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CDKs Promote DNA Replication Origin Licensing in Human Cells by Protecting Cdc6 from APC/C-Dependent Proteolysis

Niels Mailand[1] and John F.X. Diffley*
Cancer Research UK London Research Institute
Clare Hall Laboratories
South Mimms
Hertfordshire EN6 3LD
United Kingdom

Summary

Cyclin-dependent kinases (CDKs) restrict DNA replication origin firing to once per cell cycle by preventing the assembly of prereplicative complexes (pre-RCs; licensing) outside of G1 phase. Paradoxically, under certain circumstances, CDKs such as cyclin E-cdk2 are also required to promote licensing. Here, we show that CDK phosphorylation of the essential licensing factor Cdc6 stabilizes it by preventing its association with the anaphase promoting complex/cyclosome (APC/C). APC/C-dependent Cdc6 proteolysis prevents pre-RC assembly in quiescent cells and, when cells reenter the cell cycle from quiescence, CDK-dependent Cdc6 stabilization allows Cdc6 to accumulate before the licensing inhibitors geminin and cyclin A which are also APC/C substrates. This novel mechanism for regulating protein stability establishes a window of time prior to S phase when pre-RCs can assemble which we propose represents a critical function of cyclin E.

Introduction

DNA replication in eukaryotic cells initiates from large numbers of replication origins distributed on multiple chromosomes. A two-step mechanism regulates the initiation of DNA replication, ensuring that the entire genome is precisely duplicated in each cell cycle (Bell and Dutta, 2002; Blow and Dutta, 2005; Diffley, 2004). In the first step, Cdc6 and Cdt1 collaborate with the origin recognition complex (ORC) to load the putative replicative helicase (Mcm2-7) into pre-RCs at replication origins. In the second step, two protein kinases, Cdc7 and the S phase CDKs, convert pre-RCs into bidirectional replisomes at each origin. DNA replication is limited to once per cell cycle because these two steps are temporally segregated: pre-RC assembly can only occur from late mitosis through G1 phase, and once cells enter S phase, pre-RCs can no longer assemble. Thus, origins that have fired once cannot be relicensed until they pass through the following mitosis.

In budding yeast, CDKs directly prevent pre-RC assembly by inhibiting the function of all of the pre-RC components thus limiting pre-RC assembly to G1 phase (Diffley, 2004). CDKs appear to play a similar role in metazoans: inactivation of the mitotic CDK, cdc2, causes endoreduplication (Itzhaki et al., 1997), and treatment of postreplicative cells with a CDK inhibitor induces relicensing (Ballabeni et al., 2004; Coverley et al., 1996, 1998). However, metazoans also contain an additional licensing inhibitor called geminin (Blow and Dutta, 2005; McGarry and Kirschner, 1998). In human cells, geminin is targeted for proteolysis by the APC/C E3 ubiquitin ligase (McGarry and Kirschner, 1998), ensuring that the licensing inhibitors geminin and cyclin A are degraded from late mitosis through G1 phase, allowing pre-RCs to assemble during this period.

In addition to targeting licensing inhibitors for proteolysis, the APC/C also targets the essential pre-RC assembly factor, Cdc6, for proteolysis (Petersen et al., 2000). Coregulation of licensing inhibitors and activators, however, would make it impossible for cells to achieve a state where pre-RC components are all present but licensing inhibitors are absent. Cyclin E can either promote or inhibit pre-RC assembly, depending on context (see Diffley [2004] for discussion). Here we show that CDKs, including those containing cyclin E, promote licensing by phosphorylating a regulatory domain of Cdc6, thus preventing its degradation by the APC/C. This allows Cdc6 to accumulate before the licensing inhibitors geminin and cyclin A when quiescent cells re-enter the cell cycle. We propose a simple model to explain the positive role of cyclin E in licensing and discuss how context might also allow cyclin E to be a licensing inhibitor.

Results

CDKs Regulate Cdc6 Stability

We noticed that levels of endogenous Cdc6 protein in human tissue culture cells were significantly reduced very quickly after treatment with the CDK inhibitor, roscovitine. Other APC/C substrates including geminin, cyclin A, and cyclin B were unaffected by roscovitine during the same time interval. At the concentrations used, roscovitine should primarily inhibit CDKs (Meijer et al., 1997). Figure 1B shows that the levels of endogenous Cdc6 were also dramatically reduced after cdk2 levels were reduced using siRNA. The effects of roscovitine on Cdc6 are seen after just 2 hr (Figure 1A), before any significant alteration in cell cycle distribution can occur, and depletion of cdk2 by siRNA did not affect the distribution of cells in the cell cycle (see Figure S1A in the Supplemental Data available with this article online). To determine whether the reduction in Cdc6 levels is due to changes in protein stability, we examined the rate of Cdc6 disappearance after addition of cycloheximide (CHX) to prevent new Cdc6 synthesis. Endogenous Cdc6 was relatively unstable, with a half-life of approximately 1 hr based on initial rates of disappearance, and became even more unstable after roscovitine treatment, with a half-life significantly less than 1 hr (Figure 1C). A similar decrease in half-life was seen after depletion of either cyclins E1 and E2 or cdk2 (Figure

*Correspondence: john.diffley@cancer.org.uk
[1]Present address: Institute of Cancer Biology and Centre for Genotoxic Stress Research, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen Ø, Denmark.

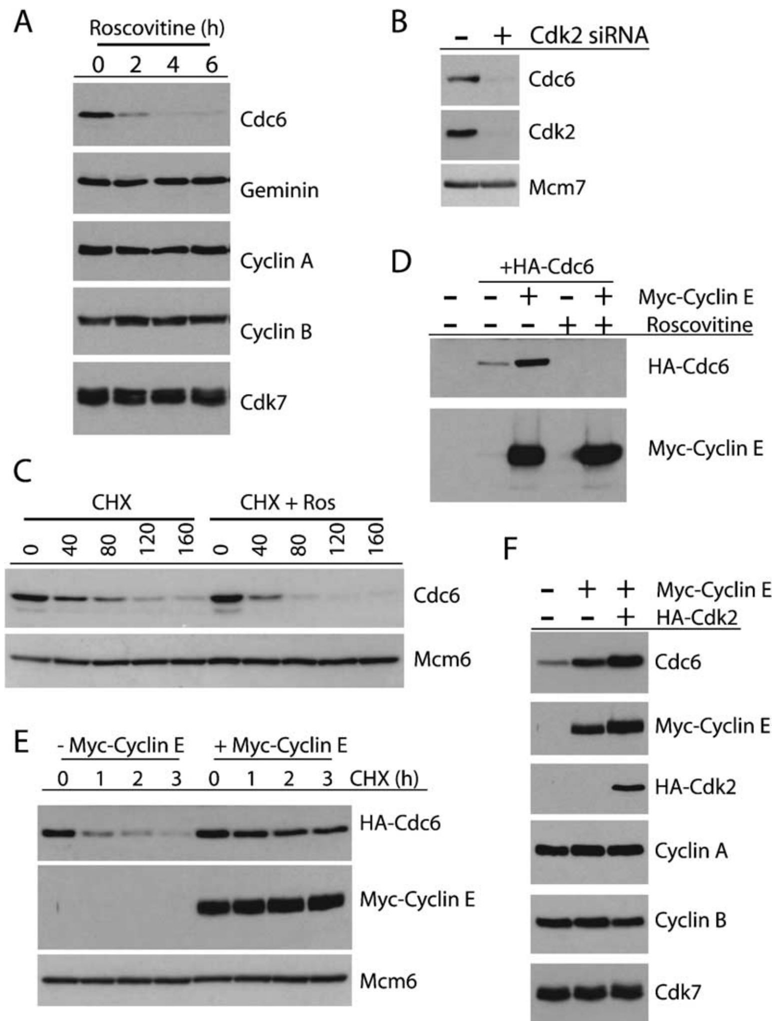

Figure 1. Cdc6 Stability Is Regulated by CDK Activity

(A) Loss of Cdc6 expression in response to CDK inhibition. Exponentially growing U2OS cells were treated with Roscovitine and total lysates were immunoblotted for the indicated proteins. cdk7 was included as a loading control.

(B) Cdc6 protein levels decline in response to cdk2 depletion. U2OS cells were left untreated or transfected with cdk2 siRNA for 72 hr, and total cell lysates were probed for Cdc6 and cdk2. Mcm7 was included as a loading control.

