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Cell Cycle News & Views

DDA3: A new dancer at the growing end?

Comment on: Jang CY, et al. Cell Cycle 2009; 8:3165-71.

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Microtubules are dynamic fibrous polymers that are required for maintaining the physical property and functional plasticity of cells, as well as having remarkable roles in numerous biological processes, such as cell division and morphogenesis. Microtubules undergo dynamic inter-conversion between growth and shrinkage both in vivo and in vitro, which is orchestrated by an array of microtubule binding proteins that associate with microtubule lattice or ends.[1] The lattice-associating proteins include microtubule stabilizers such as TPX2,[2] microtubule severing protein katanin[2] and kinesin motor CENP-E.[3] The end-binding proteins include minus-end-binding protein such as Nezha,[4] plus-end-binding depolymerase such as MCAK and its associating proteins.[5] Dynamic turnover of the spindle microtubules is a driving force for governing mitotic chromosome congression and segregation; however, the molecular control of the spindle microtubule plasticity and dynamics is not well understood. To identify novel regulators governing spindle dynamics and chromosome movements, Fang and colleagues had initiated a genomic analysis to identify candidate mitotic genes in human cells based on their expression profiles during mitosis followed by phenotype-based characterization using siRNA-mediated knock-down.[6,7,8] This approach has successfully identified a collection of microtubule regulators such as HURP, Cep55 and DDA,[3,9,10] which are essential for accurate chromosome segregation in mitosis.

DDA3 (differential display and is activated by p53) was initially identified as a downstream target of p53 via differential mRNA display approach;[11] however, its precise physiological function has remained elusive. Fang and colleagues have recently identified DDA3 as a novel regulator of mitotic spindle essential for accurate chromosome segregation via analysis of genetic profiling of mitotic genes.[9] Depletion of DDA3 perturbs chromosome congression and alignment at the metaphase equator. The DDA3-suppressed cells exhibit reduced inter-kinetochore tension and elongated spindle length, suggesting that DDA3 regulates microtubule dynamics. Indeed, suppression of DDA3 reduces the turnover rate of the spindle microtubule by increasing the rate of microtubule polymerization. The phenotype of DDA3-repressed cells mirrors what was seen in microtubule-depolymerizing KinI motor KIF2A-suppressed cells,[12] which prompted Fang et al. to examine if there is a mechanistic link between KIF2A and DDA3. As predicted, DDA3 interacts with the microtubule depolymerase KIF2A in vitro and is essential for recruiting KIF2A to the mitotic spindle.

To delineate the structure-functional relationship between DDA3 and KIF2A, Jang and Fang further analyzed the functional domains in DDA3 and revealed that the C-terminal domain of DDA3 exerts microtubule-binding activity in vitro and localizes to spindle in vivo.[10] Surprisingly, N-terminal domain of DDA3 carrying little microtubule-binding activity exhibits a dominant-negative effect in vivo. Strikingly, KIF2A failed to localize to the spindle in those DDA3 N-terminal overexpressing cells, suggesting that the N-terminal domain of DDA3 may bind and absorb the endogenous DDA3 from the spindle, which mimics the phenotype seen in DDA3-suppressed cells. It would be of great interest to test whether the N-terminal DDA3 forms a heterodimer with endogenous DDA3 given the fact that N-terminal DDA3 and endogenous DDA3 proteins distribute exclusively in the cytoplasm. Given the interaction between KIF2A and DDA3, it would be of critical importance to ascertain how DDA3 binds to the microtubule ends. Interestingly, a brief computational analysis reveals that DDA3 exhibits a typical EB1-binding motif, suggesting that DDA3 perhaps localizes to the microtubule ends by which DDA3 anchors KIF2A for microtubule length and dynamics control. In fact, the mitotic distribution profile of DDA3 is reminiscent of a recently characterized microtubule plus-end-binding protein TIP150.[13] The finding of EB1-binding motif in DDA3 and possibility of DDA3 function at the plus-end are consistent with the early observation of DDA3-EB3 interaction,[14] and elongated spindle in the absence of DDA3-KIF2A.[9] In this regard, future experiments will be directed to ascertain whether DDA3 interacts with EB1-interactive network at the plus-ends and if suppression of DDA3 alters MCAK depolymerase distribution and function. Given the phosphorylation profile of DDA3 in mitosis, it would be equally important to uncover its regulatory mechanism and evaluate how mitotic phosphorylation regulates DDA3 molecular plasticity. In any event, the current study by Fang and colleagues revealed a new class of microtubule regulators which is essential for mitotic chromosome plasticity. In sum, the findings of DDA3-KIF2A interaction in regulation of spindle dynamics provide a new direction in delineating spindle microtubule plasticity and dynamics.

