Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in *Xenopus* egg extracts

Emily E. Arias and Johannes C. Walter[1]

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA

In eukaryotes, prereplication complexes (pre-RCs) containing ORC, Cdc6, Cdt1, and MCM2-7 are assembled on chromatin in the G1 phase. In S phase, when DNA replication initiates, pre-RCs are disassembled, and new pre-RC assembly is restricted until the following G1 period. As a result, DNA replication is limited to a single round per cell cycle. One inhibitor of pre-RC assembly, geminin, was discovered in *Xenopus*, and it binds and inactivates Cdt1 in S phase. However, removal of geminin from *Xenopus* egg extracts is insufficient to cause rereplication, suggesting that other safeguards against rereplication exist. Here, we show that Cdt1 is completely degraded by ubiquitin-mediated proteolysis during the course of the first round of DNA replication in *Xenopus* egg extracts. Degradation depends on Cdk2/Cyclin E, Cdc45, RPA, and polymerase α, demonstrating a requirement for replication initiation. Cdt1 is ubiquitinated on chromatin, and this process also requires replication initiation. Once replication has initiated, Cdk2/Cyclin E is dispensable for Cdt1 degradation. When fresh Cdt1 is supplied after the first round of DNA replication, significant rereplication results, and rereplication is enhanced in the absence of geminin. Our results identify a replication-dependent proteolytic pathway that targets Cdt1 and that acts redundantly with geminin to inactivate Cdt1 in S phase.

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To maintain genome integrity, it is essential that origins of replication initiate DNA synthesis only once per cell cycle. Work in yeast has revealed that origin firing is regulated at the level of the prereplication complex (pre-RC) (Diffley 1996, 2001; Bell and Dutta 2002). Pre-RC assembly involves the stepwise binding of the six-subunit origin recognition complex (ORC) to origins of replication, followed by the recruitment of two additional proteins, Cdc6 and Cdt1. Together, these three proteins recruit the minichromosome maintenance 2-7 (MCM2-7) complex, a likely candidate for the replicative DNA helicase (for review, see Labib and Diffley 2001; Pacek and Walter 2004; Shechter et al. 2004). Pre-RCs assemble during the G1 phase, when Cyclin-dependent kinase (Cdk) activity is low, and they are transformed into active replication forks when Cdk activity rises at the G1/S transition. Cdk, and a second kinase, Cdc7, stimulate association of the replication factor Cdc45 with pre-RCs. Cdc45 activates the helicase activity of the MCM2-7 complex, promoting unwinding of DNA at origins, recruitment of the single-stranded DNA-binding protein RPA, and synthesis of an RNA primer by DNA polymerase α (pol α). Replication Factor-C (RFC) then loads the processivity factor, PCNA, onto the DNA, and this allows recruitment of DNA polymerase δ (Hubscher et al. 2002). As a result of origin firing, the pre-RC is disassembled. Critically, reassembly of pre-RCs is prohibited until the cell has completed mitosis, and thus, each origin of replication supports only one initiation event per cell cycle.

Cdks not only stimulate replication initiation, they also prevent reinitiation. In budding yeast, inactivation of Cdk induces rereplication (Dahmann et al. 1995) via multiple, redundant mechanisms (Nguyen et al. 2001), including inactivation of Cdc6 (Drury et al. 1997), stimulation of nuclear export of Cdt1, and MCM2-7 (Labib et al. 1999; Nguyen et al. 2000; Tanaka and Diffley 2002), and inhibitory phosphorylation of ORC (Nguyen et al. 2001). In fission yeast, inactivation of Cdk also causes rereplication (Broek et al. 1991), and here the targets are ORC, Cdc6, and Cdt1 (Nishitani and Nurse 1995; Nishitani et al. 2000; Vas et al. 2001). In mammalian cells,

[1]**Corresponding author.**
E-MAIL johannes_walter@hms.harvard.edu; FAX (617) 738-0516.
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inactivation of Cdc2 or expression of Cdk inhibitors during G2 phase can also cause rereplication (Itzhaki et al. 1997; Bates et al. 1998). Additionally, Cdk2/Cyclin E activity is inhibitory for MCM2-7 loading in human cells and *Xenopus* egg extracts (Hua et al. 1997; Ekholm-Reed et al. 2004).

Unlike yeast, metazoans contain geminin, an additional inhibitor of pre-RC formation (McGarry and Kirschner 1998). Geminin inhibits pre-RC assembly by binding to and inactivating Cdt1 (Wohlschlegel et al. 2000; Tada et al. 2001). In *Xenopus* egg extracts, geminin is regulated by its abundance and by post-translational modification. During mitotic exit, geminin is ubiquitinated by the anaphase-promoting complex (APC), resulting in degradation of a fraction of the protein, while the remainder is maintained in an inactive state, but not destroyed. The inactive pool of geminin reacquires its ability to bind Cdt1 upon nuclear import in telophase (Hodgson et al. 2002; Li and Blow 2004). In *Xenopus* egg extracts, geminin is a key inhibitor of pre-RC assembly (Hodgson et al. 2002). However, geminin does not appear to be the only block to rereplication, because removal of geminin from egg extracts or embryos does not cause rereplication (McGarry and Kirschner 1998; McGarry 2002). Therefore, the importance of geminin in preventing rereplication in *Xenopus* has not been formally demonstrated. In contrast, in certain *Drosophila* and human cells, the loss of geminin leads to partial rereplication of the DNA (Quinn et al. 2001; Mihaylov et al. 2002; Melixetian et al. 2004).

In addition to being inhibited by geminin, metazoan Cdt1 is also regulated by proteolysis. In *Caenorhabditis elegans*, *Drosophila*, and humans, Cdt1 is degraded at the onset of S phase (Nishitani et al. 2001; Zhong et al. 2003; Thomer et al. 2004). Degradation of human Cdt1 is mediated by the SCF^{Skp2} E3 ubiquitin ligase (Li et al. 2003; Liu et al. 2004; Sugimoto et al. 2004), and overexpression of wild-type or nondegradable Cdt1 is sufficient to induce some rereplication in human cell lines (Vaziri et al. 2003; Nishitani et al. 2004). In *C. elegans*, Cdt1 destruction requires Cul4, the loss of which produces cells with up to 100C DNA content (Zhong et al. 2003). Finally, Cdt1 is rapidly degraded in human and *Drosophila* cells in response to UV or γ-irradiation, and both Cul4 and SCF^{Skp2} have been linked to this process (Higa et al. 2003; Kondo et al. 2004). The relative roles of Cdks, geminin, and Cdt1 destruction in preventing rereplication are poorly understood.

Here, we report that Cdt1 is degraded in *Xenopus* egg extracts by ubiquitin-mediated proteolysis. Destruction of Cdt1 is dependent on the initiation of DNA replication, as it requires Cdk2/Cyclin E, Cdc45, RPA, and pol α. Cdt1 is ubiquitinated on chromatin, and this process also requires initiation. Once replication has initiated, Cdk2 is not required for Cdt1 destruction, suggesting that direct phosphorylation of Cdt1 by Cdk2/Cyclin E is not necessary. When recombinant Cdt1 is supplied following the first round of DNA replication, MCM2-7 reloads onto chromatin and rereplication is observed. This rereplication is enhanced in the absence of geminin. Therefore, replication-dependent proteolysis of Cdt1 and binding of geminin to Cdt1 both insure that no functional Cdt1 is present during S phase in *Xenopus* egg extracts. Our results show that the first round of DNA replication actively prevents additional rounds by stimulating destruction of Cdt1.

