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The APC regulator CDH1 is essential for the progression of embryonic cell cycles in *Xenopus*

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Abstract

The orderly progression of cell cycle depends on timely destruction of key regulators through ubiquitin-mediated proteolysis. The anaphase-promoting complex (APC) is a major component of this degradation machinery and its activation is regulated by CDC20 and CDH1. We demonstrate here that CDH1 mRNA is ubiquitously expressed in *Xenopus* embryos of all developmental stages. Loss of CDH1 function during early embryonic cell cycles leads to an immediate and prolonged arrest with low cyclin-dependent kinase activity. In contrast, ectopic overexpression of CDH1 induces cell cycle arrest during the first G_1 phase at the midblastula transition. CDH1-dependent degradation of cyclin A is likely involved in this G_1 arrest. Our findings establish the essential roles of CDH1 in embryonic cell cycles.

Keywords: Anaphase-promoting complex (APC); CDH1; Cell cycle arrest; *Xenopus* embryonic cell cycles; Cyclin degradation

The ubiquitin-dependent proteolysis precisely controls the order and timing of cell cycle. The anaphase-promoting complex (APC) is a major cellular ubiquitination system and it governs anaphase onset, mitotic exit as well as G_1 events [1–3]. APC contains multiple evolutionarily conserved subunits and acts as an ubiquitin ligase, which targets cyclins and other cell cycle regulators for proteolysis. Biochemical and genetic analyses have characterized CDC20 and CDH1 as APC activators and specificity factors [4–7].

CDC20 and CDH1 bind to APC transiently and facilitate substrate-specific ubiquitination. Many of the substrates recognized by CDC20 and CDH1 contain destruction (D) box [8]. Some proteins targeted by CDH1 have a distinct motif called KEN box [9]. It remains to be fully understood how CDC20 and CDH1 regulate APC. In one perspective, they are thought to mediate the temporal and spatial activation of APC through direct binding to different sets of substrates [10–12].

CDH1 is regulated both transcriptionally and post-transcriptionally. CDH1 transcript and protein rise and fall when cells enter and exit mitosis [13]. CDH1 is ubiquitously expressed in differentiated mammalian tissues, but is not detected in the early embryos of frogs [14]. The inhibitory phosphorylation of CDH1 has been widely accepted as a major regulatory mechanism [15–18], while it remains in dispute whether CDC20 phosphorylation can stimulate [19], repress [20], or has no influence [13] on the APC activity.

The APC has emerged as a downstream target of cell cycle checkpoints, which prevent the onset of anaphase in response to DNA damage or microtubule disruption [21]. Checkpoint-related proteins such as MAD2L1, MAD2L2, MAD3, BUB1, and BUB3 can associate with and inhibit CDC20 and CDH1, thereby transducing the checkpoint signal to the APC pathway [22–26]. We have previously identified human MAD1 and MAD2 [27–29]. Given that CDC20/CDH1 may be MAD1/MAD2 effectors, we sought to characterize CDC20/CDH1 using *Xenopus* embryos. Here we show that amphibian CDH1 mRNA is present at all developmental stages. Our loss-of-function and gain-of-function studies indicate an

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essential role of CDH1 for cell cycle progression both in early embryogenesis and after the midblastula transition (MBT).

Materials and methods

RNA and protein analysis. Total RNAs from *Xenopus* oocytes and embryos were isolated using TRIzol (Life Technologies). RT-PCR was performed with a reagent kit (Advantage RT-for-PCR) from CLONTECH.

Frog oocytes or embryos were homogenized in 4 volumes of an extraction buffer (25 mM Tris–HCl, pH 7.5, 70 mM KCl, 1 mM EDTA, 20% glycerol, 5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Homogenates were centrifuged at 12,000 rpm for 10 min. The supernatant was mixed with equal volume of SDS gel loading buffer.

Proteins were separated by SDS–PAGE, and electroblotted onto Immobilon-P membrane (Millipore) using a semidry blotter (Hoefer). Blots were visualized by chemiluminescence (ECL, Amersham). Rabbit antisera against *Xenopus* cyclin B1 and cyclin A2 [30] were kindly provided by Dr. Ulrich Strausfeld, Universität Konstanz, Germany. These antibodies were used at 1:1000 dilution.