(C) The degradation of Cdc6 is accelerated by CDK inhibition. Cycloheximide was added with or without Roscovitine to U2OS cells for the indicated times, and the abundance of Cdc6 was monitored by immunoblotting.

(D and E) Stabilization of Cdc6 by elevated cyclin E levels.

(D) U2OS cells were transfected with indicated combinations of HA-Cdc6 and myc-cyclin E for 24 hr and incubated for an additional 3 hr in the presence or absence of Roscovitine. Expression of the transfected proteins was analyzed by immunoblotting.

(E) U2OS cells transfected as in (D) were treated with CHX for the indicated times and the stability of HA-Cdc6 was assayed by immunoblotting.

(F) Endogenous Cdc6 but not other APC/C substrates accumulates in response to cyclin E overexpression. Cells were transfected with the indicated constructs and CD20 expression plasmid. Productively transfected cells were then isolated with magnetic beads coated with CD20 antibody and processed for immunoblotting.

S1B). These experiments indicate that CDK inhibition reduces Cdc6 protein levels at least in part by destabilizing the Cdc6 protein.

Overexpression of cyclin E (Figure 1D) or cyclin A (Figure S3A) caused an increase in levels of cotransfected Cdc6 tagged with the HA epitope (HA-Cdc6). This accumulation of Cdc6 was blocked by roscovitine, suggesting that CDK catalytic activity is required for Cdc6 accumulation (Figure 1D). Cyclin E overexpression dramatically stabilized HA-Cdc6, increasing its half-life to far greater than 3 hr (Figure 1E). Because transfected Cdc6 can be regulated differently from the endogenous Cdc6 (Alexandrow and Hamlin, 2004), we cotransfected cells with a cell-surface marker (CD20) which allowed us to specifically purify transfected cells. In these purified cells, endogenous Cdc6 levels were significantly increased after cotransfection with myc-cyclin E (Figure 1F). This effect was enhanced by cotransfection of myc-cyclin E with HA-cdk2. Figure S1A shows that overproduction of cyclin E had little or no effect on cell cycle distribution. As with the roscovitine treatment, myc-cyclin E with or without HA-cdk2 had no effect on levels of other APC/C substrates such as cyclin A or cyclin B (Figure 1F). Taken together, these experiments indicate that the stability of both endogenous and transfected Cdc6 is regulated by cyclin E-cdk2.

Stabilization Is Mediated by CDK Sites in the Cdc6 NTD

Stabilization could be due to direct phosphorylation of Cdc6 or to some indirect effect of CDKs. Eukaryotic Cdc6 proteins contain a regulatory N-terminal domain (NTD) that is not directly involved in pre-RC assembly (Drury et al., 1997). Cdc6 is a good substrate for cyclin E-, A-, and B-associated CDKs (Herbig et al., 2000), and indicated in boxes in Figure 2A are three serine residues known to be phosphorylated by CDKs in human cells (Jiang et al., 1999; Petersen et al., 1999). Two of these lie in close proximity to conserved APC/C destruction boxes (gray; Petersen et al., 2000). The first (serine 54) is next to an RXXL-type destruction box while the second (serine 74) is near a KEN-type destruction box. The RXXL motif is found in all of the vertebrate Cdc6 sequences, while the KEN box is present in all of the vertebrate Cdc6 sequences except XlCdc6a, an isoform of Cdc6 specific for early *Xenopus* embryos

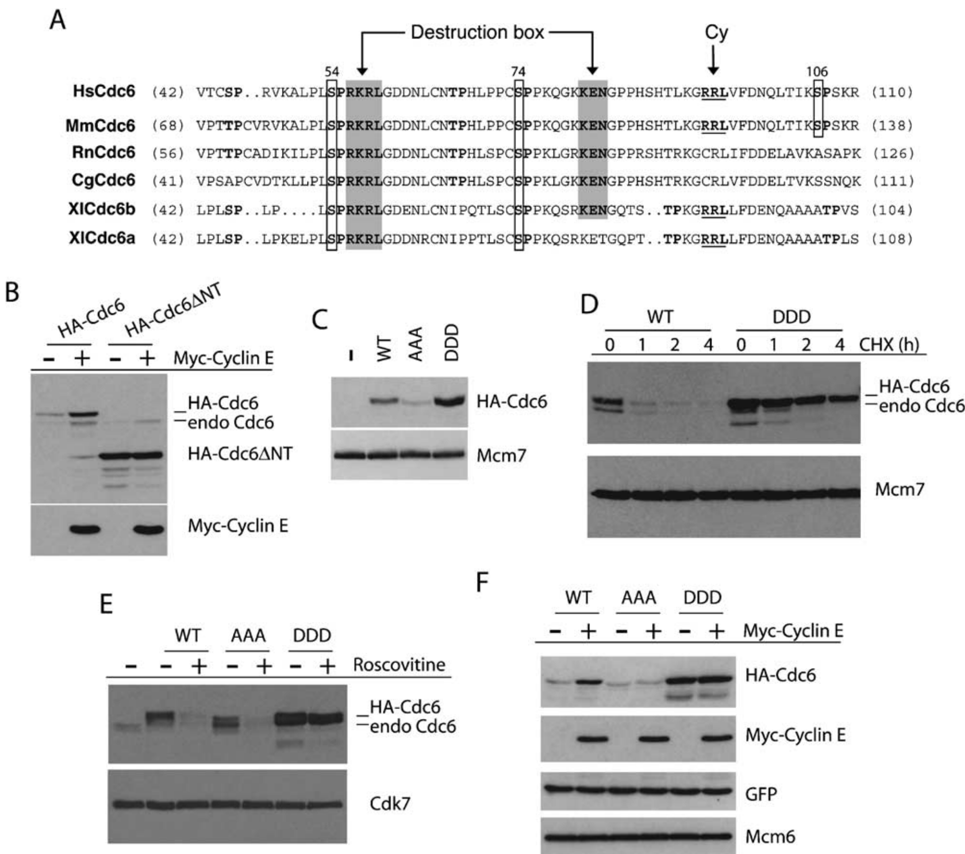

Figure 2. Cdc6 Is Stabilized by Phosphorylation of In Vivo CDK Sites

(A) Conserved regulatory motifs in Cdc6 from higher eukaryotes.

(B) Sensitivity of Cdc6 to modulation of CDK activity is mediated by the N terminus. U2OS cells were transfected with full-length or N-terminally truncated HA-Cdc6 (ΔNT) in the presence or absence of myc-cyclin E and lysates were probed for the indicated proteins.

(C) Expression level of Cdc6 CDK phosphorylation site mutants. U2OS cells were transfected with the indicated HA-Cdc6 constructs and processed for immunoblotting.

(D) Stabilization of Cdc6 by CDK phospho-mimicking mutations. U2OS cells transfected with HA-Cdc6 wt or DDD were treated with CHX for the indicated times and analyzed for total Cdc6 levels by immunoblotting.

(E and F) Phospho-mimicking mutations in Cdc6 render it insensitive to CDK deregulation.

(E) U2OS cells treated as in (C) were incubated with or without Roscovitine for the final 3 hr of transfection and analyzed for Cdc6 levels by immunoblotting.

(F) U2OS cells were transfected with combinations of HA-Cdc6 constructs, myc-cyclin E, and GFP as indicated and processed for immunoblotting.

(Tikhmyanova and Coleman, 2003). *Xenopus* embryos also lack Cdh1 (Littlepage and Ruderman, 2002), which is involved in recognizing the KEN box (Pfleger et al., 2001). There are several other potential CDK sites (bold) and an RXL or Cy motif (underlined) which acts as a docking site for cyclins (Figure 2A).