References

1. Gadde S, et al. Curr Biol 2004; 14:R797-805.
2. Manning AI, et al. Cell 2008; 134:694.
3. Cleveland DW, et al. Cell 2003; 112:407-21.
4. Meng W, et al. Cell 2008; 135:948-59.
5. Wordeman L. Curr Opin Cell Biol 2005; 17:82-8.
6. Wong J, et al. J Cell Biol 2006; 173:879-91.
7. Zhao WM, et al. Mol Biol Cell 2006; 17:3881-96.
8. Seki A, et al. J Biol Chem 2007; 282:15103-13.
9. Jang CY, et al. J Cell Biol 2008; 181:255-67.
10. Jang CY, et al. Cell Cycle 2009; 8:3165-71.
11. Lo PK, et al. Oncogene 1999; 18:7765-74.
12. Ganem NJ, et al. J Cell Biol 2004; 166:473-8.
13. Jiang K, et al. EMBO Rep 2009; 10:857-65.
14. Hsieh PC, et al. Oncogene 2007; 26:4928-40.

Quinacrine: New anti-tumor application for an old anti-malaria drug

Comment on: Neznanov N, et al. Cell Cycle 2009; 8:3960-70.

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The Heat Shock Response (HSR) allows cells to combat stressful and protein-damaging conditions (proteotoxic stress). These stresses can be physical, such as heat shock or hypoxia/ischemia or chemical, such as acidosis, ATP depletion or free radical generation. HSR is an ancient and evolutionarily conserved protective mechanism that involves synthesis of heat shock proteins (HSP), (primarily molecular chaperones such as Hsp90, Hsp70 and Hsp27) via activation of the stress-inducible transcription factor Heat Shock Factor 1 (HSF1). HSR is also characterized by attenuation of the general translation machinery, which contributes to minimization of proteotoxic stress by reducing the rate of newly synthesized, and potentially misfolding, proteins.[1] Although HSF1 is itself not an oncogene, it organizes a broad network of cellular functions that act globally to support tumorigenesis.[2,3] Therefore, inhibition of HSR may provide a potential anti-cancer target.

In a paper published in Volume 8, Issue 23 of *Cell Cycle*, Neznanov and co-workers have examined the HSR inhibitory effects of several anti-malarial drugs, including quinacrine (QC), 9-aminoacridine (9AA) and emetine. These investigators hypothesized that the three drugs may be effective in malaria because of their shared ability to inhibit HSP synthesis, thereby sensitizing *Plasmodium falciparum* to fever induced by the host (e.g., heat shock). Indeed, the authors show that QC, 9AA and emetine suppressed HSR. Emitine is a general inhibitor of translation. However, QC inhibits NFκB-mediated transcription in a fashion similar to its effect on HSF1.[4] In both cases, the drug does not interfere with activation, nuclear translocation or DNA binding of the transcription factors, but it effectively blocked transcription initiation. This suggests that nuclear localization of QC is important for its ability to suppress HSR.

Although more specific than emetine, QC was recently reported to inhibit the phorbol ester induced upregulation of matrix metalloproteinases MMP1 and MMP8 in leukocytes.[5] These findings indicate that the inhibitory effects of QC are not restricted to HSF1 and NFκB; nevertheless, QC may have selective effects on inducible transcription since the drug also stimulates p53-mediated transcription.[4]

Neznanov and colleagues' interesting observation raises further questions that are important to address. For example, what is the global transcriptional response of QC treatment in tumor cells? How exactly does QC block HSF1 transcription initiation? In most human tissues, all three known HSF isoforms (HSF1, HSF2 and HSF4) are expressed, complicating the assignment of their individual functional roles and regulatory responses.[6] For this reason yeast may provide a valuable system to dissect and better understand the inhibitory effects of QC on HSR.

