

## **Differential expression, localization and activity of two alternatively spliced isoforms of human APC regulator CDH1**

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Abbreviations used: APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; HA, hemagglutinin; GFP, green fluorescent protein; RT-PCR, reverse transcription PCR

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The human CDH1 $\alpha$ , human CDH1 $\beta$  and mouse CDH1 nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers AF083810, AF083809 and AF433157.

**Short title:** Differential expression and activity of CDH1 isoforms

## Synopsis

The timely destruction of key regulators through ubiquitin-mediated proteolysis ensures the orderly progression of cell cycle. The anaphase-promoting complex (APC) is a major component of this degradation machinery and its activation is required for the execution of critical events. Recent studies have just begun to reveal the complex control of APC through a regulatory network involving WD40 repeat proteins CDC20 and CDH1. Here we report on the identification and characterization of human CDH1 $\beta$ , a novel alternatively spliced isoform of CDH1. Both CDH1 $\alpha$  and CDH1 $\beta$  can bind to APC and stimulate the degradation of cyclin B1, but they are differentially expressed in human tissues and cells. CDH1 $\alpha$  contains a nuclear localization signal which is absent in CDH1 $\beta$ . Intracellularly, CDH1 $\alpha$  appears in the nucleus while CDH1 $\beta$  is a predominantly cytoplasmic protein. The forced overexpression of CDH1 $\alpha$  in cultured cells correlates with the reduction of nuclear cyclin A, but the steady-state amount of cyclin A does not change noticeably in CDH1 $\beta$ -overexpressed cells. In *Xenopus* embryos, ectopic overexpression of human CDH1 $\alpha$ , but not of CDH1 $\beta$ , induces cell cycle arrest during the first G<sub>1</sub> phase at the midblastula transition. Taken together, our findings document the differential expression, subcellular localization and cell cycle-regulatory activity of human CDH1 isoforms.

Key words: anaphase-promoting complex (APC), CDH1, cell cycle control, alternative splicing

## INTRODUCTION

The anaphase-promoting complex (APC) is a major cellular ubiquitination system, which controls the precise order and timing of eukaryotic cell cycle by targeting mitotic cyclins, anaphase inhibitors and other regulators for degradation by proteasome [1,2]. The composition of APC is highly conserved in eukaryotes. The APC activity is tightly regulated during the cell cycle through phosphorylation and dephosphorylation of its subunits [3,4]. In addition, biochemical and genetic analyses in yeast [5,6], fruit flies [7-9] and frogs [10] have identified homologous proteins CDC20/Fizzy and CDH1/Fizzy-related/HCT1 as activators and specificity factors of APC.

CDC20 and CDH1 are members of the WD40 repeat protein superfamily. They bind transiently to APC and are thought to facilitate ubiquitination through direct interaction with different sets of targets [11,12]. Many of these targets share a recognition motif known as D box [13,14]. Distinct motifs termed KEN box [15,16] and A box [17] have also been identified in substrate proteins recognized by the CDH1-activated APC.

The abundance of CDC20 and CDH1 plateaus in G<sub>2</sub>-M and drops abruptly at the exit from mitosis [4,14,18,19]. CDH1 is widely expressed in differentiated tissues including postmitotic neurons [20], but its expression is significantly reduced during malignant progression of a B-lymphoma cell line [21]. In keeping with this, genetic analysis in *C. elegans* indicates that CDH1 regulates cell proliferation [22]. Both CDC20 and CDH1 can be phosphorylated [12]. While it remains controversial as to whether phosphorylation of CDC20 may stimulate [3], inactivate [23], or have no influence on APC [4], the inhibitory phosphorylation of CDH1 has been demonstrated by several groups [3,24,25].

The APC has emerged as a downstream target of the mitotic checkpoint, which prevents the onset of anaphase until all chromosomes are properly aligned [26]. To date, more than six components (MAD1, MAD2, MAD3, BUB1, BUB2, and BUB3) of the mitotic checkpoint have been identified. Both MAD2 and BUBR1 associate with and inhibit CDC20, thereby transducing a stop signal to APC [27]. In addition, yeast BUB2 and human MAD2B/MAD2L2 can target CDH1 [28-30]. However, the mechanisms by which BUBR1, MAD2 and MAD2B inhibit CDC20/CDH1 remain to be elucidated.

We have previously characterized human MAD1 and MAD2 [31]. Given that CDC20 and CDH1 are effectors of MAD1 and MAD2, we set out to characterize CDC20 and

CDH1 in human cells. In the present study, we identified and characterized a novel alternatively spliced isoform of human CDH1. We demonstrated the differential expression, subcellular localization and APC-activating activity of the two functional isoforms of CDH1 designated CDH1 $\alpha$  and CDH1 $\beta$ . Our findings suggest an additional level of spatial regulation in the activation of APC.

## EXPERIMENTAL

### Antibodies

Rabbit polyclonal anti-CDH1 antibody  $\alpha$ -CDH1C was raised against a C-terminal peptide (amino acids 472-493 of human *CDH1* $\alpha$ , underlined in Figure 1A) conjugated to keyhole limpet hemocyanin. Rabbit polyclonal anti-CDH1 antibody  $\alpha$ -CDH1N was raised to a keyhole limpet hemocyanin-conjugated peptide corresponding to the N-terminus of human CDH1 (amino acids 2-21; underlined in Figure 1A). Mouse monoclonal anti-FLAG (M2) was from Sigma. Mouse monoclonal anti-HA (F-7), rabbit polyclonal anti-cyclin A (H-432) and rabbit polyclonal anti-CDC27 (H-300) were from Santa-Cruz.

### RNA and protein analyses

Human multiple tissue Northern blots (CLONTECH) were probed with a 307-bp  $^{32}\text{P}$ -labeled *NcoI-NaeI* fragment shared by both *CDH1* $\alpha$  and *CDH1* $\beta$  cDNAs. A 2 kb human  $\beta$ -actin cDNA was used as a control for RNA loading. Northern blotting was carried out as per manufacturer's protocol.

Poly(A)<sup>+</sup> RNAs from human whole brain, heart, liver and spleen were purchased from CLONTECH. Poly(A)<sup>+</sup> RNAs from cultured cells were isolated using the TRIzol reagent and oligo(dT) cellulose (Invitrogen). RT-PCR was performed in the presence of 5% dimethyl sulfoxide with a reagent kit (Advantage RT-for-PCR from CLONTECH) using the manufacturer's protocol. Synthesis of the first strand cDNA was primed with oligo(dT) and random hexamers. Image of ethidium bromide-stained gel was obtained in a FOTODYNE gel documentation system.

HeLa and HepG2 cells were cultured as described [32]. Protein samples from HeLa cells were solubilized directly in SDS gel loading buffer (60mM Tris base, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.1% bromophenol blue), separated on 12% SDS-PAGE, and electroblotted onto Immobilon-P membrane (Millipore) using a semidry blotter (Hoefer). Blots were visualized by chemiluminescence (ECL, Amersham). Co-immunoprecipitation was performed as previously described [33]. Expression vectors for

CDH1 $\alpha$  and CDH1 $\beta$  (pHACDH1 $\alpha$  and pHACDH1 $\beta$ ) were derived from plasmid pHA [34].

