potential, colony morphology, and the expression of specific representative markers, were generated from differentiated somatic cells. In recent, the mitochondria of iPS cells were reported to be rejuvenated to that of ES cells, however it is not known if the mitochondria have same potential for differentiation as ES cells. We investigated mitochondrial energetic potentials during the differentiation process of iPS and ES cells. We have established the murine ES/iPS cell-derived in vitro hepatic organogenesis model, consisting of not only hepatocytes but also endothelial networks together with cardiac mesoderm differentiation, previously. Mitochondrial energetic potentials can be followed by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). OCR is an indicator of mitochondrial respiration, and ECAR is the concentration of acids in the medium, predominantly lactic acid. The assay for mitochondrial energetic potentials was performed using an XF24 Extracellular Flux Analyzer. By measuring OCR and ECAR in the culture medium, we compared the mitochondrial energetic potentials of both ES and iPS cells during the hepatic differentiation. The mitochondrial energetic potentials of the in vitro hepatic organogenesis from iPS cells accorded with each differentiation steps, from proliferation stage as the initiation, spontaneously beating cardiac differentiation in the next, and finally liver tissue-formation, as well as that from ES cells. Our results indicate that the mitochondrial energetic potentials of the *in vitro* hepatic organogenesis model derived from the ES and iPS cells correspond to the proliferation stage in the initiation, spontaneous beating cardiac and hepatic differentiation. Both ES and iPS cells were differentiated into liver-like tissue with similar mitochondrial development.

1846

Epicardial TGFb Signal Is Required for Myocardial Maturation Before the Onset of Coronary Circulation.

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Developing epicardium originates from the proepicardial organ (PE) and spreads over the external surface of myocardium. Thereafter the epithelial epicardium seeds mesenchyme into the subepicardial space to form a multi-potent progenitor for cardiac fibroblasts, coronary smooth muscle and endothelial cells. Surgically ablated PE-deficient hearts in chick embryos as well as mutant mice hearts with defective epicardium show a thin myocardial compact layer before the onset of coronary circulation. Using a chick model with mechanically ablated PE, we investigated mechanisms that lead to thin compact myocardium. In ED6 hearts with defective epicardium, myocardial compact layer was significantly thinner than that of sham-operated hearts. Neither BrdU incorporation nor immunostaining for Phospho-Histone H3 indicated that there was significant difference in the mitotic index between the epicardium deficient myocardium and control hearts. Immunohistochemical detection for sarcomeric proteins showed that myofibrillogenesis was established in epicardium-deficient myocardium, but the average distance between the Z-lines was shorter than that in control. In addition, cell density in epicardium-deficient compact myocardium was significantly higher than that in controls. Western blot analysis showed that PSmad2, but not PERK or PSmad1/5/8, was decreased in epicardium-deficient hearts. In organ culture, primitive ventricle without epicardium from ED2.5 heart was co-cultured with or without PE. After 2 or 3 days of incubation, cultured myocardium

without PE established striated myofibrils, however the average space of Z-lines was shorter and the cell density was higher in comparison with those in co-cultured ventricles. The immaturity and hypotrophy seen in the cultured ventricle without PE were reversed by treatment of conditioned medium harvested from PE culture or recombinant TGFb protein. Co-cultured ventricle treated with LY364947 (TGFb type I receptor kinase inhibitor) showed shortened spaces of Z-lines and hypotrophic cardiomyocytes. These results suggest that the thin compact myocardium in the epicardium-deficient hearts is attributed to impaired cardiomyocyte hypertrophy in the developing myocardium, and myocardial maturation is facilitated by epicardium-secreted trophic factors such as TGFb before the onset of coronary circulation.

1847

Heparanase-1 knock-down delays lumen formation during rat ventral prostate morphogenesis in the first postnatal week.

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Branching morphogenesis is an important step in ventral prostate (VP) development. This event occurs in rats after the initiation of bud formation that happens during the last few days of gestation and in the first three postnatal weeks. The testosterone surge around birth is important for stromal and epithelial differentiation through andromedins in a paracrine way. FGF-10 is one of the candidate andromedins. We reported previously that cell death is associated to luminal epithelial cell polarization during the epithelium canalization process in the VP development. Extracellular matrix (ECM) remodeling is also necessary in this process. Matrix metalloproteinase-2 (MMP-2) was shown to contribute to organ growth and morphogenesis. Heparanase-1 (HPSE1) is an endoglucuronidase that has been reported on the releasing of heparan sulfate (HS) fragments and growth factors from ECM stores. It is also known that HPSE1 expression is compensated by MMP expression, as showed for different organs of HPSE1 knock-out mouse. Previous results demonstrated that Hpse1 is expressed mainly by epithelial cells during the first post natal week of VP development and its knock-down by siRNA delays VP epithelial outgrowth and branching morphogenesis. Thus we hypothesized that HPSE1 might be involved in VP morphogenesis of epithelial tubes. In order to test this hypothesis, we have cultured PND 0 (postnatal day zero, the day of birth) rat VP in a membrane floating system with siRNA against Hpse1 (siRNA against Gfp was used as control) and followed the development every two days until PND6 for organ and epithelial area measurements and tips counting. These VP were further processed in historesin for morphological analysis. We also dissected PND10 rat VP and isolated the cells with collagenase followed by trypsin/EDTA digestion and cultured the cells on growth factor-reduced matrigel with the same knock-down treatment described above. This material was labeled with phalloidin-TRITC and DAPI for spheroid analysis under confocal microscopy. Counting of luminated epithelial structures on PND2 demonstrated a 6.2-fold decrease in the 50 nM siHPSE (1.8%) as compared to the 100 nM siGFP control (11.3%), while the 100nM siHPSE resulted in completed absence of lumen formation. This was associated with reduced pERK1/2, which is thought as a first evidence for compromised FGF-10 signaling in this process. Spheroid cultures showed a decrease on the percentage of lumen-bearing spheroids in the group treated with siHPSE (100 nM siGFP control [62%]; 50 nM siHPSE [40%] and 100 nM siHPSE [39%]). These results indicate that HPSE1 plays a role in epithelial tube morphogenesis. It will be interesting to dissect the mechanism through which HPSE exerts this effect focusing on the FGF10 pathway, as it has been explored by others that it may act through an enzyme activity-dependent pathway by releasing HS and the bound growth factors or through an activity-independent pathway by clustering syndecan on cell surface and acting on Rac1 pathway that acts on epithelial cell polarity. Funding by FAPESP (Proc.09/16150-6).

1848

Aurora Kinase-A deficiency impairs skin stratification.

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Aurora Kinase-A (Aurora-A) is a mitotic regulator that promotes the timely entry into mitosis, centrosome maturation and formation of bipolar spindles. Although highly studied as an oncogene implicated in epithelial cancers, its role in developing skin or tissue homeostasis remains poorly understood. To characterize the role of Aurora-A in skin development, we deleted Aurora-A by interbreeding *K14.Cre* mice with mice containing a floxed Aurora-A allele (*Aurora-A^{fl}*). *Aurora-A$^{fl/fl}$;Krt14.Cre* mice died shortly after birth. The skin of these mice was translucent and histological evaluation showed the epidermis was thin, lacked hair follicles and displayed areas of frank erosion. Although marker analysis revealed the expression of basal layer markers, Krt5 and Krt14 and differentiation markers, Krt1, Krt10, and loricrin in *Aurora-A$^{-/-}$* skin, there was a marked reduction in the number of basal and suprabasal layers and dye exclusion assays showed defects in barrier function. To further characterize the effects of Aurora-A deficiency, we examined embryonic skin between 13 to 14 dpc at the stage when the epidermis begins to stratify. Unlike wildtype cells, *Aurora$^{-/-}$* basal progenitors were delayed in forming two layers as revealed by histological examination and by the late appearance of Krt1 and Krt10 positive subrabasal cells. We observed a concomitant increase in cells in mitosis as well as induction of apoptosis. Furthermore, *Aurora-A$^{-/-}$* keratinocytes were polyploid, indicating that proliferating cells did not complete mitosis but exited prematurely. *Aurora-A$^{-/-}$* keratinocytes also displayed centrosome abnormalities including apparent monopolar spindle structures. At later stages of development, the position of the nuclei relative to the centrosome appeared random, instead of the predominant perpendicular orientation to the dermis of basal keratinocytes seen in *Aurora-A$^{+/+}$* skin. Thus, the deletion of Aurora-A in the developing epidermis may alter the polarity of basal cells and markedly impaired their ability to divide and stratify.

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EB1, p150Glued and Clasp1 control apical polarization of acetylated tubulin during vascular tube formation in 3D matrices.

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Endothelial cell (EC) polarity is a necessary aspect of vascular morphogenesis. However, the molecular mechanism of vascular endothelial cell polarity during this event remains elusive. Here, we show that microtubule tip complex proteins are fundamental regulators of EC lumen formation by affecting EC apical-basal cytoskeletal polarity and tubulin post-translational modifications which are necessary for these events in 3D matrices. Using confocal microscopy, we find a striking cytoskeletal polarity during this process whereby filamentous actin is oriented basally while tubulins including acetylated and detyrosinated tubulin are oriented apically. Also, acetylated and detyrosinated tubulins are strongly induced during lumen formation. Furthermore, elevated SIRT2 and histone deacetylase 6 levels leads to reduced EC lumen formation and concomitant loss of acetylated tubulin while siRNA suppression of SIRT2 leads to increased lumen formation. These observations motivated the hypothesis that a primary mechanism for EC lumen formation is the establishment of asymmetric EC polarity which creates a polarized cytoskeleton that differentially supports the apical (microtubule-rich) versus basal (actin-rich) plasma membrane. siRNA suppression of EB1, p150Glued and Clasp1 leads

to marked blockade of vascular lumen formation, including inhibition of a Src, Pak, Raf and Erk protein kinase cascade. In addition, there is marked blockade of tubulin acetylation and detyrosination which accompanies a loss in EC apical-basal cytoskeletal polarity in 3D collagen matrices. In addition, we observe sub-apical co-localized staining for p150Glued and Clasp1. Re-expression of EB1 fully restores vascular tube formation in EB1 siRNA treated ECs but EB1 mutants fail to rescue this phenotype. We, therefore, propose that EB1, p150Glued and Clasp1 regulate vascular morphogenesis by affecting tubulin acetylation and apical-basal endothelial cell polarization.

1850

Roles of Noggin, a BMP antagonist, in proper development of the mammalian notochord and foregut.

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The mammalian trachea and esophagus, parallel tubes with distinct identities and functions, arise during development from a single anterior foregut tube. Although malformations of the trachea and esophagus are common human birth defects (~1:3,000 live births), the morphogenetic and cellular processes that govern anterior foregut development are understudied. A growing number of rodent models now exist with which to study both the normal compartmentalization of the trachea and esophagus, and the abnormal development that leads to foregut malformations. One model, the Noggin (*Nog*) null mouse, very closely recapitulates the common foregut malformation esophageal atresia with tracheo-esophageal fistula (EA/TEF) with 75% penetrance. This particular malformation consists of an esophagus that ends rostrally in a blind pouch, and a communication between the trachea (or lungs) and the stomach. NOG is a secreted Bone Morphogenetic Protein (BMP) antagonist, but the mechanism by which loss of *Nog* causes EA/TEF is unknown. One possibility is that *Nog* expressed near the time of foregut compartmentalization is essential for this process. Another, arising from the fact that *Nog* mutants have large and disorganized notochords, is that resolution of the notochord from the dorsal foregut endoderm (future esophagus) early in embryogenesis can impact the potential of the foregut to compartmentalize later. Therefore, there are two *Nog* expression domains that conceivably play a role in the proper compartmentalization of the anterior foregut into the trachea and esophagus: 1) the dorsal foregut endoderm, and/or 2) the notochord. We have used tissue- and time-specific manipulation of *Nog* and the BMP pathway in mouse embryos to determine the spatio-temporal requirements for BMP antagonism in the process of foregut compartmentalization. Our results indicate a key role for NOG secreted from early axial structures in attenuating BMP signaling within these axial structures, rather than only in the developing foregut. In conjunction with other published results and careful histological and immunological observations of the *Nog* mutant notochord, these data suggest that an early embryological event, the improper resolution of the notochord from the dorsal foregut endoderm, can cause EA/TEF. Ongoing and future work aims to determine the cell biological consequences of abnormally high BMP signaling on notochord cells as they resolve from the dorsal foregut endoderm.

1851

Could a Malformed Feather be a Melanocytes' Immune Response?

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Avian feathers are very complex epidermal appendages (Lucas & Stettenheim, 1972). During its development longitudinal cell planes will form barb ridges and barbule plates in the follicle (Maderson *et al*, 2009). Between these plates, supporting cells are source of cellular structures for the adult feather. Structures such as melanosomes are produced by the supporting cells

(melanocytes) and distributed to the barb ridge and barbule cells (keratinocytes) at early stages of the feather development (Maia *et al*, 2011). The melanosomes are rod or spherical structures composed primarily by melanin, although during its development it is possible to observe many internal membranaceous structures (Durrer & Villiger, 1967). Thus, in a Transmission Electron Microscopy (TEM) micrograph it is possible to see that the melanosome is not equally electron dense, there is some internal organisation. The use of Scanning Transmission Electron Microcopy (STEM) enhances the perception of an internal texture. In a simple coloured feather, melanosomes will not differ greatly, therefore similar texture and density is seen in its melanosomes. Another aspect of melanocytes, other than the production of melanosomes, is its capability to respond to an inflammation or a microbial infection (Mackingtosh, 2001). Melanogenesis is stimulated and further production of melanin is a signal of an immune response (Le Poole *et al*, 1993). As any tissue, feathers are subjected to inflammation or microbial during its development. We prepared flight feather barbs of Procellariiformes for TEM following Shawkey *et al* (2003) and observed it in STEM. Inflammatory-like tissue showed melanosomes different to barb melanosomes. This shows that in birds, melanocytes may have immune system properties, as observed in reptiles (Jonhson *et al*, 1999). The melanosomes present in such outgrowth showed different internal texture and different morphology. Some melanosomes were greatly fused, not being possible to distinguish them as rod or spherical. Even the ones that could be individualised were larger than those observed in the barb. Differently from the normal barb melanosomes, these had a random orientation. Therefore, it is possible to speculate that inflammatory responding melanocytes will produce melanosomes with different characteristics than melanocytes which produce melanosomes for the feather tissue. Further investigation on melanocytes of deformed or microbial-attacked feathers will help to understand the role of melanocytes in feather development.
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Connective Tissue Growth Factor Expression in Post-natal Epididymal Development.

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Objective: To determine epididymal cell type, region and segment specific expression of the connective tissue growth factor (CTGF) gene promoter and protein throughout murine post-natal development.
Methods: CTGF-enhanced green fluorescent protein (CTGF-EGFP) transgenic male mice were sacrificed at 7, 14, 21, 28, 35, 42, 49, and 56 days post natal (DPN) and analyzed by fluorescence microscopy. CTGF promoter activity was determined by presence of EGFP. CTGF protein, aquaporin 9 protein (AQP9) – a principal cell marker, and vacuolar proton ATPase subunit B2 (B2) protein - a clear cell marker, were detected by immunofluorescence. CTGF mRNA expression was assessed by in situ hybridization (ISH).
Results: At 7 DPN, weak CTGF promoter activity was detected in 80 ± 2% of luminal epithelial cells (LECs) in all epididymal segments. CTGF promoter activity diminished in LECs of only the initial segment and corpus in a time dependent manner beginning at 14 DPN, decreasing to 5 ± 4% of LECs at 56 DPN. In contrast, EGFP staining intensified in the caput and cauda, with 80% of the LECs staining. However, segments 5 and 8 were characterized by a small reduction in the number of positively stained LECS, (35-60%). A large number of cells which were positive for EGFP also exhibited apical AQP9 protein staining, marking these LECs as principal cells. Clear cells expressed B2 protein, but lacked CTGF promoter activity. CTGF protein was detected at the apical surface of LECs and in peritubular muscle of all regions. ISH of 7 and 28 DPN tissue revealed CTGF mRNA expression in LECs in a region and segment specific pattern.

Conclusions: CTGF promoter activity appears to be tightly regulated in principal cells in different epididymal regions and during the stages of sexual maturation. The increased intensity of EGFP staining post 21 DPN in principal cells of the caput and cauda along with apical CTGF protein accumulation coincides with LEC differentiation. This, plus the gradual loss of CTGF promoter activity in the initial segment and corpus with increasing development, possibly indicates a role for CTGF in establishing divergent states of differentiation in principal cells of different segments. CTGF promoter activity was observed in a vast majority of principal cells in the caput and cauda, but not in clear cells. mRNA expression appeared to mimic promoter activity.
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1853

Ectoderm mediated nitric oxide signaling regulates early skeletal muscle (myotome) formation in somites of chicken embryos.

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Nitric Oxide (NO), a short-lived messenger molecule, plays significant roles in animal physiology. NO is produced by nitric oxide synthases (NOS), utilizing L-arginine with oxygen and NADPH, and signals primarily by the NO/sGC/cGMP pathway. In adult muscle, NO stimulates activation of muscle stem cells in muscle fiber regeneration and regulates myoblast differentiation. In the chicken embryo, dynamic NO increases were previously reported in the ectoderm layer, but its role is unknown in the regulation of skeletal myogenesis (or primary myotome formation) in somites. To investigate, the ectoderm layer of chicken embryos was treated with 50-100 mM L-NAME, a competitive inhibitor of NOS, for a brief 6-hr period with subsequent myotome formation assessed by titin immunofluorescence and confocal microscopy. The L-NAME treatment period allowed formation of 4 new somites and embryos tested had between 6-38 somites (Hamburger and Hamilton stages 8-20). L-NAME treated embryos (n=53) produced delays in the start of myotome formation by 1-3 somites over the control embryos (n=87) in the range of embryos tested. The trends for both populations with best-fit line equation was y=1.233x-12.18, R^2=0.99 for control and y=1.223x-13.40, R^2=0.96 for L-NAME, with x and y representing embryo age in somite numbers and last somite level expressing titin, respectively. Embryos between 11-14 somites expressed myotome for the first time and only in the 3 cranial-most somites (n=15). Myotome formation in control and L-NAME treated embryos was also biphasic for titin expression in somites and contrasted with previously reported triphasic myotome expression in somites based on desmin immunofluorescence. A comparison of quantitative analysis of titin and desmin expression revealed that titin is expressed earlier than desmin by one somite level. We conclude that NO signaling in the embryo ectoderm layer regulates early myotome formation in somites and myogenesis is a biphasic event being prevented until HH 10-11 and later activated in the 3 cranial-most somites then progressing caudal-ward by 1.2 somites with every new somite produced. NSF-IOS-0821324

1854

SJJ2, as a Novel Cardiac Factor, involved in Myocardial Differentiation and early embryonic heart development.

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Despite several advances in identification of cardiac transcription factors, there are still needs to find new molecules that directly control cardiomyogenesis from ESCs (embryonic stem cells) to highly efficient myocardial differentiation. To identify novel cardiac factor, we performed Illumina expression microarray. SJJ2 was significantly increased in the mESCs (mouse ESCs)-derived

cardiomyocytes, and characterized as a cardiac factor in myocardial differentiation and early embryonic development. First, we generated mESCs expressing eGFP (enhanced green fluorescent protein) under direction of ¥á-MHC (¥á-myosin heavy chain) gene promoter that specifically works in functional cardiomyocytes. After established GFP expressing mES cell line, we screened novel cardiac factors in ESCs-derived cardiomyocytes using Illumina expression microarray. 276 genes were upregulated (¡Ã 4 fold) in mESCs-derived cardiomyocytes compared undifferentiated ESCs. Among them, SJJ2 is one of the novel signaling molecule candidates, and its function has not yet been analyzed. We confirmed that SJJ2 expression is increased at the initial stage of ESCs differentiation into functional cardiomyocytes. Also, the level of SJJ2 transcription and translation were transiently increased on C2C12 myoblast differentiation into myotubes. Interestingly, we found that the secreted form of SJJ2 can have a potential to be a signaling molecule. Furthermore, we confirmed that SJJ2 is expressed in the developing heart at 9.5-11.5d.p.c embryos. Taken together, our results indicate that SJJ2 was expressed during myocardial differentiation and early embryogenesis. Through which, SJJ2 has the potential to serve as an important factor in early embryonic heart development.

1855

Rncr3 (retinal non-coding RNA 3), which encodes miR-124a, is required for hippocampal axon development and retinal cone photoreceptor survival through Lhx2 suppression.

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MiRNAs are small RNAs that regulate gene expression by base-pairing to mRNAs. Interestingly, miR-124a is completely conserved at the nucleotide level from worms to humans, and estimated to be the most abundant miRNA in the brain. In our screen identifying functionally important molecules in the retina, we isolated a mouse Rncr3 Rncr3 (retinal non-coding RNA 3) cDNA which is highly expressed in the retina, and found a stem loop of precursor microRNA-124a-1 (pre-miR-124a-1), which is encoded on an Rncr3 exon.To address the function of miR-124a in vivo, we generated Rncr3 knockout mice by replacing all Rncr3 exons with the PGK-neo cassette.We detect that miR-124a was reduced in the Rncr3$^{-/-}$ mouse central nerves system (CNS), especially in the retinal presumptive photoreceptor cell layer (PPL), where cone photoreceptor neurogenesis occurs, and the hippocampal dentate gyrus. Rncr3$^{-/-}$ mutant mice exhibit abnormalities in the CNS, including small brain size, axonal mis-sprouting of DG granule cells, and retinal cone cell death. We found that Lhx2 is an in vivo target mRNA of miR-124a. We also observed that LHX2 down-regulation by miR-124a is required for prevention of apoptosis in the developing retina and proper axonal development of hippocampal neurons. These results suggest that miR-124a plays an essential role in maturation and survival of DG neurons and retinal cones by repressing Lhx2 translation.

1856

Gender differences in social interaction but not crooked tail phenotype in prenatally valproic acid exposed rats.

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Both in human and rodents, exposure to valproic acid (VPA) during fetal developmental period causes teratogenic effects on developing nervous system and produces autistic behavioral symptoms. VPA induced autistic-like behavior such as reduced sociability and social preference as well as reduced threshold against electroconvulsive seizure only when it administered to pregnant rats during the short critical periods (E12). Prenatal exposure to VPA also induced crooked and shortened tail, which might be regarded as the mildest form of neural tube defects.

Interestingly, the altered social behavior is most prominent in males with crooked tail phenotype compared with female animals even though there was no gender difference in the occurrence of crooked tail. Regardless of the occurrence of crooked tail, rat offsprings did not show any defects in motor coordination suggesting that the crooked tail phenotype is not related to the gross motor defects. These results suggest that male rats are more vulnerable to defects in social interaction compared to female animals even with similar neural tube defects phenotype. VPA animal model of autism may provide excellent animal model to study the molecular basis of gender differences of autistic behavior.

1857

Tissue-specific stiffening of embryos parallels myosin and matrix expression and dynamically matches cell function: a focus on embryonic heart.

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During development, the establishment of mature tissue mechanics at the proper time and place is essential for cellular differentiation and function. Here we study the maturation of embryonic cardiac and neural tissue mechanics in parallel with the effects of extracellular mechanics on individual cell function and structure throughout early development. We used micropipette aspiration to measure local and bulk elastic moduli (E) of embryonic avian heart and midbrain tissue from days 2-12. We observe stiffening of the early heart tube from E = 1 kPa at day 1 to E = 2 kPa at day 4, reaching neonatal values by day 14. Brain tissue does not stiffen and behaves viscoelastically. Treating the tissues with blebbistatin led to ~30% decrease in E for heart and ~50% decrease in E for brain, indicating a significant but partial actomyosin contribution to mechanics at these stages. Treating tissues with collagenase softens heart tissue significantly, but has no effect on neural tissue mechanics, indicating the important role or ECM in establishing cardiac mechanics even at early stages. We performed a quantitative proteomic analysis of HH 3-5 embryos along with 2-4 day heart tubes and midbrains by mass spectrometry to and find that many cytoskeletal protein and collagen levels follow these mechanical trends. Isolated cardiomyocytes from 3-4 day chick embryos were cultured on collagen-coated PA gels of various stiffnesses. Beating magnitude and cytoskeletal organization was modulated by substrates with E~2 kPa, similar to physiological E at those stages.

1858

ROCK 1-directed basement membrane positioning coordinates epithelial tissue polarity.

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The basement membrane underlying epithelial cells is critical for epithelial tissue organization and function. However, the mechanisms by which the basement membrane is restricted to the basal periphery of epithelial tissues are not well defined, and basement membrane-mediated signals regulating coordinated tissue organization remain poorly understood. Here, we report that a Rho kinase (ROCK)-mediated, myosin-independent pathway controls coordinated tissue organization by restricting basement membrane to the epithelial basal periphery in the developing submandibular salivary gland (SMG). This ROCK-mediated pathway coordinately aligns individual cell polarity within the tissue via basal basement membrane positioning, and yet is not required for designation of an apical membrane in individual epithelial cells. PAR-1b functions downstream of ROCK 1 to regulate tissue polarity and is specifically required for basement membrane positioning since inhibition of PAR-1b kinase activity with either targeted siRNA knockdown or overexpression of a kinase dead PAR-1b construct prevents basement

membrane deposition and disrupts overall salivary gland epithelial tissue organization. Significantly, culture of salivary epithelial cells and organ explants in the presence of exogenous basement membrane (Matrigel) rescues epithelial organization in the presence of ROCK 1 and PAR-1b inhibition. This basement membrane-mediated, outside-in signal requires functional integrin beta1 to maintain epithelial cell adhesions since they are partially disrupted with integrin beta1 function-blocking antibodies. Taken together, these studies indicate that a ROCK 1/Par 1b pathway is required to maintain tissue polarity in developing epithelium through regulation of basement membrane placement.

Stem Cells and Pluripotency

1860

Post-Translational Regulation of Microtubules during Human iPS Reprogramming.