Results

A paradox: egg extracts lacking inhibitors of pre-RC formation support only one round of DNA replication

We use two related *Xenopus* egg-extract systems to identify the mechanisms that prevent rereplication in vertebrates (Supplementary Fig. S1). In the nucleus-dependent replication system, sperm chromatin is added to a low-speed supernatant (LSS) of egg cytoplasm, leading to pre-RC assembly and nuclear envelope formation (Lohka and Masui 1983; Blow and Laskey 1986; Newport 1987). Upon nuclear import of Cdk2/Cyclin E and other replication factors, DNA replication initiates, and a single round of replication occurs. In the second approach, DNA replication takes place in the absence of a nucleus (Walter et al. 1998). Here, replication is carried out by incubating DNA sequentially with two extracts: A high-speed supernatant (HSS) of LSS supports pre-RC assembly, and a concentrated nucleoplasmic extract (NPE) added subsequently supports origin firing from these pre-RCs because it contains high concentrations of Cdk2/Cyclin E, Cdc7, and other factors (Walter 2000; Prokhorova et al. 2003). Critically, this system supports precisely one round of DNA replication, because NPE contains potent inhibitors of de novo MCM2-7 loading (Walter et al. 1998; Fig. 1B, lane 2). The nucleus-free replication system provides a simplified setting in which to study the control of rereplication, because there is no opportunity for nuclear exclusion of pre-RC components.

We first asked which factors in NPE inhibit MCM2-7 loading. Two likely candidates are Cdk2/Cyclin E and geminin, because they are ~25-fold more concentrated in NPE than in HSS (Walter et al. 1998; Hodgson et al. 2002; Yamaguchi and Newport 2003), and because recombinant geminin and Cdk2/Cyclin E inhibit MCM2-7 loading onto chromatin when added to HSS (Hua et al. 1997; McGarry and Kirschner 1998). We removed >98% of Cdk2 or geminin from NPE using specific antibodies (Fig. 1A), and asked whether the depleted extracts still inhibited MCM2-7 loading when mixed with an equal volume of HSS. Cdk2-depleted NPE was just as competent at inhibiting MCM2-7 loading in HSS as mock-depleted NPE (Fig. 1B, cf. lanes 2 and 3), likely due to the high concentrations of geminin in NPE. Surprisingly, geminin-depleted NPE was not inhibitory for MCM2-7 loading, even though it clearly retained high Cdk2 activity, because it supported Cdc45 loading (Fig. 1B, lane 4) and efficient DNA replication (Fig. 1D, column 2). These results demonstrate that geminin is the principal inhibitor of MCM2-7 loading in NPE. A similar conclusion was reached by Blow and colleagues (Hodgson et al.

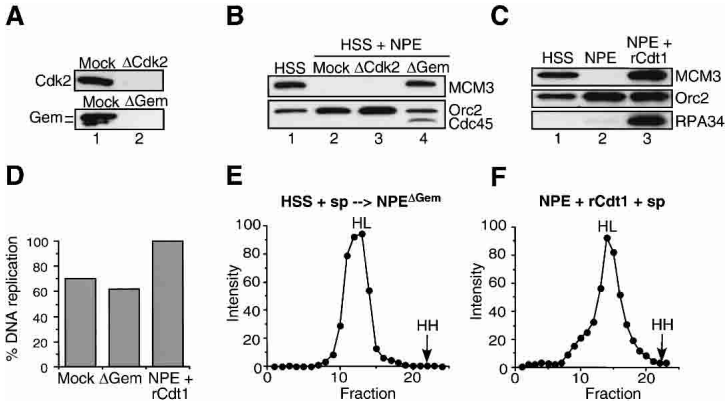

Figure 1. Geminin is the principal inhibitor of MCM2-7 loading in NPE. (*A*) A total of 1 µL of mock-, Cdk2-, or geminin-depleted NPE was analyzed by Western blotting using antibodies to Cdk2 and geminin. (*B*) Sperm chromatin (4000/µL) was incubated in HSS or a 1:1 mixture of HSS and mock-, Cdk2-, or geminin-depleted NPE that contained 50 µg/mL aphidicolin. The DNA was isolated after 30 min, and chromatin-bound proteins were analyzed by Western blotting using a mixture of ORC2 and Cdc45 antibodies, as well as MCM3 antibodies. (*C*) HSS, NPE, or NPE supplemented with 200 nM rCdt1 was incubated for 30 min before addition of sperm chromatin (3000/µL). Chromatin was isolated after 30 min, and chromatin-bound proteins were analyzed using antibodies against MCM3, ORC2, and RPA. In lanes *2* and *3*, the NPE contained 50 µg/mL aphidicolin. (*D*) Sperm chromatin was incubated in 4 µL HSS, followed by addition of 8 µL mock- or geminin-depleted NPE to yield 3000 sperm/µL final concentration (*left* and *middle* columns). Sperm chromatin was incubated in 12 µL NPE (3000/µL final concentration) supplemented with 200 nM rCdt1 (*right* column). The percentage of input DNA replicated after 90 min is plotted. (*E*) Sperm chromatin was incubated in HSS, followed by addition of geminin-depleted NPE to yield 3000 sperm/µL final concentration in the presence of [α-^{32}P]dATP and BrdU. Replication products were fractionated on a CsCl gradient and radioactivity across the gradient was measured. (HL) Heavy–Light DNA, 1.75 g/mL, (HH) Heavy–Heavy DNA, 1.80 g/mL. (*F*) NPE was supplemented with 200 nM rCdt1 and incubated for 15 min before addition of sperm chromatin (1500/µL) in the presence of [α-^{32}P]dATP and BrdU. Replication products were analyzed as in *E*.

2002), although they did not determine whether the geminin-depleted NPE remained replication competent.

If geminin is the principle inhibitor of MCM2-7 loading in NPE, we reasoned that it might be possible to achieve MCM2-7 loading in NPE by supplying enough Cdt1 to sequester all the geminin. NPE contains no Cdt1 (see below), whereas the concentration of geminin is ~160 nM (data not shown). When NPE was supplemented with at least 200 nM recombinant Cdt1 expressed in insect cells (rCdt1), MCM2-7 loaded onto chromatin as efficiently as in HSS (Fig. 1C). The requirement for Cdt1 to be present in excess over geminin by ~40 nM correlates well with the concentration of Cdt1 in HSS (25 nM), and the fact that ~25 nM rCdt1 is required to rescue replication in Cdt1-depleted extracts (data not shown). Cdk2/Cyclin E activity was not compromised by addition of rCdt1, because the resulting NPE supported loading of RPA (Fig. 1C), and DNA replication (Fig. 1D). As shown previously, NPE alone does not support DNA replication (Walter et al. 1998; data not shown). These experiments demonstrate that geminin is the primary inhibitor of MCM2-7 loading in NPE, and that a concentration of endogenous Cdk2/Cyclin E that is sufficient to promote initiation of DNA replication is not inhibitory for pre-RC assembly.

As shown above, depletion of geminin from NPE produces an extract that is not inhibitory for pre-RC formation in HSS, but supports origin firing. Similarly, addition of rCdt1 to NPE yields an extract that is permissive for both pre-RC assembly and origin firing. We expected that both of these systems would support multiple rounds of DNA replication, especially since there is no opportunity for nuclear exclusion of pre-RC components. To test this prediction, sperm chromatin was incubated in HSS, followed by addition of geminin-depleted NPE in the presence of bromodeoxyuridine (BrdU), and replication products were subjected to CsCl equilibrium-density centrifugation. Unexpectedly, only "heavy–light" replication products, which represent one round of semiconservative DNA replication, were observed (Fig. 1E). Similarly, sperm chromatin replicated in NPE + rCdt1 underwent precisely one round of DNA replication (Fig. 1F). The absence of rereplication was not due to an inherent inability of chromatin to undergo more than one round of DNA replication in this system (Walter et al. 1998). Therefore, we infer that mechanisms other than geminin-dependent inhibition of Cdt1 must exist to limit DNA replication in *Xenopus* egg extracts.

Cdt1 is degraded in Xenopus *egg extracts*

To explain the absence of rereplication in the above experiments, we speculated that a replication factor might be consumed in the course of the first round of DNA replication. Given that Cdt1 is unstable in *C. elegans*, *Drosophila melanogaster*, and humans (see above), we examined the fate of Cdt1 in the nucleus-free system. Sperm chromatin was incubated in HSS to form pre-RCs, followed by addition of NPE to initiate DNA replication. We examined the total levels of Cdt1 over time by Western blotting, and found that Cdt1 was completely degraded only 30 min after addition of NPE (Fig. 2A). The same result was obtained when we used geminin-depleted NPE, or when 200 nM rCdt1 was added to NPE (data not shown). The destruction of Cdt1 explains our inability to observe rereplication in the experiments shown in Figure 1E and F.