### **Luciferase assay**

Luciferase assay was performed as described [35]. Human cyclin B1 cDNA has been described [36]. pCYCB1-Luc was derived from reporter plasmid pGL3-Control (Promega) and it expresses a luciferase fusion protein with the first 119 amino acid residues of cyclin B1 at the N-terminus. Proteasome inhibitor MG-132 (Z-LLL-CHO) was purchased from Peptide Institute, Inc. based in Osaka, Japan.

### **Embryo manipulations**

Eggs were collected from *Xenopus laevis* females (Xenopus Express, Cape, South Africa), which had been injected with 500-700 units of human chorionic gonadotrophin (Sigma) 12 hours before egg collection. Eggs were fertilized in vitro with minced testis. Embryos were staged as described [37]. RNAs were synthesized and capped with reagent kits from Ambion.

### **Confocal microscopy**

Laser scanning confocal immunofluorescence microscopy was performed on a Zeiss Axiophot microscope as previously described [31,32]. Dual labeling was achieved with primary antibodies from different species and pre-adsorbed species-specific secondary antibodies: Cy5-conjugated goat anti-mouse immunoglobulin G (Zymax) and fluorescein-conjugated goat anti-rabbit immunoglobulin G (Zymax). To express a GFP fusion protein with peptide sequences encoded by exons 6 and 7 of human CDH1 $\alpha$  at the C-terminus, we PCR-amplified exons 6 and 7 using primers 5'-CCCAA GCTTATCCCTTAGCAC CAAG (forward) and 5'-CGGGATCCCTGGCTGGTACAGGCACT (reverse). The 267-bp fragment was inserted into pEGFP-C1 (CLONTECH) via restriction sites *Hind*III and *Bam*HI. GFP experiments were conducted as described [38].

## RESULTS

### **CDC20 and CDH1 represent two multigene families**

The identification of *Drosophila* and *Xenopus* CDH1 [8,10] facilitates the cloning of human and mouse orthologs. The isolation and characterization of human CDH1 has been reported previously [14,19,39]. In an early and independent attempt to assemble full-length CDH1 cDNAs, we also obtained several human and mouse EST clones sharing striking sequence homologies with frog CDH1. The human and mouse CDH1 sequences with a complete coding region were determined and deposited in GenBank under accession numbers AF083810 and AF083809 (submitted on August 12, 1998).

Human and mouse CDH1 proteins (Figure 1A) share 98.4% identity in an overlap of 493 amino acid residues. The seven WD40 repeats are identical in the two proteins except for one E-to-D substitution at the 193rd residue. The nine putative CDK phosphorylation sites (shaded in Figure 1A) are also conserved. Since a similarity search generated more CDC20-/CDH1-related sequences than expected, we constructed a phylogenetic tree of all CDC20/CDH1 homologs from various species (Figure 1B) in order to better understand the genetic relationship.

Notably, there is one additional CDC20 paralog in budding yeast. This protein named AMA1/CDC20B (Figure 1B) is required for sporulation and has been implicated as a meiosis-specific activator of APC [40]. In fission yeast, five CDC20/CDH1 homologs can be identified. In addition to the documented CDC20A (SLP1) and CDH1A (SRW1/STE9), two CDC20 paralogs (CDC20B and CDC20C) and another CDH1 paralog (CDH1B or MFR1 or FZR1) were found. In the phylogenetic tree, the fission yeast CDC20B and CDC20C cluster with budding yeast AMA1/CDC20B, while the fission yeast CDH1A and CDH1B group with budding yeast CDH1. Interestingly, CDH1B/MFR1/FZR1 has been shown to be another meiosis-specific activator of APC required for sporulation [41,42]. The bootstrap supports for these clusters are statistically very significant (100% or 95%). We postulate that different CDC20/CDH1 homologs in fission yeast might serve redundant and non-redundant functions. It would be of interest to see how they cooperate to ensure the precise spatiotemporal order of APC activation.

Multiple CDC20 and CDH1 homologs were also noted in higher eukaryotes (Figure 1B). Thus *Arabidopsis thaliana* (thale cress) has at least one CDH1 and three closely

related CDC20 homologs (CDC20A, CDC20B, and CDC20C). Likewise, *Drosophila* (fruit fly) possesses CDC20, CDH1/FZR and CDH2/FZR2 [9]. We predict that more vertebrate CDC20/CDH1 homologs will be identified as the genome projects progress. CDC20 and CDH1 represent two multigene families of WD40 repeat proteins critically involved in the regulation of APC. It is important to compare and contrast the functions of different CDC20/CDH1-like proteins in one species.

### **Chromosomal mapping and genomic organization of human CDH1 locus**

We determined the chromosomal map location of human CDH1 gene by sequence alignment. Using cDNA information we identified two genomic clones that contain the coding and non-coding regions of human CDH1. These were cosmids R33374 and R31109 from a genomic library constructed at the Lawrence Livermore National Laboratory (GenBank AC005787 and AC005786). Both clones map to chromosome 19p13.3. The human CDH1 locus (FZR1/ HCDH1) was noted to be closely linked to nuclear factor I/C (NFIC) and Sox-like transcription factor (HMG20B or SOXL). This map location of CDH1 was confirmed by PCR analysis and by restriction mapping. We verified that the distance between NFIC and FZR1 is within 30kb, while the FZR1 and HMG20B genes are separated by no more than 37kb (Figure 2A).

Approximately 43kb of assembled sequence from cosmid R33374 was obtained. Human CDH1 locus spans 29.8kb. The sizes of all exons and introns were verified by PCR analysis. The gene contains at least 14 exons varying in size from 63bp to 1.4kb (Figure 2B). The sequences of all splice junctions and of the 5' upstream region were confirmed by direct sequencing of PCR products amplified from human genomic DNA.

### **Identification of an alternatively spliced isoform of human CDH1**

We isolated an alternative form of CDH1 from a human heart cDNA library (GenBank AF433157). We noted that this cDNA represents an alternatively spliced isoform. This novel isoform designated CDH1 $\beta$  can be produced by splicing exon 8 to exon 5, skipping exons 6 and 7 (267 nucleotides; Figure 2B). The CDH1 $\beta$  protein lost 89 residues containing one WD40 repeat, four CDK phosphorylation sites and a cluster of positively charged residues (Figure 1A). This raises the possibility that CDH1 $\beta$  might function or



be regulated in a manner different from the original isoform of human CDH1 renamed as CDH1 $\alpha$ . Noteworthy, CDH1 $\beta$  has been independently isolated by another group (GenBank AB013463) and by the Mammalian Gene Collection at the National Institutes of Health (<http://mgc.nci.nih.gov>), suggesting that it is unlikely a cloning artifact or an erroneously spliced RNA.