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Induced pluripotency (iPS) with human stem cells provides unique patient-specific, disease-bearing pluripotent cell lines (PSCs) important to biomedical investigations, and yet the underlying molecular mechanisms responsible for iPS have not been fully elucidated. Notwithstanding the progress in understanding the roles of transcription factors and epigenetic chromatin modifications being made, research on the cytoplasmic mediators of reprogramming remain largely unexplored. Here, we present evidence that post-translationally modified microtubules (PTM), including those which comprise the centrioles in the mitotic spindles poles of human pluripotent stem cells (hPSCs), are relevant to human iPS derivation. α-Tubulin can be acetylated at lysine 40 and monoclonal antibodies to acetylated α-tubulin detect centrioles of human somatic and pluripotent stem cells at mitosis, with only weak localization in spindle pole or cytoplasmic microtubules. Cold and drug depolymerization of microtubules does not abolish centriole acetylated α-tubulin, while microtubule enhancement with paciltaxel dramatically increases cytoplasmic microtubule staining with concomitant loss at the centrioles. Rescue experiments from microtubule depolymerization show unique cold and drug resistant microtubule cables distinct from the first dynamic microtubules that reassemble from the centrosomes, which are not acetylated. While the epigenetic regulation of chromatin by post-translationally modifying histones through histone acetyltransferases (HATs) and histone deacetylases (HDACs) are well known, cytoplasmic counterparts have not been explored. HDAC6 is unique in not residing within the nucleus but being localized mainly to the cytoplasm where it functions in the deacetylation of cytoplasmic targets like acetylated-microtubules, heat-shock protein (HSP)- 90 and cortactin. Tubastatin-A (TubA; 5nM-10µM), an inhibitor to HDAC6, induces the loss of centriole acetylation with a massive increase in spindle microtubule acetylation in all human PSCs but without detectable affects on histones, HSP90, or cortactin as observed by Western blots. Furthermore, TubA prevents iPS reprogramming by blocking PSC colony formation as observed by morphology and no live cell SSEA-4 pluripotency marker expression. These investigations focus on the role of the cytoplasm and cytoskeleton in mediating the events of nuclear reprogramming, and demonstrate tight cooperation between nuclear dynamics and cytoskeletal plasticity. We conclude that post-translational modifications of microtubules, in addition to transcriptional regulation, are essential for iPS derivations and represent an unappreciated example of ‘**Cytoplasmic Post-Translational Reprogramming**.

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1861

Migration of aberrantly persisting neural stem cells in Drosophila.

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Neuroblasts are the neural stem cells of the developing Drosophila brain and divide asymmetrically to produce all neurons present in the adult brain. Unlike mammals and even some insects, no new neurons are generated in the adult brain of Drosophila and neuroblasts do not persist. Previously, we determined that two pathways function in parallel to ensure that a subset of neuroblasts are properly eliminated during development, which is essential for ensuring proper adult brain size and inhibiting excess neuron production(1). Insulin/PI3 kinase signaling, which is a highly conserved cellular and organism growth signaling pathway, becomes attenuated in neuroblasts prior to the time of their disappearance, which then primes growth impaired neuroblasts for elimination via an apoptotic-dependent cell death program. Unexpectedly, we observe that some of the apoptotic-inhibited brain neuroblasts that aberrantly persist into adulthood migrate to a brain region rich in neurosecretory neurons, including those that secrete insulin-like peptides. This is unusual since neuroblasts are not migratory and are never found in this brain region. Once positioned here, aberrantly persisting neuroblasts have increased proliferation rates and survive longer than neuroblasts failing to migrate, suggesting this region may provide an environment permissive for neuroblast growth and survival. Currently we are investigating whether diffusible factors attract neuroblasts to the neurosecretory region, and if so, what these factors may be.

(1) Siegrist, S. E., Haque, N. S., Chen, C. H., Hay, B. A. & Hariharan, I. K. Inactivation of both Foxo and reaper promotes long-term adult neurogenesis in Drosophila. Curr Biol 20, 643-648, (2010).

1862

Midbodies contribute to cellular reprogramming and tumorigenicity by evading autophagy.

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The midbody (MB) is a nonmembranous organelle formed between daughter cells during cell division and is required for the final cell separation event in late cytokinesis. Over 500 proteins comprise the MB, many of which are required for cytokinesis, cell fate determination, and chromosome segregation. After cell division, the post-mitotic MB, or midbody derivative (MBd), can be retained and accumulated in cells. However, the fate and function of these MBds have not been elucidated. In this study we investigated the nature of cells that accumulate MBds, the mechanisms by which MBds accumulate, and the effects of MBd enrichment. By immunohistochemical and immunofluorescence analyses, we found that MBds selectively accumulated in stem cells, induced pluripotent stem cells (iPSCs), and potential cancer 'stem cells' (CSCs) in vivo and in vitro. While in normal proliferating non-cancer, non-stem cells MBd-accumulation was not observed. Remarkably, MBds were reduced upon differentiation of human embryonic stem cells, and increased upon induction of pluripotency in fibroblasts. By modulating protein degradation pathways, we found that MBd-accumulation was mainly controlled by receptor-mediated autophagy. In differentiating and normal dividing cells possessing high autophagic activity, MBd degradation occurred soon after cytokinesis and was mediated by binding of the autophagic receptor, NBR1, to the MB protein Cep55. In contrast, stem cells and cancer cells exhibited low autophagic activity, thus MBds evaded

autophagosome encapsulation and were accumulated. To address MBd function, we first enriched MBd+ cells by depleting NBR1. This enhanced reprogramming of fibroblasts to iPSCs and increased in vitro tumorigenicity of cancer cells. Similarly, high MBd+ cell populations enriched by FACS-isolation also formed more colonies on soft agar compared to low MBd+ populations. Together, our data suggest new roles for post-mitotic MB in maintaining or enhancing pluripotency in stem cells and CSCs. These findings also reinforce the idea of autophagy as a cell makeover process, in which MBds are a target that requires autophagic removal upon loss of pluripotency.
(Kuo et al., accepted)

1863

Ectopic expression of *Lhx2* in muscle satellite cells inhibits their myotube differentiation.

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Despite of many efforts, a clinically promising therapeutic method against congenital muscular dystrophy has not been established yet. Transplantation of muscle satellite cells derived from immunologically compatible human induced pluripotent stem cells (iPSCs) is a recently proposed strategy. Muscle satellite cells are tissue resident stem cells to replenish myoblasts to regenerate functional skeletal muscle fibers in response to muscle damage. However, only a limited amount of satellite cells can be induced from iPSCs by currently available protocols. To overcome this problem, we attempted an ectopic expression of LIM-homeobox transcription factor Lhx2, which has been recently proven effective for robust amplification of transplantable hematopoietic stem cells from mouse embryonic stem cells (ESCs) and iPSCs *in vitro*. (Blood, 117: 3748-3758, 2011). We employed the previously established *in vitro* differentiation method for ES-derived muscle satellite cells: i.e. embryoid body formation followed by subculture in matrigel-coated plates. Retroviral infection of *Lhx2* gene in differentiating mouse ESCs resulted in increased frequency of satellite cell marker positive cells. In addition, we found that overexpression of *Lhx2* completely inhibited myotube differentiation of C2C12 cells and primary culture of muscle satellite cells isolated from adult mice. Expressions of Myogenin and Myosin heavy chain were severely suppressed in *Lhx2*-transduced C2C12 cells under the culture condition for differentiation, whereas transcription of *Msx1* and *Msx2* genes encoding de-differentiation inducing homeobox transcriptional repressors was initiated. These results demonstrate that ectopic expression of *Lhx2* is effective for blocking terminal differentiation of muscle stem cells by changing the genetic program related to skeletal muscle specification in mesenchymal cell lineage. Stage-specific expression of *Lhx2* could be utilized for obtaining relatively larger amount of skeletal muscle satellite cells from differentiating iPSCs.

1864

FGFR2 Mediates Rapid and Reversible Nanog Repression through the Proximal Promoter Region.

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Nanog-positive epiblasts and Gata6-positive primitive endoderm cells co-exist and are convertible to each other within the inner cell mass of murine early blastocysts and their in vitro counterpart embryonic stem (ES) cells. Previous studies identified fibroblast growth factor receptor 2 (FGFR2) is responsible for triggering Nanog gene downregulation and subsequent differentiation into PE; however the underlying molecular mechanism responsible for reversible and fluctuating cell fate is poorly understood. Using an inducible FGFR2 dimerization system, we demonstrate that FGFR2 downregulated Nanog gene transcription rapidly within 1 hour in

ES cells, reaching about 90% inhibition within 6 hours. This downregulation of Nanog was accompanied by accumulation of RNA Polymerase II at the transcription start site and occurred without an increase in repressive histone methylation marks, implying regulation is likely in the early and transient phase of gene repression. Moreover, the proximal promoter region of Nanog containing the minimum Oct4/Sox2 binding site was sufficient for Nanog transcriptional downregulation by FGFR2 using insulated and integrated reporter constructs. Interestingly, using chromatin immunoprecipitation, we found Oct4 and Sox2 transcription factors, which are essential for positive Nanog transcription, remain bound to the proximal promoter region. Taken together, these data support a model of reversible Nanog repression at this stage. Consistent with this, addition of a specific inhibitor of FGFR readily reversed Nanog repression induced by FGFR2 dimerization. These findings illustrate well how FGFR2 induces rapid but reversible Nanog repression during early embryonic development.

1865

Syndecan-3 in the Muscle Stem Cell Niche: Implications for Tissue Maintenance, Regeneration and Muscular Dystrophy.

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Adult skeletal muscle maintenance and regeneration is carried out mainly by a population of resident stem cells named satellite cells, which can remain quiescent in their niche for long periods of time. In response to injury satellite cells activate, proliferate and differentiate to regenerate the damaged tissue in addition to self-renew in order to maintain the muscle stem cell pool throughout an entire life. In muscular dystrophy repeated cycles of degeneration/regeneration eventually exhaust satellite cells leading to progressive loss of regenerative capacity and to muscle wasting. Satellite cells express syndecan-3 that is required for Notch processing and signaling. Syndecan-3 null (Sdc3-/-) satellite cells show impaired proliferation and self-renewal, however Sdc3-/- muscles retain full regenerative capacity in response to up to three subsequent rounds of injury-induced regeneration. When Sdc3-/- mice are crossbred to dystrophic mice, syndecan-3 loss in dystrophic mice leads to improved muscle tissue integrity and function. A number of observations strongly suggest that syndecan-3 loss leads to long-term maintenance of a population of transit-amplifying muscle progenitors that accounts for enhanced regenerative response and resistance to disease progression in dystrophic mice. Syndecan-3 is a component of the satellite cell niche and our results show that manipulation of the satellite cell niche can dramatically impact satellite cell homeostasis and muscle regeneration, providing a promising model to study the molecular mechanisms that regulate satellite cell “stemness”. Furthermore, our studies represent a powerful platform to design new therapeutic strategies to treat conditions where muscle regeneration is impaired, such as: ageing, muscular dystrophy, cancer-induced cachexia and severe trauma.

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Dichotomic role of NAADP/two-pore channel 2/Ca2+ signaling in regulating neural differentiation of mouse embryonic stem cells.

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The mobilization of intracellular Ca^{2+}stores is involved in diverse cellular functions, including cell proliferation and differentiation. At least three endogenous Ca^{2+}mobilizing messengers have been identified, including inositol trisphosphate (IP_3), cyclic adenosine diphosphoribose (cADPR), and nicotinic adenine acid dinucleotide phosphate (NAADP). Similar to IP_3, NAADP can mobilize calcium release in a wide variety of cell types and species, from plants to animals. Moreover, it has been previously shown that NAADP but not IP_3-mediated Ca^{2+}increases can potently induce neuronal differentiation in PC12 cells. Recently, two pore channels (TPCs) have

been identified as a novel family of NAADP-gated calcium release channels in endolysosome. Therefore, it is of great interest to examine the role of TPC2 in the neural differentiation of mouse ES cells. We found that the expression of TPC2 is markedly decreased during the initial ES cell entry into neural progenitors, and the levels of TPC2 gradually rebound during the late stages of neurogenesis. Correspondingly, perturbing the NAADP signaling by TPC2 knockdown accelerates mouse ES cell differentiation into neural progenitors but inhibits these neural progenitors from committing to the final neural lineage. Interestingly, TPC2 knockdown has no effect on the differentiation of astrocytes and oligodendrocytes of mouse ES cells. Overexpression of TPC2, on the other hand, inhibits mouse ES cell from entering the neural lineage. Taken together, our data indicate that the NAADP/TPC2-mediated Ca^{2+}signaling pathway plays a temporal and dichotomic role in modulating the neural lineage entry of ES cells; in that NAADP signaling antagonizes ES cell entry to early neural progenitors, but promotes late neural differentiation.

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High-throughput screening identified chemical compounds regulating human embryonic stem cell pluripotency.

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The application of high-throughput screening (HTS) to the research of human embryonic stem cells (hESCs) has been hampered by many technical difficulties. In order to use HTS to improve our understandings of the molecular mechanisms underlying hESC pluripotency and differentiation while by-passing technical challenges associated with the hESC platform, we developed a human embryonal carcinoma cell-based assay suitable for discovering chemical compounds that induce hESC differentiation. Use of this assay has led to the identification of 122 chemical compounds (out of 170,000 screened) that directed differentiation of hESCs. One of the molecules specifically targeted the constitutively expressed heat-shock protein HSC70; shRNA knock-down confirmed HSC70 as an essential regulator of hESC pluripotency. Our results demonstrate feasibility of a new and improved method for adapting HTS to hESC research, and provide a pool of novel candidates of chemical compounds for manipulating hESC fate.

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A Conserved Program for Stem Cell Dormancy.

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The ability to reversibly transition to a state of quiescence allows tissue specific stem cells to persist lifelong, and cancer stem cells to resist therapy. However the mechanisms that underlie stem cell quiescence are poorly understood. Here we outline the molecular framework of stem cell quiescence in a mouse model of delayed blastocyst implantation, also known as diapause. We performed RNA sequencing to characterize the transcriptional profiles of pluripotent stem

cells extracted from embryos at three distinct developmental stages: pre-implantation, post-implantation, and during diapause. Our analysis of diapause-associated gene expression points to a program for stem cell quiescence that links the coagulation and metabolic sensing pathways with calcium influx, autophagy, cholesterol synthesis and the induction of Runx1. Portions of these same pathways support dormancy in lower organisms and quiescence in other stem cell types.

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Comparison of autophagy in mesenchymal stem cells derived from bone marrow and chorionic plate in placenta by hypoxia.

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Hypoxia triggers physiological and pathological cellular fate such as proliferation, differentiation and death in several cell types. Mesenchymal stem cells (MSCs) derived from several tissues have potentials for self-renewal activity and differentiation towards multiple lineages. However, it should be overcome that the lower yields of MSCs isolated since the rate of self-renewal and differentiating capacity decrease with age of the donor in cell therapy. Recently, it has been reported that hypoxic condition induces the balance between survival and death by hypoxia-induced autophagy, however, the underlying mechanism is not clear. Therefore, the objectives of this study are to analyze the prolifertion in bone marrow-derived mesenchymal stem cells (BM-MSCs) and chorionic plate-derived mesenchymal stem cells in placenta (CP-MSCs) under hypoxia and investigate the regulation mechanisms of self-renewal in each MSC under hypoxic condition. Furthermore, we compared the difference between PDSCs and BM-MSCs by hypoxia-induced autophagy. The effect of hypoxia on the self-renewal of PDSCs and BM-MSCs was performed by RT-PCR, FACS analysis and Western blot against self-renewal factors. Also, we carried out monodansylcadaverine (MDC) staining and immunofluorescence against LC3 for detection of autophagosomes in MSCs exposed to hypoxia. The expression of self-renewal markers (e.g. Oct4, Nanog, Sox2) was expressed in both cell lines. The expression of Oct4 was more increased in BM-MSCs than CP-MSCs under hypoxic condition. In FACS analysis, the rates of S phase in CP-MSCs and BM-MSCs under hypoxia were 8.77 % and 4.85 %, respectively. In addition, the expression of PI3K was gradually increased in CP-MSCs, otherwise, it was remarkably downregulated in BM-MSCs by hypoxia. The expressions of p-ERK and p-mTOR were induced by hypoxia compared to normoxia in CP-MSCs. The expression of LC3 II, which is a component of autophagosome and the hoof-shaped autophagosome in CP-MSCs were more rapidly progressed than BM-MSCs under hypoxia. Taken together, hypoxia triggers autophagy in CP-MSCs and BM-MSCs and autophagy more activated in CP-MSCs than in BM-MSCs.

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The Effect of Type-1 Diabetes on Differentiation of Rat Mesenchymal Stem Cells.

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According to the American Diabetes Association, 23.6 million, eight percent of the United States population, suffers from diabetes. Diabetes has a well-documented increased healing time for fracture healing (two to three times the normal rate). This delay also increases the risk of complications, such as delayed union or non-unions. The decreased bone formation may be due to an increase level of TNF-α seen in diabetes. High levels of TNF-α has been linked to an

accelerated loss of cartilage in fracture healing. Mesenchymal stem cells, or MSCs, are the source of stem cells for bone healing.
The overall objectives of this research thrust is to characterize and quantitatively compare the functionality of the MSCs from diabetic rats, as compared to non-diabetic littermates, to determine if a loss of MSC function is responsible for the decreased bone repair noted in Diabetes. The MSCs have an immunomodulatory effect and under normal conditions can decrease the levels of TNF-α in the body. We hypothesize the impairment of bone healing in diabetes is due to both a decrease in MSC function from being in a diseased environment as well as a decreased immunomodulatory effect leading to an increase in TNF-α. The function of the MSCs will be determined by quantifying the bone, fat and cartilage differentiation capabilities.
In this experiment, mesenchymal stem cells (MSCs) were isolated from diabetic rats (Streptozotocin-induced type I diabetes) and their healthy littermates for comparison. MSCs were isolated from diabetic and control-treated rats 6 weeks after Streptozotocin administration. Currently, the differences in differentiation abilities of the MSCs from diabetic rats are being compared to the MSCs from sham-treated littermates. Specifically, differentiation of bone and fat will be examined and quantified. Changes in gene expression will be analyzed during the differentiation.
Eight lines of diabetic and control rat mesenchymal stem cells have been isolated, purified and expanded. The results thus far show the diabetic MSCs have a similar if not slightly increased potential for adipogenic differentiation. Conversely, the diabetic MSCs have a decreased potential for differentiation into osteoblasts, demonstrated by a decreased mineralized matrix production and an attenuated gene expression response.

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Effect of Korean mistletoe for self-renewal activity in placenta-derived stem cells.

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Mesenchymal stem cells (MSCs) derived from various tissues are powerful sources for cell therapy in the degenerative medicine although there are obstacles to overcome their limited self-renewal. Recently, placenta-derived stem cells (PDSCs) have been reported as an alternative source to bone marrow–derived mesenchymal stem cells (BM-MSCs) due to the characteristics of multilineages, immunosuppressions and self-renewal. Korean mistletoe, which is a natural product isolated from hemi-parasitic plants, has been known to have cytotoxicity in cancer cells. However, there are still unknown whether Korean mistletoe in stem cells has cytotoxicity or not. Therefore, the objectives of the study were to analyze the effects of Korean mistletoe in the cultivation of MSCs, compare the difference according to the cell types (e.g. naïve PDSCs, immortalized PDSCs by hTERT over-expressing), and evaluate the correlation between Korean mistletoe and self-renewal of PDSCs. The cytotoxicities of naïve PDSCs, immortalized PDSCs, and HepG2 cells exposed to Korean mistletoe (1~10,000pg/ml) were analyzed using MTT assay. The cytotoxicities of HepG2 and immortalized PDSCs were induced at 1pg/ml of the mistletoe, while the proliferation of naïve PDSCs were significantly increased at low concentration of the mistletoe (1pg~10pg/ml) without cytotoxicity ($p<0.05$). Also, the expressions of cyclin D1 and phosphorylated ERK were increased in naive PDSCs exposed to the mistletoe. From these results, Korean mistletoe have cytotoxicity in cancer cells including immortalized PDSCs, however, they also enhance the self-renewal in PDSCs through the ERK signaling. Therefore, Korean mistletoe could be used as an anti-cancer drug in cancer as well as a supplement for the cultivation of MSCs to enhance self-renewal activity.

1872

MafA Restoration Improves the Expression Profile of Genes Specific for Islet β-Cells in Mouse Embryonic Progenitor-derived Insulin-producing Cells.

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Derivation of functional insulin-producing cells from stem cells is a promising approach to develop transplantation therapy for curing insulin-dependent diabetes. We generated a dozen of expansible and glucose-responsive insulin-producing cell lines (e.g. MEPI and ERoSHK) by differentiating mouse embryonic stem or progenitor cells. Though these cells could correct hyperglycemia after transplanted into diabetic animals, they revealed features of immaturity displaying higher un-regulated insulin release and moderate mitotic activity in vivo. MafA (v-maf musculoaponeurotic fibrosarcoma oncogene homolog A) is a master transcription factor implicated in controlling islet β-cell development and glucose responsiveness; its expression was significantly lower in our insulin-producing cells in comparison with isolated mouse islets. In this study, MafA levels in mouse embryonic progenitor-derived insulin-producing (MEPI)-1 cells were largely restored by transfecting vectors expressing MafA, as confirmed by real-time RT-PCR, immunofluorescence staining and immunoblotting. MafA restoration significantly enhanced (2-5 fold) expression of genes for proteins important for β-cell development and functions which are expressed low in MEPI cells. These include Nkx6.1 (a transcription factor playing an important role in pancreatic β-cell development), glucagon-like peptide-1 (GLP-1) receptor [GLP-1 is a powerful incretin protecting β-cells, promoting β-cell growth and enhancing glucose-stimulated insulin secretion (GSIS)], Ins1 and Ins2 (both for insulin biosynthesis), PCSK1 (encoding a key prohormone convertase required for processing of proinsulin into mature insulin), Glut2 (important for glucose transportation across cell membrane, the first step in GSIS), glucokinase (an essential hexokinase that catalyzes glucose metabolism to generate ATP and serves as a principal controller for insulin secretion in response to rising levels of glucose), and Kir6.2 (a major subunit of ATP-sensitive inwardly rectifying potassium channel involved in a key signaling scenario that couples glucose metabolism to GSIS). Moreover, microarray analysis of gene expression profile in MafA-restored MEPI-1 cells revealed recovering of altered levels of several transcripts for proteins involved in the signalling pathways for susceptibility to diabetes onset and development, e.g. casitas b-lineage lymphoma (Cbl), FK506 binding protein 12-rapamycin associated protein (Frap) 1/mammalian target of rapamycin (mTOR), CCAAT/enhancer binding protein (CEBP)-β, FBJ osteosarcoma oncogene (Fos) and Kruppel-like factor 10 (Klf10). Importantly, the expression of p27, an inhibitor of cyclin-dependent kinases controlling cell cycle and proliferation, was significantly enhanced by 1.5 fold in MEPI-1 cells after MafA up-regulation. Thus, our data suggest that MafA can optimize embryonic stem cell-derived insulin-producing cells and enhance their functions by improving gene expression profile resembling primary islet β-cells.

Tumor Invasion and Metastasis

1873

In Vivo Imaging of Tumor Associated Collagen Signatures.

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Stromal reorganization has been associated with the progression of breast cancer. The importance of the stroma and specifically the extracellular matrix (ECM) has been highlighted

through the identification of mechanical signaling pathways in which cell-ECM adhesion points have increased the activity of RhoGTPases and thus cell contractility and motility. These associations have implicated that stromal reorganization, and more specifically changes in collagen alignment, are a potential biomarker for the invasion and progression of breast cancer. Non-linear optical techniques, including Second Harmonic Generation (SHG) and Multiphoton Laser Scanning Microscopy (MPLSM), have been a useful tool in studying disease progression and ECM reorganization. These imaging modalities are not only compatible with genetically tagged fluorescence markers and intrinsic signatures such as that from collagen but can also detect these changes non-invasively with cellular scale resolution deep within intact tissue. Thus together MPLSM and SHG are powerful non-invasive methods to study cancer cell and collagen architecture interaction. Recently we have been able to identify Tumor Associated Collagen Signatures (TACS) in vivo by use of MPLSM, SHG, and a Mammary Imaging Window (MIW). These intravital imaging techniques allow us to optically section approximately 300 microns into a progressing breast tumor in vivo in our mouse model. We have also been able to map out the entire collagen layout in excised breast tumor slices from the mouse models through stitching multiple fields of view together into one large image montage. These new techniques are helping to elucidate the role that ECM reorganization has in the invasion and progression of breast cancer.

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Role of stiffness and force response in integrin mediated signaling and metastasis.

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In cancer, changes in cellular phenotypes are characterized by altered cytoskeletal architecture as well as disruption of signal transduction pathways controlling proliferation, angiogenesis and apoptosis. Recent studies have shown that mechanical properties of the cytoskeleton as well as its force sensing capability are critical in maintaining
normal functions and the mechanism for this balance is currently an active area of research.We recently showed that in ovarian cancers, mechanical stiffness of cells show an inverse relationship with their invasive potential and by altering this relationship, we can alter the phenotype. We also have shown that the Rho signaling pathway is critical in force sensing and recently discovered 2 RhoGEFs, LARG and GEFH1 which regulate the mechanical stiffness and response to force on integrins. Here we combine the 2 systems to show that it is GEFH1 that regulates the mechanical stiffness dependent cell invasion behavior in ovarian cancer cells and by selectively targeting this pathway, we can alter the metastatic behavior in these cells. Our results show that the GEFH1-RhoA pathway may be a critical pathway in loss of mechanotransduction associated with cancer and a potential target for therapy.

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Lamin A/C Directs Nuclear Stiffness, 3D Cell Migration and Tumor Propagation.

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Cancer cell migration is an essential process of tumor propagation and metastasis, and therefore has significant contribution to its malignancy. The efficiency of cell migration in tissue is determined by several factors such as contractile force generated by actomyosin activity, ability to secrete enzymes remodeling extracellular matrix, and importantly mechanical properties of migrating cell. In the present study, we hypothesized that cell nucleus, the biggest and stiffest cell organelle, plays an essential role in determining cell mechanics in the context of

migration through physically constraining environment. Here strong emphasis is placed on the expression of inner nuclear membrane-associated protein lamin A/C, which is known to be a major determinant factor for nuclear stiffness. Lamin A/C down-regulation by short interfering RNA (siRNA) in A549 lung carcinoma cells resulted in softening of their nuclei and increased migration efficacy through 3 m-sized pores in the presence of nutrition gradient, whereas wound scratch healing assay showed that lamin A/C knockdown had no remarkable effect on 2D migration on cell culture dish. Furthermore, fluorescent-labeled A549 cells with initial lamin A/C knockdown by 40% were injected in immuno-deficient mice along with control cells to compare the difference in growth and expansion of tumor over 4 weeks. A549 tumors with initially decreased lamin A/C expression showed higher proliferation rate than control tumors up to 2 weeks, which nicely matched with the duration of temporal siRNA knockdown observed *in vitro*. Importantly, although lamin A/C-knockdown and control cells did not show a significant difference in protein expression profile before injection to mice, proteomic analysis revealed that A549 cells acquired new phenotype after growing as a tumor for 4 weeks *in vivo*.