Embryo manipulations. Eggs were collected from *Xenopus laevis* females, which had been injected with 500–700 units of human chorionic gonadotrophin (Sigma) 12 h before egg collection. Eggs were fertilized in vitro with minced testis. RNAs were synthesized and capped with reagents from Ambion.

Histone H1 kinase assay. Five *Xenopus* embryos were lysed and immuno-precipitated with 1 μg rabbit anti-CDK1 antibodies (Upstate). The precipitate was assayed for histone H1 kinase activity in a reaction mixture containing 20 μCi [γ-^{32}P] ATP and 2 μg histone H1. The reaction was incubated at room temperature for 15 min and then resolved by SDS–PAGE.

Site-directed mutagenesis. XCDH1-4A and XCDH1-4D mutants were generated by replacing serines 40, 151, 163, and threonine 121 with alanine or aspartate, respectively. XCDH1-AAA was constructed by substituting the arginine-valine-leucine motif (445–447) with three alanines. DNA sequencing was performed to verify that they have the desired changes and not any unexpected alterations. Details for plasmid construction are available upon request.

Results

CDH1 is expressed in Xenopus embryos of all developmental stages

Extraordinary changes in the cell cycle pattern occur during the early embryogenesis of animals [31]. In *Xenopus*, the early embryos undergo synchronous divisions up to the 12th cell cycle. Cleavage cycles 2–12 are rapid and consist of alternating S and M phases only. Then the division cycle slows down and becomes asynchronous, zygotic transcription begins, and gap phases (G_1 and G_2) are established; a change known as MBT. *Xenopus* CDH1 (XCDH1) was previously shown to be absent from the pre-MBT embryos [14]. However, it is difficult to reconcile this absence with the importance of the regulated oscillations of APC activity in early embryonic cell cycles [32]. Thus, we sought to re-address this issue using more sensitive technology for RNA detection. We performed semi-quantitative RT-PCR using histone H4 as internal control. Fig. 1 shows that CDH1 transcripts were detectable in both *Xenopus* oocytes and embryos of various developmental stages, from stage 1 (1-cell) to 18 (neural groove stage) and beyond. The relative amounts of CDH1 transcript in different stages were similar.

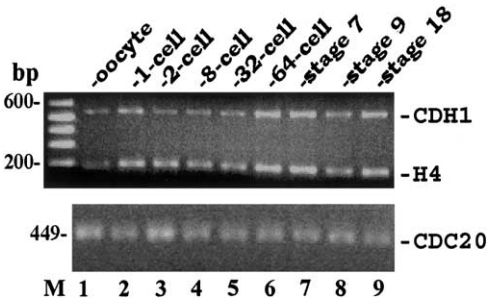

Fig. 1. Expression of CDH1 mRNA in *Xenopus* oocytes and embryos. RT-PCR was performed with sub-saturating amounts of templates and the amplification was in the linear range. Histone H4 was co-amplified as an internal control. Amplification signals for *Xenopus* CDC20 from the same samples were shown for comparison. Results are representative of duplicate amplifications of three independent preparations of RNA. Sequences for the PCR primers are: H4: 5′-CGGGATAA CATTCAGGGTA TCACT-3′ + 5′-ATCCATGGCG GTAACTGTC TTCCT-3′ (188 bp fragment); CDH1: 5′-ACAG ACTGAAGATAGA CGAC-3′ + 5′-AAGATCATCCT GTCCCTACTC-3′ (551 bp fragment); CDC20: 5′-CGTCTTCGTAATATGATCAG -3′ + 5′-AGACC ATACTATG GAGCAC-3′ (449 bp fragment).