We examined the effects of modulating CDK activity on mutant Cdc6 proteins. Overexpression of myc-cyclin E increased the levels of both HA-Cdc6 and endogenous Cdc6 but did not affect the levels of HA-Cdc6ΔNT (Figure 2B), a version of Cdc6 lacking the entire NTD, indicating that the NTD is required for CDKs to modulate Cdc6 stability. We constructed mutant versions of Cdc6 in which the three known CDK-phosphorylated serines were replaced with either alanine (AAA) to mimic the unphosphorylated state or aspartic acid (DDD) to mimic the phosphorylated state. Expression of these mutant proteins had no effect on the distribution of cells in the cell cycle (Figure S2). The DDD mutant was expressed at levels higher than the wild-type protein, while the AAA mutant was expressed at levels lower than the wild-type protein (Figure 2C). The DDD mutant was significantly more stable than the wild-type protein after CHX addition (Figure 2D). Moreover, the levels of both wild-type HA-Cdc6 and endogenous Cdc6 but not the DDD mutant were sensitive to roscovitine treatment (Figure 2E). The unphosphorylatable AAA mutant was destabilized even further, probably because roscovitine activates the APC/C (Listovsky et al., 2000). Finally, Figure 2F shows that, in contrast to wild-type Cdc6, both the AAA and DDD mutants were unaffected by cyclin E overexpression together, indicating that cyclin E regulates the stability of Cdc6 by direct phosphorylation.

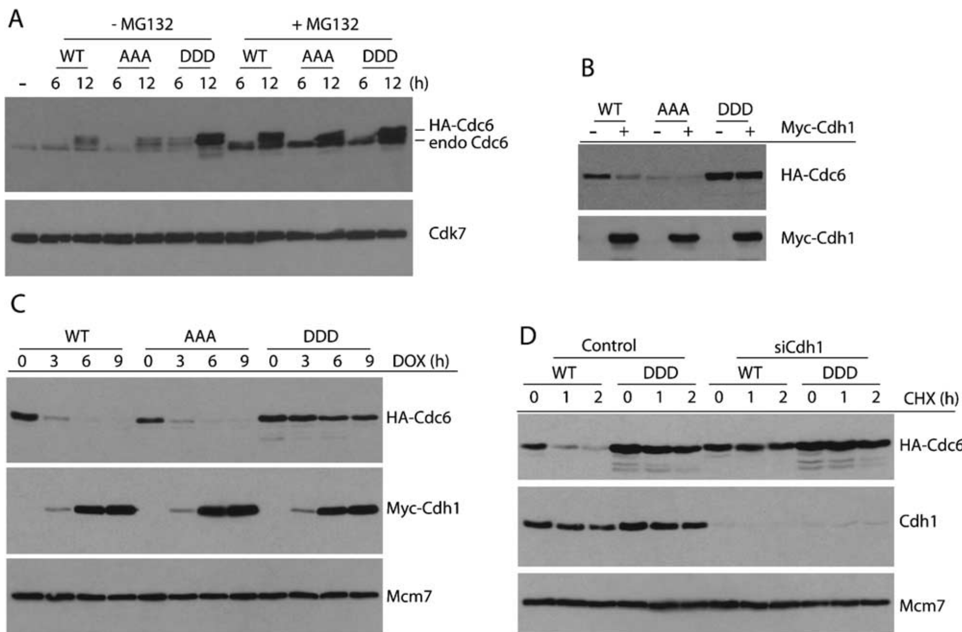

Figure 3. Phospho-Mimicking Mutations Prevent APC/C^{Cdh1}-Mediated Degradation of Cdc6

(A) The differential stability of phosphorylation site mutants of Cdc6 is proteasome-dependent. Exponentially growing U2OS cells were transfected with HA-Cdc6 constructs in the presence or absence of MG132, harvested at the indicated times after transfection, and the accumulation of ectopic Cdc6 was analyzed by immunoblotting.

(B and C) Phospho-mimicking mutations in Cdc6 protect it from APC/C^{Cdh1}-mediated proteolysis.

(B) U2OS cells were transfected with HA-Cdc6 constructs and myc-Cdh1, and total cell extracts were probed for the ectopic proteins.

(C) U2OS T-REx cells were transfected with HA-Cdc6 and myc-Cdh1/Tet-On, incubated for 24 hr to allow the accumulation of HA-Cdc6, after which Doxycycline was added to induce myc-Cdh1 expression for the indicated times. Cells were processed as in (B).

(D) The stability difference between Cdc6 wt and DDD depends on Cdh1. U2OS cells were transfected with control or Cdh1 siRNAs for 24 hr, transfected with HA-Cdc6 wt or DDD for an additional 24 hr, and the half-lives of the transfected proteins were assessed by CHX treatment for the indicated times and immunoblotting.

CDK phosphorylation sites of the NTD have been previously implicated in causing relocalization of transfected (but not endogenous) Cdc6 protein to the cytoplasm (Alexandrow and Hamlin, 2004; Jiang et al., 1999; Petersen et al., 1999). Although our experiments clearly show that the stability of the endogenous as well as transfected Cdc6 is regulated by CDKs, we wanted to address the possibility that altered subcellular localization might contribute to stabilization of the transfected proteins. Addition of a cassette containing three constitutive nuclear localization sequences (3X-NLS) to the DDD mutant caused it to reside almost entirely in the nucleus. Under these conditions, the Cdc6 DDD protein was still extremely stable (Figure S3). Thus, the effects of CDKs on Cdc6 stability are independent from the effects of CDKs on subcellular localization.

CDK Phosphorylation Blocks APC/C-Dependent Proteolysis In Vivo and In Vitro

Cdc6 has previously been shown to be a substrate for the APC/C (Petersen et al., 2000). Transfected wild-type and AAA HA-Cdc6 as well as endogenous Cdc6 accumulated to higher levels after treatment with MG132 (Figure 3A), while the DDD mutant was unaffected by MG132 treatment, indicating that the differences in the stability of the Cdc6 mutants are mediated by the proteasome. Overexpression of the APC/C activator Cdh1 is sufficient to activate the APC/C irrespective of cell cycle position and destabilizes wild-type Cdc6 (Petersen et al., 2000; Sorensen et al., 2000; Figure 3B). Cdh1 overexpression also reduced AAA HA-Cdc6 levels but had no effect on the DDD mutant (Figure 3B). To examine this in more detail, the HA-Cdc6 constructs were cotransfected with tetracycline-inducible myc-Cdh1 constructs. Transfections were adjusted so the initial levels of mutant proteins were similar. After accumulation of the HA-Cdc6 proteins, doxycycline was added to induce myc-Cdh1. This induced rapid degradation of wild-type and the AAA mutant but not the DDD mutant, which remained at high levels after Cdh1 induction (Figure 3C). Even when targeted to the nucleus with the 3XNLS cassette, the DDD mutant was resistant to Cdh1-dependent degradation (Figure S3H). Conversely, reduction of Cdh1 by siRNA significantly stabilized wild-type HA-Cdc6 but had no effect on the already stabilized DDD mutant (Figure 3D). Figure 4A shows that polyubiquitylated forms of the wild-type but not DDD Cdc6 were detected following expression of Cdh1 and His-tagged ubiquitin, indicating that, in contrast to the wild-type protein, the DDD mutant is not polyubiquitylated in vivo. Thus, the wild-type and AAA mutant are APC/C targets, but the DDD mutant is resistant to APC/C -mediated degradation.