Finally, HSF1 transcriptional activity is normally suppressed by Hsp90, and this suppression is released in the context of increased proteotoxic stress.[7] Hsp90 also chaperones numerous proteins involved in carcinogenesis.[8] Several chemically distinct Hsp90 inhibitors have shown promising anti-tumor activity in vivo and one Hsp90 inhibitor, 17AAG, is currently in late stage clinical trial in HER2-positive breast cancer and in multiple myeloma.[9,10] Nonetheless, Hsp90 inhibitors have proven to be less robust than expected in the clinic.[9] As Hsp90 pharmacologic inhibition releases HSF1 from chaperone-mediated suppression and leads to induction of HSR, the thought has arisen that this characteristic may provide a countervailing force which negatively impacts the broader clinical efficacy of Hsp90 inhibitors. Therefore, investigators are actively searching for ways to blunt Hsp90 inhibitor-induced HSF induction. The current data provided by Neznanov et al. supply a strong justification for further evaluating the anticancer properties of QC or similar compounds, especially in the context of Hsp90 inhibition.

References

1. Whitesell L, et al. Nat Rev Cancer 2005; 5:761-72.
2. Dai C, et al. Cell 2007; 130:1005-18.
3. Min JN, et al. Oncogene 2007; 26:5086-97.
4. Gurova KV, et al. Proc Natl Acad Sci U S A 2005; 102:17448-53.
5. Stuhlmeier KM, et al. J Rheumatol 2006; 33:472-80.
6. Liu XD, et al. EMBO J 1997; 16:6466-77.
7. Zou J, et al. Cell 1998; 94:471-80.
8. Neckers L. J Biosci 2007; 32:517-30.
9. Erlichman C. Expert Opin Investig Drugs 2009; 18:861-8.
10. Modi S, et al. J Clin Oncol 2007; 25:5410-7.

An experimental model of ovarian carcinoma: More than just genetics

Comment on: Zheng J, et al. Cell Cycle 2010; 9:140-6.

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Despite its relatively low incidence rate, ovarian cancer is an extremely lethal disease. It is the fifth leading cause of cancer death in women and is the most lethal gynecological malignancy. The reason for this is mainly because in its early stages, ovarian cancer has few, rather minor symptoms and, when diagnosed, the disease is often extensive, which current therapies have little effect on. However, the early events associated with the pathogenesis of ovarian cancer are poorly understood.

In order to elucidate the underlying disease mechanisms, the development of suitable in vitro systems and mouse models that can faithfully reproduce the pathological characteristics of human ovarian carcinomas are important.

One of the greatest challenges in diagnosis and treatment of ovarian cancer is its heterogenous nature. Serous carcinomas are the most common subtype of ovarian carcinoma, accounting for approximately 70% of all ovarian cancer. They are rarely confined to the ovaries at diagnosis and for almost 90% of stage III or IV tumors. The rapid and distant metastatic spread and progression to chemoresistance account

for an exceptionally high percentage of deaths of patients.[1]

In the last issue of *Cell Cycle*, Zheng et al. provide experimental evidence for normal human OSE to undergo neoplastic progression.[2] Histopathologic examination of these tumors revealed papillary carcinoma that resembles human papillary serous ovarian carcinoma.[2] Specifically, these authors, which was shown previously to transform human OSE using a defined set of genetic manipulations including the SV40 T/tag, hTERT and RAS,[3] were able to transfect these cells with HER2/neu to give rise to papillary differentiation. Interestingly, the development of tumors with papillary differentiation relies not only on specific genetic modifications but also on the tumor microenvironment.

There are few animal models, such as mice, rats and hens, that develop ovarian tumors spontaneously.[4] However, the low incidence and the length of time required for the development of these tumors have limited their use for studying the biology of ovarian cancer. Induced tumor models may circumvent some of these problems. In this paper, Zheng et al. have elegantly shown us that overexpression of selected oncogene can cause papillary carcinoma that closely resemble the human disease histopathologically. This effect seems to be specific to the choice of oncogene as in their previous publication, tumors derived from RAS-transformed cells remained largely undifferentiated or poorly differentiated. K-RAS mutations are only rarely seen in ovarian serous carcinomas, whereas gene amplification and overexpression of HER-2/neu are more common.[5,6] This also strongly implies that malignant transformation and histological differentiation are controlled via independent pathways.