CDH1 is necessary for progression of early embryonic cell cycles

To investigate how CDH1 influences early embryonic cell divisions in *Xenopus*, we performed loss-of-function studies by injecting antisense *Xenopus* CDH1 (as-XCDH1) into 2-cell stage embryos. To verify the integrity of endogenous CDH1 mRNA in as-XCDH1-injected embryos, we designed an RT-PCR that amplifies the sense XCDH1 messenger, but not the injected as-XCDH1 (Fig. 2A). From stage 3 (4-cell) up to stage 7 (64-cell), XCDH1 mRNA, as compared to the internal control (histone H4 mRNA) or to CDC20 mRNA, was either absent or significantly reduced (Fig. 2A, lanes 1–3) in embryos that had received as-XCDH1 in both blastomeres at the 2-cell stage. By contrast, diminution of XCDH1 mRNA was not observed in antisense mouse CDH- (as-MCDH1-) or mock-injected embryos (lanes 4–9). This analysis indicates the effectiveness of the antisense approach. Our attempt to detect XCDH1 protein was unsuccessful due to the lack of specific antibodies.

When we injected as-XCDH1 into one blastomere of 2-cell stage embryos, the injected cells (Fig. 2B, panel 3, see cells with an arrow) stopped dividing shortly after injection, whereas the blastomeres on the uninjected side were still dividing normally. A dynamic examination of