We next examined the degradation of an N-terminal

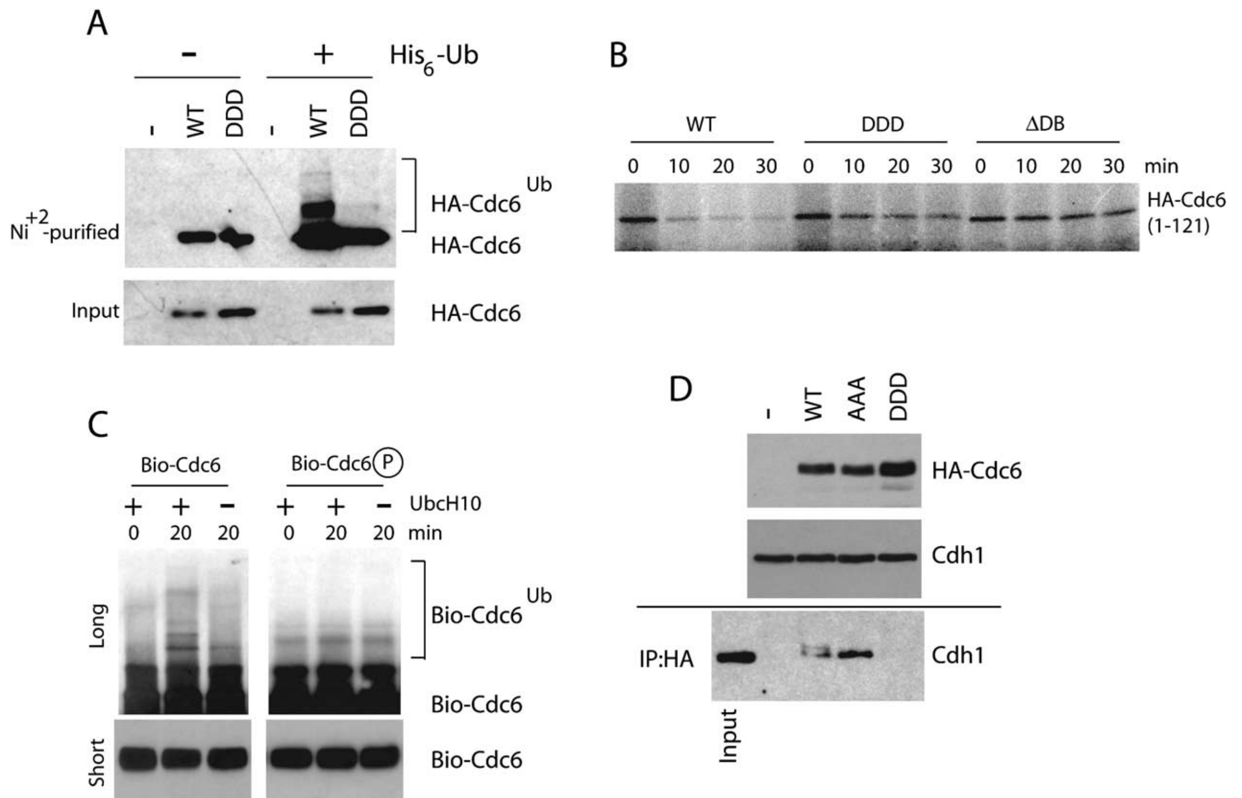

Figure 4. CDK Phosphorylation of Cdc6 Interferes with Its Recognition by APC/C^{Cdh1}

(A) Selective ubiquitination of wt Cdc6 in response to reactivated APC/C^{Cdh1}. U2OS T-REx cells were cotransfected with Cdh1 (Tet-On) and indicated combinations of Cdc6 and His-tagged Ubiquitin. Twenty-four hr after transfection, Cdh1 was induced by addition of Doxycycline, and cells were incubated in the presence of MG132 for 6 hr before denaturing lysis. Ectopic Cdc6 in total extracts and fractions purified on Ni^{2+}-NTA was detected by HA immunoblotting.

(B) Degradation of Cdc6 fragments in quiescent cell extracts. The degradation of ^{35}S-labeled N-terminal fragments of Cdc6 was analyzed over time by SDS-PAGE and autoradiography. A ΔDB mutant (R56A, L59A, K81A, E82A, and N83A) containing inactivated D- and KEN-boxes was used as a control.

(C) CDK-phosphorylated Cdc6 is insensitive to ubiquitylation by purified APC/C in vitro. Ubiquitination reactions were performed using immunopurified APC/C, E1 and E2 enzymes, and Cdh1. Unphosphorylated or Ser54/74-phosphorylated Biotin-conjugated Cdc6 peptides (125 μM) were used as substrates and detected by immunoblotting with anti-Biotin antibodies.

(D) CDK phosphorylation of Cdc6 regulates its affinity for Cdh1. U2OS cells were transfected with different amounts of HA-Cdc6 plasmids to balance their expression, and total cell extracts were subjected to immunoprecipitation with anti-HA antibodies. Cdh1 and HA-Cdc6 were detected by immunoblotting.

Cdc6 fragment in concentrated extracts from quiescent cells (Rape and Kirschner, 2004). Wild-type Cdc6 was rapidly degraded in these extracts whereas the DDD mutant as well as a construct in which the Cdc6 destruction box was mutated (ΔDB) were considerably stabilized (Figure 4B), indicating that the DDD mutant is resistant to APC/C-dependent proteolysis in vitro. We used affinity-purified APC/C from quiescent cells in a cell-free system (Kramer et al., 2000) to examine polyubiquitylation of biotinylated peptides spanning the destruction boxes in Cdc6. Polyubiquitylated forms of the Cdc6 peptide could be readily detected when incubated in the presence of the complete system, and this was greatly reduced when the E2 enzyme (UbcH10) was omitted from the reaction (Figure 4C). However, when the Cdc6 peptide was phosphorylated on Ser54 and Ser74, it was not polyubiquitylated in parallel reactions. Together, these results indicate that phosphorylation of the Cdc6 NTD by CDKs prevents its proteolysis by preventing APC/C-dependent polyubiquitylation. Finally, Cdh1 could be detected in HA immunoprecipitates with either wild-type or AAA HA-Cdc6 but not DDD HA-Cdc6 (Figure 4D). Cdh1 coimmunoprecipitated with wild-type but not AAA HA-Cdc6 always appears as a doublet. The reason for this is currently unknown. We propose the inability of DDD Cdc6 to interact with APC/C accounts for its resistance to APC/C-mediated proteolysis.

CDK-Dependent Stabilization Allows Early Cdc6 Accumulation

As shown above, APC/C-dependent proteolysis of Cdc6 is modulated by CDK phosphorylation of the Cdc6 NTD. In trying to understand why this elaborate regulatory mechanism exists, we were struck by the finding that cyclin E plays a positive, essential role in licensing specifically when quiescent cells reenter the cell cycle (Geng et al., 2003) and that cyclin E cooper-

ates with Cdc6 to stimulate licensing in vitro (Coverley et al., 2002). We considered that phosphorylation of the Cdc6 NTD by cyclin E-cdk2 might promote Cdc6 stabilization prior to the inactivation of the APC/C by cyclin A (Sorensen et al., 2000). This would ensure that, in cells reentering the cell cycle, Cdc6 would accumulate before the licensing inhibitors geminin and cyclin A.

To assess the feasibility of this idea, we examined the reaccumulation of endogenous proteins after serum-starved cells were stimulated to reenter the cell cycle. Cdc6 as well as Cdt1, geminin, Cdc20, and cyclin A were absent after serum starvation (t = 0, Figure 5A). Endogenous Cdc6 and Cdt1 both appeared approximately 10 hr after serum addition, coincident with the loading of Mcm6 onto chromatin. This was 3–4 hr earlier than the appearance of other APC/C substrates, geminin, cyclin A, and Cdc20, consistent with the hypothesis.

Two predictions of this model are that Cdc6 appearance should coincide with appearance of cyclin E kinase activity and that Cdc6 should be quantitatively phosphorylated in the NTD. To test the first prediction, cyclin E and cyclin A immunoprecipitates made from extracts of cells released from quiescence for various times were assayed for histone H1 kinase activity. Although cyclin E protein was present in the serum-starved cells, there was little or no associated H1 kinase activity (Figure 5B), perhaps due to the presence of CDK inhibitors. Cyclin E-associated histone H1 kinase activity first appeared approximately 10 hr after serum stimulation, coinciding with the appearance of Cdc6 protein and preceding the appearance of cyclin A-associated kinase activity by 4 hr.

Using a phospho-specific antibody, Figure 5B shows that as soon as Cdc6 appears, phospho-ser54 could be detected. Treatment of extracts with λ phosphatase completely eliminated recognition of endogenous and HA-Cdc6 by the phospho-specific antibody (but not anti-Cdc6 antibody) in both immunoblots and immunoprecipitations (Figure 5C). Figure 5D shows that this phospho-specific antibody can quantitatively deplete Cdc6 from extracts from either exponentially growing cells or serum-starved cells which had been serum stimulated for 10 hr (when Cdc6 first appears). Thus, upon reentry into the cell cycle, Cdc6 accumulates with cyclin E-associated kinase activity and is quantitatively phosphorylated on at least one of the residues implicated in its stabilization.

Figure 5E shows that constitutively expressed wild-type HA-Cdc6 accumulates approximately 10 hr after serum stimulation, coincident with the endogenous Cdc6 but before cyclin A (Figure 5E). Accumulation of the AAA mutant was delayed 2–4 hr relative to the endogenous protein and paralleled the appearance of cyclin A, while the DDD mutant accumulated several hours earlier than the endogenous protein. These experiments, taken with previous results, support the idea that Cdc6 is stabilized by cyclin E-cdk2 phosphorylation before other APC/C targets can accumulate upon cell cycle reentry.