1876

Physical mechanisms in cancer: the transition to metastasis.

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In cancer, one of the most significant factors that influence the prognosis for a patient is whether or not the cancer is metastatic. The transition from a non-metastatic tumor to a metastatic one is accompanied by a number of changes in the proteins that are expressed within the tumor cells. These changes must somehow conspire to produce a behavioral change in cell, and even multi-cellular, behavior: cells that were happy staying in one place begin to move, often as a group. Here we ask the question of what cell-level biophysical changes are sufficient to transition a static tumor to a metastatic one. We use a mathematical model developed to describe the biophysics of epithelial tissue to explore this problem. We find that moderate increases in cell-ECM adhesion accommpanied by a decrease in cell-cell adhesion can cause a group of cells to break free from a tumor and spontaneously migrate. This result may explain why some metastatic cells have been observed to up-regulate integrin and down-regulate cadherin.

1877

Autophagy supports migration, invasion, and differentiation driven by oncogenic Ras.

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The pro-tumor functions of autophagy, a tightly controlled cellular self-digestion process, have been largely attributed to its ability to promote tumor cell survival. Here, we demonstrate a previously unrecognized requirement for autophagy in facilitating extracellular matrix (ECM) invasion and tumor cell motility in cells transformed with oncogenic Ras. RNAi-mediated depletion of critical autophagy genes (ATGs) profoundly inhibits the invasive behavior of H-Ras^{V12} MCF10A cells in three-dimensional (3D) culture. This reduction in invasive capacity correlates with a partial restoration in cell-cell junctional integrity and polarized secretion of the basement membrane protein, laminin 5. In addition, H-Ras^{V12} MCF10A cells and MDA-MB-231 cells, a K-Ras mutant breast cancer cell line, display reduced motility following ATG knockdown. Importantly, the invasive behavior of Ras-transformed epithelial cells is associated with an epithelial-mesenchymal transition (EMT), and ATG knockdown partially reverses Ras-driven mesenchymal differentiation, by enhancing the expression of *e-cadherin* and *keratin 14*, and reducing *fibronectin* and *vimentin* expression. Interestingly, upon co-culture with autophagy

competent cells, the invasive capacity of autophagy deficient cells in 3D is restored; indicating decreased secretion of pro-invasive factors in ATG knockdown cells. In support of this finding, we observe decreased transcription of *wnt5a* and *mmp2* and reduced MMP2 activity in conditioned media following autophagy inhibition. Both factors are critical mediators of this phenotype as treatment of H-Ras^{V12} MCF10A 3D structures with an MMP-2 inhibitor was sufficient to suppress invasion, and the addition of recombinant Wnt5a rescued the invasive capacity of autophagy depleted H-Ras^{V12} cells. Our results reveal new roles for autophagy in supporting tumor cell migration, invasion, and differentiation, and we propose autophagy may facilitate dissemination and metastasis during the later stages of tumorigenesis.

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Cortactin phosphorylation regulates cell invasion through a pH dependent pathway.

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Invadopodia are invasive protrusions with proteolytic activity uniquely found in tumor cells. Recent work has shown that cortactin phosphorylation is a key step during invadopodium maturation, regulating Nck1 binding and cofilin activity. The precise mechanism of cortactin-dependent cofilin regulation and the roles of this pathway in invadopodium maturation and cell invasion are not fully understood. We show that cortactin:cofilin binding is regulated by local pH changes at invadopodia that are mediated by the sodium-hydrogen exchanger NHE1 (NHE1). Furthermore, cortactin tyrosine phosphorylation mediates the recruitment of NHE1 to the invadopodium compartment where it locally increases the pH to cause the release of cofilin from cortactin. In particular, we show that this mechanism involving cortactin phosphorylation, local pH increase and cofilin activation regulates the dynamic cycles of invadopodium protrusion and retraction and is essential for cell invasion in 3D. Together, these findings identify a novel pH-dependent regulation of cell invasion.

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Invadopodia formation and microvesicle release are required for cancer cell extravasation in vivo.

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During the metastatic process, cancer cells must undergo trans-endothelial migration from the vessel lumen into underlying tissue in a process known as extravasation. Little is known about the dynamic mechanical aspects of this process. To address this, we performed real-time, sub-cellular resolution intravital imaging of human cancer cells during arrest, intravascular migration and extravasation using a shell-less avian embryo xenograft model. We find that extravasation occurs at endothelial junctions and that the majority of extravasation events occurred during the 12 hours subsequent to the intravenous injection of cancer cells. In the majority of extravasation events, long cytoplasmic extensions identified as invadopodia were observed prior to extravasation which breached underlying endothelium. We observed the release of microvesicles during these invadopodial extension events prior to extravasation, and this resulted in a ~40% reduction in cell volume post-extravasation. Based on this, we hypothesized that invadopodia are required for extravasation, and that cancer cell extravasation could be abrogated by inhibiting factors required for invadopodia function. We found that treatment with

Src kinase inhibitors significantly reduced invadopodia formation in vivo and resulted in a ~60% decrease in extravasation events when compared to vehicle treated controls. In conclusion, we determined that 1) extravasation occurs at junctions between adjacent endothelial cells, 2) cancer cells form invadopodia that breach the endothelial layer prior to successful extravasation and 3) cancer cells undergoing extravasation release cancer microparticles into both the vessel lumen and tissue interstitium with a corresponding decrease in cell volume. Pharmacological inhibition of invadopodia by Src kinase inhibition reduces cancer cell extravasation, revealing an important mechanism of action against metastatic cancers.

1880

Cytoskeletal Regulation of Transendothelial Tumor Cell Migration.

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Most cancer patients die as a result of metastasis. Spread of metastatic cancer cells by blood circulation is responsible for most of the distant metastases, thus it is important to understand the molecular mechanisms that regulate intravasation. Tumor cells and endothelial cells dynamically alter their cytoskeletons and adhesive properties during this process. Previous results have shown that rat mammary adenocarcinoma cell lines that express an invasive isoform of Mena (MenaINV), a key regulator of the actin cytoskeleton, exhibit a dramatic increase in transendothelial migration in vitro in the presence of macrophages (Roussos et al. JCS, 2011). MenaINV has also been shown to localize to invadopodia, actin-rich structures that degrade matrix. To determine whether the formation of invasive structures such as invadopodia is required for efficient transendothelial migration, we took advantage of the high incidence of transmigration exhibited by cells expressing MenaINV. We imaged transmigration in real-time by co-opting an organotropic assay (Nakatsu and Hughes. Methods Enzymol, 2008) to grow blood vessels in a 3-dimensional matrix. We found that human breast cancer cells form protrusions that exhibit oscillatory movements as the tumor cells probe the surface of the endothelium. We also found that MenaINV is enriched in the protrusions at the site of tumor cell-endothelial cell contact, and that these protrusions penetrated through the endothelium into the blood vessel. These results suggest that tumor cells form invadopodia to break through the endothelial barrier during intravasation. Further studies exploring this hypothesis as well as addressing adhesion dynamics are on-going.

1881

Dynamin 2 Potentiates Invasive Migration of Pancreatic Tumor Cells through Stabilization of the Rac GEF Vav1.

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Tumor cell migration is an important component of the metastatic process. The large GTPase Dynamin 2 (Dyn2) has recently been identified as a potent activator of pancreatic cancer cell dissemination (Eppinga, et al., Oncogene 2011), and is also known to play a role in the Rac-mediated formation of lamellipodia. The mechanisms by which Dyn2 might modulate Rac activation to upregulate the migratory process are undefined. In this study we have found that Dyn2 is required for Rac activation in pancreatic cancer cells, and that this activation is mediated through a direct interaction with the Rac GEF Vav1. Importantly, Vav1 has been shown to be expressed ectopically in pancreatic cancers, and this expression correlates with

tumor invasion and lethality (Fernandez-Zapico, et al., Cancer Cell 2005). We have identified a direct binding between Dyn2 and Vav1 in pancreatic cancer cells, and subsequently observed that disrupting this interaction markedly impairs both Rac activation and the subsequent formation of lamellipodia that are required for migration of these neoplastic cells. Surprisingly, disruption of the Dyn2/Vav1 interaction dramatically reduces Vav1 protein stability and subsequently attenuates Rac activation. The effects of Dyn2 on Vav1 protein levels and Rac activation are independent of Dyn2 enzymatic activity and identify a novel structural role for Dyn2 in Rac activation and lamellipod protrusion. Thus, the large GTPase Dyn2 can regulate the activation of a small GTPase, Rac, through a previously unreported effect on GEF protein stability, and is a potent mechanism by which pancreatic cancer cells upregulate the migratory and metastatic program. This study was supported by CA104125 to M.A.M. G.L.R. was supported by T32 CA148073.

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Pituitary tumor transforming gene 1 (PTTG 1) regulate invasion activity of human oral squamous cell carcinoma via integrin-mediated FAK signal.

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Human pituitary tumor-transforming gene 1 (PTTG1), which is a newly identified proto-oncogene, overexpress in many nonendocrine-related tumors as well as endocrine-related cancers. Also, PTTG1 has been known to involve in the regulation of cell-cycle via securin function and in progression of tumor. However, the function and the regulation mechanism of PTTG1 in oral squamous cell carcinoma (SCC) remain still unclear. The objectives of the study are to analyze the expression of PTTG1 in oral SCC and evaluate the mechanism for invasion of oral SCC cell lines (YD-10B and YD-15) depends on PTTG1 expression by siRNA. The expressions of PTTG1 in oral SCC tissues and oral SCC cell line were analyzed by immunohistochemistry and Western blot, respectively. The expression of invasion-related factors (e.g. MMP2/-9, integrins, TIMPs) and the invasion activity in oral SCC cells transfected siRNA-PTTG1 were measured by Western blot and Metrigel assay system. The expression of PTTG1 in oral SCC tissues was mainly observed in cytoplasm, although it partially localizes in the nucleus in oral SCC. The invasiveness of oral SCC cells using Metrigel was significantly increased after PTTG1 plasmid transfection comparing to Mock ($p<0.05$). There is no difference expression for MMP-2 regardless of PTTG1 siRNA treatment. However, the enzyme activity of MMP-9 was significantly decreased. In addition, migration activities of oral SCC cells were significantly decreased after PTTG1 siRNA treatment ($p<0.001$). In addition, down-regulated PTTG1 induced alterations of integrins in oral SCC cell lines through the phosphorylations of FAK1 and mTOR. These results suggest that alteration of PTTG1 could be regulated invasion activity of human oral SCC via integrin-mediated FAK signaling. Therefore, these findings provide useful guideline for the migration mechanism of oral SCC by PTTG1 expression.

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Phosphorylation Regulates TWIST1-mediated Breast Cancer Cell Invasion.

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TWIST1 belongs to the family of basic helix-loop-helix (bHLH) transcription factors. The regulatory role of TWIST1 in mesodermal tissue development has been well established in

Drosophila and mouse models. We and others have shown that TWIST1 plays an important role in cell invasion and TWIST1 expression level is aberrantly high in metastatic breast cancer cells. Phosphorylation of TWIST1 has been reported to stabilize TWIST1 protein, inhibit p53-mediated apoptosis in response to DNA damage, and regulate nuclear localization of TWIST1. We hypothesized that phosphorylation of TWIST1 regulates TWIST1-mediated cell invasion. To address our hypothesis, we mutated a conserved threonine (T) residue into alanine (A) or aspartic acid (D) to generate a phosphodeficient (T148A) or phosphomimic (T148D) TWIST1.

Our previous results demonstrated that TWIST1 promotes cell invasion by inducing the expression of Interleukin-8 (IL-8). This IL-8 induction is mediated by the physical association between TWIST1 and RELA in the NF-κB transcriptional complex. In addition, we observed changes in the cell invasive properties in response to overexpression of T148A and T148D mutants. Therefore, to investigate whether phosphorylation at T148 affects the association between TWIST1 and RELA that leads to changes in cell invasion, we performed co-immunoprecipitation (Co-IP) experiments using T148A and T148D mutants in comparison with wild-type TWIST1. Co-IP results showed significant changes in binding affinity of TWIST1 and RELA as a result of the mutations, and these results were confirmed by additional IL-8 promoter activity assays.

In conclusion, our results suggested that phosphorylation at T148 is essential for TWIST1-mediated cell invasion by regulating the physical interaction between TWIST1 and RELA. This study provides valuable information on the relationship between post-translational modifications of TWIST1 and the onset of cancer cell invasion. Knowledge gained from this study may lead to the identification of therapeutic targets that affect TWIST1 function and will be useful for developing novel anti-metastatic drugs for advanced breast cancer.

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Distinct effects of EGF and LPA on cell migration dynamics during breast cancer progression.

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During cancer progression, subsets of cancer cells break away from the primary tumor and invade distal sites within the body. This process of metastasis is associated with alterations in the migratory potential of cancer cells. Direct observation of migration behaviors of cancer cells as they progress towards metastasis is still lacking. Using an established cell-line model for breast cancer progression, we assessed the impact of both cancer progression and extracellular stimuli on the migratory abilities of cancer cells. Specifically, we focused on the consequence of activated receptor tyrosine kinase (EGF-EGFR) and G-protein coupled receptor (LPA-EDG2) signaling on cell migration. Using live-cell imaging and particle image velocimetry analysis, we monitored and measured the migration dynamics of an epithelial sheet (normal, early tumor, pre-metastatic, and metastatic cells) as it migrates into open space with or without uniform stimulation (EGF or LPA). Under basal conditions, early tumor and pre-metastatic sheet-edges infiltrate into open space at the highest rates (0.81 ± 0.11 and 0.49 ± 0.18 µm/min, respectively). Surprisingly, both normal and metastatic cells infiltrate at lower rates (0.26 ± 0.15 and 0.25 ± 0.18 µm/min, respectively). We also found that normal breast epithelial cells and early tumor cells exhibited directional and collective sheet migration, while independent and random motions were prevalent in more metastatic cells. In response to uniform EGF stimulation, both normal and metastatic cells exhibited increases in migration rate (80% and 53%, respectively),

while pre-metastatic cells showed only a modest rise (18%) and early tumor cells remained unchanged. The motion dynamics of migration in all four cell-types remained largely unchanged under EGF stimulation. LPA treatment induced a more spread morphology in all cell-types and again lead to an increases in the migration rate of normal, pre-metastatic and metastatic cells as compared to basal conditions (90%, 23% and 41%, respectively). Interestingly, some metastatic cells treated with LPA appeared to abandon their random movements and instead migrated as a cohesive sheet similar to normal breast epithelial cells. Western blotting and immunofluorescence studies suggest that differences in E-cadherin and β̃catenin expression and localization may contribute to the distinct migration behaviors observed. Together, we demonstrate that the migration dynamics of epithelial cells dramatically changes as they transition from a normal to metastatic state. In addition, our data highlight how different external cues can distinctly regulate migratory behaviors.

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Phosphatase dysregulation mediates EGF sensitivity in Mena^{INV}-positive breast cancer cells.

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Approximately 90% of human breast cancer deaths are due to metastasis of the original tumor. An ‘Invasion Signature’ has been reported for breast carcinoma cells collected *in vivo*, and includes a significant upregulation of the cytoskeletal associated protein Mena, a member of the Ena/VASP family of actin regulatory proteins. In particular, an alternatively spliced isoform of Mena, Mena^{INV}, significantly increases metastasis and the ability of carcinoma cells to respond to epidermal growth factor (EGF) *in vivo* and *in vitro*. The aim of this study was to determine the mechanism underlying Mena^{INV} induced metastatic behavior. We found that ectopic expression of Mena^{INV} in human breast cancer cell lines increases EGF sensitivity at the level of actin polymerization (lamellipodial protrusion) and 3D collagen invasion, extending our past work in rodent carcinoma models. The elevated sensitivity to EGF initiates with an increase in EGF receptor (EGFR) phosphorylation and is propagated through the PLCγ/cofilin pathway as measured biochemically and using live- and fixed-cell FRET imaging. Concomitant with increased growth factor response, EGFR-targeted therapeutics are less effective in Mena^{INV} positive cells. We found that the increase in EGF sensitivity cannot be accounted for by a significant increase in total or surface levels of EGFR or pre-clustering of the receptor, as measured biochemically and with electron microscopy. Alternatively, expression of Mena^{INV} facilitates mis-localization of a novel Mena interacting partner, the EGFR-targeted tyrosine phosphatase PTPN1 (PTP1B). Inhibition of PTP1B activity increases lamellipodial protrusion, 3D collagen invasion and EGFR signaling pathway activation in vector control cells, phenocopying the expression of Mena^{INV}. Additionally, ectopic expression of Mena^{INV} limits the access of PTP1B to EGFR upon EGF stimulation as measured by proximity ligation assay, providing a mechanism for decreased PTP1B activity. Finally, we found that inhibition of PTP1B significantly increased the EGF sensitivity of chemotaxing MTLn3 carcinoma cells *in vivo* like that measured upon ectopic expression of Mena^{INV}. These results demonstrate that phosphatase dysregulation is a key component of Mena^{INV}-mediated metastasis. We are currently using mass spectrometric approaches, combined with signaling network analysis, to determine how PTP1B dysregulation rewires the EGF-stimulated signaling pathway upon Mena^{INV} expression.

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Fibronectin matrix-mediated cohesion suppresses invasion of prostate cancer cells.

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Invasion is an important early step in the metastatic cascade and is the primary cause of death of prostate cancer patients. To invade, cells must first detach from the primary tumor. Cell-cell and cell-ECM interactions are important regulators of cohesion, a property previously demonstrated to mediate cell detachment and invasion. The studies reported here propose a novel role for α5β1 integrin, the principle mediator of fibronectin matrix assembly (FNMA), as an invasion suppressor of prostate cancer cells. Using a combination of biophysical and cell biological methods, and well-characterized prostate cancer cell lines of varying invasiveness, we explore the relationship between cohesion, invasiveness, and FNMA. We show that cohesion is inversely proportional to invasive capacity. We also show that more invasive cells lack the capacity for FNMA and that this is associated with decreased α5β1 integrin expression. We generated cells over-expressing either wild-type α5 integrin or an integrin in which the cytoplasmic domain of α5 was replaced with that of α2. This construct does not promote FNMA. We show that only wild-type α5 integrin was able to promote aggregate compaction, to increase cohesion, and to reduce invasion of aggressive cells. These effects could be blocked by the 70 kDa fragment of fibronectin. This suggests that the mechanism of invasion suppression involves a fibronectin matrix-mediated increase in intercellular cohesion that reduces tumor cell detachment. This is a first demonstration that the fibronectin matrix can act as an invasion suppressor of prostate cancer cells. Restoring FNMA may discourage invasion and subsequent metastasis.

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Delta-catenin promotes angiogenesis in prostate cancer cells.

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δ-Catenin has been well known to be abundantly expressed in brain being implicated in the regulation of dendrogenesis and cognitive function. It has also been proved by accumulated evidence to express in a variety of cancer tissues, especially in prostate tumors. Recently, it has been found to express in vascular endothelium being involved in pathological angiogenesis. The discoveries above led us to ask whether δ-Catenin overexpressed in prostate tumor cells is able to induce angiogenesis contributing to the prostate tumor growth. In this study, we discovered that, similar to what has been found in endothelium, δ-Catenin overexpressed in prostate tumor cells was able to induce angiogenesis as well. We found that not only did δ-Catenin stimulate new blood vessel formation but also it accelerated tumor growth in CAM assay. We also observed that δ-Catenin increases the expression of Vascular Endothelial Growth Factors (VEGF) and promotes the secretion of VEGF protein into the media where tumor cells grew through increased HIF-1a protein expression. Furthermore, we found that -Catenin nuclear localization is increased in δ-Catenin over-expressed prostate tumor cells compared with in control prostate tumor cells under both normoxia and hypoxia condition. Taken together, our findings suggest that δ-Catenin may contribute to prostate tumors growth by inducing angiogenesis through increasing β-Catenin nuclear localization to function on HIF-1a mediated transcription or on VEGF directly. Our current study adds the novel evidence to the involvement of δ-Catenin in prostate cancer progression and also provides better understanding about how δ-Catenin promotes prostate tumors growth.

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Glutamate Effects on 2-Dimensional and 3-Dimensional Brain Tumor Cell Cultures.

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Gliomas account for approximately three-fourths of all malignant brain tumors. Gliomas release the neurotransmitter glutamate by the X-c system, a sodium independent cystine-glutamate exchanger. Glutamate is also known to act as an autocrine signaling mechanism for gliomas, believed to result in tumor expansion. We have shown that glioma cells in vitro release a greater amount of glutamate compared to normal brain astrocytes, and that cystine stimulates glutamate release from glioma cells by 6.4 fold more than from astrocytes by using a novel glutamate biosensor. Here we are measuring the effect of glutamate on intracellular calcium concentration ([Ca2+]i) dynamics of 2-dimensional (2D) glioma cells in real-time, metabolic analysis of glioma cells in response to high levels of glutamate, and long term effects of 1mM and 3 mM glutamate treatments on 3-dimensional (3D) glioma spheroids. Induction of [Ca2+]i oscillations with glutamate in 2D glioma systems correlate with past publications; however, when the same concentrations of glutamate are introduced in 3D spheroid, 2D cultures undergoing deadhesion, or 2D glioma cultures over 24 hours, we observed retardation of growth, [Ca2+]i quenching, and a decrease in metabolic activity, respectively. While neurons in mixed neuronal/glial cell cultures responded with [Ca2+]i increases when stimulated with glutamate concentrations as low as 100 nM, glioma cells in culture treated with glutamate as high as 5 mM demonstrated only variable [Ca2+]i oscillations over the same time period of recording, 10 minutes. However, high concentration glutamate treatment of glioma cultures for 24 hours was toxic to these cells, resulting in a 50% toxicity effect (EC-50) of 2.6 mM glutamate. Using a novel 3D model for brain tumor growth, we have successfully demonstrated that glioma spheroids using this model grow over 400% in area over 8 days in vitro; however, subjecting spheroids to repeated additions of 1mM glutamate appear to sustain or increase spheroid area, while 3mM glutamate inhibited spheroid growth by 50% compared to controls. Using confocal microscopy combined with the vital dye calcein, we observed that mature glioma spheroids in vitro have an outer, viable cell shell with an internal necrotic region. Combined with in vivo observations and our data presented here, this raises the possibility that high concentrations of glutamate for sustained periods in brain tumors may account for necrotic core regions.

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Hypoxia-inducible factor 1 alpha expression and its correlations with p53, c-Myc and CD31 in glioma.

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As a key regulator of the cellular response to hypoxia, hypoxia-inducible factor-1 alpha (HIF-1 alpha) is a well-known transcriptional factor that controls genes important for the growth and metabolism of tumors. In this study, we investigated the expression of HIF-1 alpha in glioma cells and glioma tissues utilizing western blot (WB) and immunohistochemistry (IHC). We also examined the relationships of HIF-1 alpha with p53, c-Myc and CD31 in the same cell and tissue samples. WB analysis of various glioma cell lines showed a high expression of HIF-1, whereas no HIF-1 alpha expression was detected in normal brain lysate. Similar results were found with p53 expression in glioma cells. Glioma cells displayed weak expression of c-Myc. To determine whether HIF-1 alpha expression associates with glioma progression, we also performed IHC analysis of paraffin-embedded human glioma tissues of different histological grades (#14). The IHC results showed that 90% of grade 3-4 gliomas displayed strong HIF-1 alpha staining of cancer cells, compared to only 40% of grade 2 gliomas which showed HIF-1 alpha expression. As the marker of microvessels density, CD31 exhibited a high level of

expression in the late stages of glioma tissues. IHC staining of gliomas did not show a direct correlation of HIF-1 alpha with c-Myc in vivo. In summary, our results suggest that HIF-1 alpha expression strongly correlates with glioma progression and invasion, supporting the reports that HIF-1 alpha could be a potential target for glioma therapeutic research. Our data also indicated a high correlation between HIF-1 alpha and apoptotic marker p53 in glioma cells. IHC analysis on CD31 demonstrated that HIF-1 alpha affects angiogenesis in gliomas by increasing microvessels density. In the contrary to the recent HIF-1 alpha-c-Myc axis reports, we did not detect any significant HIF-1 alpha-c-Myc correlation.

1890

Fra-1 Mediated Regulation of CD44 Expression in Malignant Brain Tumor Cells.

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Glioblastoma multiforme (GBM) is the most common form of primary brain tumors. It is a highly aggressive tumor, characterized by an increased proliferation rate and a high capacity to invade surrounding tissues. Currently, GBM treatment includes surgical resection followed by radiation and/or chemotherapy. However, these therapeutic options often do not alter the infiltrative and migratory capacity of GBMs. The invasive migration of glioblastoma multiforme has been associated with CD44, a cell surface glycoprotein and the principle receptor for hyaluronic acid (HA). The molecular basis of CD44 regulation regulated in GBM is not fully understood. However, CD44 regulation has been shown to be by an AP-1 transcription factor family member, fos-related antigen (Fra-1) in an equally aggressive cancer, mesothelioma. To better understand how Fra-1 regulates cell adhesion and migration, we performed adhesion and wound healing assays on human GBM cell lines. To determine whether malignant brain tumor cell adhesion is mediated by Fra-1 expression, we analyzed GBM cell adhesion to HA. U-1242 MG cells were transfected with siRNA directed against Fra-1 and treated with epidermal growth factor (EGF, 20 ng/ml). Adherence to HA increases in U-1242 MG cell lines following growth factor simulation, but was inhibited when the cells were transfected with Fra-1 siRNA. Using another GBM cell line A-172, and its clones, A-172 MW6 (Fra-1 dominant negative) and A-172 MW12 (Fra-1 overexpressors) we examined the migratory capacity of these cells. A-172 MW6 cells exhibit a slower migration rate than cells with higher levels of Fra-1 expression. Collectively, our results suggest that Fra-1 regulates CD44 expression in human GBM cells, which in turn affects GBM cell adhesion and migration.

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TWIST WR Domain Induces NF-κB Transcriptional Activity and Promotes Cellular Invasiveness through IL8 Production.