In order to consider effects of the nuclear envelope on the control of DNA replication, we performed most subsequent experiments in the nucleus-dependent replication system. Thus, we first examined the stability of Cdt1 in LSS and found that it was completely degraded within 50 min of sperm addition (Fig. 2B). Our finding that Cdt1 is completely degraded in LSS explains our

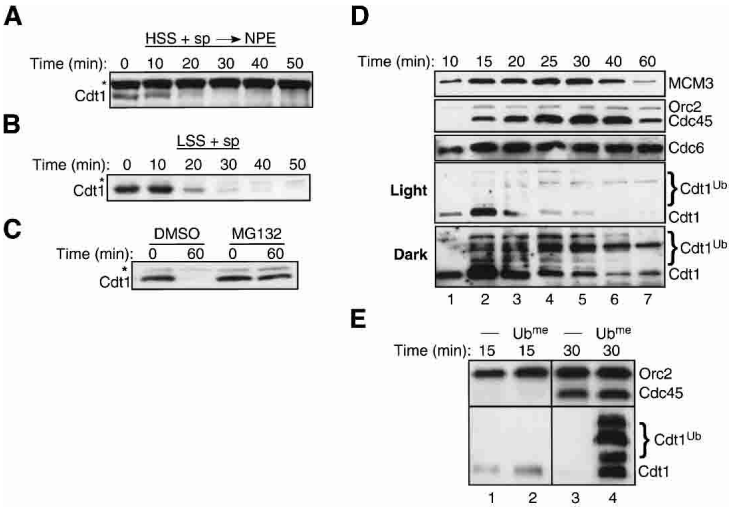

Figure 2. Cdt1 is degraded by ubiquitin-mediated proteolysis in *Xenopus* egg extracts. (*A*) Sperm chromatin (10,000/μL) was incubated in HSS for 30 min, followed by addition of NPE. A total of 1 μL of extract was withdrawn at the indicated times and blotted for Cdt1. Asterisk denotes a cross-reacting protein that served as a loading control. This protein is more abundant in NPE than in LSS. (*B*) Sperm chromatin was incubated in LSS and analyzed as in *A*. In all experiments using LSS, sperm chromatin was present at 3000/μL, unless otherwise stated. (*C*) Sperm chromatin was incubated in LSS in the presence of DMSO or 1 mM MG132 and analyzed as in *A*. (*D*) Sperm chromatin was incubated in LSS and isolated at various times after sperm addition. The indicated chromatin-bound proteins were analyzed by Western blotting. (*E*) As in *D*, except that lanes *2* and *4* contained 2 mg/mL methylated ubiquitin.

observation that NPE, which is extracted from nuclei formed in LSS for ~75 min, contains no Cdt1 (data not shown). Therefore, Cdt1 is degraded in both the soluble and nucleus-dependent replication systems.

We next explored the mechanism of Cdt1 degradation. Cdt1 destruction in LSS was blocked by MG132, indicating a requirement for the 26S proteasome (Fig. 2C). However, in the presence of MG132, we were unable to detect ubiquitinated forms of Cdt1 in total extract (data not shown), and this prompted us to examine Cdt1 on chromatin. Sperm chromatin was incubated in LSS, and at various times, chromatin was isolated. Similar to previous reports (Maiorano et al. 2000), Cdt1 reached a maximal level on chromatin 15 min after sperm addition, and it was largely absent by 25–30 min (Fig. 2D, lanes 1–5, Cdt1-Light). The rapid chromatin binding and release of Cdt1 is in contrast to other pre-RC components such as Cdc6, which remained on chromatin throughout the time course, consistent with a recent report (Oehlmann et al. 2004), and MCM3, which was not released from chromatin until 60 min. Loss of Cdt1 from chromatin preceded maximal loading of Cdc45 (Fig. 2D, lane 4), indicating that Cdt1 is removed from chromatin at an early stage during DNA replication. Importantly, a darker exposure of the Western blot (Fig. 2D, Cdt1-Dark) revealed a ladder of slowly migrating forms of Cdt1, which we suspected might be ubiquitinated Cdt1.

To determine whether Cdt1 is ubiquitinated on chromatin, we incubated sperm chromatin in LSS in the presence of methylated ubiquitin, a chemically modified form of ubiquitin that is conjugated to target proteins, but deficient in polyubiquitin chain formation (Hershko and Heller 1985). Equal amounts of chromatin-bound Cdt1 were observed in the presence or absence of methylated ubiquitin 15 min after sperm addition to LSS (Fig. 2E, lanes 1,2). However, after 30 min, when Cdc45 had loaded, multiple discrete forms of Cdt1 were evident in the presence of methylated ubiquitin, whereas Cdt1 was lost from chromatin in the corresponding control reaction (Fig. 2E, lanes 3,4). We conclude that Cdt1 is degraded by ubiquitin-mediated proteolysis in *Xenopus* egg extracts, and that polyubiquitination of Cdt1 takes place on chromatin during S phase.

Degradation of Cdt1 requires entry into S phase

Given that Cdt1 is ubiquitinated on chromatin, chromatin might be important for Cdt1 destruction. Consistent with this idea, Cdt1 degradation was significantly enhanced by the presence of a DNA template, although we often observed detectable degradation in the absence of a chromatin template (Fig. 3A, cf. top and middle). We next asked whether a nuclear environment is essential for Cdt1 degradation. Sperm chromatin was incubated in LSS in the presence or absence of Wheat Germ Agglutinin (WGA), an isolectin that binds to nucleoporins and inhibits nuclear transport (Finlay et al. 1987). Inhibition of nuclear transport by WGA also stabilized Cdt1 (Fig. 3A, bottom), indicating that rapid degradation of Cdt1 requires a nuclear milieu.

Cdk2/Cyclin E, the major Cdk present in interphase *Xenopus* egg extracts, becomes highly concentrated

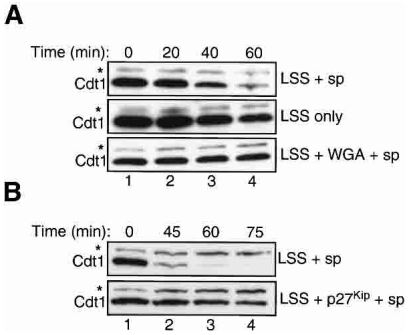

Figure 3. Cdt1 degradation requires entry into S phase. (*A*) LSS was incubated in the presence or absence of sperm chromatin. Where indicated, 0.2 mg/mL WGA was included. Reactions were stopped at the indicated times, and 1 μL of the total extract was blotted for Cdt1. (*B*) Sperm chromatin was incubated in LSS in the presence or absence of 1 μM $p27^{Kip}$ and analyzed as in *A*.

within nuclei (Hua et al. 1997). Therefore, we asked whether Cdk2/Cyclin E is required for the chromatin-dependent destruction of *Xenopus* Cdt1. Inhibition of Cdk2/Cyclin E by $p27^{Kip}$ greatly stabilized Cdt1 (Fig. 3B). Similar results were obtained with the general Cdk inhibitor roscovitine (data not shown). In summary, the requirement for a chromatin template, nuclear transport, and Cdk2/Cyclin E activity indicate that Cdt1 degradation depends on entry into S phase.

Degradation of Cdt1 requires the initiation of DNA replication

The requirement for Cdk2/Cyclin E activity to promote efficient Cdt1 proteolysis could be explained in two ways. First, destruction of Cdt1 might require direct phosphorylation of Cdt1 or the proteolysis machinery by Cdk2/Cyclin E. Indeed, human Cdt1 is targeted for degradation following phosphorylation by Cdk2/Cyclin A (Liu et al. 2004; Sugimoto et al. 2004). Second, the requirement for Cdk2/Cyclin E activity could be indirect, reflecting a role in stimulating DNA replication.