While it is widely believed that ovarian carcinoma develops de novo from the OSE, this view has recently been questioned. Alternative cellular origins such as the fallopian tube have been proposed based on the discovery that dysplastic lesions and in situ carcinomas in the fallopian tube fimbria, but not OSE, in most cases of prophylactic salphingo-oophorectomy specimens of BRCA mutation carriers, and that women with ovarian serous carcinomas also harbor tubal intraepithelial carcinomas with the presence of identical p53 mutations.[7-9] In this connection, it is interesting to see that the work of Zheng et al. shows normal human OSE can undergo neoplastic progression to papillary carcinoma when modified with specific genetic elements. The presence of SV40 T/tag positive tumor cells is in support of tumors originating at the transformed OSE. The introduction of various other genetic alterations in the OSE has also been shown to give rise to ovarian carcinoma in other mouse models.[4] However, most of the tumors developed in these models were poorly differentiated, suggesting that mechanisms other than genetic alterations may control the histological differentiation.

What is also attractive about Zheng et al. study is the cell line can only develop into papillary differentiation in a peritoneal microenvironment, which closely duplicates the well-established progression of ovarian cancer. Although carcinogenesis has long been considered a cell autonomous event in which progressive genetic alterations transform cells independent of external cues, there is increasing evidence to suggest that the tumor microenvironment plays an important role in cancer development and progression.[10] Clearly, there must be something special about the interaction between ovarian carcinoma and peritoneal epithelium that promotes the neoplastic growth and differentiation of the transformed OSE. Indeed, human peritoneal cells have been shown to release regulatory chemokines, cytokines and growth factors in response to inflammation induced by epithelial ovarian cancer implantation.[11] It will be interesting to identify those cytokines and factors in the peritoneal microenvironment that are critical to papillary growth and differentiation.

In summary, these new findings suggest an attractive model of papillary differentiation to explain the importance of tumor microenvironment on the genetic contributions in ovarian tumor development. This provides a preclinical model to test developing therapeutics and will also allow analysis of ovarian tumor progression at the cellular and molecular levels.

References

1. Bast RC, et al. Nat Rev Cancer 2009; 9:415-28.
2. Zheng J, et al. Cell Cycle 2010; 9:140-6.
3. Liu J, et al. Cancer Res 2004; 64:1655-63.
4. Garson K, et al. Mol Cell Endocrinol 2005; 239:15-26.
5. Kurman RJ, et al. Int J Gynecol Pathol 2008; 27:151-60.
6. Landen CN, et al. J Clin Oncol 2008; 26:995-1005.
7. Piek JM, et al. J Pathol 2001; 195:451-6.
8. Kindelberger DW, et al. Am J Surg Pathol 2007; 31:161-9.
9. Levanon K, et al. J Clin Oncol 2008; 26:5284-93.
10. Bissell MJ, et al. Nat Rev Cancer 2001; 1:46-54.
11. Freedman RS, et al. J Transl Med 2004; 2:23.

Cancer stem cells: Just sign here!

Comment on: Comment on: Hussenet T, et al. Cell Cycle 2010; 9:321-7.

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Taxonomy based on genome-wide molecular portraits of tumors has become a mainstay of personalized medical care for cancer patients. Microarray-based multigene assays, such as Mammaprint® or MapQuant® designed to individualize treatment for patients with breast cancer, are already integrated in clinical practice.[1] However, using whole tumor molecular signatures as prognostic or predictive markers might be in contradiction with the emerging cancer stem cell concept.

This concept is based on the existence of a subpopulation of undifferentiated cancer cells responsible for tumor initiation, maintenance and therapeutic resistance, the cancer stem cells (CSCs).[2-4] The number of stem cells within a tumor is thought to be low as compared to the bulk of their cancer cell progeny. Thus, the scarcity of the CSC population might not be reflected in the molecular portrait established on the global cancer cell population.