Cdc6 Stabilization Promotes Pre-RC Assembly

To determine whether this mechanism can contribute to the ability of Cdc6 to support licensing, we needed to develop licensing assays that would allow us to examine the functionality of Cdc6 mutants in the absence of endogenous Cdc6. We utilized the fact that quiescent T98G cells lack both Cdc6 and Cdt1. Figure 6A shows that wild-type and AAA mutant HA-Cdc6 were unable to accumulate in quiescent cells, while the DDD mutant (as well as the Cdc6 destruction box mutant, ΔDB—Figure 6B) accumulated to high levels, consistent with the idea that the DDD mutant is resistant to APC/C-dependent proteolysis. Wild-type Cdt1 can readily accumulate in quiescent cells (Figure 6C), and overproduction of Cdh1 does not affect Cdt1 levels (Figure S4), indicating that Cdt1 is not an APC/C substrate. The DDD mutant but not wild-type HA-Cdc6 promoted the loading of Mcm6 onto chromatin in quiescent cells when coexpressed with Cdt1 (Figure 6C). Coexpression of Cdt1 was required for this loading in G0 cells (Figure 6D). Our results contrast slightly with previously published work showing that expression of wild-type Cdc6 from an adenovirus expression vector promoted Cdc6 accumulation and Mcm2-7 loading in quiescent cells (Cook et al., 2002). The difference between our results is likely due to higher expression levels from the adenovirus vector. To extend our findings, we serum-stimulated quiescent cells in the presence of roscovitine, which prevents the accumulation of endogenous Cdc6 and Cdt1, presumably in part by interfering with E2F activation (Figure 6E). Ectopic expression of Cdt1 was sufficient to promote its accumulation; however, neither wild-type nor the AAA mutant HA-Cdc6 could accumulate under these conditions (Figure 6E). Figure 6F shows that these Cdc6 constructs were also unable to act with Cdt1 to support Mcm2-7 loading significantly above background levels. By contrast, the DDD mutant accumulated efficiently upon serum addition in the presence of roscovitine (Figure 6E) and could support significant Mcm2-7 loading (Figure 6F). Therefore, only the DDD mutant can efficiently accumulate and support licensing in the presence of active APC/C.

To examine the role of cyclin E in promoting Cdc6 stabilization and licensing during G1 phase, we used siRNA to deplete cyclins E1 and E2. Figure S5 shows that endogenous Cdc6 levels drop significantly a few hours after release from a nocodazole arrest consistent with previous work (Mendez and Stillman, 2000), presumably reflecting APC/C-dependent proteolysis. Depletion of cyclins E1 and E2 from nocodazole-arrested cells prevented the reaccumulation of Cdc6 during the subsequent G1 phase after release from the nocodazole arrest (Figure S5). This delay could have been a direct effect on Cdc6 stability or an indirect effect on Cdc6 transcription during G1 phase. So, we examined the effect of cyclin E depletion on accumulation of tetracycline-inducible Cdc6. Cyclins E1 and E2 were depleted in nocodazole-arrested cells and doxycycline was added upon release from the nocodazole arrest. Figure 6G shows that the accumulation of wild-type HA-Cdc6 was significantly delayed in the absence of cyclin E. By contrast, the accumulation of the DDD mutant was unaffected by cyclin E knockdown (Figure 6H).

To examine the importance of cyclin E-dependent stabilization of Cdc6 on licensing, we used siRNA to deplete endogenous Cdc6 in nocodazole-arrested cells.

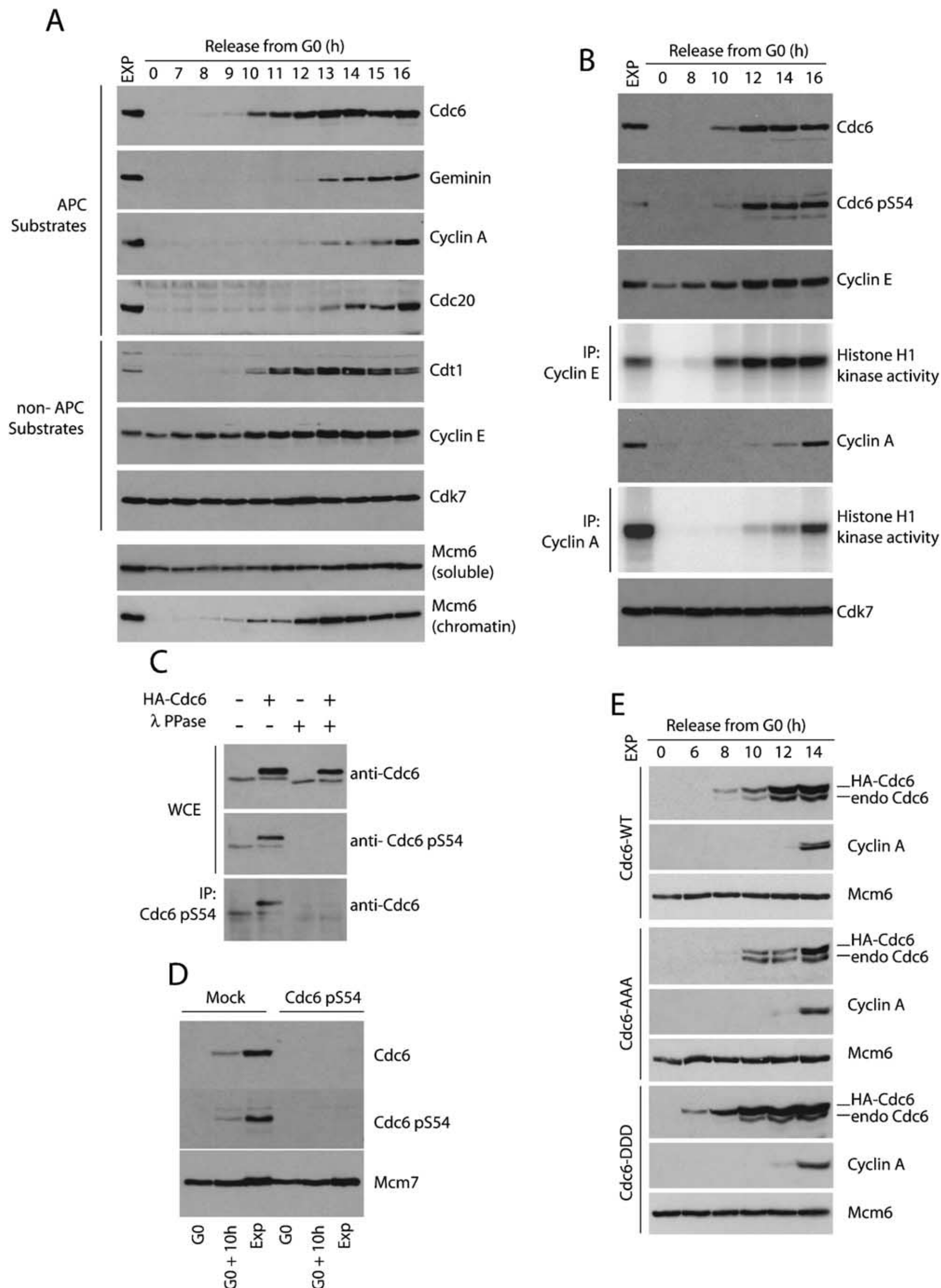

Figure 5. Kinetics of Cdc6 Accumulation after Serum Stimulation

(A) Cdc6 accumulates earlier than other APC/C targets following cell cycle reentry. T98G cells rendered quiescent by serum starvation were released into the cell cycle by serum stimulation, harvested at various times, and total cell extracts were immunoblotted for APC/C and non-APC/C substrates. In parallel, cells at each time point were subjected to chromatin fractionation to monitor pre-RC assembly in G1 (lower panels).

(B) CDK phosphorylation of Cdc6 in G1 correlates with cyclin E- but not cyclin A-associated kinase activity. Total extracts from cells treated as in (A) were processed for immunoblotting or subjected to kinase activity measurements for cyclin E and A as indicated.

(C) Characterization of a phospho-Ser54 antibody to Cdc6. Lysates from exponentially growing T98G cells were left untreated or incubated with λ phosphatase. Lysates were then subjected to immunoblotting or immunoprecipitation with Cdc6 pS54 antibody.