### **Differential expression of CDH1 $\alpha$ and CDH1 $\beta$ in human tissues and cancer cells**

To reassess the expression pattern of CDH1 $\alpha$  and CDH1 $\beta$  mRNA and in human tissues and cancer cells, we performed Northern blotting (Figure 3A) and RT-PCR (Figure 3, B-D). CDH1 $\alpha$  is the major isoform detected in Northern blot analysis (Figure 3A, highlighted by arrows). We did not observe the 2.7-kb CDH1 $\beta$  mRNA in most human tissues and cells by Northern blotting with a probe that was expected to hybridize equally well to both CDH1 $\alpha$  (3.0 kb) and CDH1 $\beta$  (2.7 kb) transcripts (Figure 3A). However, a weak band of >2.4 kb in size (highlighted by asterisks) was reproducibly seen in brain (Figure 3A, lane 1), HeLa cells (lane 14), and A549 cells (lane 19). This transcript possibly derived from CDH1 $\beta$  is much less abundant than the CDH1 $\alpha$  mRNA. Using antibodies raised against the N-terminal and C-terminal sequences shared by CDH1 $\alpha$  (55 kDa) and CDH1 $\beta$  (45kDa), we can't detect the 45-kDa CDH1 $\beta$  protein in HeLa and HepG2 cells by immunoblotting (data not shown, see Figure 5A below for an example). These results implicate CDH1 $\alpha$  as a predominant isoform in many tissues and cells. The possibly low abundance of CDH1 $\beta$  transcript and protein in cells prompted us to use more sensitive and accurate technology for RNA detection. To this end, we tested several sets of primers for amplification of human CDH1 $\alpha$  and CDH1 $\beta$  in model experiments using CDH1 $\alpha$  and CDH1 $\beta$  plasmids as template. In our experiments primer sets A and B were able to differentially amplify CDH1 $\alpha$  and CDH1 $\beta$ . Figure 3B shows that primer set A can specifically amplify CDH1 $\alpha$  from templates containing either CDH1 $\alpha$  alone (lane 1) or CDH1 $\alpha$  plus CDH1 $\beta$  (lane 4). Likewise, primer set B is specific for CDH1 $\beta$  from templates containing CDH1 $\beta$  alone (lane 2) or CDH1 $\alpha$  plus CDH1 $\beta$  (lane 3). Next, we tested RNAs from various human tissues and cells using primer sets A (Figure 3C) and B

(Figure 3D). We noted that both CDH1 $\alpha$  and CDH1 $\beta$  were detected in multiple sources. The CDH1 $\alpha$  transcript is relatively more abundant in heart, liver, spleen, HeLa cells, HepG2 cells and H1299 cells (Figure 3C). In contrast, the CDH1 $\beta$  mRNA is expressed in heart, spleen, and H1299 lung cancer cells (Figure 3D). The identity of the CDH1 $\alpha$  and CDH1 $\beta$  amplification products was verified by DNA sequencing. Thus, CDH1 $\beta$  can be found in some tissues and cells albeit in low abundance. The distinct expression patterns of CDH1 $\alpha$  and CDH1 $\beta$  suggest that they might serve different and complementary functions.

### **APC-binding and APC-activating activity of CDH1 $\beta$ in HeLa cells**

While our study is in progress, four CDH1 homologs have been identified in chick [43]. All of them bind and activate APC in vitro and in cultured cells. To our surprise, all four chicken CDH1 homologs contain sequences corresponding to exons 6 and 7 of human CDH1 gene. They represent four different gene loci and were not produced by alternative splicing. Thus, none of them are structurally equivalent to human CDH1 $\beta$ . However, vertebrate CDH1 variants generated through distinct mechanisms could still be functionally related. Based on this reasoning, human CDH1 $\beta$  isoform and chicken CDH1 homologs could serve similar or related functions intracellularly. To test this hypothesis, we asked whether human CDH1 $\beta$  binds to and activates APC in HeLa cells.

First, we expressed hemagglutinin (HA)-tagged CDH1 $\alpha$  and CDH1 $\beta$  proteins in HeLa cells. As expected, cells transfected with CDH1 $\alpha$  and CDH1 $\beta$  expression plasmids produced 55kDa and 45kDa proteins reactive to anti-HA antibody, respectively (Figure 4A, compare lane 2 to lane 1). This provides the direct evidence that a CDH1 $\beta$  protein distinct to CDH1 $\alpha$  can be translated from the CDH1 $\beta$  transcript we have identified. We next performed co-immunoprecipitation experiments with extracts of CDH1 $\alpha$ - and CDH1 $\beta$ -expressing cells. Figure 4B shows that the precipitates prepared with an irrelevant antibody against the FLAG tag did not contain CDC27, a subunit of the APC complex. In contrast, CDC27 was found in the protein complex pulled down by the anti-HA antibody (Figure 4C). These data suggest that CDH1 $\beta$  associates with CDC27 in HeLa cells as tightly as CDH1 $\alpha$  (Figure 4C, compare lane 2 to lane 1).

To determine whether association of CDH1 $\beta$  with APC leads to activation of proteolysis, we constructed a reporter plasmid expressing the firefly luciferase fused to the D box of cyclin B1. This plasmid (pCYCB1-Luc), when transfected into cells, can reflect the in vivo activity of the APC-dependent ubiquitination and degradation system in real time [24,44]. We observed that the overexpression of either CDH1 $\alpha$  or CDH1 $\beta$  protein significantly increased the degradation of the cyclin B1-luciferase fusion protein (Figure 4D). APC-dependent proteolysis is through proteasome. If CDH1 $\alpha$  and CDH1 $\beta$  specifically target APC, their stimulatory effect on cyclin B1 degradation should be responsive to proteasome inhibitors. Indeed, when we incubated the CDH1 $\alpha$ - and CDH1 $\beta$ -expressing cells with proteasome inhibitor MG-132, the CDH1 $\alpha$ -/CDH1 $\beta$ -induced degradation of cyclin B1-luciferase was prevented (Figure 4D, compare filled with unfilled columns). Thus, both CDH1 $\alpha$  and CDH1 $\beta$  are specific activators of APC-dependent and proteasome-mediated destruction of cyclin B1 in HeLa cells.

### **Differential localization of CDH1 $\alpha$ and CDH1 $\beta$**

Next we queried for the subcellular localization of CDH1 $\alpha$  and CDH1 $\beta$ . We generated two specific antisera against CDH1 ( $\alpha$ -CDH1N and  $\alpha$ -CDH1C, Figure 1A) and stained cultured cells using the purified antibodies. As a first step, the specificity of the anti-CDH1 antibodies was verified by Western blotting. For one example, HepG2 cell lysates were probed with either  $\alpha$ -CDH1C or  $\alpha$ -CDH1C pre-incubated with excessive amount of immunizing peptide (Figure 5A). A single protein species corresponding to the 55-kD CDH1 $\alpha$  was detected from HepG2 cells (Figure 5A, lane 1) and the band was not seen with a pre-absorbed serum (lane 2). Then we stained HepG2 cells with either  $\alpha$ -CDH1C or  $\alpha$ -CDH1C pre-absorbed with immunizing peptide. The endogenous CDH1 $\alpha$  in HepG2 cells localized to the nucleus and the specificity of this nuclear staining was corroborated by peptide blocking experiment (data not shown). These results generally agree with recent findings on the nuclear localization of CDH1 [45,46].

We were concerned that CDH1 $\beta$  was not detected in Western blotting and immunostaining due to the low abundance (Figure 5A). To remedy this and to distinguish the CDH1 $\beta$  staining from that of CDH1 $\alpha$ , we stained for epitope-tagged CDH1 $\alpha$  and

CDH1 $\beta$  expressed from exogenously introduced plasmids (Figure 5B). HeLa cells transiently transfected with expression plasmids for HA-tagged CDH1 $\alpha$  (Figure 5B, panels 1 and 2) and CDH1 $\beta$  (panel 3) were stained with  $\alpha$ -CDH1N (panel 1) or anti-HA ( $\alpha$ -HA; panels 2 and 3) antibodies (Figure 5B). These antibodies reacted with HA-CDH1 $\alpha$ /HA-CDH1 $\beta$  in a highly specific manner. Notably, a nuclear localization pattern of CDH1 $\alpha$  was consistently seen with both  $\alpha$ -CDH1N and  $\alpha$ -HA. By sharp contrast, CDH1 $\beta$  localized homogenously to the cytoplasm (Figure 5B, panel 3). The identity of the HA-CDH1 $\beta$  protein was further verified by immunostaining with  $\alpha$ -CDH1N and  $\alpha$ -CDH1C. Both antibodies reacted with the overexpressed HA-CDH1 $\beta$  as well as the endogenous CDH1 $\alpha$  (data not shown).