To combine molecular portraits of tumor and the CSC concept many groups have sought to determine whether the presence of

molecular imprints of stemness [embryonic stem cells (ESC) or adult tissue-specific stem cells signatures] in tumors might be detected and whether they could represent accurate prognostic markers.[5-7] In this issue, using molecular signatures enrichment analyses, Hussenet et al. demonstrates that an ESC-like molecular phenotype is enriched in tumor compared to normal tissue but is not different between CSCs and non-CSCs. Interestingly, several studies have described an association between an ESC-like phenotype and poor prognosis in various types of cancer (including breast, lung, bladder and brain tumors). These observations are interpreted by Hussenet et al. as a hallmark of poor differentiation rather than a surrogate of cancer stem cell activity, as suggested by similar studies.[5-7] Thus, the ESC-like signature in cancers is more likely the results of re-activation of an ESC-like phenotype during the course of tumor progression rather than an inherited phenotype from a cell-of-origin. Consequently, this ESC-like signature failed to link CSCs biology with the whole molecular portrait of the tumor.

The question of stemness signature within a tumor as a valid molecular marker for cancer prognosis remains open. In other words, does the number or quality of CSCs reflect cancer clinical evolution? CD44+CD24- phenotype and ALDH1 expression, both associated with tumor-initiating cells, have been investigated as prognostic markers. Only the presence ALDH1 positive cells is associated with poor clinical outcome.[8,9] Even though these data appear contradictory, one can expect that a more complete approach based on genome-wide profiling of CSCs might be more successful for prognostic prediction. Exploring this approach, Hussenet and colleagues show that a normal adult tissue-specific stem cell molecular phenotype, previously defined, is enriched in epithelial cancer stem cells compared to non tumorigenic cancer cells. Moreover, they provide evidence that enrichment in CSC-signatures is correlated with poor prognosis in breast and lung carcinomas.

The development of CSC molecular portraits is promising in translating the CSC concept to clinical practice, even though it remains critical to further validate the subsequent signatures at the functional level. Moreover, characterization of CSC genomic profiles will contribute to open the Pandora box of CSC targeted therapies.

References

1. Sotiriou C, et al. N Engl J Med 2009; 360:790-800.
2. Reya T, et al. Nature 2001; 414:105-11.
3. Creighton CJ, et al. Proc Natl Acad Sci USA 2009; 106:13820-5.
4. Phillips TM, et al. J Natl Cancer Inst 2006; 98:1777-85.
5. Ben-Porath I, et al. Nat Genet 2008; 40:499-507.
6. Hassan KA, et al. Clin Cancer Res 2009; 15:6386-90.
7. Wong DJ, et al. Cell Stem Cell 2008; 2:333-44.
8. Ginestier C, et al. Cell Stem Cell 2007; 1:555-67.
9. Honeth G, et al. Breast Cancer Res 2008; 10:R53.

A genome wide view of hunchback-like-1 targets

Comment on: Comment on: Niwa R, et al. Cell Cycle 2009; 8:4147-55.

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Development of a multicellular organism involves coordinated cellular division and differentiation programs to generate the appropriate cell types and tissues. This complex orchestration depends on gene regulatory pathways that function in the right place and at the right time. Genetic screens in *C. elegans* identified a series of genes that regulate developmental timing.[1] Mutant worms that reiterate or skip earlier cell fates are classified as heterochronic, or developmental timing, mutants because their cell lineages are temporally transformed.[2] Several genes in this pathway, including *lin-14*, *lin-29* and *hbl-1*, encode transcription factors and potentially act as master regulators in multiple cell types to promote gene expression patterns appropriate for specific stages in development. Hence, identification of the genes under the control of these transcription factors will help elucidate the networks that drive cellular progression in tune with the developmental clock.

In a new study by Niwa et al., *Cell Cycle* 2009, dozens of genes affected by *hbl-1* activity were identified in developing worms.[3] The hunchback-like-1 (*hbl-1*) gene in *C. elegans* is the homolog of the *Drosophila melanogaster* hunchback (hb) transcription factor.[4] Hb was first identified in Drosophila as a member of the gap class of segmentation genes and is a key regulator of embryonic segmentation, pattern formation and neuroblast identity. In Drosophila, hb binds to well characterized A-rich DNA motifs and can function as a transcriptional activator or repressor depending on its concentration and interaction partners.[5] Homologs of hunchback are present from worms to humans, but the function of these genes in animal development has not been deeply investigated beyond ecdysozoans.