To determine whether the initiation of DNA replication is required for Cdt1 proteolysis, we immunodepleted the replication initiation factor Cdc45 from LSS. As expected (Mimura et al. 2000), depletion of Cdc45 severely inhibited DNA replication, and the effect was rescued by recombinant Cdc45 (Fig. 4A). Strikingly, depletion of Cdc45 also stabilized total Cdt1 levels, and addition of rCdc45 restored rapid degradation (Fig. 4B). Finally, the ubiquitination of Cdt1 on chromatin in the presence of methylated ubiquitin required Cdc45 (Fig. 4C). We conclude that Cdt1 degradation requires Cdc45, and therefore depends on an early step in the initiation of DNA replication.

To further delineate which event in DNA replication is required to trigger Cdt1 degradation, we examined the next known initiation step after Cdc45 loading, the chromatin-recruitment of RPA to the unwound origin (Walter and Newport 2000). Depletion of RPA inhibited DNA synthesis (Fig. 4D), stabilized Cdt1 (Fig. 4E), and abolished ubiquitination of Cdt1 on chromatin (Fig. 4F), demonstrating that this replication factor is critical for efficient Cdt1 degradation. In the absence of RPA, Cdc45 was still loaded onto chromatin (Fig. 4F), demonstrating that the RPA-depleted extract retained activity, and that Cdc45 loading is insufficient for Cdt1 ubiquitination or degradation.

The next factor that binds to chromatin after RPA is DNA polymerase α. Depletion of pol α inhibited DNA replication by 98% relative to a control-depleted extract (Fig. 4G). Degradation of Cdt1 was partially inhibited by loss of pol α, and addition of recombinant human pol α fully restored efficient Cdt1 degradation (Fig. 4H), even though it only modestly rescued DNA replication (Fig. 4G). Moreover, ubiquitination of Cdt1 on chromatin was almost completely inhibited by depletion of pol α, and it was rescued by addition of recombinant pol α (Fig. 4I). It is currently unclear whether the residual ubiquitination and degradation of Cdt1 in pol α-depleted extracts is due to trace amounts of DNA synthesis. Nevertheless, highly efficient ubiquitination and degradation of Cdt1 clearly require the presence of pol α.

A trace amount of DNA replication is required for efficient Cdt1 degradation

To determine whether DNA synthesis by pol α is required for Cdt1 degradation, we titrated the DNA polymerase inhibitor aphidicolin into LSS. At concentrations

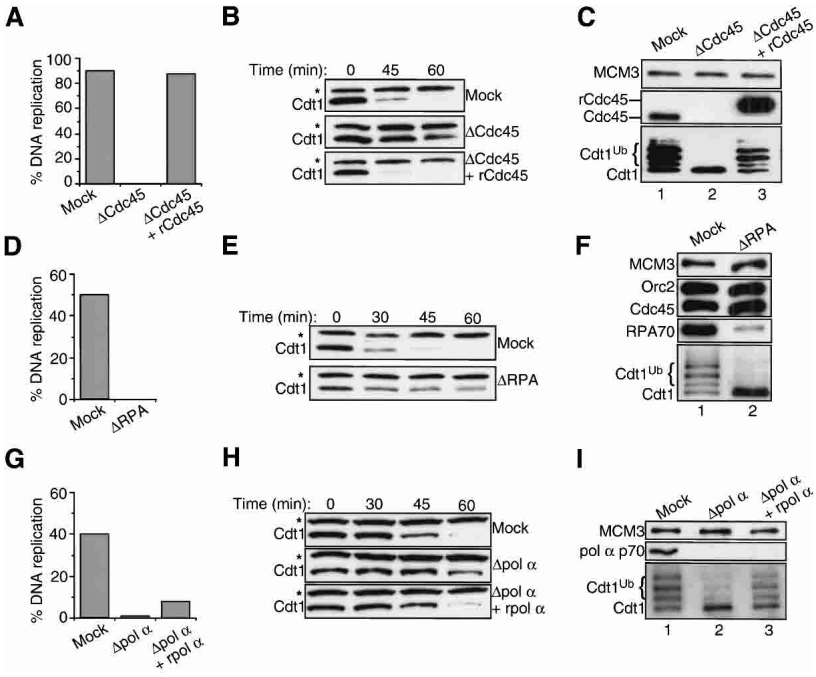

Figure 4. Cdt1 degradation requires initiation of DNA replication. LSS was immunodepleted of Cdc45, RPA, or pol α, as indicated. Where indicated, extracts contained 1.6 μM recombinant 6xhis-Cdc45 (+rCdc45) or 50nM recombinant human pol α complex (+rpol α). (*A*,*D*,*G*) Sperm chromatin was incubated in the indicated LSS in the presence of [α-^{32}P]dATP. The percentage of input DNA replicated after 90 min is plotted. (*B*,*E*,*H*) Sperm chromatin was incubated in the indicated LSS, and at various times, reactions were stopped and 1 μL of extract was blotted for Cdt1. (*C*,*F*,*I*) Sperm chromatin was incubated in the indicated LSS supplemented with 2 mg/mL methylated ubiquitin. Chromatin was isolated after 45 min, and the indicated proteins were visualized by Western blotting. Note, the *Xenopus* pol α antibody does not react with recombinant human pol α.

of aphidicolin up to 75 μM, the rate of Cdt1 degradation was unaltered, even though DNA replication was reduced to 0.3% of the control (Fig. 5A). Interestingly, although Cdt1 degradation was not inhibited by 75 μM aphidicolin, its ubiquitination was clearly reduced (Fig. 5B, Cdt1-Light). These results suggest that proteolysis of Cdt1 by the proteasome is slower than the ubiquitination of Cdt1, and that ubiquitination is therefore not rate-limiting for Cdt1 destruction. When the aphidicolin concentration was increased up to 600 μM, DNA replication was further inhibited, dropping below the detection limit. At these higher concentrations of aphidicolin, Cdt1 ubiquitination was further reduced (Fig. 5B, Cdt1-Dark), and inhibition of Cdt1 degradation became evident (Fig. 5A).

The key function of pol α is to synthesize a short RNA primer, followed by a ~30-nucleotide DNA primer, which allows the RFC-dependent chromatin recruitment of PCNA, a processivity factor for DNA polymerase δ (Hubscher et al. 2002). We therefore postulated that PCNA assembly might stimulate Cdt1 degradation. As an initial test of this idea, we blotted chromatin for PCNA and ubiquitinated Cdt1. Over the entire range of aphidicolin concentrations tested, chromatin binding of PCNA correlated with Cdt1 ubiquitination (Fig. 5B, cf. PCNA and Cdt1-Dark), and maximum stabilization of Cdt1 occurred when PCNA binding to chromatin was undetectable (Fig. 5, cf. A and B). To test the requirement for PCNA directly, we attempted to remove this protein from extracts. However, PCNA is present at micromolar concentrations in egg extracts (Mattock et al. 2001), and we were not successful in achieving the high degree of replication inhibition that would be necessary to observe a defect in Cdt1 destruction. Therefore, at present, we are able to conclude that while extensive DNA replication is clearly not required for Cdt1 degradation, synthesis of a short DNA primer by DNA pol α appears to be an important prerequisite.

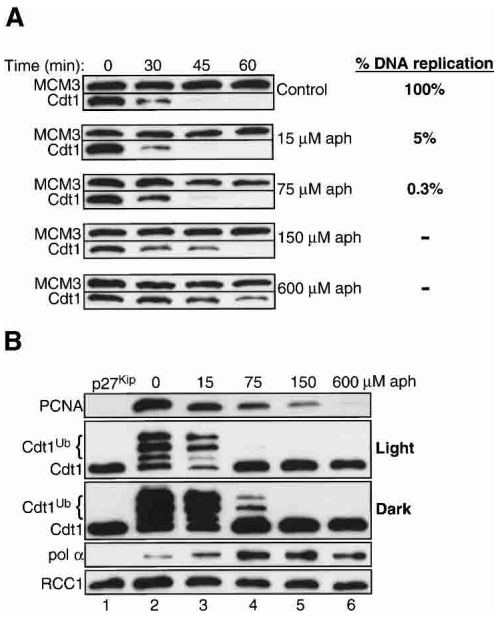

Figure 5. Degradation of Cdt1 does not require extensive DNA synthesis. (*A*) Sperm chromatin was incubated in LSS containing the indicated concentrations of aphidicolin. At various times, reactions were stopped and 1 μL of extract was blotted for Cdt1 and MCM3. Parallel reactions were carried out in the presence of [α-^{32}P]dATP and the percentage of input DNA replicated after 120 min is indicated. (*B*) Sperm chromatin was incubated in LSS containing 2 mg/mL methylated ubiquitin and the indicated concentrations of aphidicolin or 1 μM p27^{Kip}. Chromatin was isolated after 45 min and blotted for various replication proteins and RCC1 as a loading control.