Both CDH1 $\alpha$  and CDH1 $\beta$  bind to and activate APC (Figure 4), but they localize to different subcellular compartments (Figure 5B). In this setting, CDH1 $\alpha$  and CDH1 $\beta$  could encounter distinct sets of APC substrates at different locales. For example, they are able to activate cyclin B1 destruction (Figure 4B) and might cooperate with each other to mediate the timely and orderly degradation of nuclear and cytoplasmic cyclin B1. In another case, they might act differentially on the nuclear cyclins such as cyclin A and cyclin E. To explore how expression of CDH1 $\alpha$  and CDH1 $\beta$  might influence the proteolysis of cyclin A, we overexpressed HA-CDH1 $\alpha$  and HA-CDH1 $\beta$  in HeLa cells and co-stained for CDH1 and cyclins (Figure 5C). In agreement with previous studies [47], ambient cyclin A is predominantly nuclear (Figure 5C, panels 2 and 5). We observed that CDH1 $\alpha$  co-localized with cyclin A to the nucleus (Figure 5C, panel 3). In addition, the forced overexpression of CDH1 $\alpha$  correlated with the reduction of nuclear cyclin A (Figure 5C, panel 2, compare cell with an arrow with cells without arrow). This observation is generally consistent with reported findings [44]. On the other hand, CDH1 $\beta$  and cyclin A were found in different subcellular locations (Figure 5C, panel 6) and CDH1 $\beta$  overexpression did not lead to noticeable degradation of cyclin A (panel 5, compare cell with an arrow with cells without arrow). These results support the notion that human CDH1 $\alpha$  and CDH1 $\beta$  fulfill different functions at different locales of the cell.

**CDH1 $\alpha$  contains a nuclear localization signal which is absent in CDH1 $\beta$**

The differential localization of CDH1 $\alpha$  and CDH1 $\beta$  (Figure 5) suggests that the peptide fragment present in CDH1 $\alpha$  but absent in CDH1 $\beta$  might dictate nuclear import. Indeed, a cluster of nine positively charged residues can be found in that fragment (Figure 1, residues highlighted by “#”). This cluster might act as a nuclear localization signal in CDH1 $\alpha$ . To test this hypothesis, we fused the peptide sequence encoded by exons 6 and 7 of CDH1 $\alpha$  to green fluorescent protein (GFP). When overexpressed in HeLa cells, GFP alone was diffusely found in both the cytoplasm and the nucleus (Figure 6, panel 1). In contrast, when we overexpressed the chimeric protein GFPE67 in cells, the heterologous GFP protein was targeted into the nucleus by the nuclear localization signal resided apparently in the sequences encoded by exons 6 and 7 of CDH1 $\alpha$  (Figure 6, panel 2). Thus, CDH1 $\alpha$  harbors a nuclear localization signal which is absent in CDH1 $\beta$ . Identification and further characterization of this nuclear localization signal are reported elsewhere [46].

### **Differential activity of human CDH1 $\alpha$ and CDH1 $\beta$ in *Xenopus* embryos**

The co-localization studies shown above suggest that human CDH1 $\beta$  might be a poor activator of the APC-mediated degradation of nuclear cyclin A (Figure 5C). To compare the global influence of human CDH1 $\alpha$  and CDH1 $\beta$  on cell cycle progression, we used the *Xenopus* embryo microinjection system. We and others have shown that the injection of *Xenopus* CDH1 RNA into one blastomere of two-cell stage *Xenopus* embryos sufficiently induced a cell cycle arrest at the midblastula transition (MBT) due to continued activation of APC [30,48]. As such, the overexpression of CDH1 was phenotypically silent before the MBT (stage 8.5) and the cell division cycles seemed normal. However, immediately after the MBT the cells on the CDH1-injected side were significantly larger than those on the uninjected side. Indeed, when we overexpressed human CDH1 $\alpha$  in early *Xenopus* embryos by microinjecting CDH1 $\alpha$  RNA into one of the two blastomeres in stage 2, we observed a post-MBT arrest (Figure 7A, panel 3) exactly as described for *Xenopus* CDH1 (Figure 7B). As shown in a CDH1 $\alpha$ -injected embryo at late stage 9 (Figure 7A, panel 3), the cells overexpressing CDH1 $\alpha$  were more than five times bigger, implicating that the inhibition lasted for at least several cycles of

cell division. In control groups, the uninjected, mock-injected, and antisense CDH1 $\alpha$ -/CDH1 $\beta$ -injected embryos did not arrest cell division during the MBT (Figure 7A, panels 1 and 2; Figure 7B). Interestingly, the injection of human CDH1 $\beta$  RNA is phenotypically silent (Figure 7A, panel 4 and Figure 7B) and the injected embryos were indistinguishable from the controls (Figure 7A, compare panel 4 with panels 1 and 2). Thus, human CDH1 $\alpha$  and CDH1 $\beta$  have differential APC-activating activity in *Xenopus* embryos. CDH1 $\beta$  is incapable of inducing the post-MBT cell cycle arrest.

## DISCUSSION

Here we documented the differential expression, subcellular localization and activity of CDH1 $\alpha$  and CDH1 $\beta$ , two functional isoforms of human CDH1. CDC20 and CDH1 represent two multigene families of WD40 repeat proteins that are key regulators of APC (Figure 1). A novel alternatively spliced isoform of human CDH1, named CDH1 $\beta$ , was identified from a heart cDNA library (Figure 1 and Figure 2). The two CDH1 isoforms were expressed to different levels in various human tissues and cells (Figure 3). Both isoforms associate with APC subunit CDC27 and stimulate the APC-dependent proteolysis of cyclin B1 in cultured cells (Figure 4). CDH1 $\alpha$ , which harbors a nuclear localization signal absent in CDH1 $\beta$  (Figure 6), localizes to the nucleus (Figure 5). In contrast, CDH1 $\beta$  is cytosolic (Figure 5). Finally, CDH1 $\alpha$ , but not CDH1 $\beta$ , co-localizes with nuclear cyclin A, activates cyclin A degradation, and induces a post-MBT arrest in cell cycle progression in *Xenopus* embryos (Figure 5 and Figure 7). Our work suggests a complex spatial and temporal control of APC activation during the cell cycle.

One salient finding in our study is the differential expression, localization and activity of human CDH1 $\alpha$  and CDH1 $\beta$  generated through alternative splicing. Alternative splicing is very common in human cells and approximately 60% of human genes are estimated to have two or more transcripts [49]. Among the various types of alternative splicing that give rise to multiple isoforms of a gene, exon skipping can be caused by exonic and intronic mutations [50]. In this context, it would be of interest to identify and characterize the signals and mechanisms through which the production of CDH1 $\beta$  is induced.