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Metastasis is the primary cause of death for cancer patients. TWIST, an evolutionarily conserved basic helix-loop-helix (bHLH) transcription factor, was shown to promote metastatic spread to the circulatory system in a mouse model and displays elevated expression in many advanced human carcinomas. However, the molecular events triggered by TWIST to cause cancer cell dissemination are not completely understood. Here we show a novel mechanism by which TWIST induces the production of interleukin 8 (IL8), which signals autocrinely to cause metalloproteinases activation and cell invasion, key feature of a metastatic cancer. In this mechanism, the TWIST carboxy-terminal WR (Trp-Arg) domain stimulates the IL8 promoter, which is independent of the TWIST bHLH DNA binding domain. Unexpectedly, the TWIST WR

domain forms a transcriptional complex with the NF-κB subunit RELA (p65/NF-κB3) and activates the transcriptional activity of NF-κB. This protein complex associates with the IL8 promoter and increases the DNA binding affinity of RELA, thus inducing IL8 expression. Suppression of IL8 or inhibition of its receptors resulted in reduced invasiveness of breast epithelial and cancer cells. Taken together, these data demonstrate that the TWIST WR region is a functional domain that controls the activity of NF-κB and promotes cellular invasiveness.

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Urokinase plasminogen activator receptor induced non-small cell lung cancer invasion and metastasis requires NHE1 expression and transport activity.

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Non-small cell lung cancers (NSLC) are aggressive cancers that are insensitive to chemotherapies and accounts for nearly 33% of all cancer deaths in the United States. Two hallmarks of cancer that allow cells to invade and metastasize are sustained proliferation and enhanced motility. In this study we investigate the relationship between urokinase plasminogen activator (uPA)/uPA receptor (uPAR) signaling and Na^+/H^+ exchanger isoform 1 (NHE1) expression and activity. The addition of 10nM uPA increased the carcinogenic potential of three NSCLC cell lines, NCI-H358, NCI-H460, and NCI-H1299. The rate of cell proliferation was increased in an NHE1-dependent manner 1.87, 1.76, and 1.63 fold respectively for the three cell lines. The percentage of cells displaying stress fibers was also increased by uPA addition 3.17 (H358), 3.05 (H460), and 3.09 (H1299). In each case this increase was blocked when the experiments were performed with NHE1 inhibited by 10 μM EIPA (ethylisopropyl amiloride). Similarly, uPA increased anchorage-independent growth as measured by a soft-agar assay 2.0, 1.84, and 1.64 fold respectively for H358, H460, and H1299 cells. To further evaluate the role of uPA/uPAR and NHE1 in tumor progression we completed two additional studies. First, evaluating signaling events using full-length uPA compared to the uPA amino terminal fragment (ATF). Second using two new cell lines, one with reduced NHE1 expression (H460 NHE1 K/D) and one with reduced uPAR expression (H460 uPAR K/D). When comparing uPA and ATF signaling in H460 cells, we found that both uPA and ATF increased stress fiber formation 2.00 and 1.94 fold respectively, while uPA increased matrix metalloproteinase 9 (MMP9) activity 5.44 fold compared to 2.81 fold for ATF. Using the K/D cell lines we found that neither uPA nor ATF could stimulate stress fiber formation or MMP9 activity in cells with dramatically decreased NHE1 or uPAR expression. Finally, using *in vivo* tumor formation studies in athymic mice we found that when mice were injected with H460 cells 80% of mice formed tumors with an average volume of 390 mm^3. This was compared to 20% of H460 uPAR K/D injected mice forming tumors with an average volume of 15 mm^3 and 10% of H460 NHE1 K/D injected mice forming tumors with an average volume of 5 mm^3. Taken together, these data demonstrate that uPA/uPAR-mediated tumor progression and metastasis requires NHE1 in NSCLC cells and suggests a potential therapeutic approach to blocking cancer progression. This work supported by NIH 1R15CA135616-01.

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Cell fusion between cancer cells induces chromosomal instability.

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Recent studies suggested the possibility that tetraploid cells induce aneuploidy, one of the major characteristics of most cancer cells. One of the ways to tetraploidy formation is cell fusion,

which eventually leads to a wide range of chromosomal aberrations. Notably, cell fusion increases chromosomal instability which probably is involved not only in tumorigenesis but in tumor progression. We tested whether cell fusion between cancer cells induces chromosomal instability, and whether fused cells get more aggressive phenotypes. HeLa cells were fused by electric fusion. After fusion, about 90 percent of the fused cells eventually died within 1 week. However, we could observe increased centrosome number as well as enhanced multi-polar spindles during mitosis. Therefore, the fused cells showed increase of chromosome segregation defects that are major cause of chromosomal instability. Moreover, Giemsa staining and interphase fluorescence in situ hybridization (FISH) revealed high frequency of alteration in chomosomal number, evidencing the presence of chromosomal instability. Regarding tumor progression, agarose colony generation assay and in vivo tumorigenesis assay showed wide variation between stable colonies of fused cells, but not overt increase of tumor progression. However, wound-healing assay showed 2 fold increase of cell migration in fused cells, suggesting the possibility that fusion between cancer cells can contribute cancer progression possibly via enhancement of cell migration.

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High-fat diet-induced obesity increases solid tumor growth and metastasis of melanoma cells in C57BL/6 mice.

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Epidemiological evidence indicates that overweight and obesity increase the risk of developing several types of cancers, including colon, breast, liver cancer, and melanoma. In this study, in an effort to evaluate the effect of high-fat diet (HFD)-induced obesity on the progression of malignant melanoma, the solid tumor growth and metastasis of B16-F10 mouse melanoma cells were monitored in syngeneic C57BL/6N mice. Mice were fed on an HFD or control diet (CD) for 16 weeks and subdermally injected with B16-F10 cells into the right rear flank. The body weights and the growth of solid tumors were markedly increased in mice fed on HFD. In order to determine whether obesity stimulates cancer cell growth in mice, B16-F10-luciferase cells stably expressing firefly luciferase were injected into mice and luciferase activity was monitored using an in vivo imaging system. The mean intensity of the bioluminescence signal emitted from the B16-F10-luciferase cells was significantly higher in mice fed on the HFD as compared to those fed on CD. Immunofluorescence staining results of tumor tissue sections revealed that HFD feeding increased the protein expression levels of Ki67, CDK4, cyclin A, cyclin D1, VEGF, and VEGF receptor 2. Western blot analysis results demonstrated that the protein levels of COX-2, iNOS, p-Akt, p-ERK1/2, p-p38 MAPK, p-SAPK/JNK, p-STAT3, p-p65, p-c-Jun, and HIF-1(were increased in the tumor tissues of mice fed on the HFD. Furthermore, HFD feeding increased the number of F4/80- and PECAM-positive cells in tumor tissues. In order to determine whether HFD feeding increases metastasis, the subcutaneously injected cells were allowed to grow and establish themselves as a solid tumor for 3 weeks, and the tumor was subsequently surgically removed to prevent the rapidly growing primary tumor from causing an end-point termination of the experiment. 3 weeks later, the mice evidenced lung and lymph metastasis, which was increased in the mice fed on HFD. The serum levels of pro-inflammatory cytokines/chemokines were increased significantly in mice fed on the HFD. The results of this study demonstrate that HFD-induced obesity stimulates melanoma growth and metastasis in mice, which may be mediated via the induction of inflammation and angiogenesis.

Cancer Therapy II

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Viability screens in cancer cells with pooled shRNA libraries identify potential therapeutic targets and synergistic lethal interactions.

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Genes modulating proliferation and survival in oncogenic cells have been identified using pooled lentiviral-based libraries expressing many thousands of shRNAs. A viability assay with leukemic and prostate adenocarcinoma cell lines transduced with these shRNA libraries targeting thousands of genes identified a few hundred essential genes for each panel of cells. Subsequent validation using single shRNA-expressing constructs showed that about 80% of the shRNAs identified in the complex library screen did in fact lead to cell death when transduced in cells. Analysis of the identified essential genes for known biological interactions revealed several non-random clusters of interacting proteins that provide some insight into signaling pathways and protein networks specific to these cancers. Also, we have recently adapted the approach to combinatorially screen shRNA sequences targeting hundreds of genes to discover additive and synergistic combinations that generate a synthetic-lethal phenotype. Analysis of the lethal combinations indicates redundant, complementary, and compensatory responses in cancer cells. This presentation will provide an overview of the screening platform, our approach to shRNA library design, and results from the screens for cancer cell-specific lethal and synthetic-lethal genes.

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Automated morphometric analysis of spheroid cultures.

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Spheroid cultures are very important *in vitro* models in the field of cancer research, and are believed to better represent three-dimensional cell behavior as compared with conventional two-dimensional monolayer cultures. Morphometric analyses of tumor spheroids are of particular interest in oncology research to identify new drug candidates for chemotherapy-induced apoptosis and reduction of metastasis. Morphometric analysis of these large, non-adherent multi-cellular objects is predominantly done by manual visual inspection, typically on a small subset of the total sample population. This subjective approach is slow, very inconsistent, not scalable to large samples, not amenable to high-throughput analyses (e.g., screening), or analysis over extended periods (e.g., kinetics). To address these issues, a Colony Counting application was developed on the Celigo Adherent Cell Cytometer featuring uniform full-well brightfield imaging via rapid scanning mirrors with slow gentle plate movements to reliably image spheroid cultures in multi-well (96-well to 6-well) plates and novel software for high throughput brightfield image analysis (e.g., <15 min to scan and analyze an entire 12-well plate). Results showed successful imaging and characterization of mammospheres created from several different breast cancer cell lines. Spheroid counts, sizes, and growth tracking over time were used to evaluate spheroid forming efficiency, tumorigenicity, and self-renewal of a variety of breast cancer tumor-initiating cells. In addition, the effects of several cytotoxic drugs, including doxorubicin and paclitaxel, were evaluated by repeated non-destructive and non-invasive brightfield imaging of serially-passaged mammospheres. The Colony Counting application on Celigo therefore provided an efficient, reproducible, and automated method for

imaging and assessing the number, size, and morphology of non-adherent spheroids within multi-well plates.

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Differentiation of Reactive Stroma in a Novel 3D Organoid Culture Model.

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Prostate cancer is a disease that is prevalent among older men. The tumor microenvironment in prostate cancer is inhabited by different types of cells, such as cancer epithelial cells and stromal myofibroblasts. The mechanism whereby the interactions of these different cells promote the growth and progression of a tumor is not fully understood. Therefore, the primary objective is to better understand how the differentiation of normal stromal cells to myofibroblasts can lead to the formation of a tumor microenvironment. Our hypothesis was that there was an over-expression of certain stromal markers, within LnCAP (prostate cancer) organoid model compared to PNT1A (human prostate tissue). To test our hypothesis, normal human prostate tissue, PNT1A organoids, and LnCAP organoids were stained, using immunohistochemical techniques, to identify specific epithelial and stromal markers. These stromal markers consisted of AR (androgen receptor), which can be found in both the epithelial and stromal cells, tenascin C, vimentin, SMA (smooth muscle alpha-actin), and FAP (fibroblast activating protein); while pan-cytokeratin is an epithelial marker. After performing immunohistochemistry for cytokeratin, a control protein, our results showed that in PNT1A and LnCAP organoids, the epithelial cells made up the outer lining of the organoid, and the stromal cells were found in the inner part of the organoid. Although both organoids have epithelial cells and stromal cells within, the structure of LnCAP organoids were far less uniform than PNT1A which creates different expression standards between the two organoids. With this immunohistochemical data, identification of each of these stromal markers is feasible. Therefore, these data may help us understand the mechanism by which prostate cancer cells interact with myofibroblasts, thus leading to new treatments that could control or eradicate prostate cancer.

1898

Breast Cancer Cell Interactions.

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In this study, a cell culture approach is employed to identify patterns of pair-wise interaction between cancer cells over a panel of 20 established epithelial breast cancer cell lines. It is our hypothesis that different clones within a tumor, as well as different cell lines in cultured conditions, interact in one of two ways: a) by competitive growth or, b) by synergistic growth. We investigate this by culturing cell lines in serum-free conditioned medium (CM), i.e. medium which has already been used on other cell lines. Cell population growth is monitored by incubating cultures with 5-Ethyl-2'-deoxyuridine (EdU). Cell apoptosis is obtained by labeling fixed cultures with a cleaved Poly ADP Ribose Polymerase (c-PARP) monoclonal antibody. Results yield two 20x20 matrices of cell to CM interaction, and confirm that cancer cells interact through secreted substances in the medium. In an effort to trace these phenomenological interactions back to specific biochemical pathways, the interaction matrices are correlated to gene expression data available for these cell lines. Furthermore, the experiment reveals two cell lines, HCC38 and BT20, whose media produce a strong apoptotic and strong anti-proliferative effect respectively. By employing molecular weight fractionation, we attempt to isolate specific components from these media retaining the strong interaction activity.

1899

Finding novel chemical compounds with selective cytotoxic activity on human cancer cell lines using automated cellular imaging.

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Hematological malignancies are a life-threatening group of neoplastic diseases that affect a large population from children to adults. This situation is more aggravated by the fact that cancer cells develop resistance to the drugs used for their currently undergoing treatments. Moreover, drug therapies are known to cause serious side effects on patients, especially due to their non-selective activity. For these reasons, more efficiency and effectiveness therapies are needed to improve the patient's quality of life, reduce mortality, and contribute to the fields of cell biology and cancer research. The main goal of this study was to discover novel drugs with selective cytotoxicity on lymphomas, exhibiting minimal side-effects on normal cells. As proof of concept, a small library of novel chemical compounds was tested on a panel of human B- and T-lymphoma cell lines, as well as human non-tumorigenic control cells. A recently developed Differential Nuclear Staining (DNS) high throughput screening technique and flow cytometry were used to perform the analyses in triplicate using Propidium iodide to quantify loss of membrane integrity. The mode of action of the most promising cytotoxic compounds was subsequently elucidated via flow cytometry. Interestingly, the majority of experimental compounds were found to induce apoptosis with moderate specificity toward either T or B lymphomas. Ongoing experiments should reveal the cell death pathway(s) activated after compound treatment.

1900

Exploring novel diagnostic marker for breast cancer by autoantigen screening.

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Although diagnosis marker has a potential to raise survival rate of breast cancer, no diagnosis marker is applicable for early stage breast cancer. Recently several autoantigen proteins have been found in cancer patients and they attracted attention as a novel diagnosis marker candidate. For global exploration of cancer-specific autoantigens, we developed a high-throughput screening system using AlphaScreen and biotinylated protein library (BPL) expressed by wheat germ cell-free expression system. We conducted the first screening using pooled sera from breast cancer patients, and selected 41 proteins for the second screening. The second screening revealed that seven autoantigens strongly reacted with patient□fs sera. Next, we examined expression of these autoantigens in breast cancer. His-tagged proteins were synthesized by wheat germ cell-free system, purified, and immunized to mice. Finally we obtained polyclonal antibodies for Five autoantigens. Immunoblotting using these polyclonal antibodies demonstrated that these antigens expressed in MCF-7 cell and two antigens expressed in T-47D cell but not in PrEC (normal human prostate epithelium cell).

1901

Identification of FDA-approved drugs that computationally bind to MDM2.

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The integrity of the p53 tumor suppressor or its pathway is compromised in the majority of cancers. In 7% of cancers, p53 is inactivated by abnormally high levels of MDM2, an E3 ubiquitin ligase that polyubiquitinates p53, marking it for degradation. The p53 protein binds to a hydrophobic cleft of MDM2 and blockage of that cleft can re-establish p53 tumor suppressor activity. Some small molecule MDM2 inhibitors have been developed but de novo drug development is marked by high costs and a lengthy timeframe. This report describes an approach for the identification of FDA-approved compounds that are suitable candidates repurposing as lead drugs for MDM2 inhibition. To this end, 241 conformers of 1,125 FDA-approved drugs were generated computationally. Drug conformer similarities to 41 computationally generated conformers of MDM2 inhibitor Nutlin 3a were ranked by shape and charge distribution. Tanimoto combo scores ranged from 0.716 to a high of 0.801 for Bepridil. In silico docking of the drugs to MDM2 was used to calculate binding energies and to visualize contacts between the top-ranking drugs and the MDM2 hydrophobic cleft. We present a subset of FDA-approved drugs that are predicted to inhibit p53/MDM2 interaction.

1902

S100A4 Inhibition: An Approach for the Prevention of Metastatic Disease.

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The leading cause of mortality in cancer patients is the consequence of malignant cells leaving the primary tumor, traveling to distant sites within the body and forming secondary tumors. From a clinical standpoint, the prevention or inhibition of metastasis is vital to the treatment of cancer. S100A4 is a member of the S100 family of calcium-binding proteins that regulates tumor cell motility via interactions with myosin-IIA and has a direct role in promoting metastatic disease. These observations suggest that S100A4 is an attractive candidate for therapeutic development. We developed three assays to screen for S100A4 inhibitors; a novel fluorescent biosensor (Mero-S100A4) that reports on the conformational rearrangements associated with Ca^{2+}–binding and S100A4 activation, a fluorescence polarization assay that monitors the interaction of S100A4 with a peptide derived from the minimal S100A4 binding site on the myosin-IIA heavy chain and a turbidity assay that directly reports on S100A4-mediated depolymerization of myosin-IIA filaments. These approaches allowed us to screen a small library of FDA-approved compounds, the LOPAC and MayBridge Hitfinder collections and approximately 20,000 ChemDiv compounds. We identified small molecules that disrupt S100A4 function by a variety of mechanisms, including small molecule-induced oligomerization and covalent modification. Our studies demonstrate that small molecule approaches can be used to disrupt the S100A4/myosin-IIA protein-protein interaction.

1903

Association of Polymorphisms and Expression of ERCC1 and XPA with the Response of Patients with Testicular Germ Cell Tumors treated with Cisplatin.

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Testicular germ cell tumors (TGCT) are the most common solid tumors among young men. Cisplatin-based chemotherapy cures over 80% of metastatic TGCT; nucleotide excision repair (NER) is one of the factors that modify the sensitivity to this anticancer drug. In the present study we analyzed the influence of two single nucleotide polymorphisms, which occur in two genes that codify proteins involved in NER, ERCC1 (8092C>A) and XPA (5'UTR), in the response of 101 TGCT patients treated with cisplatin (CPt). In addition, we determined the effect of these polymorphisms in the expression of ERCC1 and XPA using real time PCR in tumor samples from 31 patients. We analyzed 101 TGCT patients; DNA was extracted and genotyped by RFLP's method. The expression of ERCC1 was analyzed by Real Time PCR. We found a statistically significant association between the ERCC1 8092C>A polymorphism and the sensitivity to CPt (p=0.02); the A allele was associated with an under-expression of ERCC1 (p=0.04). There was no association between the XPA 5'UTR polymorphism and the response to chemotherapy. To confirm the association between ERCC1 and XPA expression and sensitivity to CPt, we determined mRNA levels in CPt-sentitive, and CPt-resistant testis tumor cell lines after treatment with CPt. Our results show that ERCC1 mRNA levels increased after 1h, 15h and 21h post-treatment with CPt in resistant cell lines. There was no significant change in XPA expression. The ERCC1 8092C>A polymorphism has a direct effect in the response of TCGT patients to cisplatin. We hypothesize that this polymorphism may influence the mRNA stability promoting its degradation, and resulting in a diminished DNA repair. It is important to notice that these associations have not been previously described, so the determination of the polymorphism of ERCC1 8092C>A and the ERCC1 mRNA levels could be used as predictive markers of response to CPt-based chemotherapy in TCGT patients. This work was supported by CONACYT 83959 and PAPIIT IN213311-3.

1904

Caspase 8 cleavage is inhibited downstream of p38 MAPK in cisplatin resistant cells.

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Cisplatin is a platinum based anti-cancer drug that is used to treat a variety of cancers such as lung, head and neck, ovarian, and testicular. Although initial responsiveness to cisplatin therapy is high, the majority of patients relapse with development of tumors that are resistant to treatment. Therefore, we wanted to investigate the apoptotic signaling events occurring in cisplatin responsive cells with the goal of determining how these signaling events are inhibited in resistant cells. In this study we examined apoptotic signaling in the *ras* transformed A549 lung carcinoma cell line. Following 48 hour treatment of cells with 10uM cisplatin there was a significant increase in caspase 8 cleavage and DNA fragmentation. These events were independent of one another since inhibiting caspase 8 activity did not prevent DNA fragmentation, but did prevent breakdown of the nuclear envelope. We also determined that caspase 8 cleavage, but not DNA fragmentation, was dependent upon the signaling of the pro-apoptotic mediator p38 MAPK. Interestingly, cisplatin treatment selected for resistant cells that were still able to initiate p38 MAPK dependent signaling, but were unable to cleave caspase 8. Therefore, these data suggest resistance to cisplatin induced breakdown of the nuclear envelope involves inhibition of caspase 8 cleavage downstream of p38 MAPK.

1905

Photodynamic therapy-induced apoptotic cell death is mediated by a transglutaminase 2-dependent mechanism involving the calpain/Bax signaling pathway.

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We investigated the key role of transglutaminase 2 (TG2) in apoptotic cell death induced by photodynamic therapy (PDT) with the chlorin-based photosensitizer DH-II-24 in human gastric adenocarcinoma cells. PDT-induced apoptotic cell death was prevented by cystamine and TG2-specific siRNA, both of which inhibited TG2 activation by PDT. Moreover, overexpression of EGFP-TG2 (wild-type TG2) potentiated apoptotic cell death via enhanced activation of TG2 in response to PDT, while EGFP-[C277S]TG2 (transamidation-defective mutant form of TG2) attenuated apoptotic cell death because of reduced TG2 activation. PDT resulted in translocation of Bax and collapse of the mitochondrial membrane potential that caused the release of cytochrome c and apoptosis-inducing factor (AIF). Released AIF translocated to the nucleus and subsequently led to apoptotic cell death synergistically with the caspase-dependent pathway induced by PDT. Both the caspase cascade and the action of AIF in PDT were mediated by TG2 activation. In addition, PDT-activated calpain was responsible for Bax translocation, the collapse of the mitochondrial membrane potential, caspase-3 activation, and AIF translocation, but not TG2 activation. Together, these results demonstrate that PDT with DH-II-24 targets TG2 by activating calpain-induced Bax translocation, which induces apoptotic cell death simultaneously through both caspase-dependent and AIF-mediated pathways. Moreover, these results suggest TG2 as a possible therapeutic target for PDT treatment of cancer.

1906

The role of beta-tubulin isotypes in microtubule drug resistance.

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Epothilones are a novel group of microtubule binding drugs, of which ixabepilone (Ixempra®) is in clinical use for treatment of breast cancer. In this study we evaluated the importance of various human beta-tubulin isotypes in the epothilone drug response in lung and breast cancer cell lines. Silencing experiments of TUBB2A-C, TUBB3 and TUBB5 showed that reduction of TUBB3 levels significantly sensitized the cells to sagopilone, epothiloneB and ixabepilone. In contrast, diminishment of TUBB2 or TUBB5 did not influence the cellular drug response. TUBB3 deficient cells displayed an increased number of drug induced abnormal multipolar mitotic spindles and a decreased rate of cell proliferation. In addition, reduction of TUBB3, but not TUBB5 isotype stabilized the microtubule dynamics which may, at least partly, explain the increased sensitivity of TUBB3 deficient cells to the microtubule stabilizing epothilones. Exogenous expression of TUBB3 in epothilone resistant A549EpoB40 cell line reversed the drug resistant phenotype most likely by replacing the mutated TUBB5 isotype expressed in these cells. Together, our results indicate that the TUBB3 isotype functions as epothilone binding target and the expression levels of TUBB3 define the cellular response and sensitivity to epothilones.

1907

ATM-NBS1 Interaction Is Essential for Subsequent ATM Functions after Ionizing Radiation.

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The Ataxia-Telangiectasia Mutated (ATM) kinase and the Nijmegen Breakage Syndrome 1 (NBS1) are critical elements in regulation of optimal DNA damage responses. ATM is a major kinase that functions by phosphorylating a number of substrates to facilitate cell cycle checkpoints, DNA repair and apoptosis. NBS1, on the other hand, is a component of the MRN complex required for DNA repair. NBS1 interacts with and recruits ATM to DNA damage sites. The constitutive interaction of ATM with NBS1 at the extreme C-terminus is required for initial ATM autophosphorylation at Serine 1981. However, it is less clear whether the interaction is essential for ATM function at later time points after DNA damage. Using a small inhibitory peptide that can disrupt the ATM-NBS1 interaction, we found that IR-induced ATM activation is significantly delayed in the absence of the interaction. While ATM activation was later restored, disruption of the ATM-NBS1 interaction after IR-induced DNA damage impaired subsequent DNA repair processes, leading to an extended retention of H2AX foci. Abrogation of the ATM-NBS1 complex before IR leads to the loss of the early G2/M checkpoint but sustain functional G2/M accumulation in response to IR. However, a prolonged G2 accumulation with a moderate radiosensitivity was induced when the ATM-NBS1 interaction was disrupted after IR. Together, these observations have highlighted an essential role of ATM-NBS1 interaction in the process of ATM activation as well as radiosensitivity.

1908

Understanding the functions and therapeutic significance of the poly(ADP-ribose) polymerase (PARP) enzyme family.

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Post-translational protein modifications such as phosphorylation and ubiquitination have well-established roles in a range of cellular processes. One less-understood modification is the covalent addition of ADP-ribose groups to proteins, termed ADP-ribosylation. The poly(ADP-ribose) polymerase (PARP) family of proteins synthesizes this modification onto acceptor proteins, using NAD+ as substrate. Human cells express 17 different PARP proteins, which play critical roles in cell division, DNA damage repair and stress responses including stress granule formation and ER stress sensing mechanisms. One sub-family of PARPs has also been linked to the regulation of cell motility. Recently, PARP proteins have proven to be attractive targets for disease therapy, with PARP inhibitors in over 16 clinical trials. For example, inhibitors of PARP-1, which plays critical roles in single strand DNA damage repair, are promising targets for cancer therapeutics, particularly in cancers, such as BRCA-deficient tumors, with genetic lesions in double-strand DNA damage repair, where PARP inhibitors are thought to function via synthetic lethality. Interestingly, PARP inhibitors appear to also be effective in treating specific cancers that lack defects in double-strand DNA damage repair, suggesting that some therapeutic benefits of current PARP inhibitors are due to inhibition of PARPs other than PARP-1. To gain a deeper understanding of the mechanism of PARP function, we identified the major binding partners of each PARP via LC/MS-MS. This analysis suggested novel roles for many of the PARPs and provided support for PARP functions in cell migration. We then developed an assay to test the effects of a variety of PARP inhibitors on the enzymatic activity of each of the 17, full-length PARP proteins purified from human somatic cells and a cell-based assay to explore the effects of PARP inhibitors on cell motility. Our work allows a more complete

comprehension of the cellular effects of PARP inhibitors, and of their functions in cancer therapy.