(D) The total pool of Cdc6 is phosphorylated on Ser54. Total cell extracts were prepared from cells kept quiescent, released for 10 hr from quiescence, or growing exponentially. Lysates were then subjected to three rounds of depletion with Cdc6 pS54 antibody or rabbit preimmune serum (mock) and immunoblotted for the indicated proteins.

(E) Accumulation of Cdc6 alleles after cell cycle reentry. Cells treated as in (A) were transfected with HA-Cdc6 constructs upon release into the cell cycle, harvested at various times after serum stimulation, and immunoblotted for the indicated proteins in total cell extracts.

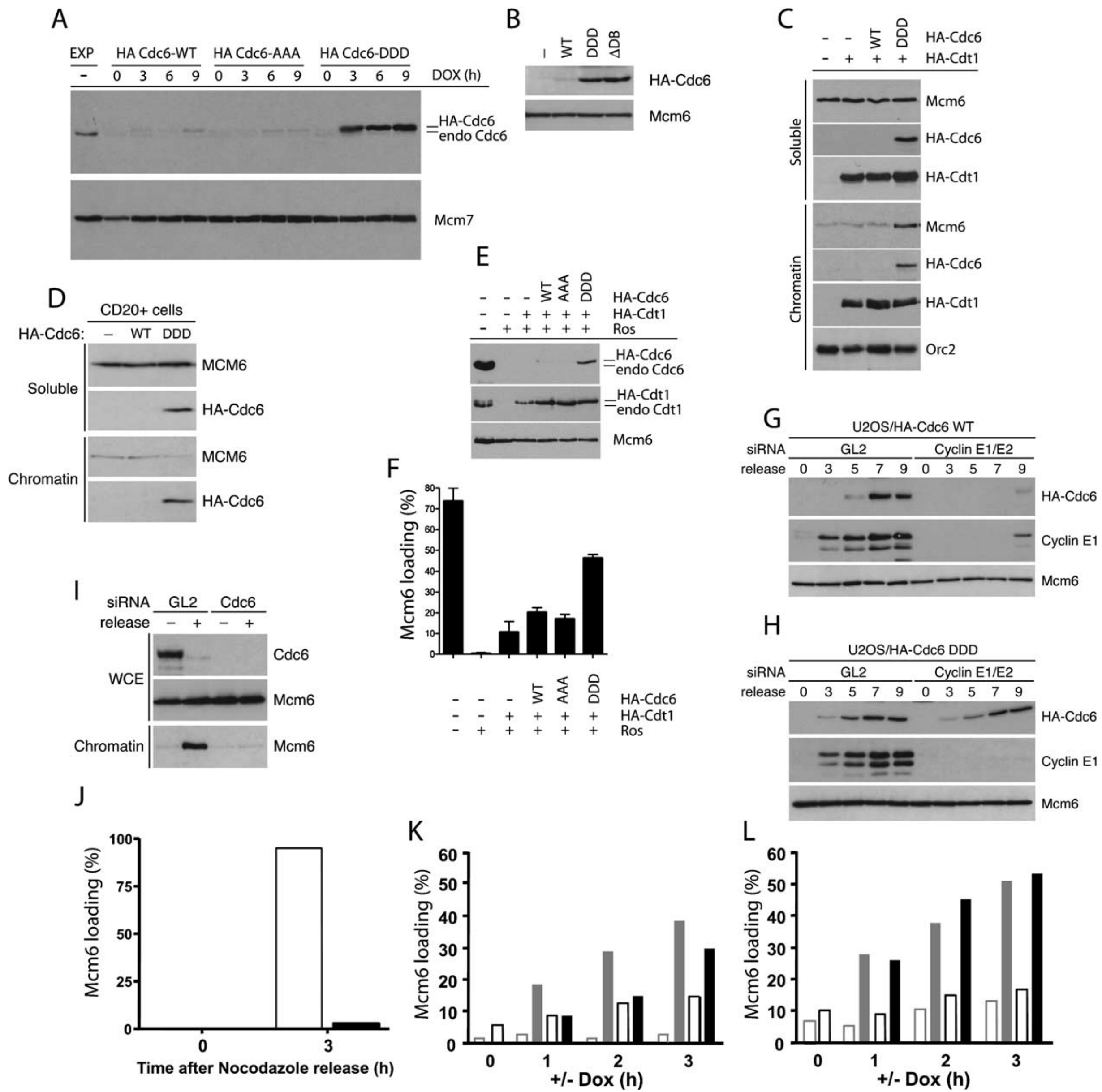

Figure 6. Phospho-Mimicking Cdc6 Drives pre-RC Assembly in the Absence of CDK Activity

(A) Phospho-mimicking mutations allow efficient expression of Cdc6 in G0. T98G-T cells were transfected with Tet-On constructs for HA-Cdc6, serum-starved for 48 hr, and ectopic Cdc6 expression was induced for the indicated times by addition of Doxycycline. Expression of ectopic Cdc6 was analyzed by immunoblotting.

(B) Expression of Cdc6 in G0 by phospho-mimicking or destruction box mutations. Quiescent T98G cells transfected with indicated constructs were assayed for the expression of ectopic proteins by anti-HA immunoblotting.

(C and D) Cdc6 DDD drives pre-RC assembly in G0 in the presence of Cdt1. T98G cells were serum-starved for 72 hr and cotransfected with indicated Cdc6 constructs and CD20 expression plasmid in the presence (C) or absence (D) of Cdt1 for an additional 24 hr. Positively transfected cells were then isolated with CD20-coated beads and processed for chromatin fractionation and immunoblotting.

(E and F) Cdc6 DDD selectively stimulates pre-RC assembly in CDK-inhibited G1 cells. Quiescent T98G cells were transfected with the indicated constructs and H2B-GFP 4 hr before serum stimulation. Two hours after G0 release, Roscovitine was added where indicated, and cells were incubated for an additional 14 hr, harvested, and processed for immunoblotting (E) or immunofluorescent detection of chromatin bound Mcm6 in transfected cells, as a measure of pre-RC assembly (F). Results show the mean of two independent experiments. Approximately 200 transfected cells were counted in each sample. Error bars in (F) represent the standard error of the mean.

(G and H) The accumulation of Cdc6 in G1 depends on its phosphorylation mediated by cyclin E. U2OS lines inducibly expressing Cdc6 wt (G) or DDD (H) were transfected with control or cyclin E1/E2 siRNA for 24 hr and arrested in mitosis with Nocodazole for an additional 12 hr. Cells were then released from the block in the presence of Doxycycline to induce the ectopic proteins, and their accumulation at various times after the release was monitored by immunoblotting.

(I and J) Depletion of Cdc6 prevents pre-RC assembly upon exit from mitosis. Asynchronous U2OS cells were transfected with control (open bars) or Cdc6 (closed bars) siRNA for 24 hr, after which Nocodazole was added for an additional 12 hr. Pre-RC formation in Nocodazole-arrested cells or cells released from the block for 3 hr was then assessed as in (E) and (F).

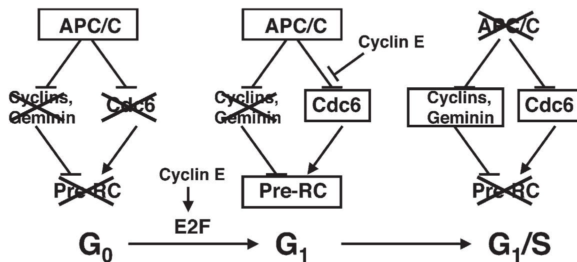

Figure 7. Model of the Regulation of pre-RC Assembly after Cell Cycle Reentry

Details of the model are described in the text.

Figures 6I and 6J show that this prevented loading of Mcm6 onto chromatin after release from the nocodazole arrest. We next examined the effects of cyclin E depletion on the capacity of tetracycline-inducible, siRNA-resistant Cdc6 constructs to promote licensing. Figure 6K shows that Mcm6 loading by wild-type Cdc6 was significantly delayed after depletion of cyclin E (compare solid gray and black bars). Again, by contrast, the DDD mutant showed no delay in licensing after cyclin E depletion (Figure 6L). Therefore, cyclin E contributes to licensing during G1 phase by stabilizing the Cdc6 protein.