Both CDH1 $\alpha$  and CDH1 $\beta$  can bind to the APC complex and activates the APC-dependent degradation of cyclin B1 in cultured cells (Figure 4). However, the presence of CDH1 $\alpha$  and CDH1 $\beta$  in different abundance (Figure 3) and at different locales (Figure 5) suggests that they might play different roles in cell cycle regulation. In line with this, we observed that they showed differential activity in the activation of APC both in cultured human cells (Figure 5C) and in *Xenopus* embryos (Figure 7). As yet the mechanism of the differential APC-activating activity of the two isoforms is not fully understood. One possibility is that CDH1 $\alpha$  and CDH1 $\beta$  may be active on different sets of substrates. We

note that some of our data are in support of this model. Thus, both CDH1 $\alpha$  and CDH1 $\beta$  bind to APC and stimulate the degradation of cyclin B1 (Figure 4). Cyclin B1 shuttles between the nucleus and the cytoplasm [47] while the cyclin B1-luciferase fusion protein used in this study is both cytoplasmic and nuclear (data not shown). This localization pattern is compatible with the observed degradation of cyclin B1 by both CDH1 $\alpha$  and CDH1 $\beta$ . It is also noteworthy that cyclin B1 destruction also takes place at metaphase when the nuclear envelope has been broken down [51]. In this scenario, cyclin B1 and possibly other cyclins are accessible to both CDH1 $\alpha$  and CDH1 $\beta$ . On the other hand, CDH1 $\beta$  is apparently a poor activator of the APC-mediated proteolysis of nuclear cyclin A (Figure 5). Plausibly, CDH1 $\alpha$  and CDH1 $\beta$  may have differential activity on cyclin A and other cell cycle regulators. In this regard, further biochemical analyses are required to compare in parallel the ubiquitination-stimulating activity of CDH1 $\alpha$  and CDH1 $\beta$  on different targets.

Four CDH1 homologs in chick derived from four different loci have recently been documented [43]. These chicken CDH1 homologs share 62%-95% identical amino acid residues with human CDH1 $\alpha$ , but are differentially expressed and localized. Importantly, they exhibit quantitatively different APC-stimulating activity on different substrates [43]. These CDH1 homologs are plausibly derived from gene duplications. None of the four chicken CDH1 homologs were generated through alternative splicing. Neither were they structurally equivalent to human CDH1 $\beta$  identified in this work. Up to date human orthologs of the four chicken CDH1 proteins have not been described except for the original human CDH1 $\alpha$  isoform and another EST clone possibly orthologous to the most divergent ChkCDH1-D. With the working draft of human genome sequence available, we are still uncertain whether there are four human CDH1 loci corresponding to those in chick. It is not impossible that at least some of the gene duplication events leading to the production of four CDH1 homologs in chick might have taken place after the separation of birds from other vertebrates. On the other hand, it remains to be seen whether CDH1 isoforms orthologous to human CDH1 $\beta$  exist in other species including chick. Nevertheless, our working model is that the various CDH1 isoforms in vertebrates have differential activity on different sets of targets. In this regard, gene duplication, which



produced four CDH1 homologs in chick, and alternative splicing, which led to the formation of human CDH1 $\beta$ , likely represent two major mechanisms by which CDH1 isoforms could be generated. According to our model, the differential activity of CDH1 $\alpha$  and CDH1 $\beta$  in *Xenopus* embryos is attributed to their differential ability to activate the degradation of different targets such as cyclin A.

The subcellular localization pattern (Figure 5B) implicates human CDH1 $\alpha$  as a nuclear activator of APC. Consistent with this, the forced overexpression of CDH1 $\alpha$  in the nucleus correlates with the degradation of nuclear cyclin A (Figure 5C). The nuclear localization of human CDH1 $\alpha$  ensures its accessibility to nuclear regulators and targets such as CDC14 [52], Emi1 [53], APC subunits [54] and cyclins [47]. For example, the colocalization of CDH1 $\alpha$  and cyclin A (Figure 4C) provides the opportunity for the suggested regulation of CDH1 $\alpha$ -APC by cyclin A [24,55]. In contrast to the nuclear pattern of CDH1 $\alpha$ , CDC20 resides ambiently in the cytoplasm [18]. Thus, CDC20 and CDH1 $\alpha$  are activated in different compartments of a cell. This adds another level of complexity to the regulation of APC. In another perspective, the steady-state localizations of many cell cycle regulatory proteins are determined by the relative rates of nuclear import and export, because they shuttle continuously between the nucleus and the cytoplasm [47,56,57]. In this regard, it will be of great interest to investigate the dynamic nucleocytoplasmic shuttling of CDH1 $\alpha$  and CDH1 $\beta$ .

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## FIGURE LEGENDS

**Figure 1 CDC20 and CDH1 represent two families of WD40 proteins. (A)** Amino acid sequence of human and mouse CDH1. The human sequence is in capital. The mouse sequence different from the human is indicated with small letters in brackets lying on the top. Sequences of the N-terminal and C-terminal synthetic peptides used to raise antisera in rabbits are underlined. Putative CDK phosphorylation sites are shaded. Residues absent from alternatively spliced variant CDH1 $\beta$  are boxed. The positively charged residues that act putatively as a nuclear localization signal are highlighted by “#”. The seven WD40 repeats are indicated. **(B)** Consensus phylogenetic tree of all CDC20-/CDH1-related proteins in the extant databases. Phylogenies were inferred from protein sequences aligned with the ClustalW program ([www.ch.embnet.org/software/clustalw.html](http://www.ch.embnet.org/software/clustalw.html)). Kimura’s distances were computed with the PROTDIST program in the PHYLIP package (version 3.573, J. Felsenstein, University of Washington). Tree reconstruction was based on the neighbor-joining method (NEIGHBOR). SEQBOOT and CONSENSUS programs in the same package were used to perform bootstrap replication and to produce the majority rule consensus tree from 100 replicates. Numbers on the nodes are bootstrap confidence probabilities (%). GenPept (gp), EMBL (emb), PIR (pir), or SWISS-PROT (sp) protein accession numbers of the sequences are: CDC20B-thale cress, gp/AF160760; CDC20C-thale cress, pir/T01768; CDC20-rape, emb/CAA11819.1; CDC20A-thale cress, gp/AAF14048.1; CDC20-carrot, pir/T14352; CDC20-rat, gp/AAC14741.1; CDC20-human, gp/AAD16405.1; CDC20-frog, gp/AAC41376.1; CDC20-clam, gp/AAC06232.1; CDC20A-fission yeast, pir/T41719; CDC20-budding yeast, gp/BAA03957.1; CDC20-nematode, pir/T27762; CDC20-trichomonad, gp/AAB51112.1; CDC20B-fission yeast, pir/T41034; CDC20C-fission yeast, pir/T41148; AMA1/CDC20B-budding yeast, gp/NP\_011741.2; CDH1-mouse, gp/AAD52029.1; CDH1-human, gp/AAC62835.1; CDH1-frog, emb/CAA74576.1; CDH1-fly, gp/AAF45973.1; CDH1-nematode, pir/T27730; CDH2-fly, gp/AAF47111.1; CDH1-barrel medic, gp/AAD22612.1; CDH1-thale cress, pir/T09351; CDH1 $\beta$ -fission yeast, pir/T40614; CDH1 $\alpha$ -fission yeast, pir/T37680; CDH1-budding yeast, gp/NP\_011512.1.