The *C. elegans* homolog of hunchback, *hbl-1*, is expressed during embryogenesis and is required for later larval development, but not for early pattern formation.[4,6,7] *Hbl-1* was identified as a member of the heterochronic pathway because mutations in this gene result in the precocious differentiation of hypodermal cells.[6,7] Expression of *hbl-1* is observed in multiple embryonic cell lineages but becomes predominantly neuronal after hatching.[4] During larval development, temporal down-regulation of *hbl-1* in hypodermal and neuronal cells is mediated at least in part by the miRNA pathway. The *let-7* miRNA and its related "sister" miRNAs, *mir-48*, *mir-241* and *mir-84*, regulate *hbl-1* expression via multiple complementary sites in its 3'UTR.[6-8] Recently, *hbl-1* and *let-7* were reported to be in a negative feedback loop.[9] This model is based on evidence that transcription of *let-7* miRNA is repressed by HBL-1 protein in hypodermal cells until the *let-7* sister miRNAs inhibit *hbl-1* expression during the transition from the second to third larval stages.[8,9] The resulting increase in *let-7* miRNA can then bolster repression of *hbl-1* by the miRNA pathway.[6-9]

The study by Niwa and colleagues demonstrates that *let-7* is probably just one of many transcriptional targets of *hbl-1* in developing worms.[3] To detect genes that respond to HBL-1 protein, the authors induced expression in live worms using a heat-shock promoter fused to the *hbl-1* gene. A pulse of *hbl-1* expression during the last larval stage (L4) was sufficient to induce heterochronic defects, prompting the investigation of target genes that might contribute to the phenotypes. Using microarray analyses, the authors detected significant up and down regulation of 26 and 53 genes, respectively, in worms induced to express *hbl-1* during L4. This genome wide analysis revealed a common A-rich motif upstream of both the positively and negatively regulated sets of genes.

Remarkably, this motif is comparable to the well-established Hunchback recognition site in Drosophila.[10]

By investigating potential genetic interactions between *hbl-1* and each of the genes mis-regulated upon induction of HBL-1 protein, the authors identified *sym-1*, which encodes a protein with multiple leucine-rich repeats.[3] Interestingly, the genetic data indicate that *hbl-1* directs opposite cellular fates through *sym-1* during the larval and adult stages, inhibiting then promoting differentiation of hypodermal cells. This complexity may be expected if regulation by *hbl-1* is modulated by graded HBL-1 levels and combinatorial actions with other transcription factors, as is the case in Drosophila.[5]

The identification of potential direct targets of *hbl-1* transcriptional regulation, including a genetically validated target, is an important contribution towards understanding the common and distinct pathways controlled by hunchback homologs across species. The finding that similar numbers of genes positively and negatively responded to ectopic *hbl-1* expression suggests that this transcription factor may act as an activator or repressor, although further experiments are needed to determine direct versus indirect effects. Additionally, all of the mis-regulated genes were found to contain A-rich motifs in their promoter regions, indicating that context and other factors may govern whether *hbl-1* activates or represses specific targets. Considering the spatially and temporally regulated expression pattern of HBL-1 protein, future studies may reveal entirely different networks of genes under the control of this dynamic transcription factor.

References

1. Moss EG. Curr Biol 2007; 17:R425-34.
2. Ambros V, et al. Science 1984; 226:409-16.
3. Niwa R, et al. Cell Cycle 2009; 8:4147-55.
4. Fay DS, et al. Dev Biol 1999; 205:240-53.
5. Payankaulam S, et al. Curr Biol 2008; 18:R653-R655.
6. Abrahante JE, et al. Dev Cell 2003; 4:625-37.
7. Lin SY, et al. Dev Cell 2003; 4:639-50.
8. Abbott AL, et al. Dev Cell 2005; 9:403-14.
9. Roush SF, et al. Dev Biol 2009; 334:523-34.
10. Stanojevic D, et al. Nature 1989; 341:331-5.

Circadian rhythms: Phosphorylating the CLOCK

Comment on: Comment on: Spengler ML, et al. Cell Cycle 2009; 8:4138-46 .