Inhibition of replication by aphidicolin activates the ATR-dependent checkpoint in *Xenopus* egg extracts (Guo et al. 2000). To rule out that the destruction observed in the presence of aphidicolin in Figure 5 occurred via a checkpoint-dependent pathway, we verified that caffeine, an inhibitor of ATR and ATM checkpoint kinases (Abraham 2001), had no effect on the rate of Cdt1 destruction, and this was true whether or not aphidicolin was present (data not shown). A checkpoint-independent Cdt1 destruction pathway induced by stalled forks also appears unlikely, since Cdt1 destruction is blocked at high, but not low aphidicolin concentrations (Fig. 5A).

We also addressed what happens when fresh Cdt1 is added to nuclei that have completed DNA replication. Our results show that while rCdt1 added early is degraded efficiently, degradation of rCdt1 added after the first round of DNA replication has gone to completion is significantly slower (Supplementary Fig. S2). This result indicates that the replication-dependent Cdt1 degradation machinery loses activity after the first round of DNA replication is completed.

Inhibition of Cdk2/Cyclin E after replication initiation does not inhibit Cdt1 destruction

In addition to promoting Cdt1 destruction indirectly by stimulating replication initiation, Cdk2/Cyclin E might also play a more direct role in this process, for example, by phosphorylating Cdt1. To address whether Cdk2 is required independently of initiation, we synchronized replication forks after replication initiation in LSS by incubating sperm chromatin in the presence of 15 μM aphidicolin. We then added buffer or p27^{Kip}, followed by rCdt1. We found that p27^{Kip} did not inhibit the ubiquitination or destruction of Cdt1, suggesting Cdk2 is not required independently of its role in replication initiation (data not shown). To avoid the use of aphidicolin, we also used the nucleus-free system, in which replication forks can be synchronized after initiation by incubating sperm chromatin in NPE at 19°C instead of 22°C (Pacek and Walter 2004). We then added buffer or p27^{Kip}, and after Cdk2 activity had been inactivated (Pacek and Walter 2004), rCdt1 was supplied, and the rate of Cdt1 degradation was measured. In these experiments, the rCdt1 was first treated extensively with λ phosphatase to abolish any phosphorylation of Cdt1 that might have occurred in the insect cells from which it was purified. Figure 6 (right) shows that the rCdt1 added after initiation was destroyed rapidly, even when p27^{Kip} was pres-

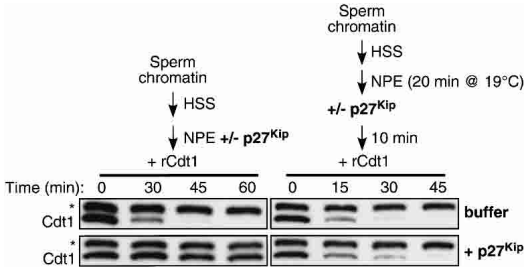

Figure 6. Cdk2 activity is not required for Cdt1 degradation following initiation of replication. (*Left*) Sperm chromatin (10,000/µL) was incubated in HSS followed by addition of NPE supplemented with $p27^{Kip}$ or buffer, and 60 nM rCdt1 was added immediately. Reactions were incubated at 19°C for 20 min, and then transferred to 22°C. (*Right*) Sperm chromatin was incubated in HSS followed by addition of NPE. Reactions were incubated at 19°C for 20 min, followed by addition of $p27^{Kip}$ or buffer and transfer to 22°C. A total of 60 nM rCdt1 was added after an additional 10 min. In both panels, reactions were stopped at the indicated times after addition of rCdt1 and 0.5 µL of extract was blotted for Cdt1.

ent. We observed the same result using 500 µM roscovitine instead of $p27^{Kip}$ (data not shown). Importantly, destruction of rCdt1 added at the start of the reaction was Cdk2 dependent (Fig. 6, left), and it also required Cdc45 (data not shown). Together, these results strongly suggest that the replication-dependent destruction of Cdt1 does not require direct phosphorylation of Cdt1 by Cdk2. Interestingly, the presence of $p27^{Kip}$ (Fig. 6) or roscovitine (data not shown) reproducibly caused a slight delay in the destruction of rCdt1 added after initiation, suggesting that Cdk2 might stimulate the destruction of Cdt1 in *Xenopus* egg extracts.

Cdt1 addition to replicated nuclei stimulates rereplication

To examine the significance of Cdt1 destruction, we tested the effects of adding fresh rCdt1 after the first round of DNA replication in LSS. Sperm chromatin was incubated in LSS for 70 min, followed by addition of rCdt1. MCM3 and Cdc45 were released from chromatin within 70 min after sperm addition (Fig. 7A, lanes 1,2), indicating that the first round of replication was complete. Addition of rCdt1 after a further 20 min resulted in reloading of MCM3 and Cdc45 onto chromatin (Fig. 7A, lanes 3,4), and the reloading of Cdc45 was inhibited by $p27^{Kip}$ (data not shown). Together, the results suggest that Cdt1 is sufficient to stimulate pre-RC assembly and replication initiation after the first round of DNA replication has been completed.

The nuclear envelope has been implicated in limiting DNA replication to a single round per cell cycle in *Xenopus* egg extracts (Blow and Laskey 1988). Therefore, one caveat was that the experimental manipulations involved in adding rCdt1 to the extract might compromise nuclear integrity, potentially causing two undesired effects as follows: dilution of pre-RC inhibitors concentrated in the nucleus, and/or exposure of chromatin to pre-RC components normally excluded from the nucleus, either of which could wrongly lead us to conclude that the lack of Cdt1 following DNA replication is the only block to MCM2-7 reloading in nuclei. To address whether addition of rCdt1 to replicated nuclei disrupts nuclear integrity, we asked whether reloading of MCM3 following rCdt1 addition requires nuclear transport. When the nuclear transport inhibitor WGA was added after DNA replication, but before rCdt1 addition, reloading of both MCM3 and Cdc45 was blocked (Fig. 7A, lanes 5,6), suggesting that nuclei remained intact

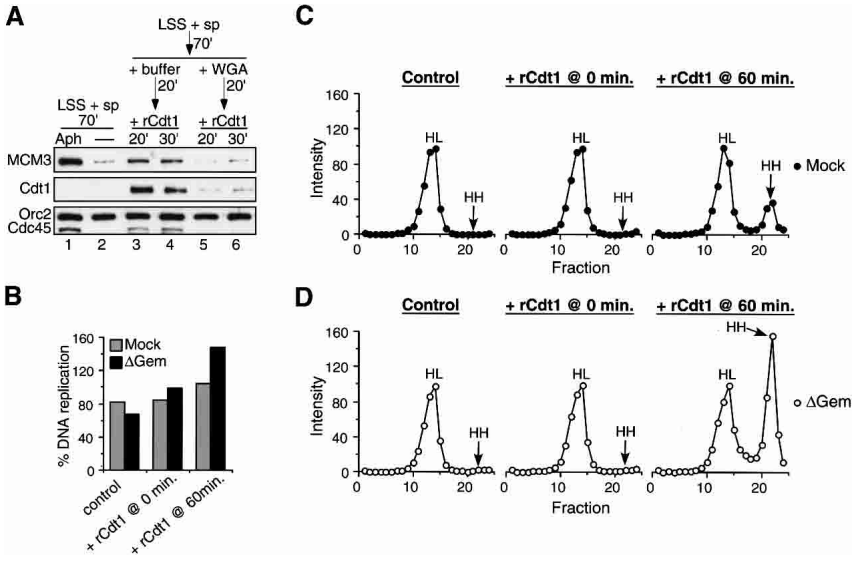

Figure 7. Addition of Cdt1 to replicated nuclei results in reloading of MCM2-7 and rereplication. (*A*) Sperm chromatin was incubated in LSS for 70 min, and chromatin was isolated (lanes *1,2*) or samples were supplemented with control buffer (lanes *3,4*), or 0.2 mg/mL WGA (lanes *5,6*) and incubated further. After 20 min, reactions were supplemented with 100 nM rCdt1. DNA was isolated after a further 20 or 30 min, and chromatin-bound proteins were analyzed. Reaction 1 contained 50 µg/mL aphidicolin to retain MCM3 and Cdc45 on chromatin. (*B,C,D*) Sperm chromatin (2000/µL) was incubated in mock- or geminin-depleted LSS in the presence of [α-^{32}P]dATP and BrdU. Reactions were supplemented with 80 nM rCdt1 immediately or 60 min after addition of sperm or with control buffer at the time of sperm addition. (*B*) The percentage of input DNA replicated after 180 min is plotted. Parallel reactions were stopped 180 min after sperm addition and replication products in mock (*C*) or geminin-depleted extracts (*D*) were separated on a CsCl gradient. The radioactivity in the peak Heavy–Light fractions among the six conditions did not differ by >10%, and was adjusted to a value of 100.