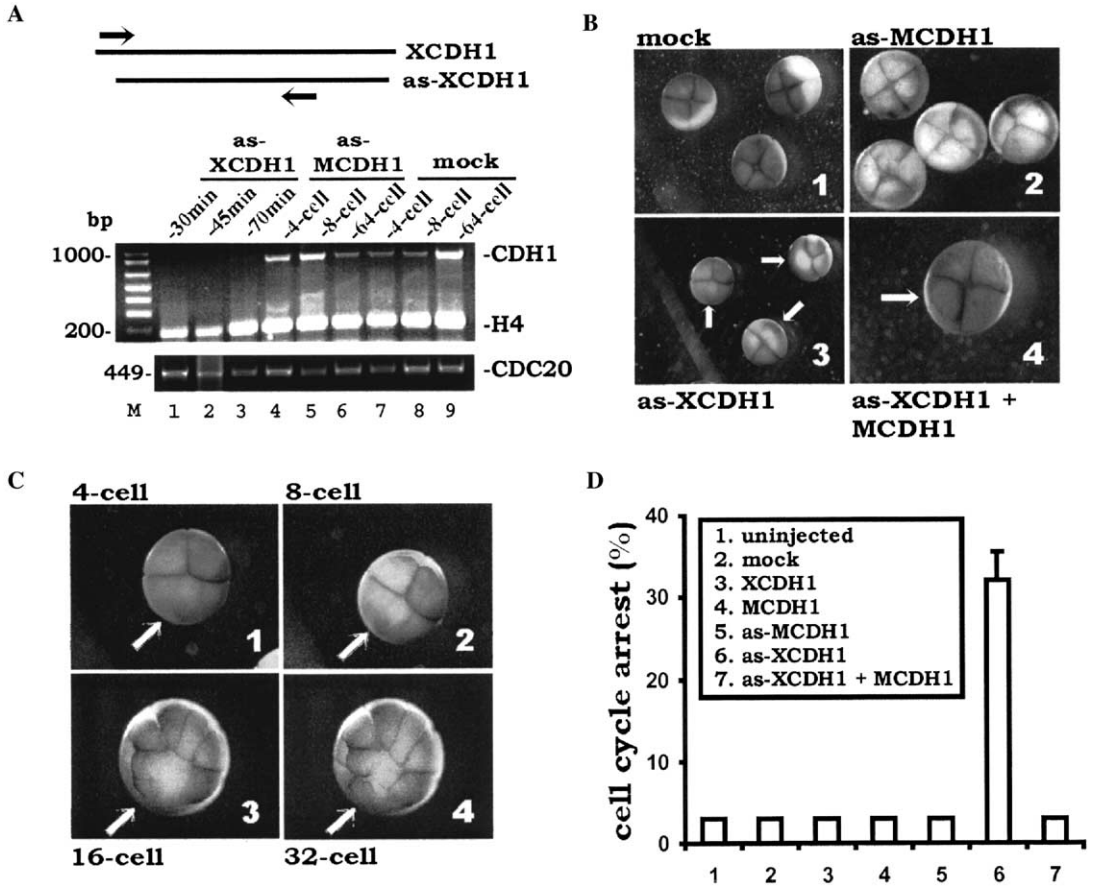

Fig. 2. *Xenopus* CDH1 is essential for early embryonic cell cycles. (A) Depletion of endogenous XCDH1 transcript by as-XCDH1 RNA. Both blastomeres of the 2-cell stage embryos were injected with 4 ng as-XCDH1 RNA (lanes 1–3), 4 ng as-MCDH1 RNA (lanes 4–6), or PBS (mock, lanes 7–9). Injected embryos were collected at indicated time (30, 45, and 70 min after injection) or stages (4-cell, 8-cell, and 64-cell), and RT-PCR was performed as in Fig. 1. Sequences for the XCDH1 primers are 5′-CTACGTGTCT CTATCTGCAA-3′ and 5′-AAGATCATCCT GTCCCTACTC-3′ (900 bp fragment). Arrows indicate the annealing positions of the primers. It is noteworthy that the forward primer anneals to the endogenous XCDH1 mRNA, but not to the injected as-XDH1 RNA. (B) Loss of XCDH1 function induces cell cycle arrest: representative images of embryos. One blastomere of the 2-cell stage embryos was injected with PBS (mock; panel 1), 4 ng as-MCDH1 RNA (panel 2), 4 ng as-XCDH1 RNA (panel 3), or 4 ng as-XCDH1 RNA + 4 ng MCDH1 RNA (panel 4). Images were photographed at the 4-cell stage. Arrows indicate the injected side. (C) Persistent cell cycle arrest induced by XCDH1 depletion. An as-XCDH1-injected embryo was followed for 4 consecutive divisions (4-cell, 8-cell, 16-cell, and 32-cell). (D) Graphic quantitation of the cell cycle arrest phenotype induced by XCDH1 depletion. Each of the 7 indicated groups had 20–30 embryos. Percentages of embryos that showed the phenotype of cell cycle arrest as in panel 3 of B were calculated. Each bar represents the average values from four experiments.

the injected embryos indicates that the inhibition of cell division by as-XCDH1 lasted for more than 4-cell cycles (Fig. 2C). To verify the specificity of action, we used as-MCDH1 RNA as a negative control. Since MCDH1 and XCDH1 mRNAs share less than 80% homology, as-MCDH1 RNA cannot efficiently deplete the XCDH1 messenger (Fig. 2A, lanes 4–6). Fig. 2B, panel 2 shows that the cleavage of blastomeres injected with as-MCDH1 RNA is normal, as compared to that of the mock-injected embryos (panel 1). The effects of as-XCDH1 RNA were further tested by a cross-species functional complementation assay. We showed that the endogenous XCDH1 mRNA is uninhibited by as-MCDH1 (Fig. 2B, panel 2; Fig. 2D, column 5). By the same reasoning, MCDH1 mRNA should not be deprived by as-XCDH1. Since the MCDH1 and XCDH1 proteins share 96% identity and 98% similarity in their amino acid sequences, they are expected to be functionally interchangeable. Indeed, the co-injection of MCDH1 sense RNA with as-XCDH1 rescued the arrest phenotype (Fig. 2B, compare panel 4–panel 3; Fig. 2D, compare column 7–column 6). Thus the expression of MCDH1 protein counteracts the cell cycle inhibitory effect of as-XCDH1 RNA. Collectively, our data suggest that as-XCDH1 inhibits cell division through deprivation of endogenous XCDH1 mRNA.

To shed light on the underlying mechanisms, we queried for the cyclin-dependent kinase (CDK) activity in the injected embryos. The phosphorylation of histone H1 by the active cyclin–CDK complex reflects the status

of APC. This activity goes up and down in mock-injected embryos as cells enter and exit mitosis (Fig. 3A, upper curve). By sharp contrast, the CDK1 activity precipitated from embryos injected with as-XCDH1 drops abruptly after the injection and remains low persistently (Fig. 3A, lower curve). Western blot analysis verified that cyclin B1 and cyclin A2 were absent or significantly reduced in the 16-cell and 64-cell embryos injected with as-XCDH1 (Fig. 3B, compare lanes 2 and 3 with lanes 5 and 6). Results from both the histone H1 kinase assay (Fig. 3A) and the Western blotting (Fig. 3B) consistently demonstrate that loss of XCDH1 function in early embryos leads to an immediate cell cycle arrest with low CDK activity.