**Figure 2 Genomic characterization of human CDH1.** (A) Schematic delineation of human CDH1 at 19p13.3. Arrows underlying the genes indicate orientation of transcription. FZR1/HCDH1, human fizzy-related 1 or CDH1; INSR, insulin receptor; NF1C, nuclear factor I/C; HMG20B, high mobility group 20B or Sox-like transcription factor; Tel, telomeric; Cen, centromeric. (B) Genomic structure of the human CDH1 gene. Non-coding (open boxes) and coding exons (filled boxes) are shown.

**Figure 3 Expression of CDH1 mRNA in human tissues and cells.** (A) Northern blot analysis of CDH1 mRNA in human tissues and cells. CDH1-specific probe hybridizes to a predominant mRNA migrating with a size of ~3 kb (arrow). This size corresponds to CDH1 $\alpha$  (3.0 kb). A band possibly derived from CDH1 $\beta$  (2.7 kb) is highlighted by asterisk (\*). The hybridization signal of CDH1 $\beta$  is much weaker (~ 10-fold) than that of CDH1 $\alpha$ , but it is reproducibly seen in some tissues and cells.  $\beta$ -actin-specific hybridization was performed on the same blot after stripping the CDH1 $\alpha$  signal in order to verify equivalent loadings. Blots were from CLONTECH and all lanes contain ~2  $\mu$ g of polyadenylated RNA. Similar results were obtained with three different probes corresponding to the N-terminal, C-terminal, and central parts of CDH1 $\alpha$ . (B) Isoform-specific PCR analysis of CDH1 $\alpha$  and CDH1 $\beta$ . The PCR reactions contain the indicated primer sets (set A for lanes 1 and 4, and set B for lanes 2 and 3) and templates (plasmid pCDH1 $\alpha$  for lane 1, pCDH1 $\beta$  for lane 2, and pCDH1 $\alpha$  + pCDH1 $\beta$  for lanes 3 and 4). Results are representative of triplicate experiments. Sequences for the PCR primers are 5'-GTG CAG GAC CCG CAG ACT GAG-3' plus 5'-CTC CGT GTA CTG CTG CAC GGG-3' (primer set A, size of CDH1 $\alpha$  amplification product: 740bp), and 5'-GAG GAC CGC AGG CTG CAG CCC-3' plus 5'-CTG CTG CAC GGG GCT CAG GCT CGA-3' (primer set B, size of CDH1 $\beta$  amplification product: 443bp). M: molecular weight marker. (C) RT-PCR analysis of CDH1 $\alpha$  mRNA in human tissues and cell lines. The PCR reactions contain primer set A and total RNAs from the indicated sources (HepG2: hepatoma cells, U2 OS: osteosarcoma cells, H1299: lung cancer cells, AGS: gastric adenocarcinoma cells, MKN: stomach adenocarcinoma cells, Chang: Chang cells possibly established via HeLa cell contaminant). Results are representative of duplicate

amplifications of two independent preparations of RNA samples. **(D)** RT-PCR analysis of CDH1 $\beta$  mRNA in human tissues and cell lines. The PCR reactions contain primer set B and total RNA from the indicated sources.

**Figure 4 Both CDH1 $\alpha$  and CDH1 $\beta$  associate with APC and activate APC-dependent degradation of cyclin B1.** **(A)** Expression of CDH1 $\alpha$  and CDH1 $\beta$  in HeLa cells. Expression plasmids for HA-tagged CDH1 $\alpha$  and CDH1 $\beta$  were transiently into cells. Extracts of transfected cells were probed with a monoclonal anti-HA antibody. **(B-C)** Co-immunoprecipitation experiments. HeLa cells expressing HA-tagged CDH1 $\alpha$  (lane 1) and CDH1 $\beta$  (lane 2) were immunoprecipitated with mouse anti-FLAG (panel B) or anti-HA (panel C). The precipitates were resolved on SDS-PAGE and the Western blots were probed with rabbit anti-CDC27. **(D)** CDH1 $\alpha$  and CDH1 $\beta$  stimulate cyclin B1 degradation. Cells were co-transfected with pCYCB1-Luc reporter plus empty vector, or plus CDH1 $\alpha$  expression plasmid, or plus CDH1 $\beta$  expression plasmid. Transfected cells were untreated ( $\square$ ) or treated ( $\blacksquare$ ) with proteasome inhibitor MG-132 (25  $\mu$ M) for 12 hours before harvest.

**Figure 5 Differential localization of CDH1 $\alpha$  and CDH1 $\beta$ .** **(A)** Western blotting. Extracts of HepG2 cells (~20  $\mu$ g) were resolved by SDS-PAGE. Immunoblotting was performed using purified  $\alpha$ -CDH1C serum. The position of CDH1 $\alpha$  (55 kDa) is highlighted. The  $\alpha$ -CDH1C antibody was purified through a HiTrap NHS-activated affinity column (Pharmacia) coupling to the immunizing peptide. A duplicate blot was separately probed with  $\alpha$ -CDH1C pre-incubated with 3 $\mu$ g of immunizing peptide ( $-\alpha$ -CDH1C w/ pep.; lane 2). Similar results were obtained using another antibody  $\alpha$ -CDH1N raised against the N-terminal sequences shared by CDH1 $\alpha$  and CDH1 $\beta$  (data not shown). **(B)** Subcellular localization of exogenously expressed CDH1 $\alpha$  and CDH1 $\beta$  in HeLa cells. Cells were transfected respectively with expression vectors pHACDH1 $\alpha$  (panels 1 and 2) and pHACDH1 $\beta$  (panel 3), in which the expression of HA-tagged CDH1 $\alpha$ /CDH1 $\beta$  is driven by the SV40 promoter. Cells were fixed 36 hours after transfection and stained with  $\alpha$ -CDH1N (panel 1) or  $\alpha$ -HA (panels 2 and 3) antibodies.

The same fields of the cells were also monitored by phase contrast microscopy (panel 1', 2' and 3'). vec.: expression vector. ab.: antibody. Bar, 20 $\mu$ m. The patterns shown in panels 2 and 3 are representative of 85% and 79%, respectively, of 200 CDH1 $\alpha$ -/CDH1 $\beta$ -expressing cells. (C) Differential co-localization of CDH1 $\alpha$  and CDH1 $\beta$  with cyclin A. HeLa cells were transfected with pHACDH1 $\alpha$  (panels 1-3) or pHACDH1 $\beta$  (panels 4-6). Cells were co-stained with mouse  $\alpha$ -HA (panels 1 and 4) and rabbit  $\alpha$ -cyclin A (panels 2 and 5). In panels 3 and 6, the CDH1 (green; probed with  $\alpha$ -HA) and cyclin A (red) fluorescent signals were overlaid by computer assistance. Co-localization is shown in yellow. The same fields are shown in panels 1-3 and 4-6. Bar, 20 $\mu$ m. The patterns shown in panels 3 and 6 are representative of 68% and 63%, respectively, of 200 CDH1 $\alpha$ -/CDH1 $\beta$ -expressing cells.

**Figure 6 Peptide sequences encoded by exons 6 and 7 of human CDH1 $\alpha$  target heterologous GFP protein to the nucleus.** HeLa cells were transfected with pEGFP-C1 (panel 1) or pEGFPE67 (panel 2). Unfixed cells were observed directly under the fluorescence microscope 24 hours after transfection. The same fields of the cells were also monitored by phase contrast microscopy (panel 1' and 2'). Arrows indicate transfected cells. The pattern shown in panel 2 is representative of 62% of 200 GFPE67-expressing cells. Bar, 20 $\mu$ m.