during manipulations and that rCdt1 entered nuclei via the nuclear pore. From these results, we conclude that the principal block to MCM2-7 reloading after the first round of replication is the absence of functional Cdt1 within the nucleus.

Since addition of rCdt1 promoted rebinding of MCM2-7 and Cdc45 to chromatin, we expected it might also promote another round of DNA synthesis. We tested this hypothesis by incubating sperm chromatin in LSS in the presence of BrdU. Total DNA synthesis was measured (Fig. 7B, gray bars) and replication products were fractionated on a CsCl gradient. Whereas a control reaction yielded only "Heavy–Light" DNA (Fig. 7C, left), addition of 80 nM rCdt1 60 min after the start of the reaction resulted in 16% rereplication, as seen by the presence of a "Heavy–Heavy" peak (Fig. 7C, far right). The DNA was rereplicated within one cell cycle because extracts contained cycloheximide, which prevents entry into mitosis. Importantly, when the same concentration of rCdt1 was added at the start of the reaction, no rereplication was observed (Fig. 7C, middle). Because rCdt1 added to LSS is efficiently degraded in a replication-dependent manner (data not shown), this result argues that replication-dependent destruction of Cdt1 is sufficient to completely remove all functional Cdt1 from the extract.

We next examined why only limited rereplication is observed following rCdt1 addition after DNA replication. We postulated that geminin, present at high concentrations within nuclei, inhibits some of the rCdt1 that enters the nucleus. To test this idea, sperm chromatin was incubated in geminin-depleted LSS. As expected (McGarry and Kirschner 1998), depletion of geminin alone did not lead to rereplication (Fig. 7D, left). However, addition of rCdt1 to geminin-depleted extract after 60 min resulted in rereplication of 50% of the DNA, suggesting that geminin inhibits rCdt1 added after the first round of DNA replication (Fig. 7D, right). Moreover, addition of rCdt1 at the start of the reaction did not result in any rereplication (Fig. 7D, middle), suggesting that, in the absence of geminin, the Cdt1 degradation machinery is fully active and sufficient to restrict DNA replication to a single round.

A prediction of our results is that in geminin-depleted extracts, inhibition of the proteasome during S phase will stimulate rereplication, because endogenous Cdt1 is stabilized. Indeed, we found that MG132, which stabilizes Cdt1 (Fig. 2C), stimulated 20% rereplication in geminin-depleted extracts (data not shown). Addition of MG132 to mock-depleted extracts had no effect, presumably because Cdt1 was neutralized by geminin.

Degradation of Cdt1 does not require geminin

The observation that depletion of geminin does not result in rereplication (McGarry and Kirschner 1998; Fig. 7D, left), even when excess rCdt1 is added at the start of the reaction (Fig. 7D, middle), suggests that Cdt1 destruction occurs independently of geminin. To test this, LSS was depleted of geminin (Fig. 1A) and degradation of Cdt1 was monitored over time. As shown in Figure 8A, Cdt1 is efficiently degraded in the absence of geminin (note that ~50% of the endogenous Cdt1 is codepleted with geminin). Therefore, as reported in human cells (Nishitani et al. 2004), Cdt1 degradation in *Xenopus* egg extracts is independent of geminin.

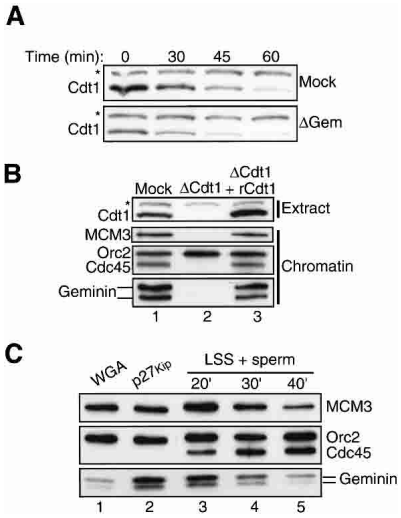

Figure 8. Replication-dependent Cdt1 degradation and geminin-mediated inhibition of Cdt1 occur independently of one another. (*A*) Sperm chromatin was incubated in mock- or geminin-depleted LSS. At the indicated times, reactions were stopped and 1 µL of extract was blotted for Cdt1. (*B*) (Chromatin) Sperm chromatin was incubated in mock-depleted LSS, Cdt1-depleted LSS, or Cdt1-depleted LSS containing 50 nM rCdt1. All samples contained 50 µg/mL aphidicolin. Chromatin was isolated after 30 min and blotted for the indicated factors. (Extract) One microliter of the indicated extract was blotted for Cdt1. (*C*) Sperm chromatin was incubated in LSS containing 0.2 mg/mL WGA (lane *1*), 1 µM $p27^{Kip}$ (lane *2*), or no additions (lanes *3–5*). Chromatin was isolated at the indicated times (lanes *3–5*) or after 40 min (lanes *1,2*) and blotted for the indicated factors.

Geminin binds to chromatin before initiation of DNA replication

Cytoplasmic geminin is activated to bind to Cdt1 upon nuclear import, and its activation is independent of Cdk2 activity (Hodgson et al. 2002), suggesting that geminin should be able to associate with Cdt1 before initiation of DNA replication. As a means to measure the binding of geminin to Cdt1 in S phase, we examined its loading onto chromatin. As previously reported (Gillespie et al. 2001; Maiorano et al. 2004), we found that chromatin binding by geminin was Cdt1 dependent (Fig. 8B). Thus, geminin likely loads onto chromatin via a direct interaction with Cdt1. We next asked when geminin associates with chromatin relative to the initiation of DNA replication. A time course revealed that geminin reached a maximal level on chromatin 20 min after sperm addition, and it was largely lost from chromatin after 40 min (Fig. 8C, lanes 3–5). Geminin dissociation from chromatin is dependent on Cdt1 destruc-

tion, since methylated ubiquitin blocked Cdt1 destruction and geminin release (data not shown). Importantly, inhibition of nuclear transport by addition of WGA reduced geminin binding to chromatin, but inhibition of Cdk2/Cyclin E activity by $p27^{Kip}$ did not (Fig. 8C, lanes 1,2). Therefore, geminin binding to chromatin requires nuclear transport, but not Cdk2/Cyclin E activity, consistent with the requirements for geminin activation (Hodgson et al. 2002). These experiments indicate that geminin binds to Cdt1 on chromatin and inhibits its activity before initiation of DNA replication, prior to activation of the Cdt1 destruction machinery.

Discussion

In this study, we report that rereplication does not occur in *Xenopus* egg extracts, due to the absence of functional Cdt1 during S phase, a condition that is established by geminin and proteolysis of Cdt1. Surprisingly, the initiation of DNA replication stimulates Cdt1 degradation, demonstrating that the first round of DNA replication actively prevents subsequent rounds.