1909

Identification of Oncogenic Point Mutations and Hyperphosphorylation of

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The oncogenic property of anaplastic lymphoma kinase (ALK) plays an essential role in the pathogenesis of various cancers and serves as an important therapeutic target. In this study, we identified frequent intragenic loss of heterozygosity (LOH) and six novel driver mutations within ALK in lung adenocarcinomas. Overexpression of H694R or E1384K mutant ALK leads to hyperphosphorylation of ALK and activation of its downstream mediators STAT3, AKT and ERK resulted in enhanced cell proliferation, colony formation, cell migration and tumor growth in xenograft models. Furthermore, the activated phospho-Y1604 ALK was increasingly detected in 13 human lung cancer cell lines and 263 lung cancer specimens regardless of tumor stages and types. Treatment of two different ALK inhibitors, WHI-P154 and NVP-TAE684, resulted in down-regulation of aberrant ALK signalings, shrinkage of tumor, suppression of metastasis and significantly improved survival of ALK mutant-bearing mice. Together, we identified novel ALK point mutations possessed tumorigenic effects mainly through hyper-phosphorylation of Y1604 and activation of downstream oncogenic signalings. The up-regulated phospho-Y1604 ALK could serve as a diagnostic biomarker for lung cancer. Furthermore, targeting oncogenic mutant ALKs with inhibitors could be a promising strategy to improve therapeutic efficacy of fatal lung cancers.

1910

Comparative effects of TNFR1 and TNFR2 on the early TNF signaling.

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Tumor necrosis factora (TNFα) is a proinflammatory cytokine with pleiotropic immunological and biological activities. TNFα signaling is triggered by the engagement of soluble TNFα to two types of cell surface receptors, TNFR1and TNFR2. TNFR1 and TNFR2 resemble each other in their extracellular, cysteine-rich domains. TNFR1 contains a cytoplasmic death domain (DD) that binds to the adaptor TRADD (TNFR-associated death domain). TNFR2 lacks a DD, but has cytoplasmic motif that binds TRAFs (TNFR-associated factors). Upon binding to ligated TNFR1, TRADD recruits the secondary adaptors RIP (Receptor interacting protein), TRAF2 (TNF Receptor associated factor2), FADD (Fas associated death domain), cIAP1, cIAP2. Unlike TNFR1, TNFR2 binds TRAF2 directly and TRAF2 also recruits TRAF1, cIAP1, cIAP2. Because the majority of TNFα signaling events are mediated by TNFR1 and function of TNFR2 is poorly understood, this study is subject to know comparative effects of TNFR1 and TNFR2 on the early TNFa signaling.
First, we purified wild type TNF, TNFR1-specific TNF, TNFR2-specific TNF. Then we show endogenous TNFR-interacting proteins in vivo through wild type TNF, mutant TNF, when both TNF receptors were stimulated simultaneously or respectively. Analysis of data demonstrated that ubiquitin E3 ligase cIAP1 and cIAP2 are associated with TNFR2 constitutively. But, cIAP1 and cIAP2 associated with TNFR2 was not observed, when both TNF receptors were stimulated simultaneously. These means that cIAP1 and cIAP2 could be ubiquitinated or translocated. When HeLa-R2 cells were stimulated with TNFα, endocytosis of TNFR1 occured within 10 min. Unlike this, endocytosis of TNFR2 do not occur after 1hour. These data demonstrated that TRAF2 associated with TNFR2 when both TNF receptors were stimulated simultaneously. We guess that TRAF2 is key mediator to TNFR2 signaling. We show that downregulation of cIAP2

increase cell death of TNF-stimulated cells and TNFR2 accelerates cell death of TNF stimulated cells. Understanding the regulation of TNFα-TNF receptors production and signals transmitted by TNFa is necessary for the development of effective treatments for these diseases.

Blood Cells and Vessels

1911

Angiogenic effects of C-peptide in human umbilical endothelial cell.

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The pro-insulin connecting peptide, C-peptide is a cleavage product of insulin biosynthesis that is co-secreted with insulin by pancreatic $_{\beta}$-cells following glucose stimulation. Several studies suggest that C-peptide has beneficial effects in a number of diabetes-associated complications, such as prevention of diabetic neuropathy, neuronal apoptosis, vascular dysfunction and atherosclerosis. However, the roles of C-peptide in vascular remodeling and induction of angiogenesis process remain obscure. In the present study, we investigated reactive oxygen species (ROS) and Ca^{2+} as angiogenesis mediators and induction of cell migration, actin reorganization and tube formation by C-peptide in human umbilical endothelial cells (HUVECs), and these responses were compared with those were induced by vascular endothelial growth factor (VEGF). Interestingly, C-peptide is more potent than VEGF in induction of tube formation without generation of ROS, Ca^{2+} and stress fiber. Also, C-peptide increased expression of FXIIIa, which is known regulator of angiogenesis by enhancing cell migration and cell survival. Thus, our results suggest that C-peptide has potential therapeutic application in angiogenesis and prevention of vascular leakage.

1912

Influence of dermatan sulfate in inflammatory response and migration of mononuclear cells differentiated or not into to endothelial progenitor cells after arterial lesion.

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Dermatan sulfate (DS) is an antithrombotic and anticoagulant glycosaminoglycan present in extracellular matrix that can be involved in attraction, migration and differentiation of cells after endothelial lesion. Its inflammatory capacity is not yet elucidated. Endothelial progenitor cells (EPC), first described by Asahara 1997, can migrate to lesion site in order to recover damaged endothelium. In this work, we analyzed the effect of DS in the inflammatory process and migration of mononuclear cells (MNC) obtained from mice bone marrow (BM) induced or not to differentiation into EPC, after arterial injury. Lesion was done in the carotid artery using a wire guide probe. DS [20mg/kg] was administered 10 min, 12, 24 and 48 hours after surgery. MNC extracted from BM were cultured or not for 4 days in DMEN containing VEGF, IGF and FGF (differentiation medium). MNC were labelled with PKH26-GL (2 x 10^6 cells) and MNC cultivated (MNCc - 10^6 cells) with PKH26-GL and LDL-FITC and injected 1 and 24 hours after injury. Both treatments were administered intravenously. Arteries were analyzed 7 days after injury to map the presence of these cells in the vessel. We analyzed 4 groups: MNC (1), MNCc (2), MNC + DS (3) and MNCc + DS (4). PKH-26 + cells were higher in groups 3 and 4 when compared to the others (45 or 52,33 vs. 22 or 26,25, $p<0,05$). LDL^+ cells did not differ in any treatment. Inflammatory response was analyzed by western blotting using ICAM-1, eNOS, P-selectin and SDF-1 antibodies. ELISA (TGF-β and IL-6) assays were done 1 day after lesion in injured mice

treated or not; Injured (A), DS (B), MNC (C) and not injured (D). TGF-β levels decreased in B (6571,15 vs. 10259,21 pg/ml (A), p<0,05), IL-6 levels did not differ among groups. Expression of ICAM-1 in group B decreased about 52%, P-selectin 10 times and SDF-1 was abolished in DS treated animals; C increased the expression of ICAM-1 in 20% and eNOS in 133% in relation to A. We observed that DS therapy promoted a significant migration of MNC to lesion site and decreased the expression of proteins related to inflammation, suggesting DS effect can be not only related to inhibition of thrombosis, but also to decrease of inflammation in the injury site promoting a better environment for MNCs adhesion.
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1913

Expression of Nampt, an NAD+ regenerating enzyme, in the developing and adult vasculature.

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Pathways that consume NAD+, including those mediated by sirtuins, PARPs, and cADP ribose, have emerged as critical mediators of DNA repair, transcriptional regulation, calcium mobilization, and lifespan. The existence of these NAD+-consuming events has highlighted the importance of pathways that generate NAD+ locally. However, tissue expression of NAD+ biosynthetic enzymes has not been well characterized. Furthermore, there has been controversy over the identity of nicotinamide phosphoribosyltransferase (Nampt), the rate limiting enzyme for NAD+ salvage from nicotinic acid, which has also been proposed to be an adipokine. We undertook RT-PCR screening of adult mouse tissues which revealed that Nampt was expressed in heart, aorta, skeletal muscle, kidney, liver, bladder and brain. Immunostaining revealed that although Nampt was expressed in most tissues, there was cell type variability. This was particularly evident in the vessel wall, where endothelial cells expressed abundant Nampt but expression in the underlying smooth muscle cells was weak. This pattern was observed in the aorta, carotid arteries, and medium and small vessels of the heart, kidney and lung. This gradient was also observed in human aorta, particularly in the adventitial microvessels. Upon injury to the mouse carotid artery induced by distal ligation, Nampt expression was significantly upregulated in the medial SMCs. Mouse embryos revealed strong Nampt staining in the primordial aortic structures at 9.5 dpc and Nampt staining in the endothelial layer was strong throughout embryonic development. In contrast, mural cell expression declined in late embryonic stages, by e18.5. Conclusion: Nampt is a ubiquitously expressed enzyme but in adult blood vessels there is a significant gradient between the endothelial cells and underlying smooth muscle cells, implying differential requirements for NAD+ regeneration within the artery wall. In SMCs, NAD+ consumption may be particularly important during early differentiation in the embryo and following vascular injury in the adult.

1914

HDL reduces the risk of foam cell apoptosis in foam cell associated with PC-FC complex formation.

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[Objective] Phosphatidylcholine (PC)-cholesterol (FC) complex structures, a unique atheroma related antigen associating with foam cell death. To elucidate the role of antigen in the development of atherosclerosis , the relationship among PC-FC complex, cellular FC transport and role of HDL in cultured foam cell were investigated.
[Method] Foam cells were prepared by incubation with various modified LDL for 24hr, then cultured up to 3—6 days in the presence of LPDS +/- HDL. PC-FC complex and Oxidized

Phosholipid were detected by immunofluorescent microscopy using specific monoclonal antibodies. Cell Apoptosis was detected by Annexin V binding.
[Results & Discussion] We succeeded to express PC-FC complex as a cell apoptosis marker on the surface of FC-rich lipid droplets using Hyperlipidemic sera and acetylated LDL. PC-cholesterol complex expression and Foam cell apoptosis significantly reduced by HDL treatment. HDL also reduced CE deposit in lipid droplets formation in foam cell. Whereas, HDL was not effective for decreasing the frequency of foam cell apoptosis in the presence of Probcol or Glyclazide treatment via ABC transporter pathway, closely related with cellular FC transport. Probcol enhanced to express PC-FC complex and FC-rich lipid droplets in foam cells, whereas no significant change of this antigen expression and cell apoptosis with Glyclazide treatment.
This phenomena would be related to the PC-cholesterol complex formation and foam cell death via cellular FC transport. HDL played an important role in PC-FC complex formation.
Expression of PC-FC complex can be related to oxidized lipid production in cultured foam cells. Oxidized lipid accumulation might be also related to the foam cell death. These significantly relationship among the PC-FC complex formation, cellular FC transport, oxidized lipid accumulation and foam cell apoptosis can reflect to development of atherosclerosis in thickened intima.

1915

A1 adenosine receptors are involved in vasa vasorum endothelial cells barrier enhancement in a neonatal model of hypoxia-induced pulmonary arterial hypertension.

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In a neonatal model of pulmonary hypertension, hypoxia-induced pulmonary vascular remodeling involves dramatic pulmonary artery (PA) adventitial thickening, accumulation of inflammatory cells and expansion of the adventitial *vasa vasorum* network. However, the molecular and cellular mechanisms underlying these events remain elusive. The main aims of this study were i) to determine the differences in barrier function between VVEC isolated from animals exposed to normoxia or 2-week hypobaric hypoxia (P_B=430Hg), and ii) to investigate the role of adenosine receptors (ARs) in VVEC barrier regulation. The quantitative RT-PCR data indicate that VVEC isolated from control animals (VVEC-Co) express all the ARs (A1, A2a, A2b and A3). Interestingly, A1 and A3 ARs expression is decreased in VVEC isolated from calves exposed to chronic hypoxia (VVEC-Hyp) compared to VVEC-Co. We further demonstrate that the AR ligand, adenosine, strikingly enhanced the barrier function in VVEC-Co, as measured by transendothelial electrical resistance (TER) and to a lesser extent in VVEC-Hyp. Using receptor-specific agonists and antagonists, as well as specific siRNAs we could demonstrate that A1 ARs are responsible for the adenosine-induced TER increase. We subsequently investigated the role of cytoskeletal proteins in the observed barrier enhancement effect of adenosine in VVEC. Our results show that adenosine failed to increase the TER in cytochalasin B (an inhibitor of actin polymerization)-treated VVEC. In contrast, adenosine was able to increase the TER in nocodazole (an inhibitor of microtubule polymerization)-treated VVEC, suggesting a critical role of actin polymerization in VVEC barrier enhancement. Moreover, adenosine significantly increased the cortical actin in the cell-cell junctions in VVEC-Co, but to a lower extent in VVEC-Hyp, possibly due to observed decreased levels of A1 ARs expression in the latter. Further, we showed that TNFα, which is produced in hypoxic vessels, increases the permeability of VVEC-Co, but not VVEC-Hyp, an effect blunted by adenosine pre-treatment in VVEC-Co. In summary, we demonstrate for the first time that Gi-coupled A1 ARs enhance barrier function in VVEC,

probably via actin microfilament remodeling. We propose that A1 ARs are a possible therapeutic target in regulating endothelial barrier function, crucial in pulmonary arterial hypertension.

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Myosin-II inhibition leads to functional enrichment of long-term hematopoietic stem cells.

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Non-muscle myosin-II (NMM-II) promotes cell division, membrane rigidity and adhesion to a rigid matrix; hence it seems predictable that NMM-II activity is low in dormant hematopoietic stem cells (HSCs) and increased during differentiation. Deletion of NMM-II is known to be embryonically lethal, but its role in adult HSC differentiation is not known. Here, reversible inhibition of NMM-II sustained over several cell cycles enriches long-term HSCs up to ~20 fold by selective elimination of proliferating progenitors. Molecular profiling and functional analyses indicate that NMM-II isoforms may play distinct roles during HSC differentiation. NMM-IIA dictates the early stage of HSC differentiation as indicated by the lower expression in HSCs than committed progenitors, which is consistent with higher membrane elasticity of HSCs. In contrast, NMM-IIB may dictate the late stage of HSC differentiation as indicated by the higher expression in HSCs and progenitors than differentiated CD34- cells – its functional implication in matrix elasticity sensing and migration is under investigation. HSC and progenitor number is sensitive to matrix elasticity in a NMM-II dependent manner, with the maximal number observed on soft and high-density fibronectin matrices. In contrast, the extent of HSC enrichment is more sensitive to matrix density than elasticity under NMM-II inhibition. Deactivating NMM-IIA phorphorylation at the Ser1943 residue is highest in HSCs and decreased during differentiation. Phospho-mimetic mutation of NMM-IIA at Ser1943 shows decreased cytoskeletal integrity and increased membrane elasticity, indicating that physical properties of HSCs can also be regulated by HSC-specific signaling via NMM-II heavy chain phosphorylation. Myosin-inhibited CD34+-derived bone marrow cells maintain functional long-term HSCs in vivo in the marrows of xenografted mice with an added benefit to increase platelet circulation simultaneously. Therefore, NMM-II is a critical node for HSC differentiation.

1917

Defective retinal angiogenesis in isoxanthohumol-treated mice.

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Angiogenesis, the formation of new blood vessels from an established vascular network, occurs in many physiological conditions, such as wound healing or pregnancy. Pathological angiogenesis, conversely, is associated with several diseases, like cancer, diabetes or ischemia. Identification and characterization of dietary compounds, able to modulate angiogenesis may constitute an important strategy for prevention of these pathologies. Isoxanthohumol (IXN), a flavonoid of beer, is one of the main xanthohumol metabolites. Several studies demonstrated anti-inflammatory, anti-oxidant and anti-angiogenic properties.
To further evaluate the effects of IXN on developmental angiogenesis, the neonatal retinal neovascularization model was used. In the developing retina, endothelial sprouting and organization of vascular networks occur in a reproducible temporal and spatial pattern and can be easily imaged to study the interactions of EC with growth factors, astrocytes and mural cells and to analyse several angiogenic steps, namely EC survival, proliferation, migration and vascular pruning and normalization.
C57BL/6 mice pups were injected intraperitoneally with 20µL of 50µM IXN or control (PBS), daily, until post-natal day (P)4. Then, pups were euthanized and eyes enucleated, fixed in 4% p-

formaldehyde overnight, and retinas were dissected out and stained with FITC-conjugated isolectin. Finally, retinal endothelium was visualized using fluorescent and confocal microscopy, photographed and vascular quantification measurements were performed.
At P4, IXN group displayed a significant decrease in the vascular length and increase in the width of the capillary free zone of main arteries and no differences in the number of artery side branches. Furthermore, it was observed a decline in the number of endothelial tip cells and filopodia, and decreased filopodia length in the IXN-treated mice. Taken together these results suggest that IXN negatively modulates EC migration (tip cells and filopodia) and vessel sprouting (vascular length) and endothelial cell survival (with of capillary free areas).
Analysis of IXN treatments on later stages retinal angiogenesis at P5 and P7 pups seems to confirm that IXN modulates tip cells behaviour and vessel morphology.
Altogether, the accumulated evidence proposes IXN as an interesting anti-angiogenic compound that might have therapeutic and preventive applications in angiogenic and inflammation associated diseases.
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1918

Analysis of evolutionary repurposing predicts an orally available vascular disrupting agent.

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In the course of systematically identifying genetic modules that are repurposed during the course of evolution (phenologs), we discovered a pathway that was both relevant to restructuring of yeast cell walls in response to stress and at the same time responsible for regulating angiogenesis in vertebrates (1). By analyzing such repurposed networks, we reasoned that small molecule inhibitors targeting the pathway in yeast might act as angiogenesis inhibitors suitable for chemotherapy in vertebrates. We computationally prioritized candidate angiogenesis inhibitors based upon measured synthetic genetic interaction of known drugs. This strategy led to the finding that thiabendazole (TBZ), an orally available antifungal drug in clinical use for 40 years, also potently inhibits angiogenesis. We found that TBZ treatment severely impaired angiogenesis in the frog, *Xenopus*, an accurate and tractable model vertebrate for *in vivo* studies of angiogenesis. We also found that TBZ inhibited angiogenesis in a dose-dependent manner in human cells (HUVECs) *in vitro*. Among various growth factors and cell adhesion molecules, the vascular endothelial growth factor (VEGF) pathway is perhaps the best-characterized signaling pathway associated with angiogenesis. Ectopic VEGF potently induces ectopic angiogenesis in frog embryos, and this effect was blocked by TBZ, suggesting that the drug acts downstream of this key regulatory node. In TBZ treated embryos, we noted disconnected and scattered arrays of cells in which vascular gene expression persisted. The ability of TBZ to disrupt pre-existing vasculature in our subsequent tests suggests such defects in vascular morphogenesis in the absence of changes to vascular cell fates may stem from impairment of junctional integrity in the vasculature. This is especially significant because disruption of more established vasculature may also prove beneficial for cancer therapy, and a new class of drugs called Vascular Disrupting Agents (VDAs). Though promising, no VDAs have as yet been approved for use in humans. *In vivo* time-lapse imaging reveals that TBZ reversibly disassembles newly established blood vessels, marking it as one of the few known vascular disrupting agents. Finally, we also showed that TBZ slows tumor growth in preclinical fibrosarcoma xenografts. Notably, a TBZ dose for these experiments was close to the U.S. Food and Drug Administration-approved maximum recommended daily dose in humans. Thus, the prospects are excellent for human chemotherapeutic use of TBZ. In sum, a

direct test of the evolutionary repurposing of gene networks has revealed a viable therapeutic agent, already approved for human use in other contexts.

1. K. L. McGary et al., Proc Natl Acad Sci U S A 107, 6544 (2010).

1919

Chronic insulin exposure uncovers a tyrosine phosphatase dependent pro-inflammatory phenotype of endothelial cells.

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Objective: To elucidate intracellular mechanism mediating chronic insulin induced endothelial inflammation.

Methods: Human umbilical vein derived endothelial cells were treated with different concentrations of endotoxin free insulin for varying periods of time prior to performing leukocyte adhesion assays. Endothelial inflammation was assessed by measuring adhesion of PKH26 labelled peripheral blood derived mononuclear cells to insulin treated HUVECs. Changes in gene expression were assessed via reverse transcriptase PCR (RT-PCR) and enzyme activity was measured through immunophosphatase assay.

Results: 48 hours of insulin treatment increased the adhesion of leukocytes to the endothelial monolayer even at sub-optimal concentration of 1nmole/L. This effect of insulin was accompanied by time dependent decrease in intracellular nitric oxide as measured via DAF-2DA imaging. Addition of exogenous nitric oxide through NO donor DETA-NO attenuated insulin-mediated leukocyte adhesion to endothelial cells. These effects were due to intracellular changes in response to chronic insulin treatment and not due to secretion of pro-inflammatory cytokines. Increased leuckocyte adhesion was accompanied by increased expression and activity of protein tyrosine phosphatase PTP1D in a time dependent manner, which in turn induced a time-dependent increase in expression of adhesion molecules E-selectin, ICAM-1 and VCAM-1. Use of NSC87877 and down-regulation of endogenous levels ofPTP1D via siRNA significantly attenuated chronic hyperinsulinemia mediated adhesion of leukocytes to the endothelium.

Conclusions: Chronic insulin exposure elicits inflammatory phenotype in primary cultures of endothelial cells via a mechanism involving increased expression and activity of PTP1D.

1920

The nuclear liver X receptor and its role in smoke exposed rat lungs.

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Background: Chronic obstructive pulmonary disease (COPD) is one of the leading causes of mortality worldwide and currently there are no efficient treatments. Liver X receptor (LXR) plays a role both in lipid metabolism and inflammation. Objectives: To investigate the effects of cigarette smoke (CS) on LXR activation and target gene regulation in vivo and in vitro and to study potential protective effects of LXR activation against CS-induced emphysema. Methods: Sprague Dawley rats were exposed to second hand CS for two months. The lung tissue and bronchoalveolar lavage (BAL) cells were examined for LXR target gene expression and emphysema development was measured by mean linear intercept (MLI). Rat alveolar macrophages (AM) and rat pulmonary endothelial cells (EC) treated with CS extract, LPS or

DMHCA (a steroidal LXR agonist) were examined for changes in LXR target gene and protein expression. Results: Whereas expression of ATP binding cassette transporter A1, a known LXR target gene, was not changed in the lung, it was significantly downregulated in the BAL cells from CS-exposed rat lungs and in AM treated with CS extract. Real-time PCR showed that LXRalpha was significantly downregulated in BALf cells after 1 month of CS exposure and similarly in rat pulmonary micro- and macrovascular cells upon 1% CS extract treatment. Activation of LXR attenuated the expression of LPS-induced genes such as COX2, CCL5 and IL18. We also found that IL18 is increased in BALf cells of CS exposed rats, whereas IL18 binding protein (IL18bp), an endogeneous IL-18 inhibitor, was not changed. Interestingly, activation of LXR lead to upregulation of IL-18BP in AM. Additionally, we saw emphysematous changes in LXR-deficient mice, implying a protective role for this nuclear receptor. Conclusions: CS inhibits regulation of LXR target genes, but activation of LXR attenuated the LPS-induced production of pro-inflammatory cytokines in AM. We also saw an increase in IL18bp, which might be a potential new LXR target gene playing a role in the anti-inflammatory signaling pathway of LXR. Our data suggest that LXR agonist treatment might be beneficial to prevent and treat CS-induced emphysema.
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New Technologies for Cell Biology

1921

Encapsulation of functional cytoplasmic extract within giant unilamellar vesicles.

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Cytoplasmic extracts and purified proteins can be used to identify and reconstruct the molecular mechanisms underlying a broad range of cellular behaviors, including mitotic spindle assembly and actin-based motility. Despite the promise of these *in vitro* approaches, many fundamental cellular processes depend on or are influenced by confinement within membranes and are thus challenging to reconstitute. Although giant unilamellar vesicles (GUVs) offer a promising platform for addressing this challenge, traditional techniques for forming GUVs provide limited flexibility for encapsulating protein solutions, often have limited yield, and may produce highly polydisperse or multilamellar vesicles. Here we show that functional cytoplasmic extracts can be loaded into GUVs of controlled size by temporal control of microfluidic jetting-based encapsulation. This technique uses the liquid impulse expelled from a small (~10 μm) nozzle to deform a planar lipid bilayer into spherical vesicles. Because the solution expelled from the nozzle ends up inside the GUV, this technique offers excellent control over the internal composition of vesicles and can be used to create vesicles over a wide range of sizes containing biological solutions up to fourfold higher in viscosity than water, including whole-cell extracts. We achieve this flexibility by shaping the electrical signal that actuates the liquid jet so as to minimize the mechanical stress imparted on the membrane. As a demonstration of this technique, we encapsulated cytoplasm from *Xenopus laevis* eggs and showed that this extract retains its ability to form mitotic spindles in the presence of sperm chromosomes after encapsulation in a GUV. The precise control over microfluidic jetting demonstrated here is a step towards the reconstitution of increasingly complex biological phenomena within membrane compartments.

1922

Sub-cellular scaled multiplexed protein patterns for single cell co-cultures.

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Cell culture studies are hampered by the heterogeneous nature of cell populations. Further, bulk measurement techniques currently average cellular responses and are not capable of discerning whether an observed phenomenon is a result of cell type differences or other variables within a population. Tip-based direct protein printing is a relatively new technique useful for controlling the cellular microenvironment with sub-cellular resolution. Here this technique demonstrates spatial control of multiple cell types at single cell levels on a substrate. Specifically, 3T3 fibroblasts and C2C12 myoblasts and their respective binding dynamics with fibronectin and laminin demonstrate the single cell co-culture concept.