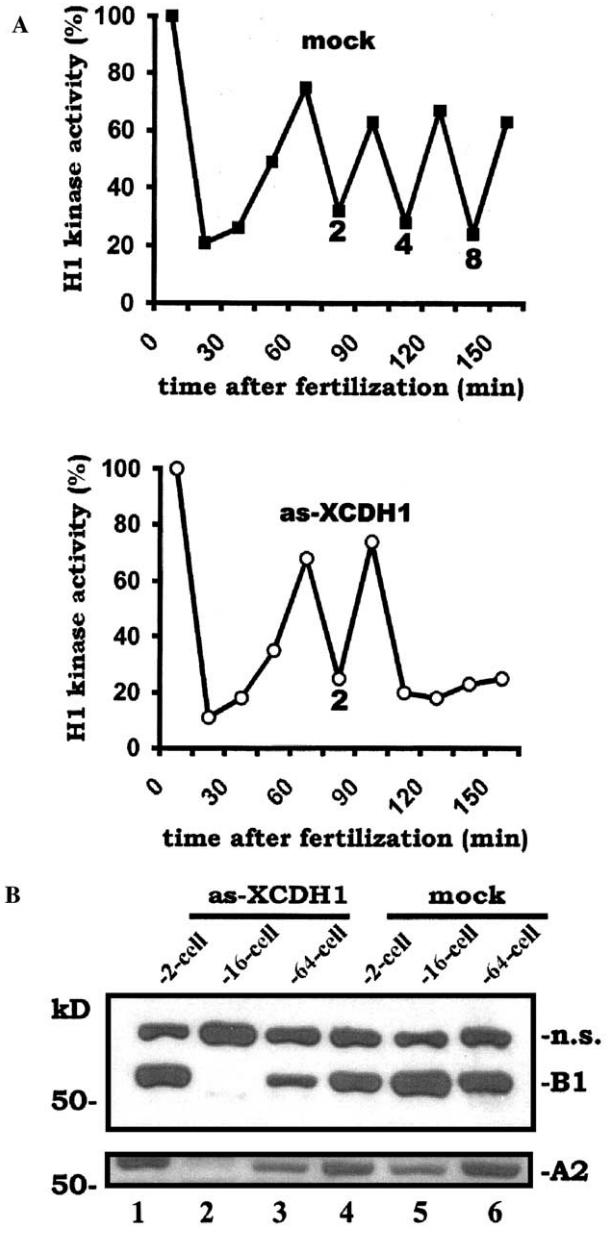

Fig. 3. Loss of CDH1 function in early embryonic cell cycles leads to prolonged arrest with low CDK activity. (A) Time course of histone H1 kinase activity immuno-precipitated with an antibody to CDK1. Both blastomeres of the 2-cell stage embryos were mock-injected with PBS (mock; upper panel), or injected with 4 ng as-XCDH1 RNA (lower panel). Five embryos from each group were collected and lysed every 15 min. The lysates were immunoprecipitated with anti-CDC2/CDK1 antibody and the precipitates were assayed for relative histone H1 kinase activity. Cell numbers of the embryos are indicated. Experiments were repeated twice with similar results. (B) Western blot analysis of cyclins. Five embryos were collected at each of the indicated stages. B1: cyclin B1. A2: cyclin A2. n.s.: non-specific band. Experiments were repeated twice with similar results.