Initiation of DNA replication stimulates Cdt1 degradation

Our results show that inhibition of Cdk2/Cyclin E activity and Cdc45 depletion greatly reduced the rate of Cdt1 degradation. Therefore, replication initiation is required for efficient Cdt1 destruction. However, when rCdt1 was added to extracts after replication initiation, it was degraded independently of Cdk2 activity, suggesting that phosphorylation of Cdt1 by Cdk2 is not essential for its degradation in S phase. In mammalian cells, where Cdt1 is also destroyed in S phase (Nishitani et al. 2001), overexpression of p21 or p27 completely stabilizes Cdt1 (Liu et al. 2004). However, while deletion of the cyclin-binding motif of human Cdt1 renders it refractory to Cdk2 phosphorylation and Skp2 binding, the protein is only partially stabilized in S phase (Liu et al. 2004; Sugimoto et al. 2004). More dramatic stabilization of Cdt1 by expression of cdk inhibitors than mutation of cyclin-binding motifs in Cdt1 has now also been reported in *Drosophila* (Thomer et al. 2004). These results suggest that in other metazoans, Cdk2 contributes to Cdt1 destruction in ways that do not involve direct phosphorylation of Cdt1, and we suggest this may be due to a replication-dependent destruction pathway.

We showed that Cdc45, RPA, and pol α are all required to trigger efficient Cdt1 ubiquitination and proteolysis. To assess the role of DNA synthesis by pol α, we titrated aphidicolin into extracts. Below 75 μM aphidicolin, there was no effect on Cdt1 degradation, but Cdt1 ubiquitination was greatly reduced. Above 75 μM, Cdt1 degradation slowed significantly, and Cdt1 ubiquitination became undetectable. At all concentrations of aphidicolin, the extent of Cdt1 ubiquitination correlated with PCNA binding to chromatin. We conclude that primer synthesis by pol α is essential for Cdt1 destruction, perhaps reflecting a requirement for chromatin-bound RFC or PCNA.

The identity of the ligase that carries out Cdt1 ubiquitination in *Xenopus* egg extracts is unknown. In human cells, SCF^{Skp2} contributes to Cdt1 destruction (Li et al. 2003), and the interaction of Skp2 with Cdt1 is completely Cdk2 dependent (Liu et al. 2004; Sugimoto et al. 2004). Our data indicate that replication-dependent Cdt1 destruction does not require phosphorylation of Cdt1 by Cdk2, suggesting that SCF^{Skp2} is not involved, or that it can recognize Cdt1 independently of phosphorylation in the context of replication. In worms, and in human cells suffering DNA damage, Cul4 mediates Cdt1 ubiquitination and destruction (Li et al. 2003; Zhong et al. 2003). We cannot rule out that an undetectable level of DNA damage occurs during DNA replication that promotes Cdt1 destruction, and thus, Cul4 remains a candidate for the ligase. Whatever the relevant E3 ubiquitin ligase, it ubiquitinates chromatin-bound Cdt1, and thus, may itself be tethered to chromatin. Since all of the endogenous Cdt1, as well as large amounts of added rCdt1 are efficiently degraded in a replication-dependent manner, it is clear that not only the Cdt1 originally bound to pre-RCs is degraded. We suggest that the large majority of ubiquitinated Cdt1 observed on chromatin is not binding to pre-RCs, but rather to a chromatin-bound E3 ligase or some other structure. Indeed, a Cdt1 destruction mechanism in which Cdt1 must first bind to pre-RCs would be self-defeating. We speculate that the initiation of DNA replication generates a structure on DNA that leads to colocalization of Cdt1 and a Cdt1-specific E3 ligase, thereby facilitating destruction of Cdt1.

A potential dilemma arises from the replication-dependent Cdt1 destruction model. While the MCM2-7 complex begins unwinding DNA upon Cdc45 loading (Walter and Newport 2000), and thereby presumably vacates its binding site, Cdt1 is not destroyed until after pol α has synthesized a DNA primer. Therefore, in the absence of geminin, a window of time could theoretically exist before Cdt1 destruction, during which a new MCM2-7 complex could load onto an origin that already fired. Although we cannot rule out this scenario because our assays would not detect rereplication at a small subset of origins, we prefer to think that this window of opportunity does not exist, and we speculate that after replication initiation, the origin is rendered temporarily inactive, perhaps due to the presence of single-stranded DNA.

The replication-dependent destruction of Cdt1 shows parallels with the destruction of human ORC1, as well as Xic1, a *Xenopus* inhibitor of Cdk2 that is similar to $p27^{Kip}$. When recombinant Xic1 (rXic1) is added to *Xenopus* egg extracts, it is ubiquitinated on chromatin and efficiently degraded (Yew and Kirschner 1997; Furstenthal et al. 2001; You et al. 2002). As in the case of Cdt1, degradation of rXic1 requires Cdk2 and Cdc45, and after replication initiation, Cdk2 becomes dispensable (You et al. 2002). Similar to Cdt1 and Xic1, human ORC1 is degraded in S phase, and it is ubiquitinated on chromatin (Mendez et al. 2002). Moreover, a mutant of ORC1 whose phosphorylation by Cdks is dramatically reduced is still efficiently ubiquitinated and presumably de-

graded. The requirement for Skp2 in ORC1 degradation might reflect Skp2-mediated ubiquitination of ORC1, or the requirement for Skp2 in S-phase entry. Thus, it is tempting to speculate that Cdt1, ORC1, and Xic1 can all be degraded by a common, chromatin-based mechanism that requires replication initiation, but not direct phosphorylation by Cdks.

Two mechanisms restrain Cdt1 activity in S phase

To explore the significance of Cdt1 degradation and its relationship to geminin-mediated inhibition of Cdt1, we added rCdt1 to mock-depleted and geminin-depleted egg extracts. We showed that addition of rCdt1 to mock-depleted extracts following DNA replication resulted in 16% rereplication. Importantly, no rereplication occurred when the same quantity of rCdt1 was added before the first round of DNA synthesis. These results strongly argue that the absence of functional Cdt1 in replicated nuclei is the primary barrier to rereplication, and that proteolysis during the first round of DNA replication is capable of removing all functional Cdt1. Consistent with this view, we found no evidence that Cdc6 is negatively regulated during S phase, since it remained bound to chromatin, as recently reported (Oehlmann et al. 2004). The ability of rCdt1 to stimulate rereplication in *Xenopus* egg extracts has recently also been observed by three other laboratories (J. Blow, H. Takisawa, and M. Mechali, pers. comm.).

Addition of rCdt1 to replicated nuclei in geminin-depleted extract resulted in threefold more rereplication than in mock-depleted extract, demonstrating that geminin plays an important role in restraining Cdt1 activity in nuclei. Importantly, supplementing geminin-depleted extracts with rCdt1 before DNA replication caused no rereplication. Therefore, replication-dependent Cdt1 degradation is sufficient to prevent detectable rereplication in the absence of geminin. Indeed, Cdt1 was degraded with normal kinetics in geminin-depleted extracts. Conversely, it is important to ask whether geminin is sufficient to block rereplication in the absence of Cdt1 degradation. We have shown that geminin loads onto origins in a Cdt1-dependent fashion before the Cdk2-step, strongly suggesting that geminin can bind to and inhibit Cdt1 before the Cdt1 degradation step. Moreover, we have found that addition of the proteasome inhibitor MG132 to LSS, which stabilizes Cdt1, results in rereplication only when geminin is absent, indicating that geminin can restrict rereplication when the Cdt1 proteolytic machinery is blocked. It is important to note that our assays are only equipped to detect significant amounts of re-replication. Therefore, while geminin and Cdt1 destruction have the appearance of being independent and redundant mechanisms to prevent rereplication, it may be that both mechanisms must be present to insure that no rereplication whatsoever occurs. Since our experiments are performed in interphase-arrested egg extracts, they do not contain mitotic cyclins, which may provide additional safeguards against rereplication.