Co-culture studies have been useful for mimicking the in vivo environment and studying effects on stem or progenitor cell function. However there are many experimental variables that cannot be properly controlled and may lead to confounding results. Several different techniques have been developed in an effort to address the heterogeneity of cell populations including single cell analysis. However, most current single cell analysis is just that, analysis of single cells within a population, using high resolution imaging and tracking technologies. Although cell populations can be monitored at the single cell level, it is still difficult to discern the effect of neighboring cells on cell behavior. Newer technologies such as microfluidics have been used to isolate single cells and then analyze their genomic or proteomic contents. However, these are destructive techniques making longitudinal studies and causal-effect relationships difficult to determine.

Tip based lithography is used to control cell morphology, cell cluster size and differential cell binding at single cell levels. Co-culture of NIH 3T3 fibroblasts with C2C12 myoblasts is demonstrated with greater than 70% pattern binding efficiency with 80% of those being single cell patterns. These proof-of-concept experiments demonstrate a single cell co-culture technique which may be useful for studying cell signaling, differentiation and other stem and primary cell interactions.

1923

Uncovering the Diversity of Individual Cells: Protocol for High Throughput Gene Expression Profiling of Single Cells.

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Fluctuations in gene expression at the single cell level could be key for generating developmental signals and for understanding the progression of tumors. Data needs to be collected from a statistically significant number of single cells in order to determine the range of gene expression present in a population of cells. Furthermore, transcripts need to be quantified for a number of genes in order to obtain meaningful cell signatures.

We describe a new protocol that enables analysis of 96 gene transcripts in 96 individual cells. The method entails collecting single cells directly into reverse transcriptase buffer in a 96-well PCR plate. Following a reverse transcriptase reaction with random primers, multiplex Specific Target Amplification (STA) is performed to enrich samples for the 96 targets of interest. STA retains relative abundance between loci and permits quantitative Cq information to be derived. The 96 individual qPCR assays are then performed for all 96 single cell samples in a 96.96 Dynamic Array™ IFC (Fluidigm). The qPCR assays use DNA binding dye detection in order to

reduce the upfront cost of obtaining 96 assays relative to probe-based assays. The qPCR thermal cycling protocol is immediately followed by acquisition of melting curves (Tm) in order to assess reaction quality.

The results obtained using this protocol demonstrate that individual mammalian cells exhibit a great deal of heterogeneity in transcript levels. For all genes analyzed, transcript levels varied 10- to 100-fold among individual cells even for a homogeneous population of tissue culture cells. These results support the model that eukaryotic transcripts are produced in short but intense bursts interspersed with relatively long intervals of inactivity of random duration. For a population of single cells, this leads to what has been called a lognormal distribution of transcripts but is more accurately described as a Gamma distribution.

1924

KillerRed-Rab7 induces light-dependent cell death in fibroblasts.

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Objectives:

Phototoxic fluorescent protein KillerRed opens new perspectives for application of a genetically encoded photosensitizer in various fields of experimental biology for light-induced (and thus strictly controlled in space and time) inactivation of target proteins and killing of target cell populations. However, low phototoxic activity impedes wide application of KillerRed and finding sensitive targets for KillerRed-induced phototoxicity is necessary. Lysosomes are known to be an important target for photosensitizer-induced oxidative stress. Cell damage by lysosomotropic photosensitizers is mediated by lysosomal cathepsins. We study phototoxic effects caused by KillerRed localized to the cytoplasmic side of lysosomal membrane.

Methods:

Standard molecular cloning techniques; transient transfection; preparation of cell lines stably expressing KillerRed-Rab7 by lentiviral transduction; wide-field fluorescence microscopy; Hoechst33342, propidium iodide staining;

Results:

We targeted KillerRed to lysosomes by fusing it to the fragment of Rab7 protein. KillerRed-Rab7 (KR-Lyso) fusion co-localized with EGFP-Rab7, a known marker of lysosomal localization. We found that REF52 cells transiently expressing KR-Lyso undergo morphological changes after illumination with KillerRed-activating green light. These cells showed either nuclear fragmentation (as seen after Hoechst33342 staining) and no propidium iodide staining, or normal nuclear morphology and propidium iodide staining. Propidium iodide staining coincided with the loss of EGFP fluorescence in cells co-expressing cytoplasmic EGFP. These changes could be prevented by preincubation with cathepsin inhibitors E-64 and pepstatin A, implying that lysosomal membrane damage is responsible for the KR-Lyso phototoxicity. We further studied the influence of illumination intensity on the mode of cell death that occurs after photosensitization with KR-Lyso. We transiently co-transfected REF52 cells with KR-Lyso and EGFP-Baxα. After 20 min illumination with 77 mW/cm2 EGFP-Bax redistribution from diffuse cytoplasmic fluorescence to punctate loci immediately preceded cell shrinkage. On the other hand, after 5 min illumination with 680 mW/cm2 no Baxα redistribution was observed either before or after cell shrinkage. Also we used FRET-based caspase sensor Casper3-GR to determine whether caspase-3 activation is involved in KR-Lyso-induced phototoxicity. With the same illumination intensity we observed both cells where caspase activation preceded cell shrinkage, and cells where caspase activation wasn’t observed. We conclude that different

modes of cell death occur after photosensitization with KR-Lyso depending on the illumination intensity and other factors, possibly, level of KR-Lyso expression in each particular cell.

1925

Imaging-based proteomic maps of neuron.

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We present logistics to conduct systematic surveys of protein-protein interaction networks as they operate in their native environment of a cell. Our isPIN (in situ protein-protein interaction networks) project employs advanced Drosophila genetics, sophisticated molecular imaging technology, and high-performance computation. FRET (Förster resonance energy transfer) offers a visual indication of molecular distance and behavior. Direct association of two interacting proteins brings them typically within 8 nm from each other. When they are each tagged with spectrally matched fluorescent molecules, this nanoscopic distance induces FRET. Because the tagged proteins are not artificially tethered to each other, fluorescent lifetime measurement is the preferred method of FRET quantification. As a starter, we chose to focus on 141 neuronally expressed cytoplasmic proteins. Each protein is tagged in four-ways: with EGFP on the N-terminus, mCherry on the N-terminus, EGFP on the C-terminus, and mCherry on the C-terminus. Resulting GAL4-responsive transgenes are inserted at pre-specified acceptor sites on the second chromosome though PhiC31 integrase-mediated transgenics. These fluorescently tagged proteins are expressed under the control of cell-specific GAL4 drivers, either singly or in pairs, and examined for localization and interactions within model neurons in vivo. Whereas the localization of each protein is assessed through four different tags, the interactions among them are quantified through eight FRET-able tag pairs. Of 9,941 possible interactions among 'humanized' complement of the 141 proteins, only <0.5% are reported in literature. Our project will be able to not only confirm some of these interactions in real-life but also reveal when and where they occur within a single complex cell. Of particular interest are the dynamics of signal convergence and divergence being expected among Rho GTPases and their CRIB effectors. By subdividing the data into twelve functionally distinct subcellular compartments at eight developmental stages, up to ninty-six context-specific proteomic maps will be created for these and many other proteins. While the current phase of the project focuses on well-known pairs of signaling proteins in identified neurons under normal conditions, future experiments may include stress or disease-affected states as well as non-neuronal cells. Ultimately, the project aims to add information-rich contexts to proteomics. Supported by NIH/NINDS.

1926

A photoactivatable small molecule inhibitor for light-controlled spatiotemporal regulation of Rho kinase in live embryos.

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To uncover the cellular and molecular mechanisms of embryonic development, the ideal loss-of-function strategy would be capable of targeting specific regions of the living embryo with both temporal and spatial precision. To this end, we sought to develop a novel pharmacological agent that can be light-activated to achieve spatiotemporally limited inhibition of Rho kinase activity *in vivo*. A new photolabile caging group, 6-nitropiperonyloxymethyl (NPOM), was installed on a small molecule inhibitor of Rho kinase, Rockout, to generate a “caged Rockout”

derivative. Complementary biochemical, cellular, molecular and morphogenetic assays in both mammalian cell culture and *Xenopus laevis* embryos validate that the inhibitory activity of the caged compound is dependent on exposure to light. Conveniently, this unique reagent retains many of the practical advantages of conventional small molecule inhibitors, including delivery by simple diffusion in the growth medium, and concentration-dependent tuneability, but can be locally activated by decaging with standard instrumentation. Application of this novel tool to the spatially heterogeneous problem of embryonic left-right asymmetry revealed a differential requirement for Rho signaling on the left and right sides of the primitive gut tube, yielding new insight into the molecular mechanisms that generate asymmetric organ morphology. As many aromatic/heterocyclic small molecule inhibitors are amenable to installation of this caging group, our results indicate that photocaging pharmacological inhibitors may be a generalizable technique for engendering convenient loss-of-function reagents with great potential for wide applicability in cell and developmental biology.

1927

SPASM: A novel, genetically encoded technique to systematically regulate protein-protein interactions.

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Cellular functions of proteins are strongly influenced by their interactions with other proteins. The frequency of protein-protein interactions, in turn, is a function of the local concentration of two proteins. When two proteins are tethered together, the link between them influences the effective concentration of both and therefore the frequency of their interaction. Currently, in the absence of detailed structural information of the interaction interface, no methods exist to systematically vary the effective concentration within this intramolecular interaction. Here we outline a modular, genetically encoded linker, namely an ER/K single α-helix that can be used to regulate the frequency of interaction between two proteins, or between a protein and a peptide, one at each end. We exploit the wide range of interaction affinities between calmodulin and its binding peptides, combined with Forster's resonance energy transfer (FRET) for a detailed characterization of the role of the ER/K α-helix in regulating protein-protein interactions. We find that regardless of the strength of the bi-molecular interaction, increasing the length of the ER/K α-helix reduces the on-rate of the intramolecular interaction, without significantly affecting the off-rate. We demonstrate a generalized, genetically encoded method to determine the dissociation constant for both moderate (μM Kd) and strong (nM Kd) interactions between proteins, that combines FRET-based detection of protein-protein interactions with an ER/K α-helix linker to make stoichiometric amounts of the two interacting proteins. We apply this technique to develop novel FRET biosensors for signaling proteins and to control auto-inhibition in protein kinases.

1928

An Intein with Genetically Selectable Markers Provides a New Approach to Internally Label Proteins with GFP.

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Inteins are proteins that catalyze their own removal from within larger precursor proteins. The *Pch* PRP8 mini-intein of *Penicillium chrysogenum* was modified four ways to create inteins that could confer 1) G418 resistance 2) hygromycin resistance or 3) histidine prototrophy in appropriate genetic backgrounds or 4) express the transcriptional activator LexA-VP16. Splicing efficiencies for the modified inteins were high when they were expressed in either *E. coli* or *S.*

cerevisiae. The genetically marked inteins were coupled to GFP at their N-termini. The GFP-intein-marker-intein DNA was used as a template to create a PCR product with ends homologous to the interior of a target gene. Transformation and genetic selection yielded cells with the intein inserted in frame into the target gene. Following expression, protein splicing removed the intein, leaving behind an embedded GFP tag. As a proof of principle the central linker of the yeast calmodulin gene *CMD1* was tagged with GFP and shown to be fully functional.

1929

PSI:Biology-Materials Repository: A Biologist's Resource for Protein Expression Plasmids.

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The Protein Structure Initiative:Biology-Materials Repository (PSI:Biology-MR; MR; http://psimr.asu.edu) sequence-verifies, annotates, stores, and distributes the protein expression plasmids and vectors created by the Protein Structure Initiative (PSI). DNASU (http://dnasu.asu.edu), a freely searchable database, stores the plasmid annotations, which include the full-length sequence, vector information, and associated publications for over 140,000 plasmids created by our laboratory, by the PSI and other consortia, and by individual laboratories for distribution to researchers worldwide. Each plasmid links to external resources, including the PSI Structural Biology Knowledgebase (http://sbkb.org), which facilitates cross-referencing of a particular plasmid to additional protein annotations and experimental data. To expedite and simplify plasmid requests, the MR uses an expedited material transfer agreement (EP-MTA) network, where As of July 2011, over 43,000 protein expression plasmids and 85 empty vectors from the PSI are available upon request from DNASU. Overall, the MR's repository of expression-ready plasmids, its automated pipeline, and the rapid process for receiving and distributing these plasmids more effectively allows the research community to dissect the biological function of proteins whose structures have been studied by the PSI. With the added biological focus in PSI:Biology, the distribution of these materials will help researchers expand the knowledge of the role of proteins both in normal biological processes and in disease.

1930

Cationic carrier-mediated mRNA delivery.

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We complexed mRNA encoding luciferase (mLUC) to Lipofectamine (LF), DOTAP/DOPE, or to linear poly(ethyleneimine) (linPEI). Luciferase activity after transfecting HeLa cells with the resulting lipo- and polyplexes was determined by a bioluminescent assay. Interestingly, extent and duration of luciferase expression was determined by the type of complex used. Luciferase activity could be determined for over a week if mLUC was complexed with Lipofectamine. We further transfected the cells with an mRNA or pDNA encoding Green Fluorescent Protein (GFP). This allowed us to estimate the number of transfected cells and directly compare the results for the two nucleic acids. mRNA complexed to Lipofectamine or DOTAP/DOPE transfected a substantially larger fraction of cells (>80%) than pDNA (40%).

We also tested the carriers for their ability to deliver mRNA encoding CXCR4, a receptor binding stromal derived factor 1, into mesenchymal stem cells. With the mRNA-cationic

lipoplexes the fraction of CXCR4-positive cells was around 80%, and with the lin-PEI polyplexes about 40%. These results encouraged us to employ this technology to re-program fibroblasts into stem cell-like cells (induced pluripotent stem cells or iPS). To that end we subjected fibroblasts to either a single, double or triple transfection with 4 different mRNAs, each encoding a transcription factor (Oct4, Sox2, Klf4, cMyc). Flow cytometry following immunostaining demonstrated successful transfection of all introduced factors. Moreover, expression of other transcription factors typically up-regulated during the reprogramming process (such as Nanog) was also significantly increased. Preliminary results, based on morphology and immunohistochemistry, confirmed the formation of iPS colonies.

1931

Novel discrete PEG-based crosslinking reagents for conjugation of antibodies and proteins to biotin, fluorochromes, enzymes and gold that eliminate aggregation, improves solubility, reduces non-specific binding and enhances low level detection limits.

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This research describes the preparation of antibodies in which various labels are linked to the antibodies via a short, uniform (> 3) polyethylene glycol (PEG) chain. The PEG substitution for the traditional hydrocarbon linker results in a conjugate that is more resistant to aggregation and has far superior solubility properties.

As one specific example, biotin modification (biotinylation), results in covalent attachment of one or more biotin groups to biomolecules, (e.g. antibodies). Such biotinylated proteins are capable of binding avidin or streptavidin with very high specificity and thus biotin-tagged molecules can be easily detected using an appropriate streptavidin/avidin detection reagent. Biotinylation reactions are widely used for labeling peptides, proteins, antibodies and cell surface receptors. Standard biotinylation procedures can have drawbacks, most notably, aggregation and non-specific interactions with other biomolecules.

One reason for aggregation of biotinylated proteins may be that commercial reagents, such as sulfo-NHS-LC-biotin and NHS-LC-biotin, introduce hydrophobic aliphatic chains along with the biotin tag. Both reagents contain an (amine-reactive) sulfo-NHS ester with a aliphatic spacer region which separates the protein/antibody from the biotin group. The aliphatic chain (LC group) amplifies the tendency of biotinylated proteins to aggregate in aqueous solution and may cause protein precipitation over time. For this reason, hydrophilic PEG-based biotin compounds are a better alternative for maintaining water solubility of modified proteins. We compared sulfo-NHS-LC-biotin with a unique biotinylation reagent, NHS-dPEG12-biotin, in which the LC group is replaced with a short PEG spacer arm. The results of side-by-side biotinylation reactions of immune affinity purified goat anti-rabbit IgG antibodies shows that NHS-dPEG12-biotin requires no special changes in biotinylation conditions and easily substitutes for sulfo-NHS-LC-biotin. The biotinylated proteins are indistinguishable from each other except in streptavidin-accelerated aggregation of antibodies. The NHS-dPEG12-biotin dramatically improved the solubility properties of the Streptavidin-biotinylated IgG complex.

Our results show that dPEG reagents increase the solubility, increase sensitivity and decrease the aggregation of proteins and that these benefits are likely due to an increased hydrophilic character of the modified biomolecules or surfaces.

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Quantitative *in situ* Measurement of Target Biomolecules by Metal Nano-Particle Label Sets and "Adaptive SEM" Technology.

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1. Objective

Identification of cellular distributions having different phenotypes in a tissue is essential for understanding functions of the tissue originated each cellular spatial arrangement. One useful way is *in situ* detection of expressed biomarkers for each cell in the tissue using a lot of labels, because each cellular phenotype is generally reflected expression patterns of key biomarkers. However, fabrication and identification of such label sets are still challenging. We propose a new sensing technology, which is a comprehensive development of fabrication and identification of nano-particle (NP) labels for simultaneous *in situ* measurements of a lot of expressed biomarkers in a cell.

2. Methods

The NP labels were fabricated as follows. Various sizes of polystyrene spheres were used as casts of NPs, placed on a substrate, and the size was furthermore processed as desired diameter with oxygen plasma treatment. Next, metals were deposited on the spheres by thermal evaporation. Then, metal NPs composed of various sizes and elements were fabricated. When polystyrene casts were interfering with measurements, UV-excited ozone oxidization was performed, and cup-shaped metal NP shells were also obtained. For the identification of fabricated NPs, field emission scanning electron microscopy (FE-SEM) was used. Both diameters and elements of NPs were identified with observations of secondary electron (SE) and backscattered electron (BE) in the FE-SEM.

3. Results and discussion

By using our fabrication method, more than 400 kinds of NPs were fabricated. Metal shell layers were formed by thermal evaporation; therefore, multi-layered NPs can be easily fabricated with sequential evaporation. We used double-layered NPs; outer layer is Au for easy immobilization of probe molecules to use these NPs as labels of biomarkers, and inner layer is various to apply label varieties. Spatial distributions and diameters of reacted NP labels were identified by SE observation with high spatial resolution, and metal kinds were identified by BE observation as the difference of intensities in the image caused by the difference of atomic number of inner metal. We call it "adaptive SEM" technology (i.e., NP identification is "adaptive" for various samples), and at least six different metals can be simultaneously discriminated by this technology. These results indicate a possibility for quantitative *in situ* detection of expressed biomarkers in a cell by the suggested technology.

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Targeted NF-κB Inhibitory Nanoparticle Decreases Melanoma Cell Proliferation by Preventing P65 nuclear translocation.

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Although death rates from all cancers are declining in US, the incidence of new cases and deaths from melanoma continue to rise. Malignant melanoma exhibits approximately a 10-20% response rate to conventional chemotherapy. Adjuvant immunotherapy with interferon-α and/or interleukin-2 modestly increases the response rate in a small group of patients, but excessive toxicity from immunotherapy may outweigh the benefit. Therefore, seeking for the alternative therapeutic approaches continues. It has been reported that abnormal activation of NF-κB signaling pathway contributes to the melanoma progression and metastasis. NBD peptide, a NF-κB inhibitory peptide, has been shown to inhibit abnormal NF-κB activation. The NBD peptide delivery has been reported by fusing it to cell penetrating peptide. But lacking cellular specificity calls for a constrained delivery system. In this study, we report a post-formulation technology for generating targeted NF-κB Inhibitory nanoparticle and its application on potential melanoma treatment.
Methods and Results: Targeted NF-κB inhibitory nanoparticles were generated by loading NBD peptide, a NF-κB inhibitory peptide, into $\alpha_\gamma\beta_3$-integrin targeted perfluorocarbon (PFC) nanoparticles by using a linker peptide, which enables post-formulation self-assembling process. Physic characterization of NF-κB inhibitory nanoparticles was performed by measuring particle size and zeta potential. Dose dependent manner of NF-κB nuclear translocation inhibition was demonstrated by ELISA-based transcription factor assay. XTT cell proliferation assay results indicated that proliferation of human melanoma cell (C32) was inhibited by 40%. Moreover, the *in vivo* stability of the NF-κB inhibitory nanoparticles was demonstrated by using both radioactive and magnetic resonance spectroscopy readouts.
Conclusion: This targeted NF-κB inhibitory nanoparticle prevents melanoma cell proliferation by NF-κB signaling pathway inhibition. We anticipate that this new nano-therapeutic system could benefit melanoma management. Moreover, this post-formulation strategy could help to accelerate translation of cell biology findings into clinic applications.

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Silicone gels for cell rigidity sensing and traction force microscopy: robust mechanical properties, rigidity sensing assays in a multi-well format, and TIRF microscopy on soft substrates.

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Substrate rigidity impacts cellular migration, gene expression, development, and tumorigenesis, especially in a range of elastic moduli, *E*, of 0.1 to 100 kPa. Soft substrates commonly used for cell rigidity sensing studies are hydrogels, such as polyacrylamide gels. These gels also enable cell traction force microscopy (TFM), where forces exerted by cells on a substrate with known *E* are evaluated from deformation of the substrate. Elastic moduli of thin gels are usually measured with atomic force microscopy (AFM), which requires expensive equipment. Elastic moduli of hydrogels can change due to drying, swelling, aging, and hydrolysis of molecular bonds. Here, we formulated and tested a series of silicone gels with *E* covering a range from 0.4 to 300 kPa that were all prepared by mixing different proportions of two components of a commercial silicone, Sylgard 184. Silicone pre-polymers were spin-coated onto microscope cover glasses to ~35 um thickness. Mechanical properties of the silicone gel layers were

consistent and robust, with ~3% variability within a batch and ~10% change after 6 months of storage. Elastic moduli of the gels were measured with 5-10% absolute accuracy by tracking displacements of beads on surfaces of the gels, when known hydrodynamic shear stresses were applied to the surfaces using a specially built microfluidic device. We made 6-well plates with gels of different E at the well bottoms. We found that mouse embryonic fibroblasts spread over significantly larger areas on gels with moderate E of 30 kPa than on gels with $E = 0.5$ and 3.5 kPa. Cells were harvested from the wells and analyzed for biochemical changes. Western-blot analysis revealed varying levels of phosphorylated Focal Adhesion Kinase (FAK) in fibroblasts grown on silicone gels with different E.
We also formulated and prepared layers of silicone gels with E from 0.4 to 130 kPa and with refractive index, n, of 1.49. The high value of n made the gels fully compatible with through-the-lens Total Internal Reflection Fluorescence Microscopy (TIRFM) using TIRF objectives with numerical apertures (NA) of up to 1.49. TIRFM enables selective visualization of the dynamics of substrate adhesions, vesicle trafficking, and biochemical signaling at the cell-substrate interface. For TFM, we also covalently bound to gel surfaces 40nm far-red fluorescent beads that were distributed nearly evenly with a high surface density. We plated mouse endothelial cells with fluorescently labeled F-actin on the $n = 1.49$ silicone gels and successfully performed concurrent TIRFM and TFM. We obtained high-quality TIRF images of the cells showing maps of F-actin localization at the substrate and juxtaposed them with high-resolution traction force maps obtained with TFM.

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Nanofluidics-based antibody microarray for application of single cell Immunoassay.

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Cellular heterogeneity that arises from stochastic transcription and translation processes is a fundamental phenomenon in cell biology. However, demands to quantitatively measure multiple proteins within individual cells beyond current capability of proteomics technology. This may be particularly true for stem cell research. For example, while the study of induced pluripotency frequently rely on experimental assays that average measurements among a large population of cells, the majority of them do not become pluripotent. Although fluorescence microscopy is commonly employed to observe individual stem cells, the number of species can be observed simultaneously is limited to the spectrum overlapping of fluorescent dyes.

For the purpose to quantitatively measure protein contents in single cells, we invented a novel nanofluidic device for immunoassays, combining conventional antibody microarray with state-of-art Lab-on-Chip technology. In our device, antibody spots with diameters of 200 micrometers were imbedded in fluidic channels with depths of less than one micrometer. The binding activity and the specificity of immobilized antibodies were confirmed using target proteins conjugated with fluorescent dye. To characterize the performance of the device, real-time reaction kinetics was studied in experiments using DNA hybridization and ligand-protein (biotin-streptavidin) interactions. The results, combining with theoretical works, show that the shallow depths shorten the diffusion time and thus significantly improve the overall binding efficiency.

To sum up, we present a nanofluidic platform combined with antibody microarray, and thus could identify multiple target proteins from extremely small volume comparable to single or few cells. Not only for better understanding of cellular heterogeneity, this technique should also

contribute to the fields where starting materials are extremely limited, including single cell analysis and stem cell research.

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Spatio-temporal profiling of IgE receptor-mediated pathway using an integrated microfluidic platform.

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Traditional biochemical techniques for studying cellular behavior rely on multiple manual steps leading to hard-to-reproduce results, loss of sample, and poor temporal resolution. We are developing a microfluidic platform that integrates and automates the various steps required (e.g., cell culture, cell preparation, challenge, and measurement) to systematically interrogate cellular signaling process at a single cell level with high-sensitivity and minimum reagent consumption. The monolithic serpentine microfluidic chip is about 250 m in width and 30 m in depth, which holds up to a thousand cells per chamber. The accessories including pneumatic control units, electronic control valves, digital heaters and optical detectors are assembled into a bench-top, semi-automated platform that maintains cell viability and enables quantitative measurement of signaling events in single cells using high-resolution imaging and on-chip flow cytometry. The platform is being validated using rat basophilic leukemia cells (RBL-2H3), a mast cell model used to understand fundamental mechanisms that lead to allergic responses and asthma. Following on-chip cross-linking of the high-affinity receptor FcεRI for IgE by multivalent antigen, we examined early, intermediate, and late stages of signaling events including protein phosphorylation, calcium mobilization and the release of inflammatory mediators and synthesis and secretion of cytokines, demonstrating the ability of our platform to make quantitative measurements on a cell by cell basis from just a few hundred cells. The new platform enables analysis of the heterogeneity of cellular responses and makes it possible to analyze rare primary cells and tissue samples.

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Delivery of Epidermal and Dermal Stem Cells to a Burn Wound via a Spray Device - Exploring Human Fetal Skin Progenitor Cells for Regenerative Medicine Cell Based Therapy.

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Introduction

Alternatives to autologous mesh grafting are not available in high demand. Skin progenitor cells derived from human fetal skin tissue are believed to have an interesting potential for regenerative cell-based therapy in acute and chronic skin disease treatment due to their relatively undifferentiated basal nature. We propose to address the need for innovative cell-based therapies by focusing on fetal skin derived (non-embryonic) and adult stem cells for a cell therapy for 2nd and 3rd degree burns.