Xenopus CDH1 induces G_1 arrest at the mid-blastula transition

To further characterize XCDH1, a series of gain-of-function studies were carried out. First, we overexpressed XCDH1 in early *Xenopus* embryos by microinjecting XCDH1 RNA into one of the two blastomeres at stage 2. Notably, overexpression of XCDH1 was phenotypically silent before the MBT (stage 8.5) and the cell cycle progression appeared normal (Fig. 2D, column 3). However, we noted that immediately after MBT the cells on the XCDH1-injected side were significantly larger than those on the uninjected side. This phenotype reflects a post-MBT inhibition of cell cycle progression. As shown in an XCDH1-injected embryo at late stage 9 (Fig. 4A, panel 3), the XCDH1-overexpressing cells were more than five times bigger, implicating that the inhibition lasted for at least several cycles of cell division. Injection of MCDH1 RNA generated a similar phenotype after the MBT—a prevalence of large cells, which had stopped dividing, on the injected side. A representative picture of an MCDH1-injected embryo at stage 9.5 was shown in Fig. 4A, panel 4. In control embryos, the mock- and the as-MCDH1-injection did not induce post-MBT arrest (Fig. 4A, panels 1 and 2; Fig. 4B, columns 2 and 3). Our results are consistent with recent findings that injection of XCDH1 RNA results in a post-MBT arrest with nuclei that stain diffusely for DNA [23]. Thus, overexpression of XCDH1 likely induces continued activation of APC leading to G_1 arrest.

It is generally accepted that CDK phosphorylation of CDH1 inhibits its APC-activating activity in yeast and humans [15–18]. In light of this, we asked whether CDH1 phosphorylation influences the induction of post-MBT arrest in *Xenopus*. We used XCDH1-4A and XCDH1-4D, two previously characterized [17] hypo- and hyper-phosphorylated mutants of XCDH1, in which the four conserved CDK phosphorylation sites were mutated to alanine and aspartate, respectively. Interestingly, both mutants were as active as XCDH1 wild type in the induction of post-MBT arrest (Fig. 4B, columns 6 and 7), suggesting that in this setting phosphorylation at these sites do not regulate the APC-activating activity of CDH1.

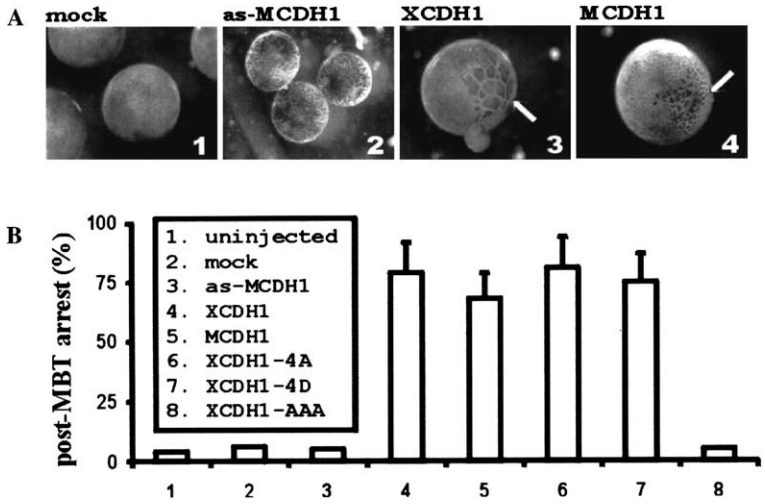

Fig. 4. Overexpression of CDH1 induces post-MBT arrest. (A) Representative images of embryos. One blastomere of the 2-cell stage embryos was mock-injected with PBS (mock; panel 1) or injected separately with 4 ng of the indicated RNAs: as-MCDH1 (panel 2), XCDH1 (panel 3), and MCDH1 (panel 4). Images were photographed at stage 8.5–9.5. The arrow indicates the arrested large cells on the injected side. (B) Graphic quantitation. For each indicated group 25–30 embryos were counted. Each bar represents the average values from three experiments.

CDH1 binds directly to cyclin A through a conserved cyclin-binding motif [33]. This interaction is essential for the cyclin A-dependent regulation of APC [17] and the APC-mediated proteolysis of cyclin A [33]. To investigate whether and how far the interaction with cyclin A is involved in the CDH1 induction of post-MBT arrest in *Xenopus*, we tested the effects of a previously known cyclin binding-deficient XCDH1 mutant with alanine substitutions in the RVL motif (XCDH1-AAA; Fig. 4B, column 8). One interpretation for the failure of the XCDH1-AAA mutant to induce post-MBT arrest is that CDH1-dependent degradation of cyclin A is required for maintenance of G_1.