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The molecular circadian clock is a cell-autonomous system organized to create a conserved transcriptional-based negative feedback loop. This feedback loop is initiated when a transcription factor complex (positive arm) induces the expression of negative elements which, after a time-delay, feed back to inhibit the positive arm. The kinetic properties of circadian systems are set through post-translational modifications (PTMs) acting on nearly every component.[1] Phosphorylation is generally regarded as the PTM primarily responsible for setting the 24-hour period length through altering the stability or accessibility of the negative (repressor) complex. While a number of studies have focused on comprehensive dissection of these negative element PTMs,[2-4] the molecular rhythms community has just begun to dig into the regulation and function of PTMs in the positive arm. In a previous issue of *Cell Cycle*, Spengler et al.[5] fill in some of the story by investigating the role of phosphorylation on the transcription factor CLOCK in the mammalian circadian system and present data on its possible regulation by the GSK-3β kinase.

CLOCK forms a heterodimeric complex with another transcription factor, BMAL1, and together they regulate expression of circadianly regulated genes. Both trigger each other's phosphorylation but little is known about the function of these PTMs. The authors have taken a bioinformatic approach to identify potential phosphorylation sites on CLOCK, narrowing down the possibilities within the 885-residue protein to focus on a region encompassing 36 conserved residues, 425–461, containing predicted kinase targets. CLOCK proteins harboring serine/threonine to alanine mutations in this region result in decreased phosphorylation and an increase in protein stability, thus defining a discreet region on CLOCK important for regulated turnover, termed a "phospho-degron." The authors show that these phopho-deficient mutants still interact with BMAL1 and that BMAL1-mediated degradation of CLOCK requires the phospho-degron. Circadian physiology is also impacted as the mutant CLOCK proteins have attenuated transcriptional activity and an apparent phase delay in synchronized cells.

Previously a mass-spectrometry based study had shown that S427, within the phospho-degron, is phosphorylated in vivo;[6] however, that study did not establish function to the site. Spengler et al. further focus on two residues, including the in vivo phosphorylated S427, and S431. Mutation of S431A almost completely blocks all BMAL1-dependent CLOCK phosphorylation, while S427 is one of two predicted GSK-3β target sites within the phospho-degron. These data led the authors to hypothesize a conserved role for GSK-3β in regulating CLOCK specifically at S427, with S431 acting as its priming site. Along these lines, they present compelling evidence that GSK-3β can phosphorylate CLOCK in vitro and manipulation of upstream activators/inhibitors of GSK-3β predictably alters CLOCK phosphorylation and stability. Unfortunately, S427A and S431A mutations were not assessed individually for CLOCK stability, and were not included in the mutant strains when the authors looked at BMAL1-dependent CLOCK degradation or the circadian phase delay. If the proposed model is that GSK-3β mediates these clock effects specifically through S427 it would be interesting to look at S427A and S431A using the same conditions in order to determine if they impact CLOCK functions.

While this study defines a new region important for proper phosphorylation and function of CLOCK it also raises a few interesting questions. For instance, why would reduced phosphorylation and increased stability lead to an attenuation of transcriptional activity? One possibility is that phosphorylation is structurally required for CLOCK/BMAL1 promoter occupancy or, as the authors make the case, that there is an intermediate, unstable, phosphorylated form more transcriptionally active: such a model has recently been suggested based on data from fungal circadian oscillators[7] whose molecular architecture is quite similar to the mammalian clock studied here. Additionally, given that small molecule inhibitors of GSK-3β specifically decrease period in cycling mammalian cells,[8] it is interesting that in this study the proposed role of GSK-3β is in promoting degradation of CLOCK. The authors suggest that reduction of CLOCK phosphorylation results in the observed phase delay, through longer protein

half-life. This is perhaps opposite to what one might expect when inactivation of the responsible kinase leads to period decreases. However, here the authors are looking at one specific target, CLOCK, which may not be the only circadian protein regulated by GSK-3β. While further work will be needed to understand how GSK-3β activity affects other kinetic parameters of the circadian clock, Spengler et al. have taken an important first step in the analysis.

References

1. Mehra A, et al. Trends Biochem Sci 2009; 34:483-90.
2. Chiu JC, et al. Genes Dev 2008; 22:1758-72.
3. Baker CL, et al. Mol Cell 2009; 34:354-63.
4. Vanselow K, et al. Genes Dev 2006; 20:2660-72.
5. Spengler ML, et al. Cell Cycle 2009.
6. Yoshitane H, et al. Mol Cell Biol 2009; 29:3675-86.
7. Schafmeier T, et al. Genes Dev 2008; 22:3397-402.
8. Hirota T, et al. Proc Natl Acad Sci USA 2008; 105:20746-51.