Materials and Methods

We focus on fetal epidermal progenitor cells for 2nd degree burns as well as a combined treatment using dermal mesenchymal stromal cells (MSC) for 3rd degree burns. Adult cells are used as a control.

In vitro work on cell isolation, cell culture, cell characterization and cell spraying are performed to prepare key proof of concept studies. Further, post-culture and post-cryopreservation FACS analysis are used to determine immunogenicity, proliferative activity and extent of differentiation.

Results

• Cell isolation, cell culture and cryo-preservation methods are established for dermal and epidermal stem cell types.

• Proliferation markers like ki67 confirm feasibility for cell banking.

• Fetal and adult keratinocytes exhibit CK15 marker in later passages, showing their basal keratinocyte cell capability.

• Fetal and adult dermal cells are negative for markers CD34, CD14, CD45 and also for HLA DR and are positive for CD90, CD105, CD73 representing MSC according to the ISCT definition.

• Fetal and adult dermal cells are also capable of differentiating into chondrogenic, osteogenic and adipogenic cells using in-vitro assays.

Conclusions

•The use of epidermal adult cells and MSC offers opportunities for developing an autologous stem cell spray-Tx therapy in an “intra-operative setting” isolating and spraying keratinocytes and MSC immediately after the burn injury.

• These results allow us to advance the original concept from using autologous stem cells exclusively, to include fetal cells as a cell-banking product for clinical therapy on 2nd and 3rd degree burn injuries, potentially allowing new skin development.

1938

Precise Control of O_2 for Hypoxia Studies in Multi-well Cell Culture Plates.

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Hypoxic conditions, with the concentration of oxygen, [O_2], reduced below its normal physiological level, have been shown to significantly affect the expression of numerous genes and influence cellular behavior. In addition, to emulate native physiological conditions, it is preferable to culture cells at [O_2] = 7%, normally found in the human body, as opposed to [O_2] = 21% found in the atmosphere. However, physical limitations have made the precise control of [O_2] in cell cultures difficult. Diffusive transport of gas through the cell media is much slower than in the surrounding atmosphere, greatly increasing the equilibration time of [O_2] in the cellular environment with the incubator’s (or hypoxia chamber’s) atmosphere. Further, cell respiration may consume O_2 at a faster rate than diffusion through the media can replenish it, possibly resulting in significant differences between [O_2] in the incubator and around the cells. To overcome these problems, we have built and tested several systems to impose precise [O_2] in cell culture media in 24-well plates. The systems use a specially designed computer controlled, multi-channel gas mixer to generate mixtures of N_2 and O_2 (with 5% CO_2 in all mixtures), in which [O_2] is set anywhere between 0 and 21% with 0.1% accuracy. In the first system, six different N_2/O_2 mixtures are supplied to six rows of wells of a 24-well plate using flexible strips with micro-machined gas distribution networks and four silicone plugs in each strip (the plugs are inserted into the wells on the plate). Each plug is equipped with a tube, which is immersed in the medium and slowly bubbles gas through it, thus facilitating mixing and effective O_2 transport. In this fashion, we have the ability to study the response of cells to six different levels of [O_2] in parallel, with four separate wells for each [O_2]. We have tested this system by exposing mouse fibroblasts to hypoxic conditions and subsequently performing real-time PCR to study the effect on RNA expression of several hypoxia-activated genes. In addition, we found that without

bubbling, [O_2] around the cells rapidly drops far below [O_2] in the incubator once cell density reaches a high level, whereas with the bubbling, [O_2] around the cells closely matches the [O_2] in the incubator. We have also built two other multi-well plate systems that are equally efficient, but impose a uniform [O_2] upon all wells in the plate and are easier to use. The desired level of [O_2] in one of these systems can be effectively imposed by simply placing it into an [O_2]-controlled incubator. Our systems facilitate studies of cells under hypoxic conditions by enabling precise control of the oxygen content in the cell environment.

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Improved expansion of hematopoietic progenitors with osteogenically differentiated BM MSCs conditioned medium.

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Objective: We recently reported that UCB hematopoietic stem cells co-cultured with irradiated mesenchymal stem cells (I MSCs) without contact increased cell and progenitor expansions due in part to the rapid differentiation of the MSCs into osteoblast-like cells following pre-irradiation treatment. The objective of this study was to determine whether expansion of hematopoietic cells and megakaryocyte progenitors would be recapitulated using medium conditioned with I-MSCs or osteogenically differentiated MSCs (OST).
Materials and Methods: Serum-free media were first conditioned for 7 days with I–MSCs (14 Gy) or OST cells. CD34+-enriched cells (>70%) were cultured in the conditioned media that were supplemented also with the cytokine cocktail OMPC (SCF, TPO, FL, from day 0-4) and BS1 (TPO, SCF, IL-6 and IL-9, from day 4-6), to favour progenitor expansion and megakaryocyte differentiation, respectively. Control cultures were supplemented solely with OMPC and BS1. At day-6, flow cytometry analyses and clonogenic progenitors (myeloid and megakaryocyte) assays were conducted.
Results: OST conditioned medium (CM) increased significantly the overall cell expansion achieved by CD34+ cell cultures at day-6 by 1.6-fold vs. control ($P< 0.01$) and 1.4-fold vs. I-MSC CM ($P< 0.07$). Expansions of CD34+ (1.8-fold, $P< 0.01$), CD34+41+ (megakaryocyte, 1.3-fold, $P< 0.01$) and CD235+ (erythroblast, 2.4-fold, $P< 0.05$) cells were also increased in OST CM compared to control. No significant differences in cell culture composition were observed in I MSC CM and control. Total myeloid (CFU-C) progenitors increased their expansions 1.3-fold while they were cultured in OST CM. Highest expansions (in CFU-C) were obtained for monocyte-granulocyte (CFU-G/M/GM) and granulocyte-monocyte-megakaryocyte progenitors (CFU-GEMM) such that 1.2 and 2.0-fold, non-significantly. Although I MSC CM had no impact on CFU-C, the rise expansion for CFU-G/M/GM progenitors was remarkable vs. control at day-6 (62±16 vs.45±20, $P< 0.05$). In contrast, the expansion of CD41+ cells and megakaryocyte progenitors (CFU-MK) were not promoted with I-MSCs and OST CM.
Conclusion: The present study demonstrated that OST conditioned medium increased total, CD235+, CD34+, CD34+ 41+ cell expansions and myeloid progenitors when used with our cytokine cocktail (OMPC and BS1). Future experiments will assess whether these growth advantages provided by conditioned medium translate into beneficiary in vivo reconstitution effects.

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Effect of Centrifugation on Viability of the Ciliated Protist, *Tetrahymena pyriformis.*

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Because some labs report protocols that avoid centrifugation of suspension cultures, cell viabilities in cultures of *Tetrahymena pyriformis* were compared before and after centrifugation at different RPMs for varying amounts of time in order to determine if centrifugation resulted in lower cell viability. Trypan blue was utilized to indicate viability before and after centrifugation. There were no significant differences in viabilities before and after centrifugation when cells were centrifuged at 10K RPMs for 5 minutes (p=0.28; n=6), 10K RPMs for 10 minutes (p=0.067; n=12), 13.2K for 5 minutes (p=0.13; n=3), and 13.2K for 10 minutes (p=0.24; n=12). However, there were significant differences in before and after viabilities between cells that were 3 days old and cells that were 6 days old (p=0.003; n=6). It was concluded that, while centrifugation up to 13K RPMs for 10 minutes does not negatively affect the viability of 3-day old cultures, increasing age of the culture may result in a lower viability after centrifugation.

1941

New separation method of SDS-PAGE using inverse-gradient polyacrylamide gel.

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Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used technique to separate a mixture of proteins based on their molecular size. Separation of proteins in SDS-PAGE relies on the gel concentration of acrylamide. Thus, suitable gel concentration must be chosen to separate target proteins.

I have been working on analyzing the regulatory mechanisms of tau phosphorylation catalyzed by tau kinases by Western blot analysis using phosphorylation dependent anti-tau antibodies. Phosphorylated tau proteins were separated on an acrylamide gel with similar migration distances and it is not easy to distinguish all the isoforms of phosphorylated proteins by immunoblotting because of sharing the immunoreactivity. Concerning this difficulty, better separation has been expected.

We are proposing the use of inverse-gradient acrylamide gel SDS-PAGE for the separation of the proteins with subtle differences in molecular size. This modified SDS-PAGE technique traps high molecular size proteins at the top of the gel, which contains high concentration of acrylamide. Electrophoresis of smaller size of proteins accelerates with decreasing acrylamide concentration of the bottom of the gel.

As a result, the method provided wider separation of proteins with certain molecular sizes below certain molecular weight. We found that 15-5% inverse-gradient gel is best suited for the reparation of the proteins smaller than 100 kDa. We will also demonstrate further improved method using inverse-gradient gel and the result will be discussed.

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A Novel System Enabling High Efficiency Low Toxicity Transfection of Cells in 3D Culture.

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Three-dimensional (3D) cell culture is an emerging trend in cell biology as it permits complex multicellular organization that more closely mimics the mechanical and biochemical cues present in native tissues as compared to routinely used two-dimensional (2D) cell culture. Transfection is an advantageous technique frequently used by cell biologists in 2D cell culture

and requires further development to be effectively expanded to 3D cell culture. In this study, we demonstrate a transfection system that implements the alvetex® polystyrene scaffold and a transfection formulation that enables high efficiency transfection of cells that have been adapted and grown in 3D culture. Unlike current 3D transfection protocols which require transfection of cells in suspension prior to plating in 3D matrices, we have established a system whereby cells are effectively transfected within the scaffold. To facilitate acclimation of cells to 3D culture, critical parameters such as cell density and post-seeding adaptation time were fine-tuned to obtain optimal transfection efficiencies. Thereafter, various transfection reagent formulations were tested for efficacy in terms of reporter output, e.g. luciferase and green fluorescent protein (GFP) expression, and absence of cytotoxicity in 3D culture. Transfection performance was further improved by titrating DNA dosage and reagent-to-DNA ratios. Optimization of cell growth conditions and transfection parameters has led to the development of a novel high efficiency low toxicity transfection system specific for 3D cell culture.

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Zinc-Finger Nucleases to "Chop" (Cop3) Chlamydomonas gens.

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The unicellular green algae *Chlamydomonas reinhardtii* is a promising model for fundamental and biotechnological research. A complete toolset for genetic manipulation has been developed for *Chlamydomonas* except site-specific gene targeting. By now we have developed a nuclear gene targeting system for *Chlamydomonas* which is based on the application of Zinc-Finger Nucleases (ZFNs) that function as artificial site-specific enzymes. ZFNs consist typically of 3 N-terminal ZF-modules each recognizing a DNA triplet. The C-terminal FokI domain works as a dimer and is able to induce DNA double strand breaks (DSB). Thus, two functional ZFN molecules must be created, both recognizing 9bp within the target sequence and separated by a small spacer (~6bp). DSBs stimulate homologous recombination that can be utilized to induce changes in the target sequence. However, engineering ZFNs for target sites that differ from the natural targets often results in low specificity and inefficient nuclease activity. The conclusion is that some target sites are better then others. Using available online tools the *Chlamydomonas* Channelrhodopsin1 (Cop3) genome sequence was searched for potentially good binding sites of available and evaluated ZF-modules. Although progress has been made in predicting the specificity of the assembled domains, *in vivo* testing is indispensable since unwanted off-target activity may result in cytotoxicity. Therefore we have constructed an easy-applicable model system for testing the sequence recognition of assembled ZF-modules in *Chlamydomonas*. We were able to optimize our system resulting in the successful generation of Cop3-specific Zinc-Finger Nucleases that targeted and finally deleted the non-selectable Channelrhodopsin1 gene in *C. reinhardtii*.

1944

Stabilization of Acetylcholinesterase Folding by Non-Catalytic Subunit N-Terminal Peptides Prevents Its ER Associated Degradation in Skeletal Muscle.

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Acetylcholinesterase (AChE) terminates neurotransmission at cholinergic synapses in the peripheral and central nervous systems. The synaptic, or junctional, AChE in skeletal muscle derives from two separate gene products encoding the catalytic and the non-catalytic collagenic tail (ColQ) subunits, while a secreted tetrameric form is assembled from catalytic subunits either alone or with an N-terminal fragment of the non-catalytic subunit. However the vast majority of

newly synthesized AChE molecules do not assemble into catalytically active forms and are rapidly degraded by the endoplasmic reticulum associated protein degradation system. We have previously shown that AChE in skeletal muscle is regulated in part posttranslationally by the availability of ColQ subunits, and other laboratories have shown that the 17 amino acid N-terminal proline rich attachment domain (PRAD) of ColQ is sufficient to promote tetramerization of newly-synthesized AChE. Here we show that primary muscle cells or HEK cell lines expressing AChE catalytic subunits exposed to synthetic PRAD peptides containing an endoplasmic reticulum KDEL retrieval sequence take up and retrogradely transport them to the ER where they induce oligomerization and stabilization of AChE tetramers. The peptides rescue the newly-synthesized AChE molecules from ER degradation by enhancing AChE folding thereby stabilizing the tetrameric complex. This enhanced folding and stabilization of newly-synthesized AChE occurs in the presence of protein synthesis inhibitors indicating that it is post-translational. This in turn results in increased total cell associated AChE activity and increased secretion of active tetramers. These observations are supported by isotopically-labeled methionine pulse chase experiments showing that the newly synthesized AChE molecules are rescued from intracellular degradation. These studies provide a mechanistic explanation for the large intracellular pool of inactive AChE and intracellular degradation of 80% of AChE and suggest that simple peptides alone can increase the production and secretion of this critical synaptic enzyme in muscle tissue. Moreover, they suggest a novel therapeutic approach to treat organophosphate poisoning. These studies were supported by grants from the NIH to RLR and a Ruth Kirschstein National Research Service Award to CAR.

1945

Plasticity of the Asialoglycoprotein Receptor deciphered by ensemble FRET and Single-Molecule Counting PALM.

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The composition of many multi-subunited receptor complexes at the plasma membrane is still debated as well as the impact of changes in the receptor subunit composition. Receptor plasticity involving changes in stoichiometry and receptor function has been difficult to address. Here, we introduce new spectroscopic tools to dissect receptor subunit assembly of the Asialoglycoprotein Receptor (ASGPR). Using this highly tractable receptor model system we show that ASGPR can assemble into distinct oligomers at the plasma membrane and that differential subunit assembly dictates receptor specificity. With ensemble analyses of Fluorescence Resonance Energy Transfer (FRET) and analytical modeling, we quantified receptor subunit homo- and hetero-oligomerization in the living cell. Furthermore, we established single-molecule counting using Photoactivated Localization Microscopy (PALM), visualizing an asymmetric receptor subunit assembly on the single-molecule level. Our results define a probability hierarchy of ASGPR oligomerization driven by different molecular motifs that entails distinct co-existent receptor subunit assemblies. The Asialoglycoprotein Receptor is involved in the clearance of thrombogenic material [1,2]. The variety of potential ligands and the propensity of ASGPR subunits to form distinct oligomers may explain previous inconsistent results and underscore the importance of deciphering oligomerization in the single cell and on the single-molecule level.

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1946

A specific,fast and gentle method of capturing cells expressing a protein of interest or exhibiting a specific promoter activity.

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Existing cell capture technologies rely mainly on endogenous or exogenous protein markers that are unique to the cell type of interest. Specific T-cell populations, for example, have been isolated away from other blood cells in this manner using magnetic bead-based methods. Fluorescence activated cell sorting (FACS) is another widely used approach, however the harsh and unsterile sorting procedure itself is problematic for subsequent continuous culturing steps. However, capturing a sub-population of cells, distinguished from surrounding cells by the expression of a specific exogenous protein of interest or by exhibiting a specific promoter activity has not been addressed.
A novel cell capture system has been developed that addresses these limitations and allows for isolating homogeneous cell populations characterized by a specific promoter activity, or expressed protein. The CherryPicker™ system utilizes a chimeric membrane-anchored fluorescent protein with the dual purpose of providing both the monitoring and capturing moieties. Its red fluorescence can be monitored either via fluorescence microscopy or fluorometry, while the magnetic beads capture itself occurs via an antibody recognizing the chimeric protein. Importantly, the magnetic capture beads used in this system are unique in their remarkably low rate of non-specific binding.
The strength of this system is in its ability to isolate a homogeneous cell population. Of the entire population of transfected cells, only those with the desired gene or promoter activity express the chimeric membrane-anchored fluorescent protein, and are therefore prone to capture. This is achieved by expressing the chimera either with the protein of interest from a bicistronic mRNA, or its expression is driven by activation of a upstream promoter of interest. Since the capture method preserves cell integrity, the homogeneous cells can be re-cultured or further analyzed for marker identification purposes.
Here we present the distinct features of this magnetic bead-based cell capture system and how it can be used for re-culture purposes of captured cells. We show two studies where the methodology has been used to enrich for specific cells – the first for cells with an active NFkB response element, and the second, for cells expressing a constitutively active, cytosolic kinase mutant.

1947

Use of Fully Automated Cell Culture and Imaging Systems to Increase the Capacity and Consistency of Downstream Virology Assays.

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The Cell Culture Laboratory at the NIAID Integrated Research facility is a newly designed core facility that is centralizing the production of tissue culture cells for translational virology research. The Cell Culture Lab supplies mammalian cells in flasks and plates to laboratories operating under standard Biosafety Level 2 (BSL-2) and high containment (BSL-3/4) conditions. In order to provide quality-controlled cell cultures to meet the needs of the Clinical Core Lab and molecular virologists, we are incorporating a tissue culture robot and automated imaging equipment into our workflow. The CompacT SelecT™ robot presently maintains multiple cells lines (e.g. VeroE6, BS-C-1, 293T) in T175 flasks in a controlled environment. The robot counts and checks cell viability before dispensing the cells into multi-well plates ready for downstream assays (e.g., plaque assays). Robotic plating is undergoing optimization by varying the viable

cell density, dispense volume and pattern, and incubation time. With such optimization, cell plating is completed in one quarter of the time required for manual plating. We are able to supply 2800 wells per week in confluent 6- or 24-well plates for downstream plaque assays. Comparative data from cowpox and simian hemorrhagic fever virus plaque assay show that the titers are analogous between cells plated by the robot and those plated manually. In addition, an Incucyte™ live-cell imager records cell growth properties and cell plating quality of selected plates to ensure consistency for multiple experiments. An in-house online system is under development that manages cell orders and generates valuable data about tropism of specific viruses for various cell lines. Automation of tissue culture provides both high quality cells and cost savings because of the centralization of tissue culture reagents and staff expertise.

1948

Detection of Mono-and Polyubiquitylated Proteins with Novel Ubiquitin Monoclonal Antibody.

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Work in the ubiquitin field is hampered by the lack of selective antibodies that recognize mono- and poly-ubiquitylated proteins. Many commercially available antibodies exhibit variable degrees of sensitivity and specificity against ubiquitin species in cell and tissue samples. Several ubiquitin antibodies available on the market detect predominantly polyubiquitin conjugates and have a low affinity to unconjugated ubiquitin limiting their usage in multiple applications. We have developed a panel of mouse monoclonal antibodies that recognize different configurations of ubiquitylated proteins. Here, we describe VU-1, a highly sensitive reagent for the identification of ubiquitin conjugates and free ubiquitin. The antibody was developed and tested in several applications in biochemistry and immunohistochemistry and can be used to address a wide spectrum of questions related to ubiquitin signaling. Here we demonstrate that VU-1 is a highly sensitive reagent for the identification of proteins with different degrees of ubiquitylation in cell and tissue lysates compared to other commercially available antibodies. Using tissue sections and different cell types in culture, we show that this antibody is a superior reagent for immunohistochemistry using both immunofluorescence and chromogenic substrates making it an indispensible tool for the Ubiquitome research.

1949

Improvement of Absorbance Measurements of Tetrahymena pyriformis Using the Vital Stain Acridine Orange/Ethidium Bromide.

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Numerous published studies report absorbance values of unstained *Tetrahymena pyriformis* analyzed by a Spec20 spectrophotometer. Previous attempts by this lab to determine absorbances of unstained cultures from a microplate reader had yielded very low values. So, the goal of these experiments was to determine if the vital stain acridine orange/ethidium bromide improved absorbance readings from a microplate reader for *Tetrahymena pyriformis* cells in culture. Both stained and unstained samples of *T. pyriformis* were analyzed with a microplate reader using filters of differing wavelengths ranging from 340 nm to 655 nm. The highest absorbance from stained cells was achieved using a 495nm filter (mean OD = 0.160, n=18). The highest absorbances for unstained cells were obtained using filters at 340nm and 540nm wavelengths, each achieving a mean OD of 0.080 (n=6). The average absorbances for

stained and unstained cells at 90,000 cells/mL densities were significantly different (p=0.0238). Finally, cell cultures of increasing densities (20,000 to 100,000 cells/mL) were read by the microplate reader at 495nm, yielding a linear relationship (y = 0.0218x – 0.0152; r^2=0.0971, n=3). In conclusion, the microplate reader may be a useful method for estimating cell population counts of *Tetrahymena pyriformis* when the vital stain acridine orange/ethidium bromide is used.

Biophysical Approaches to Cell Biology

1950

Subcellular distributions of traction forces in HUVECs under uniaxial stretch.

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Cellular components maintain physical balances which are collapsed with applied external forces. This collapse of the physical balances induces new physical balances and mechanotransduction. In order to understand how the collapse of physical balances occur in cells under uniaxial stretch, subcellular distributions of traction forces were measured in HUVECs before and after uniaxial stretch. Furthermore, in order to understand involvement of actin stress fiber in the collapse of physical balances, GFP-actin was expressed in the HUVECs. As a result, 12% uniaxial stretch decreased traction force at edge regions in single HUVECs. In contrast, the stretch increased traction force at internal regions in single HUVECs. The decrease of traction force correlated with ablation of actin stress fibers. Thus, it was suggested that ablation of actin stress fiber largely contribute to collapse of physical balances in HUVECs under 12% uniaxial stretch.

1951

Designed 3D polymer scaffolds to study the migration of cancer cells with altered nuclear mechanics.

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During metastasis cancer cells migrate out of the primary tumor to form sister tumors in distant organs. This cancer cell migration and invasion is not only determined by biochemical but also by physical cues of the environment. In addition, the mechanics of the cell nucleus has an impact on cell motility. Nucleus stiffness is mainly determined by the intermediate filament proteins lamin A/C.

Here, we investigate the migration and invasion behavior of cancer cells using a novel 3D cell culture system. Woodpile-like 3D scaffolds with different mesh sizes (5-15 µm) are fabricated by direct laser writing (DLW) into biocompatible photoresists. The scaffolds are manufactured by scanning a photoresist relative to the focus of a femtosecond-laser beam. Thus, DLW can be thought of as a pencil of light in three dimensions. The resulting 3D scaffolds are geometrically well-defined, have a physiologically relevant stiffness, and are surface-functionalized with ECM proteins.

The human A549 cancer cell line is used to establish a siRNA-mediated lamin A/C knockdown (KD). KD cells have significantly softer cell nuclei compared to control cells as quantified by atomic force microscopy. Furthermore, morphology of the nucleus and the actin cytoskeleton is changed for KD cells compared to control cells on 2D substrates. Wound healing assays show that cell migration rate is reduced and cell polarization is disturbed in KD cells. Concerning 3D

woodpile scaffolds, we observe morphological differences of the nucleus and the cell body in both KD and control cells. With decreasing mesh size the nucleus is divided by the 3D scaffolds into separate small but still connected compartments and cells form more protrusions. We also observe a reduced proliferation rate of KD cells on the 3D substrates compared to control cells and 2D substrates. We are currently optimizing our 3D substrates to investigate cell motility by live cell imaging.
In summary, we describe a novel 3D cell culture system that allows for the precise control over physical environmental properties. This system will be supportive for the basic understanding of cell migration and nuclear mechanics in general and in cancer metastasis.

1952

A novel culture system provides high yields of non-fibrogenic primary dermal fibroblasts for grafting applications.

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Background: The expansion of primary autologous dermal fibroblasts in culture is a pivotal step to obtain sufficiently high numbers for applications where large surface area tissue destructions, e.g., occurring in burn/trauma injuries, exceed the body's repair capacity and demand grafting of regenerative cells. Stimulated by the stiff surface of conventional culture plastic, a substantial percentage of fibroblasts spontaneously differentiate into fibrotic myofibroblasts by producing excessive amounts of collagen, *de novo* expressing α-smooth muscle actin (α-SMA), and consequently developing high contractile force. Myofibroblasts cause pathological tissue contractures characteristic of hypertrophic scars and fibrosis. Hence, suppressing this phenotype during cell culture expansion is crucial for the success of subsequent grafting. We hypothesize that culture on highly elastic surfaces will deliver better quality fibroblasts by inhibiting myofibroblast development. **Objective:** To investigate the intracellular signalling mechanisms that control the transition from beneficial reparative cells to harmful fibrogenic cells under different mechanical conditions. **Method:** To rapidly produce high numbers of fibroblasts for cell therapy we implement a novel method combining dynamic enlargement of the culture surface with fibroblast growth on a highly compliant, and extendable, silicone rubber (HESR). **Results:** 1) Attachment and proliferation of human dermal fibroblasts on functionalized HESR is similar to conventional tissue culture plastic. 2) Repeated passaging on plastic augments the percentage of fibrogenic myofibroblasts. 3) Primary fibroblasts explanted and cultured on static and dynamically expanded HESR begin to express α-SMA at later passage numbers and always at lower levels when compared to plastic culture. 4) HESR fibroblast cultures show differential gene expression of putative markers for fibrosis. 5) The pro-fibrotic cytokine TGF-β1 fails to activate myofibroblast differentiation on HESR. 6) HESR polymer rheology and not polymer chemistry is responsible for the mechanically-induced gene expression changes. 7) Single stretch events of 1-10% do not induce α-SMA on HESR, and 8) HESR dynamic expansion culture generates a multi-fold increase in daily cell yield while retaining the anti-fibrotic properties of static HESR. **Conclusion:** The low elastic modulus of the HESR membrane provides mechanical cues to primary dermal fibroblasts which are anti-fibrotic while dynamic expansion culture serves to maintain a cell density optimal for proliferation. **Support:** This research is supported by the Collaborative Health Research Program CIHR/NSERC (CHRP).