Discussion

We demonstrate that CDH1 mRNA is expressed in *Xenopus* oocytes and embryos of all stages (Fig. 1). In contrast to our results, CDH1 protein has previously been shown to be absent from *Xenopus* oocytes and early embryos [14]. We can only speculate that low abundance might account for the undetectability of CDH1. Results from our loss-of-function experiments in frog embryos (Fig. 2) strongly support that CDH1, albeit in low abundance, is indispensable for the progression of early cell cycles.

Previously, *Xenopus* CDC20 has been shown to be necessary for APC-dependent proteolysis in early embryos [14]. The absence of CDH1 in those embryos has led to the hypothesis that CDC20 alone may regulate the destruction of all APC substrates. The absolute requirement for CDH1 in early embryonic cell cycles demonstrated here (Fig. 2) challenges this view and supports the notion that cooperation of CDC20 with CDH1 is crucial for determining the temporal and spatial order of APC activation in early embryogenesis.

The sequential activation of CDC20 and CDH1 is precisely regulated by multiple mechanisms that may show differences in various species [16]. Depletion of CDH1 from *Xenopus* embryos sufficiently induces an immediate and persistent arrest (Fig. 2) with low CDK activity (Fig. 3), pointing to an essential role of CDH1 in cell cycle regulation. Our findings that CDH1 is not a major activator of cyclin destruction in *Xenopus* early embryos (Fig. 3) are consistent with the concept that cyclins are degraded in metaphase by CDC20 [14,34–36]. The arrest phenotype induced by CDH1 depletion is likely due to the presence of anaphase inhibitors that are normally degraded by CDH1. One candidate of these inhibitors is securin and its degradation has been shown to be mediated by both CDC20 and CDH1 [10]. Thus, the arrest phenotype induced by loss of CDH1 function likely reflects a failure to exit from mitosis at a step prior to cytokinesis.

We noted that a regulatory loop among CDH1, CDC20, and cyclin A has been suggested as a conserved mechanism for cell cycle control [37]. In this scenario, the persistent CDH1 activity plays an important role in preventing CDC20 expression [37]. Should this be the case, one would expect depletion of CDH1 to activate CDC20. Hence, one interpretation to our findings that deprivation of CDH1 led to increased degradation of cyclins A2 and B1 (Fig. 3) could be that CDH1 negatively controls CDC20 level or activity. In this regard, further investigations are required to clarify where CDH1 depletion results in de-repression of CDC20.

Our gain-of-function studies in *Xenopus* embryos (Fig. 4) revealed another function of CDH1 in the maintenance of G_1. Notably, the overexpression of

XCDH1 before the MBT is phenotypically silent, indicating that CDH1 alone is not sufficient to establish a G_1 phase. However, as soon as the first G_1 is established, CDH1 is capable of maintaining a long-lasting G_1 arrest. Thus CDH1 may have a rate-limiting or pacemaking role in G_1. Ectopic overexpression of CDH1 has previously been shown to induce degradation of cyclins, inhibition of mitotic entry, endoreduplication, and cell enlargement in yeast and other organisms [16]. Our observations are generally in line with these documented phenotypes of CDH1 overexpression.

It has been suggested that phosphorylation of CDC20 does not affect APC activation under certain circumstances [13]. Likewise, it is not too surprising that phosphorylation of CDH1 at four conserved CDK sites has no influence on the induction of post-MBT arrest (Fig. 4B, columns 6 and 7). On the other hand, our results implicate that cyclin A is critically involved in the maintenance of G_1 by CDH1 (Fig. 4B, column 8). However, it remains unanswered whether the regulation of CDH1 by cyclin A or the degradation of cyclin A by CDH1 is more important for mediating the long-lasting G_1 arrest. Further investigations are required to elucidate exactly how CDH1 activity is regulated in embryonic cell cycles.

In summary, CDH1 mRNA is detected in *Xenopus* oocytes and embryos of all stages. Loss of CDH1 in early embryonic cell cycles induces a prolonged mitotic arrest and overexpression of CDH1 results in a G_1 arrest at the MBT. Thus, amphibian CDH1 fulfills multiple regulatory functions at different points of the cell cycle.

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