How important is degradation of Cdt1 in other metazoans? In *C. elegans*, destruction of Cdt1 appears to be important for regulating DNA replication, because loss of Cul4 results in Cdt1 stabilization in S phase and massive over-replication, an effect that is reversed by loss of one gene copy of Cdt1 (Zhong et al. 2003). In *D. melanogaster* and humans, Cdt1 is unstable in S phase, and overexpression of Cdt1 causes rereplication (Vaziri et al. 2003; Nishitani et al. 2004; Thomer et al. 2004). However, it is unclear whether this manipulation suppresses Cdt1 degradation or the inhibitory effects of geminin. Interestingly, loss of geminin is sufficient to induce partial rereplication in some human cells (Melixetian et al. 2004; Zhu et al. 2004) but not in others (Nishitani et al. 2004). The extent of rereplication appears to correlate inversely with the efficiency of Cdt1 degradation, suggesting that Cdt1 degradation in human cells is an important mechanism to restrain rereplication. In summary, Cdt1 degradation is likely to be important in multiple organisms, but unlike in *Xenopus*, it may not always be sufficient to efficiently suppress rereplication.

Although a recent study concluded that Ran-GTP is the principal inhibitor of rereplication in *Xenopus* egg extracts (Yamaguchi and Newport 2003), experiments performed by us (E.E. Arias and J.C. Walter, data not shown), a subset of the original authors (Newport 2004), and two other laboratories (J. Blow and H. Takisawa, pers. comm.), failed to confirm key results from this report.

Does Cdk2/Cyclin E affect pre-RC components other than Cdt1?

An important question concerns whether Cdk2/Cyclin E negatively regulates pre-RC formation by a mechanism other than the indirect promotion of Cdt1 destruction. We showed that addition of rCdt1 to NPE yields an extract that supports full MCM2-7 loading and a complete round of DNA replication. This result indicates that concentrations of Cdk2/Cyclin E that promote origin firing are not inhibitory for de novo pre-RC assembly. Moreover, rCdt1 addition to replicated nuclei triggers MCM3 reloading and extensive Cdk2-dependent reinitiation. Together, the data argue that when Cdt1 is present, Cdk2/Cyclin E is not sufficient to inhibit pre-RC formation. It should be noted that rCdt1 addition to geminin-depleted extracts causes only ~50% of the DNA to undergo rereplication. Although the failure to rereplicate all of the DNA could be due to nonspecific inactivation of the extract over time, it remains possible that Cdk2/Cyclin E exerts some negative effect on rereplication. Nevertheless, the fact that we observe extensive rereplication in the presence of Cdk2/Cyclin E is surprising in light of the previous finding that when HSS is supplemented with ~1 μM recombinant human Cdk2/Cyclin E (the same concentration as endogenous Cdk2/Cyclin E present in NPE), MCM2-7 loading is blocked (Hua et al. 1997). Because rCdk2/Cyclin E added to HSS does not stimulate DNA replication, Cdt1 was unlikely to be degraded in this experiment, suggesting that Cdk2/Cyclin

E can inhibit pre-RC formation by a mechanism not involving Cdt1 destruction. The apparent discrepancy with our results might be explained by the use of human Cdk2/Cyclin E by Newport and colleagues (Hua et al. 1997).

Previous in vivo results indicate that Cdk2/Cyclin E can either inhibit or stimulate pre-RC assembly, depending on the developmental stage and point in the cell cycle. In endoreduplicating *Drosophila* tissues (Follette et al. 1998; Weiss et al. 1998), or in mammalian tissue culture cells (Ekholm-Reed et al. 2004), inappropriate Cyclin E expression inhibits replication, likely due to defective MCM2-7 loading. In contrast, in cleavage division *Drosophila* embryos (Su and O'Farrell 1997), or in human cells exiting from quiescence (Geng et al. 2003), Cyclin E stimulates MCM2-7 recruitment to chromatin. It will be interesting to understand the conditions in vitro and in vivo that determine whether Cdk2/Cyclin E affects pre-RC formation positively, negatively, or not at all. Nevertheless, our results indicate that endogenous Cdk2/Cyclin E plays no major role in preventing rereplication in *Xenopus* egg extracts.

A model for the cell-cycle regulation of DNA replication in Xenopus *embryos*

Together with previous results, our data suggest the following model for the regulation of DNA replication during the embryonic cell cycles. As cells exit mitosis, APC-mediated destruction of Cyclin B and inactivation/degradation of geminin allow pre-RCs to assemble on chromatin (McGarry and Kirschner 1998; Li and Blow 2004). As nuclei reassemble in telophase, nuclear transport of geminin leads to its reactivation, allowing geminin to bind nucleoplasmic (Hodgson et al. 2002) and chromatin-bound Cdt1 (Fig. 8), effectively neutralizing Cdt1. Concurrently, Cdk2/Cyclin E becomes concentrated within nuclei, stimulating replication initiation and rapid destruction of Cdt1. Two mechanisms likely replenish Cdt1 for the next round of pre-RC assembly. First, de novo synthesis could continually replenish the supply of Cdt1. Second, if the total pool of Cdt1 is not degraded, chromatin would simply be re-exposed to cytoplasmic Cdt1 when the nuclear envelope breaks down in mitosis. Indeed, we have observed that when the concentration of nuclei in *Xenopus* egg extracts is low, such as occurs during the first few embryonic cell cycles, Cdt1 destruction is incomplete (data not shown). It is important to note that even at low nucleocytoplasmic ratios, any Cdt1 that enters nuclei should be fully degraded. Indeed, loss of geminin in early *Xenopus* embryos does not result in detectable rereplication (McGarry 2002), suggesting that replication-dependent destruction of Cdt1 is sufficient to block rereplication during S phase in vivo.

Materials and methods

DNA replication and chromatin-binding assays

Extract preparation, replication assays, chromatin-binding assays, density substitution experiments, and low-temperature synchronization assays in the nucleus-free system were carried out as described (Walter et al. 1998; Walter 2000; Pacek and Walter 2004). LSS was prepared as described (Blow 1993). Chromatin-binding, DNA-replication, and density-substitution experiments in LSS were performed as for the nucleus-free system. In all experiments, the percentage of input DNA synthesized was calculated as described (Blow and Laskey 1986). Each replication experiment was repeated at least twice, and representative results are shown. MG132 and methylated ubiquitin were from Boston Biochem. Wheat Germ Agglutinin was from Sigma.

Immunological methods

Depletion of HSS and NPE were carried out as previously described (Walter et al. 1998). To deplete LSS, 0.2 vol of antibody-bound protein A sepharose (Pharmacia) was incubated with extract for 2 h at 4°C, and the procedure was repeated once. To generate the pol α antibody, the p70 subunit of pol α was expressed in bacteria, purified under denaturing conditions, and used to immunize rabbits (M. Michael, pers. comm.). Antibodies against Cdt1 and geminin were raised against the bacterially expressed proteins. Anti-MCM3, ORC2, Cdc45, and RPA are as described (Walter and Newport 1997, 2000). PCNA antibody was from Santa Cruz. We obtained RCC1 antibody from Mary Dasso (National Institutes of Health, Bethesda, MD).

Expression of recombinant proteins

Xenopus Cdt1 was cloned into pDONR201 using the Gateway Cloning System (Invitrogen) to generate pDONR-XCdt1. pDONR-XCdt1 was engineered to contain an N-terminal tag consisting of 6xHis-Flag, and a Prescission Protease Cleavage site (Amersham) by insertion of annealed complimentary oligos encoding the tag sequence into the Nde1 start site of Xcdt1. Baculovirus DNA was produced using the BaculoDirect Baculovirus Expression System (Invitrogen), and rCdt1 was expressed in Sf9 cells. Cells were harvested 48 h post-infection, pelleted, and lysed in 50 mM Tris (pH 7.5), 200 mM NaCl, 0.5% NP-40, 50 µM TCEP, and 20 µg/mL aprotinin and leupeptin. The soluble lysate was mixed with 200 µL M2 Flag resin (Sigma) for 2 h. Beads were washed three times with 50 mM Tris (pH 7.5), 500 mM NaCl, and 50 µM TCEP, equilibrated in 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 50 µM TCEP. rCdt1 (untagged) was eluted from the resin with 20 U of Prescission Protease (Amersham), and the supernatant was recovered. Recombinant Cdc45 was prepared as described (Mimura and Takisawa 1998). Recombinant human pol α was a gift from Dewight Williams and Ellen Fanning (Vanderbilt University, Nashville, TN).

Acknowledgments

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