1953

Patterning Cardiac Myocyte Micro-tissues for Force Analysis on Soft Poly(dimethylsiloxane) (PDMS)-Substrates Using a Lift-off Membrane.

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Microcontact printing (µCP) has become a valuable tool in the field of biology, creating surfaces with defined patterns of complex molecules helping to control cell shape in culture. However, µCP in cell biology is normally performed on rigid substrates, e. g. Petri dishes, glass, or gold surfaces using elastomeric stamps with patterns of interest transferring biomolecules to the substrates. Despite the fact that those substrates can easily be patterned by µCP they display an artificial environment for cells effecting the cell´s morphology and behavior. In addition to that disadvantage cell force analysis, e. g. on cardiac myocytes, is impossible to perform. Using tunable elastomeric substrates, mimicking the cells natural environment reveals a more natural morphology and behavior of the cell and provides the opportunity of cell force analysis. Nevertheless, µCP on soft elastomeric substrates (cross-linked silicone rubber (PDMS)) is very difficult to perform.
Here we describe the patterning of extracellular matrix (ECM) proteins and cardiac myocytes onto soft cross-linked PDMS substrates with a Young´s modulus ranging from 15- 75 kPa using a newly established lift-off membrane. The membrane features two different geometric patterning designs of squares and rectangular holes with diverse width-to-height ratios varying from 50 µm to 100 µm for the square holes and from 15 µm x 200 µm to 50 µm x 200 µm for rectangular holes. Those geometrical shaped patterns create small defined myocyte micro-tissues for the cell force analysis

1954

Conduction Slowing with Stretch in Micropatterned Cardiomyocytes.

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An optical mapping system has been developed for study of electrical conduction during biaxial stretch in cultured cardiomyocytes.

The health and disease effects of mechano-electric feedback during cardiac loading are incompletely understood. Characterizing the several conflicting mechanisms by which tissue stretch may modulate conduction velocity and arrhythmogenicity is difficult in an in situ model, where changes in tissue geometry during the cardiac cycle may confound local measurements. In fact, published reports of the nature of the effect of stretch on conduction in the whole heart disagree.1 Optical mapping techniques for imaging cardiac electrophysiology have been adapted for assessment of conduction in a two-dimensional cell culture.2 This method utilizes high temporal and spatial resolution imaging of a transmembrane voltage-sensitive fluorescent dye and avoids the electrical disturbances of electrode measurements. This system also permits application of physiological anisotropic biaxial strain.

Neonatal mouse and rat ventricular cardiomyocytes are cultured on anisotropically patterned silicone elastomer substrates.3 Grooves are created in the PDMS using a micropatterned silicon wafer mold, ensuring physiological rod-like morphology and permitting electrical coupling with axial and lateral neighbors. Custom stretch devices apply homogeneous anisotropic biaxial strain across the substrate, such that the direction of the primary axis of strain may be varied relative to the axes of the aligned cardiomyocytes. Optical imaging of excitation is accomplished

using voltage-sensitive di-8-ANEPPS dye and a CMOS camera. Conduction velocity and duration of the action potential may be quantified.

Patterned cultures of mouse cardiomyocytes exhibit arrhythmogenic conduction velocity slowing, where the magnitude of slowing increases with magnitude of strain (directed either parallel or perpendicular to cell longitudinal axis), with little effect observed for stretches of 5% or smaller. Physiological strain on the order of 10% in the major stretch axis results in a reversible conduction velocity slowing on the order of 20%, comparable with in situ results.4

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1955

Motor-driven force fluctuations in suspended cells and in cytoskeletal model systems.

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Mechanical processes, such as cell division and growth or cell locomotion, are essential in cell life and are driven and controlled by the cytoskeleton. The polymeric components of the cytoskeleton are semiflexible polymers. The activity of motor proteins creates directed forces as well as random force fluctuations.

We have studied such force fluctuations and, at the same time, mechanical properties of cells and of in vitro model systems with microrheology techniques. We have used micron-sized probe particles, embedded in the medium to be studied or attached externally to cells, and laser optical traps to confine the particles, combined with laser interferometry to detect either their spontaneous fluctuations or the particles' response to a driving force with sub-nm accuracy and bandwidths up to 100 kHz. We have applied this technology to cytoskeletal model systems consisting of crosslinked actin and myosin 2 motors as well as to osteocytes and fibroblasts. We have universally found an intriguing spectral characteristic of the motor-generated fluctuations, resembling deceptively that of Brownian motion in a viscous fluid. We show that a simple model of slow build-up and sudden release of motor-generated tension in the elastic networks can explain this observation.

1956

Using Oscillating Magnetic Fields and Magnetic Nanoparticles To Improve Transfection Efficiency and Protein Expression.

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OBJECTIVES: To evaluate the effectiveness of nanomangetic transfection combined with oscillating magnet arrays to improve gene uptake and protein expression.
METHODS: Magnetic nanoparticles complexed with either plasmid DNA or short interfering RNA (siRNA) were introduced to cells in culture and exposed to a high-field/high-gradient oscillating magnet array. For comparison, transfections were conducted on cells exposed to a static magnetic field, electroporation and cationic lipid transfection agents. Transfection efficiencies and protein yields were determined using fluorescence microscopy, flow cytometry or lucifierase assay.
RESULTS: Transfection efficiencies of 20-40% for primary hippocampal neurons and Mesenchymal Stem cells (MSCs) were achieved with GFP-nanoparticle complexes using the oscillating magnetic array, with >80% cell viability. Using a large (11kb) neuron-specific plasmid, mature neurons (>14 days *in vitro*, DIV) were successfully transfected with high cell viability. Delivery of siRNA against actin in the presence of an oscillating magnetic field was highly effective, with 75% knockdown observed in HeLa. Finally, Chinese Hamster Ovary (CHO) cells transfected with a Luciferase reporter plasmid demonstrated a six-fold increase in protein expression levels compared with conventional methods.
CONCLUSIONS: The use of oscillating magnetic fields in nanomagnetic transfection is very effective compared to other leading non-viral methods, even in hard-to-transfect cell types. High cell viabilities were maintained in all cases. This application exhibited the ability to 1) introduce large plasmids into mature primary hippocampal neurons; 2) improve protein production, and 3) silence an endogenous gene in an important cell line. The use of oscillating magnetic fields coupled with nucleic acid complexed with magnetic nanoparticles is a simple yet effective technique in gene delivery which will be an invaluable tool for cell biology research.

1957

Oxygen microscopy and high-throughput analysis of icO_2 by means of cell-penetrating phosphorescent dyes.

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The molecular oxygen (O_2) is one of the key parameters in analysis of mitochondrial function and cellular metabolism. In recent years new approaches based on the use of cell-penetrating phosphorescent O_2 sensitive probes were introduced. Among these, protein-, peptide- or nanoparticle-based probes show the potential for analysis of extra-, peri- and intracellular O_2 (icO_2).
Here, we report the development and evaluation of a number of cell-permeable intracellular phosphorescent O_2 probes which allow measurement of icO_2 in live cells: 1) the probes consisting of cell-penetrating peptide moiety (Arg- or Pro-rich) conjugated with PtCP dye which demonstrate various patterns of subcellular localization ranging from endosomal compartments to whole cell distribution and 2) PtTFPP dye embedded into positively charged nanoparticles which has increased brightness and photostability advantageous for microscopy imaging applications. The O_2-dependent luminescence of these probes can be measured on different

platforms such as TR-F microplate readers and fluorescence microscope (in intensity-based or FLIM-based modes).
Both classes of O_2 probes were tested on various cell lines in order to evaluate kinetics of cell staining, cell toxicity and possible cell-specificity specificity. A number of 'model' experiments have been performed including stimulation of mitochondrial respiration in adherent cells under different levels of external hypoxia. The cells produced the anticipated respiratory responses, with changes in probe signal and local (intracellular) O_2 concentrations reflecting dynamics of cellular respiration.
The usefulness of these new cell-permeable probes was demonstrated in physiological study of uncoupling effect of bafilomycin A1 (Baf) and ex vivo imaging applications. Altogether, the phosphorescence based cell penetrating O_2 probes represent a simple and efficient tool for analysis of icO_2 and cell bioenergetics.
Supported by Science Foundation of Ireland (SFI).

1958

BRET reports the subcellular localization and topology of membrane proteins with high spatial and temporal resolution.

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Many cellular functions require membrane proteins to be localized in a particular subcellular compartment or to move between compartments. Therefore, it is frequently necessary to determine the subcellular location of a membrane protein, or to track the movement of a protein between organelles. Here we demonstrate that bioluminescence resonance energy transfer (BRET) can report the subcellular location of membrane proteins in populations of live cells with high spatial and temporal resolution. In a typical experiment a membrane protein of interest is fused to a BRET donor (*Renilla* luciferase; Rluc8), and a membrane-bound compartment is labeled with a small targeting peptide fused to a BRET acceptor (venus fluorescent protein). A BRET signal is produced only if the membrane protein of interest is located in the acceptor-labeled compartment. For example, the temperature-sensitive vesicular stomatitis virus G protein tsO45 mutant fused to Rluc8 (VSVG-Rluc8) is retained in the endoplasmic reticulum (ER) at 40°C, and at this temperature produced BRET only with acceptors localized in the ER. After shifting to 32°C BRET between VSVG-Rluc8 and ER markers decreased, whereas BRET between VSVG-Rluc8 and markers of the golgi apparatus and plasma membrane increased. Similar results were observed when forward trafficking of misfolded G protein coupled receptors (GPCRs) was induced by pharmacological chaperones. BRET readily detected differences in the steady-state localization of the small G proteins H-Ras and K-Ras, and changes in H-Ras localization due to block of palmitoylation. BRET occured only when donor and acceptor moieties were located on the same side of a membrane bilayer, thus the method reported membrane topology, and resolved inner and outer mitochondrial membranes. The temporal resolution of BRET was sufficient to monitor translocation events that occurred in less than one second. The method yields semi-quantitative measures of membrane abundance without extensive data processing or user intervention, and can be scaled to medium-throughput with a simple plate reader. BRET is thus a versatile alternative to fluorescence microscopy and other methods to determine the subcellular location of membrane proteins. *Supported by NIH grants GM078317 and GM096762.*

1960

Use of a cell based screening device to decipher complex cell-microenvironment interactions.

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Mammalian cells exist in unique micro-environments in vivo that affect their behavior (proliferation, differentiation, death, etc.) and function. When cells are cultured in vitro, it is necessary to recapitulate such microenvironment for physiologically relevant growth. Among many factors affecting cell behavior in vivo, extra cellular matrices (ECMs) play a significant role in determining cellular functions. Currently, finding the optimal ECM for cell culture is a trial and error process requiring large numbers of cells as well as a significant time and cost commitment.
MicroStem has developed a cell screening technology that deciphers and leverages optimal ECM combinations as a method for immobilizing live cells in a physiologically relevant state for high content immuno-fluorescent study of cellular function and behavior. The technology is especially powerful when studying rare cell populations like stem cells and human primary cells extracted during surgery. MicroStem has used this technology to study human primary cells such as cancer stem cells and human mesenchymal stem cells isolated from bone marrow. In each case, use of the technology led to a determination of an optimal ECM for which respective cells would attach and maintain proper cell function in vitro. This is the first technology of its kind that allows researchers to immediately study rare physiologically relevant cell behavior in vitro with out substantial scaling of cell populations.

1961

Optimal growth of cells in 3D culture relies on the appropriate shape and form of the supporting scaffold.

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Cultured mammalian cells are important tools for providing predictions of drug activity, basic cell function, disease modeling, and toxicity in vivo. However, conventional cell culture environments are far removed from real-life tissues. In vivo, cells grown naturally in three dimensions (3D) and are supported by a complex extracellular matrix which facilitates cell-cell communication via direct contact and through the secretion of trophic factors. In contrast, cells grown in two dimensional (2D) culture are generally confined in flat monolayers. It is widely recognized that culturing cells in 3D radically enhances cell growth, differentiation and function. Authentic 3D cell cultures provide greater insight to how cells behave in the body in response to external challenges than is currently possible with existing 2D culture technologies. However, there is currently platform no technology that supports genuine 3D cell culture for routine use alongside conventional 2D cell culture methods.
Our research has resulted in the development of alvetex, a unique highly porous polystyrene scaffold that supports genuine 3D cell culture. Alvetex is developed for routine use following straightforward protocols and using conventional laboratory equipment and practices. Analysis of the resulting cultures is compatible with a broad range of standard molecular and cellular assays.
The shape and form of alvetex is an essential aspect to enable its optimal use and therefore much attention has been directed to the presentation of the scaffold and its application for 3D cell culture. For example, alvetex is engineered into a 200 micron thick membrane to enable cells to penetrate the material throughout and receive adequate nutrient and oxygen exchange. Different formats of the technology are presented for alternative uses, including a well insert for feeding cells from above and below the scaffold, and the option to include a large medium reservoir to support long term 3D cell cultures.

The 3D growth of many different cell types has been tested using alvetex technology. Cell types that are commonly used in industry such as CHO, HepG2, 3T3 fibroblasts, grow readily on alvetex. In addition, more extensive research has led to the development of customized 3D assays for liver toxicity and the development of skin equivalents. We will present evidence showing that cells grown in alvetex display enhanced cell viability, differentiation and function when compared to conventional 2D culture models.

1962

Quantifying the persistence of cell shape over time and through mitosis.

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A major unanswered question in cell biology is what mechanisms determine the shape of cells. Because cell shape results from an interplay of dynamic processes, it is best viewed as a dynamical system, with biochemical regulatory pathways acting to "steer" the trajectory of the system towards an evolutionarily advantageous shape. In order to understand cell shape from a dynamical systems perspective, it is essential to know the time scale over which cell shape can persist. Here, we have addressed this question in two ways. First, we consider whether cell shape can be transmitted during mitosis. By comparing sister cells immediately after division, we ask whether sisters show increased similarity compared to non-sisters. We compared several different shape comparison metrics, including a chamfer matching based approach, an approach based on the Procrustes algorithm, and a non-rigid shape matching method. We found that for a set of data taken with fixed RPE1 cells, all methods showed strong evidence for similarity, whereas for live cell imaging of 3T3 cells, the first two methods did not show any evidence of similarity between sisters, while the non-rigid shape matching method did show a statistical bias for sisters to be more similar than non-sisters. We also tested whether there was a bias for or against mirror symmetry and found that the similarity between sisters did not have any bias for either type of symmetry for the live 3T3 cells, while for the fixed RPE1 cells there was a clear bias in favor of mirror symmetry. We also examined the shapes of mother cells immediately before division, and found that they tend to be bilaterally symmetric, which suggests a possible origin for a mirror symmetry bias in daughters, at least in some cell types. Finally, we directly analyzed the time scale of cell shape persistence using a principal components analysis in which we could reduce cell shape variation to a small number of modes, and then measure the relaxation time of those modes.

Prokaryotic Cell Biology

1963

A small molecule stabilizer of FtsZ inhibits cell division in Gram-negative bacteria.

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In bacteria, cytoskeletal proteins play a role in various processes including cell division and cell wall remodeling. FtsZ is a homolog of eukaryotic tubulin that polymerizes in the presence of GTP. In vivo, FtsZ polymers localize into a ring-like structure at the mid-cell to initiate cell division. The FtsZ ring provides a constricting force, and recruits other division proteins. The importance of chemical tools for studying eukaryotic cytoskeletons has fueled efforts to discover small molecule inhibitors of FtsZ. However, very few compounds have been demonstrated to target FtsZ specifically, and the best-characterized compound (PC190723) is effective only in Gram-positive organisms. Using a high-throughput in vitro screen, we identified a new FtsZ inhibitor and named it Divin (*DIV*ision *IN*hibitor). We found that the treatment with Divin caused incomplete constrictions in dividing cells of Gram-negative bacteria, including *Caulobacter crescentus* and *Escherichia coli*. The Divin-induced phenotype was similar to the morphology of FtsZ-overexpression strains, which led us to hypothesize that Divin stabilizes the division machinery in vivo. We tested this hypothesis by treating MipZ-overexpression and Δ*zapA* strains with Divin: MipZ and ZapA are antagonists and agonists of FtsZ polymerization, respectively. The presence of Divin suppressed the antagonistic effect of excess MipZ, and rescued the agonist-deficient cells. These results indicate that Divin inhibits cell division by stabilizing FtsZ. To measure a direct interaction between Divin and FtsZ, we performed a competitive binding assay using a fluorescent GTP analog. The apparent dissociation constant was 7 ± 6 μM for the Divin-FtsZ complex. In summary, we report a novel inhibitor, Divin, whose function is analogous to the stabilizing effect of Taxol on microtubules. We envision that this small molecule will be a valuable chemical tool for studying the function and structure of FtsZ, and can be potentially developed as a therapeutic agent against bacterial infections.

1964

In vivo time-lapse imaging reveals the mechanism of DNA segregation by the bacterial actin-like protein AlfA.

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AlfA is an actin-like protein encoded by a plasmid from Bacillus subtilis. It forms filaments and promotes plasmid partitioning during vegetative growth and sporulation. The in vitro dynamics of AlfA filament assembly (lack of dynamic instability and anti-parallel bundling) are incompatible with proposed models of plasmid segregation driven by the actin-like protein ParM. To understand the mechanism of AlfA-dependent DNA segregation, we imaged AlfA polymers and plasmids by time-lapse fluorescence microscopy and used particle tracking to characterize DNA motions. We find that AlfA promotes two distinct plasmid motions, depending on cell growth conditions. In exponentially growing cells taken from liquid media, the alf operon positions plasmids at regular intervals along the long axis of the cell and reduces plasmid mobility. In

these cells, AlfA forms small, dynamic foci closely associated with plasmids. In cells taken from colonies grown on solid media, however, AlfA forms long-lived bundles composed of many short, dynamic filaments that promote plasmid movement. We postulate that the two modes enable the alf system to position plasmids equally between daughter cells during binary fission and increase the frequency of delivery to cell poles during sporulation. In contrast to the dynamic behavior of AlfA filaments observed in vivo, FRAP experiments performed in vitro reveal that purified AlfA filaments are much more stable. Our results both suggest a mechanism for DNA segregation AND indicate that unknown factors, provided by either the plasmid or the host cell, promote filament turnover.

1965

Structures of the RNA-guided surveillance complex from a bacterial immune system.

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Prokaryotes acquire resistance to viruses and plasmids by integrating short fragments of foreign DNA into clustered regularly interspaced short palindromic repeats (CRISPRs). The long CRISPR transcript is processed into a library of short CRISPR-derived RNAs (crRNAs) that each contain a unique sequence complementary to a foreign nucleic-acid challenger. In E. coli, crRNAs are incorporated into a multisubunit surveillance complex called Cascade, which is required for protection against bacteriophages. We used cryo-electron microscopy to determine the subnanometer structures of Cascade before and after binding to a target sequence. These structures reveal an architecture in which the crRNA is displayed along a helical arrangement of protein subunits that protect the crRNA from degradation, while maintaining its availability for base pairing. Cascade engages invading nucleic acids through high-affinity base-pairing interactions near the 5′ end of the crRNA. Base pairing extends along the crRNA, resulting in a series of short helical segments that trigger a concerted conformational change. This conformational rearrangement may serve as a signal that recruits a trans-acting nuclease (Cas3) for destruction of invading nucleic-acid sequences.

1966

Swarmer cells of Proteus mirabilis migrate faster than swimmer cells in viscous environments.

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It has been repeatedly shown that slight increases in viscosity actually lead to increases in the swimming speeds of a wide variety of organisms, which is interesting given that many bacteria, especially pathogenic bacteria, commonly exist in viscous environments such as the mucosal lining of the gut. One such bacterium is *Proteus mirabilis*, an opportunistic human pathogen that can migrate through the urinary tract. It has been suggested that swarming, a type of surface motility that allows rapid migration, is involved in urinary tract colonization. During swarming, cells of *P. mirabilis* undergo a morphological change from ~5 μm long rods with 5-15 flagella to ~10-50 μm long rods with 50 to more than 100 flagella. Prior work has shown that increasing the number of flagella on a cell does not yield the expected increase in torque – i.e., a four-fold increase in the number of flagella yields less than a two-fold increase in torque. This result suggests that the dense flagellation of swarmer cells should not convey a motility advantage. However, mutant cells of *Bacillus pseudiformis* that express an increased number of flagella do swim faster than their wild-type counterparts in viscous solutions, and it has been proposed that

this increase in speed is due to altered flagellar bundling. We are examining whether this phenomenon also occurs with the different morphological forms of *P. mirabilis*. We harvest homogenous populations of four different cell morphologies: (1) long and hyperflagellated [swarmer], (2) short and normally flagellated [vegetative], (3) short and hyperflagellated [consolidated], and (4) long and normally flagellated [elongated vegetative]. We then use microscopy and MATLAB-based cell-tracking software to measure the swimming velocity of each of these different cell types in fluids containing different amounts of polyvinylpyrrolidone, a polymer that increases the viscosity of the solution. We have found that swarmer cells of wild-type *P. mirabilis* are significantly faster ($P<0.001$) than all other cell types in media with a viscosity of 9 - 1260 cP. In media with a viscosity of 8340 cP, swarmer cells are motile while all other cell types are amotile. These results suggest a possible role for the swarm cell morphology: allowing migration through viscous mucosal layers during infection. Furthermore, vegetative/swarmer cells of a strain that over-expresses FlhDC (a transcription factor responsible for an increase in flagellar synthesis) are not significantly faster than wild-type cells with the corresponding morphology, suggesting that an increase in flagellar density alone is insufficient to produce an increase in swimming velocity.

1967

Phylogenetically-Based Classification of Newly Sequenced Haloarchaeal Rhodopsins.

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We present a broad phylogenetic analysis of 128 haloarchaeal opsins proteins encoded in the genomes of 65 newly and 14 previously sequenced species of halophilic archaea. Distinct branching within the bacteriorhodopsin (bR) and sensory rhodopsins (sR) clades suggests that greater functional segregation may be present within these two classes than has previously been appreciated. The sRs, for instance, cluster to form sub-clades beyond those of the well-known sR I and sR II. This finding lends additional support to a recent hypothesis, derived from biochemical characterizations of *Haloarcula marismortui's xop2*, which suggests a separate sR III functional group may exist. Interestingly, the sR III group is populated by at least three other sequences aside from *xop2,* making it nearly as well represented as the two previously known sR groups. Like sR, bR also diverges into multiple subgroups. We present spectroscopic data showing that the visible absorbance maximum of a secondary bR in *H.marismortui* is shifted ~20nm left of *Halobacterium salinarum's.* Mapping clade-specific amino acid substitutions onto the wild-type bR structure reveals that many of these residue substitutions occur on the interfaces between helices rather than within the cavity itself or the exterior surface. We hypothesize that these amino acid substitutions might be responsible for fine-tuning bR's spectral response curve. Finally, a novel clade of opsins has also been uncovered, whose members lack a structurally conserved lysine residue that typically functions as a covalent attachment site for a retinal chromophore. Further phylogenetic analysis performed in the context of a wider microbial opsin community, with 2000+ opsin sequences derived from non-redundant environmental samplings ("env_nr") and hypersaline metagenomes, suggests that these opsins are most closely related to fungal opsin-related proteins (ORPs), a group whose function, other than assistance in cell survival under stress, remains unknown. Cloning, expression and spectroscopic characterization of 3 of these novel haloarchaeal opsins, as well as of 23 additional bRs, is ongoing, and time permitting, may also be presented.

1968

Assembly of a bacterial cell division machine.

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Cytokinesis in Gram-negative bacteria is mediated by a multiprotein machine (the divisome) that assembles at midcell and serves to invaginate and remodel the inner membrane, peptidoglycan, and outer membrane. In the alpha-proteobacterium *Caulobacter crescentus*, the incipient division site also serves as a platform for localized peptidoglycan synthesis during elongation of the cell and recruitment of factors involved in the establishment of cell polarity. Understanding the order of assembly of proteins at midcell would inform models of the interactions among its components, their respective functions, and the mechanisms underlying integration of cytokinesis with other cell cycle events. We therefore leveraged the ability to isolate synchronous populations of *Caulobacter* cells to investigate assembly of proteins at the division site and place the arrival of each component into functional context. Additionally, we investigated the genetic dependency of localization among divisome proteins and the cell cycle regulation of their transcript and protein levels to gain insight into the control mechanisms underlying their assembly. Our results revealed a picture of assembly of proteins at midcell with unprecedented temporal resolution. Specifically, we observed 1) initial establishment of the division site, 2) recruitment of early FtsZ-binding proteins, 3) arrival of proteins involved in peptidoglycan remodeling, 4) arrival of FtsA, 5) assembly of core divisome components, 6) initiation of envelope invagination, 7) recruitment of polar markers and cytoplasmic compartmentalization, and 8) cell separation. Our analysis revealed differences in divisome assembly among Caulobacter and other bacteria that establish a framework for identifying aspects of bacterial cytokinesis that are widely conserved from those that are more variable.

1969

Entropy-driven translocation of an unstructured protein through the Gram-positive cell wall.

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Listeria monocytogenes is a Gram-positive, intracellular pathogen that recruits host factors to promote the polymerization of actin filaments at its surface. The only bacterial molecule that is required to initiate actin-based motility is the transmembrane protein ActA. Because ActA is a transmembrane protein, and thus effectively anchored to the membrane, it must find a way to extend beyond the thick, highly cross-linked Gram-positive cell wall in order to interact with host cytoskeletal factors. We offer a geometrical explanation for protein translocation through the Gram-positive cell wall that is dependent only on the energetics of the protein and the physical properties of the translocation barrier. The predictions from our hypothetical model suggest that the entropic constraint imposed by a small periplasmic space could drive the translocation of a large, unstructured protein (e.g. ActA) across a barrier with a thickness and porosity similar to that of the Gram-positive cell wall. We present three independent experimental results that support our proposed entropic model. First, we show that truncating the endogenous ActA protein below a critical length shifts the entropically beneficial state from an extended state to a periplasmic state. Second, we demonstrate that exogenous disordered proteins of sufficient size are able to translocate through the Listeria cell wall. Finally, we show that a sufficiently large, unstructured protein will translocate through the Bacillus cell wall, while a shorter unstructured protein will not. Further, bioinformatic analysis of disordered proteins in Gram-positive bacteria suggest an enrichment for large, disordered regions in surface proteins of host-associated bacteria.