Discussion

We propose a model to resolve the paradoxical situation whereby Cdc6, an essential pre-RC component, as well as geminin and cyclin A, pre-RC inhibitors, are all targeted for degradation by the APC/C (Figure 7). During quiescence, the highly active APC/C ensures that Cdc6 as well as geminin and cyclin A are absent from cells. The absence of Cdc6 contributes to preventing inappropriate pre-RC assembly in quiescence. When cells are stimulated to reenter the cell cycle, E2F-dependent transcription is activated, in part, by cyclin E-cdk2. Neither geminin nor cyclin A can accumulate initially because the APC/C is active; however, cyclin E, which is an E2F target but not an APC/C substrate, can accumulate during this period. Cyclin E-cdk2 phosphorylates the Cdc6 NTD, specifically preventing Cdc6 degradation via the APC/C, allowing Cdc6 to accumulate before geminin and cyclin A. Because Cdt1 is an E2F target but not an APC/C substrate, it can accumulate together with Cdc6. Later in G1 phase, the APC/C becomes inactivated, allowing the licensing inhibitors geminin and cyclin A to accumulate. Thus, Cdc6 stabilization ensures that all of the essential pre-RC components are present for a window of time in late G1 phase to allow licensing. Then, as cells enter S phase, this window is closed by the accumulation of licensing inhibitors.

Our results describe a novel mechanism for controlling protein degradation: CDK phosphorylation-dependent masking of destruction boxes in Cdc6 directly prevents APC/C-dependent proteolysis. CDKs control the stability of other key cell cycle regulators such as CDK kinase inhibitors by phosphorylating them and targeting them to the SCF E3 ubiquitin ligase (Ang and Harper, 2005). Thus, CDKs can both stabilize and destabilize proteins.

The model in Figure 7 applies to the regulation of licensing when cells reenter the cell cycle from quiescence. Proliferating cells must also establish a window of time (at the end of mitosis) when all of the required pre-RC components are present and active and the licensing inhibitors are absent. Our experiments indicate that CDK-dependent Cdc6 phosphorylation prevents APC/C-dependent degradation even in proliferating cells. However, it is clear from genetic analysis in mice that cyclin E is not required in normally proliferating mitotic cells (Geng et al., 2003). Moreover, we have found that depletion of cyclin E in nocodazole-arrested cells inhibits licensing if Cdc6 expression is limited to G1 phase (Figures 6G–6L) but does not inhibit licensing at the end of mitosis (data not shown). We suggest three possible, nonexclusive mechanisms to explain the lack of a cyclin E requirement for licensing on telophase chromosomes. Firstly, Cdc6 made in the previous cell cycle may be resistant to APC/C-dependent proteolysis, perhaps because it is already bound to chromatin before mitosis. This would allow Cdc6 to persist at origins after geminin and cyclin A have been degraded. Secondly, instead of cyclin E-cdk2, another CDK complex, perhaps involving one or more of the mitotic cyclins, may phosphorylate and stabilize Cdc6 during mitosis. Finally, Cdc6 may be specifically targeted for proteolysis by APC/C^{Cdh1}, whereas geminin and cyclin A are targets of both APC/C^{Cdc20} and APC/C^{Cdh1} (Geley et al., 2001; McGarry and Kirschner, 1998). Because APC/C^{Cdc20} is activated prior to APC/C^{Cdh1}, this would generate a window of time in telophase when geminin and cyclin A have already been degraded but Cdc6 is stable. Endoreduplication in giant trophoblast cells was greatly reduced, and the generation of another polyploid cell type, megakaryocytes, was compromised in the cyclin $E1^{-/-}$,$E2^{-/-}$ mice. Endoreduplication in cdc2 shut-off cells involves the periodic activation of APC/C^{Cdh1} but not APC/C^{Cdc20} (Itzhaki et al., 1997; Laronne et al., 2003). Thus, cells coming out of quiescence as well as endoreduplicating cells may both need to license origins in the absence of APC/C^{Cdc20}, and cyclin E-cdk2 may be required for this. In this regard, it is interesting that Cdc6 levels positively correlate with cyclin E expression in endoreduplicating human megakaryoblast cell lines (Bermejo et al., 2002).

Cdt1 is targeted for proteolysis after DNA damage, thus restraining pre-RC assembly (Higa et al., 2003; Kondo et al., 2004). Because CDKs are regulated in response to DNA damage, for example, by means of p53-

(K and L) Phospho-mimicking but not wt Cdc6 overrides the requirement of cyclin E for pre-RC assembly in G1 cells depleted for endogenous Cdc6. U2OS/HA-Cdc6 wt (K) or DDD (L) cell lines were transfected with Cdc6 siRNA alone (gray bars) or a combination of Cdc6 and cyclin E1/E2 siRNAs (black bars) for 24 hr, treated with Nocodazole for an additional 12 hr, and mitotic cells were then released from the block for 3 hr, after which the cells were kept repressed (open bars) or induced to express Cdc6 by the addition of Doxycycline (closed bars) for the indicated times. Pre-RC assembly was scored as in (F).

dependent synthesis of the CDK inhibitor, p21, DNA damage may also destabilize Cdc6 protein, potentially providing an additional mechanism for preventing licensing after DNA damage, perhaps contributing to the p53-dependent checkpoint pathway involved in preventing extensive rereplication when Cdt1 and Cdc6 are overexpressed (Vaziri et al., 2003).

Pre-RCs assemble in telophase, before the cell is committed to replicate its DNA (Diffley et al., 1994; Mendez and Stillman, 2000; Okuno et al., 2001). In budding yeast, pre-RCs assembled in mitosis disappear if cells exit the cell cycle due to nutrient depletion (Diffley et al., 1994). Quiescent mammalian cells also lack pre-RCs, which has formed the basis of a diagnostic test for neoplastic cells (Williams et al., 1998). Cdt1 and Mcm2-7 disappear more slowly from quiescent cells (data not shown). We suggest that, because of its rapidity, the APC/C-dependent proteolysis of Cdc6 is the primary mechanism for preventing licensing in quiescent cells. It will be of interest to know whether this mechanism contributes to either genome stability or to maintaining cells in a quiescent state. The availability of the phospho-mimicking DDD mutant should allow this to be tested in animal models.

Cyclin E can also play a negative role in licensing: overproduction of cyclin E inhibits pre-RC assembly in mammalian cells (Ekholm-Reed et al., 2004), analogous to the effect of overproducing G1 cyclins in budding yeast (Tanaka and Diffley, 2002). We suggest that cyclin E exerts its negative effect on licensing through a factor that is not present during the G0-G1 transition perhaps because it, too, is an APC/C substrate. An attractive candidate for such a factor is Skp2, a specificity factor for the SCF ubiquitin ligase that targets CDK-phosphorylated proteins (Ang and Harper, 2005) and is itself an APC/C^{Cdh1} target (Bashir et al., 2004; Wei et al., 2004). Because the pathway leading to cyclin E activation is misregulated in most cancer cells and cancers exhibiting elevated cyclin E levels generally have a poor prognosis, it will be important to determine whether the positive and/or negative roles cyclin E plays in pre-RC assembly contribute to oncogenesis.

Experimental Procedures

Cell Culture and Drugs

Human U2OS osteosarcoma and T98G glioblastoma cells were grown in DMEM containing 10% fetal bovine serum. The U2OS T-REx cell line expressing the Tetracycline repressor (TetR) protein was purchased from Invitrogen and maintained in DMEM as above containing 100 μg/ml Hygromycin B (Invitrogen). Stable cell lines expressing siRNA-resistant HA-Cdc6 constructs in a Tetracycline-responsive manner were isolated by transfecting U2OS T-REx cells with pcDNA4/TO-HA-Cdc6 and selecting productively transfected cells with 200 μg/ml Zeocin (Invitrogen). A T98G-derivative cell line (T98G-T) stably expressing the TetR protein was generated by selecting cells transfected with pcDNA6/TR in medium containing 100 μg/ml Zeocin. T98G cells were rendered quiescent by removing serum from confluent cells for 72 hr, after which cells were stimulated to reenter the cell cycle by trypsinization and replating at a 1:3 dilution in fresh medium containing 10% serum. To synchronize cells in metaphase, 50 ng/ml Nocodazole (Sigma) was added to the medium for 12 hr. Subsequently, cells were released from the block by washing twice in PBS and replating in fresh medium. The following drugs were used: 10 μM MG132 (Sigma), 10 μg/ml Roscovitine (BioMol), 25 μg/ml Cycloheximide (Sigma), and 1 μg/ml Doxycycline (Sigma).