**Figure 7 Differential activity of human CDH1 $\alpha$  and CDH1 $\beta$  in *Xenopus* embryos.** (A) Representative images of embryos. (B) Graphic quantitation of post-MBT cell cycle arrest phenotype induced by expression of CDH1 $\alpha$ . One blastomere of the 2-cell stage embryos was uninjected (uninj.; panel 1), mock-injected with PBS (mock; panel 2) or injected separately with 4 $\mu$ g of the indicated RNAs: *Xenopus* CDH1 (XCDH1), human CDH1 $\alpha$  (HCDH1 $\alpha$ , panel 3), human CDH1 $\beta$  (HCDH1 $\beta$ , panel 4), antisense human CDH1 $\alpha$  (as-HCDH1 $\alpha$ ), or antisense human CDH1 $\beta$  (as-HCDH1 $\beta$ ). For each determination thirty embryos were counted. Each bar represents the average values from three experiments. Images in A were photographed at stage 8.5-9.5. The arrow indicates the arrested large cells on the injected side.

**A**

(i) (vPcVs) (n)

1 MDQYERRLLRQIVIQNENTMPRVTEMRRLLTPASSPVS

SPSKHGDRFIPSRAGANWSVNFHRINENEKSPSQNRKAKDATSDN

(h)

GKDGGLAYSALLKNEELGAGIEKVDQDPQTEDRRLQPSITPEKKGFLT

(s) # # # # # # #

YSLSTKRSSPEDDGNDVSPYSLSFVSNKSQKLLRSPRKPTRKISKIP

#

FKVIDAPELQDDFYINLVWSSINVLVGLGTCVYLWSA

CTSQVTRLCDLSVEGDSVTSVGSERGNLVAVGTHKGFVQIWDA

(d)

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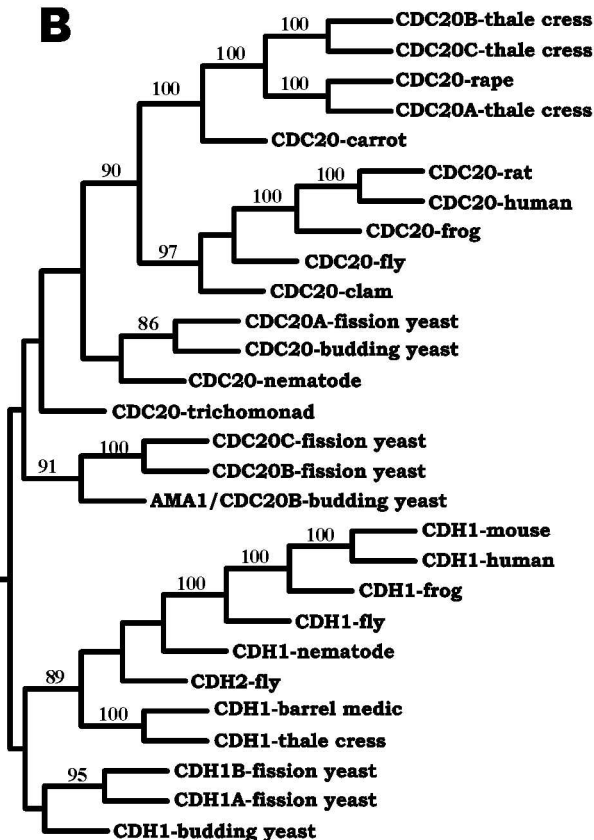
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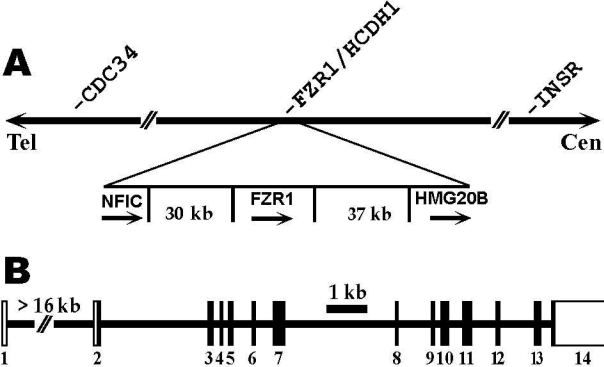
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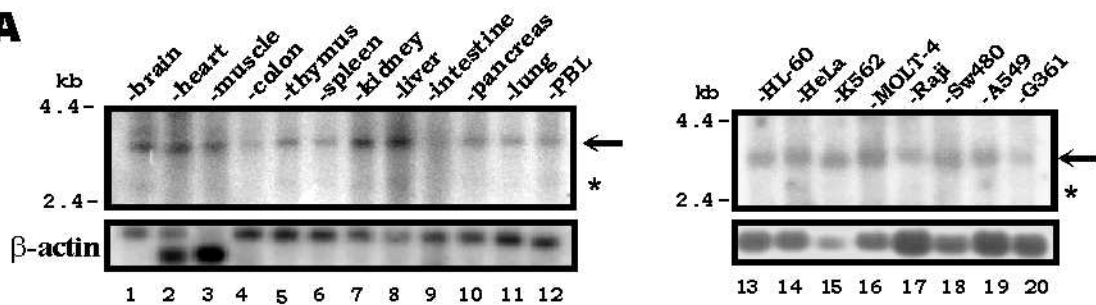
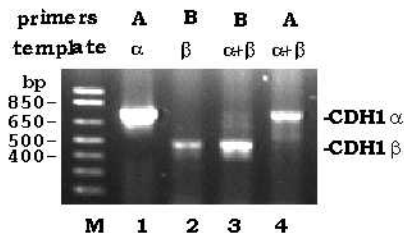
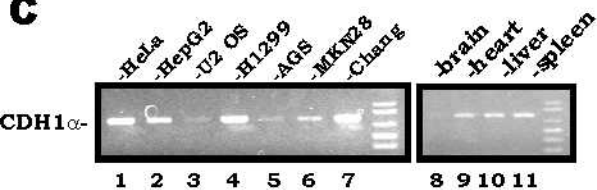
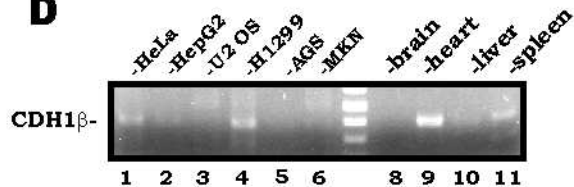
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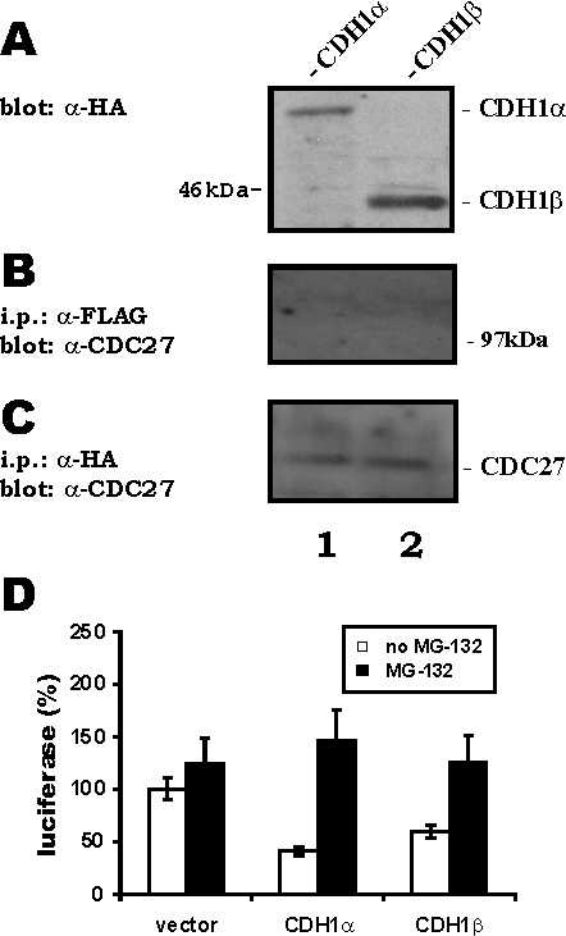
WD40 repeats