Plasmids

A pCGN.CSH.FL42 plasmid (Mendez and Stillman, 2000) encoding N-terminally HA-tagged full-length human Cdc6 was a gift from Dr. B. Stillman. For comparison of full length Cdc6 and Cdc6ΔNT (amino acids 110–560), these sequences were amplified by PCR and cloned into pcDNA3.1 (Invitrogen) harboring an N-terminal HA tag. Cyclin E and A cDNAs were subcloned into pcDNA3.1 containing an N-terminal myc tag. Plasmids expressing myc-Cdh1 (Lukas et al., 1999) and pCMV-CD20 were gifts from Dr. J. Lukas. For Doxycycline-inducible (Tet-On) expression of Cdc6 and Cdh1, HA-Cdc6 variants and myc-Cdh1 were amplified by PCR and inserted into pcDNA4/TO (Invitrogen). The Cdc6 AAA (S54A, S74A, S106A) and DDD (S54D, S74D, S106D) mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). An expression plasmid for HA-tagged Cdt1 was a gift from Dr. K. Helin. The pcDNA6/TR plasmid was obtained from Invitrogen. pBOS-H2B-GFP was from BD Pharmingen. Transfections were performed using FuGene6 (Roche) or Lipofectamine 2000 (Invitrogen) for U2OS and T98G cells, respectively, according to the manufacturers instructions. Unless otherwise stated, transfections were performed for a total of 24 hr.

Antibodies

Antibodies used in this study included mouse monoclonals to Cdc6 (sc-9964), cyclin E (sc-247 and sc-248), Mcm7 (sc-9966), Cdc27 (sc-9972), GFP (sc-9996), rabbit polyclonals to Cdc6 pSer54 (sc-12920-R), cyclin A (sc-751), Geminin (sc-13015), HA-probe (sc-805), and goat polyclonals to Mcm6 (sc-9843) and Cdc20 (sc-1906), all from Santa Cruz Biotechnology. Rabbit ORC2 antibody (559266) was from BD Pharmingen. Monoclonal Cdh1 antibody was a gift from Dr. T. Hunt, Cdt1 monoclonal antibody was a gift from Dr. K. Helin, and rabbit polyclonal Cdh1 and monoclonal cdk7 antibodies were gifts from Dr. J. Lukas. Other antibodies used were myc (9E10) and HA (12CA5).

siRNA

siRNA oligonucleotides (Dharmacon) were synthesized to the following sequences (sense strand): Cdc6 (5′-AACUUCCCACCUUA UACCAGA-3′), Cdh1 (5′-AAUGAGAAGUCUCCCAGUCAG-3′), cdk2 (5′-AAGCCAGAAACAAGUUGACGG-3′), cyclin E1/E2 (5′-AACCAA ACUUGAGGAAAUCUA-3′), and GL2 (control) (5′-AACGUACGCGG AAUACUUCGA-3′). Transfections were performed with 100 nM siRNA duplexes using Oligofectamine or Lipofectamine 2000 (both from Invitrogen) for U2OS or T98G cells, respectively, according to the manufacturer's instructions. To generate siRNA-resistant Cdc6 expression constructs, two silent mutations (underlined) were introduced into the Cdc6 siRNA target sequence (AA<u>T</u>TTCCC<u>G</u>CCTTA TACCAGA) by site-directed mutagenesis.

Total Cell Extracts and Chromatin Fractionation

To prepare total cell extracts, cells were lysed in EBC buffer (50 mM Tris [pH 7.5], 120 mM NaCl, 0.5% NP-40, 1 mM EDTA) containing 1 mM DTT, 5 μg/ml Leupeptin, 2 μg/ml Aprotinin, 0.1 mM PMSF, 10 mM β-Glycerophosphate, 1 mM NaF, and 0.1 mM Na_3VO_4, incubated for 20 min on ice, sonicated briefly, and lysates were cleared by centrifugation for 10 min at 20,000 rpm. For dephosphorylation of total cell extracts, lysates were prepared using EBC buffer without phosphatase inhibitors and incubated with λ phosphatase (New England BioLabs) for 15 min at 37°C. To obtain chromatin-enriched fractions, cells were separated into Triton X-100-soluble and -insoluble fractions essentially as described (Ballabeni et al., 2004). Briefly, cells were lysed in cytoskeleton (CSK) buffer (10 mM PIPES [pH 6.8], 100 mM NaCl, 1.5 mM $MgCl_2$, 300 mM sucrose), containing 0.5% Triton X-100, 1 mM ATP, 1mM DTT, 5 μg/ml Leupeptin, 2 μg/ml Aprotinin, and 0.1 mM PMSF for 20 min on ice. Lysed cells were then centrifuged for 4 min at 3200 rpm to obtain the soluble fraction. The pellet was washed once with CSK buffer for 5 min on ice, centrifuged for 4 min at 3200 rpm, and resuspended in 2× Laemmli Sample Buffer, then boiled for 3 min and sonicated for 10 s.

Immunoblotting, Immunoprecipitation, and Kinase Assays

Immunoblotting and immunoprecipitations were done as described earlier (Mailand et al., 2000). For immunodepletion of Ser54-phos-

phorylated Cdc6, total cell extracts were subjected to three successive rounds of immunoprecipitation with phospho-Ser54 antibody or preimmune rabbit serum for 1 hr at 4°C on an end-over-end rotator. Measurements of cyclin E- and A-associated Histone H1 kinase activities were done exactly as described (Mailand et al., 2000).

Immunofluorescence

Cells grown on glass coverslips were fixed in ice-cold 1:1 (v/v) Methanol:Acetone, and immunostained with antibodies specified in figure legends and secondary Alexa Fluor 594 reagents (Molecular Probes). To detect chromatin bound Mcm6, cells were washed with CSK buffer once, extracted with CSK buffer containing 0.5% Triton X-100, 1mM DTT, 5 μg/ml Leupeptin, 2 μg/ml Aprotinin, and 0.1 mM PMSF for 5 min at room temperature and washed again in CSK buffer before fixation. Cells were visualized using a Zeiss 510 laser-scanning microscope mounted on Axioplan 2 (Zeiss).

Ubiquitination and Degradation Assays

For detection of in vivo ubiquitin-conjugates of ectopic Cdc6, cells were cotransfected with 6× His-tagged Ubiquitin (Treier et al., 1994), and 6× His-tagged proteins were purified on Ni^{2+}-NTA spin columns (Qiagen) under denaturing conditions according to the manufacturer's instructions. The ability of immunopurified APC^{Cdh1} to ubiquitinylate Cdc6 was assessed essentially as described (Kramer et al., 2000); briefly, APC was immunoprecipitated with Cdc27 antibodies from 3 mg of quiescent T98G cell extract, washed extensively, preincubated with in vitro-translated Cdh1 for 30 min at 37°C, washed again, and incubated with 125 μM Biotin-conjugated unphosphorylated or Ser54/74-phosphorylated Cdc6 peptides (amino acids 49–88) in the presence of 1.25 mg/ml Ubiquitin (Sigma), 5 μM human E1 (Boston Biochem), 2 μM hUbcH10 (Boston Biochem), and an energy-regenerating system for 20 min at 37°C. Ubiquitinated peptides were detected by immunoblotting with anti-Biotin antibody (Vector Laboratories). In vitro degradation assays were performed as described (Rape and Kirschner, 2004), using extracts from quiescent T98G cells and ^{35}S-labeled N-terminal fragments (amino acids 1–121) of Cdc6. Aliquots were resolved by 15% SDS-PAGE and autoradiography.

Purification of CD20-Positive Cells

Transfected cells expressing the CD20 surface marker were isolated with Dynabeads (Dynal Biotech) according to the manufacturers instructions. Briefly, cells transfected at a 1:10 ratio with pCMV-CD20 were collected in PBS containing 0.1% EDTA, centrifuged and resuspended in PBS containing 0.1% BSA, and incubated with 25 μl Pan Mouse IgG Dynabeads coated with monoclonal CD20 antibody for 60 min at 4°C on an end-over-end rotator. Bound cells were washed three times using a magnetic rack and processed for total cell extract preparation or chromatin fractionation.

Supplemental Data

Supplemental Data include five figures and can be found with this article online at http://www.cell.com/cgi/content/full/122/6/915/DC1/.

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