**B****Figure 1**



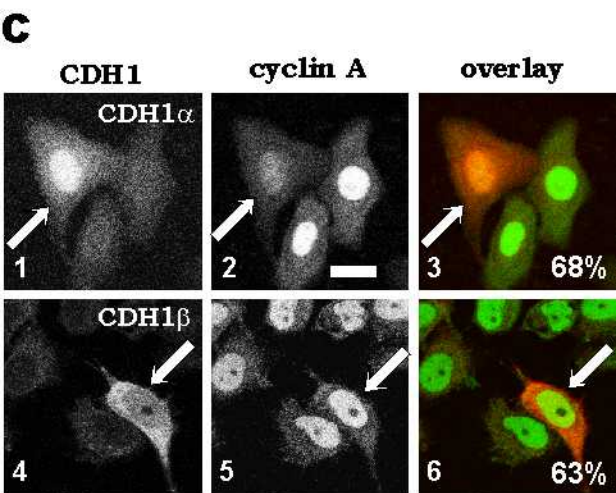
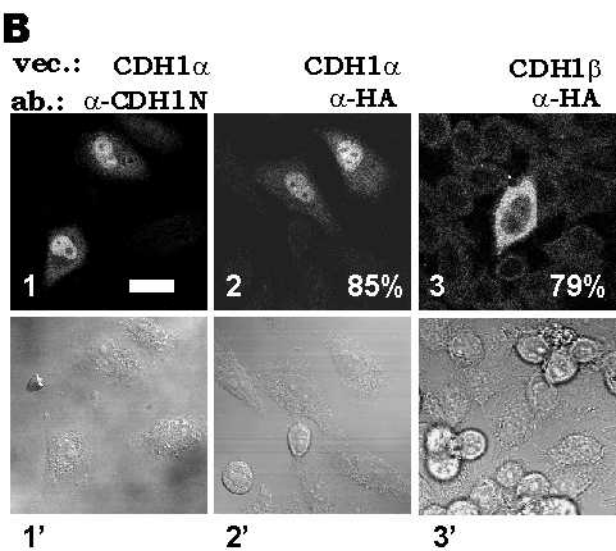
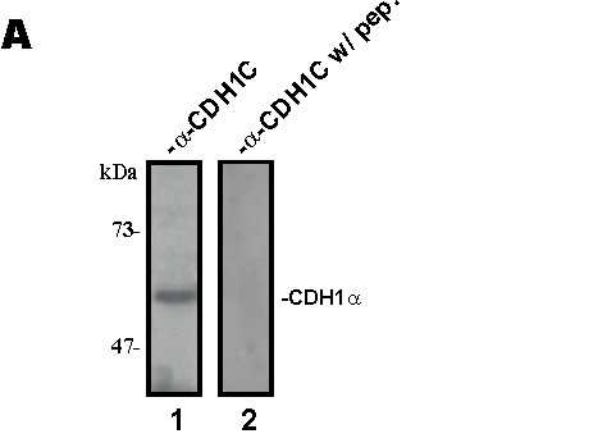
**Figure 2**

**A****B****C****D****Figure 3**



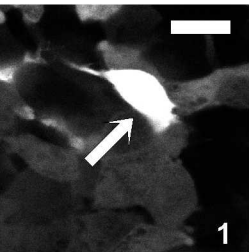
**Figure 4**



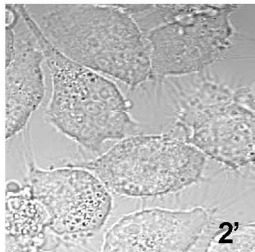
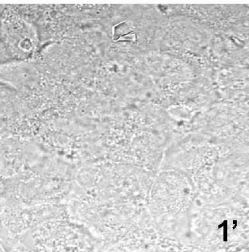
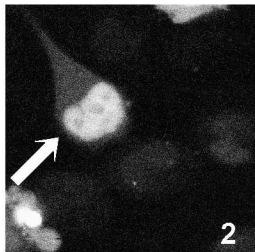


**Figure 5**

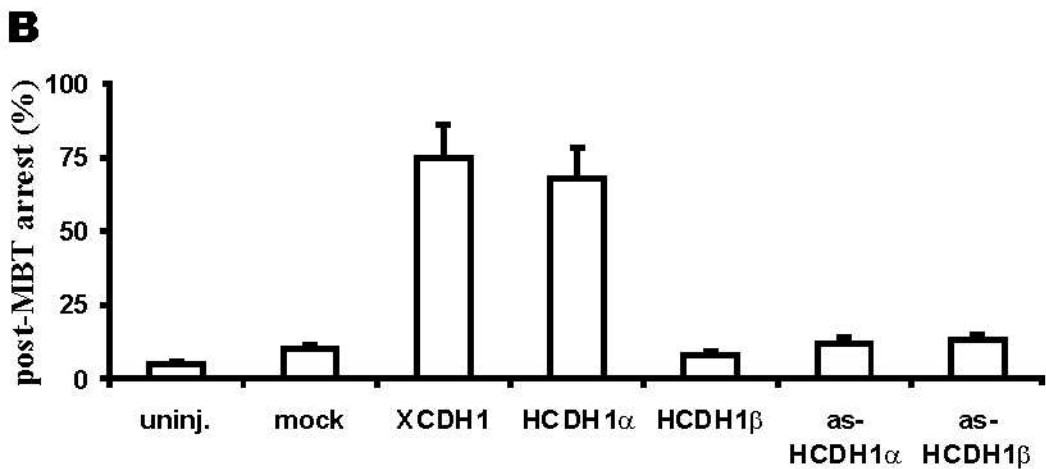
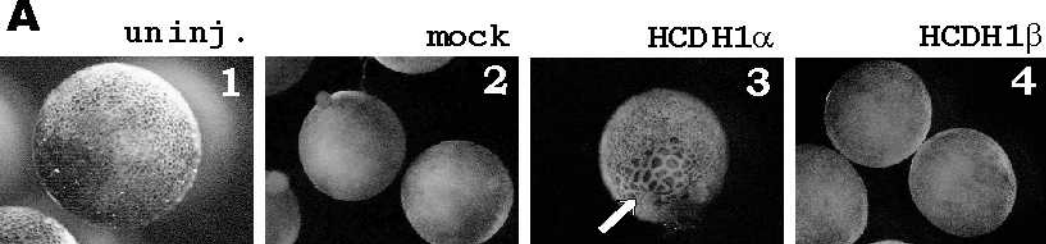
GFP



GFPE67



**Figure 6**



**Figure 7**