nature cell biology

PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication

Emily E. Arias[1] and Johannes C. Walter[1,2]

Ubiquitin-mediated proteolysis of the replication licensing factor Cdt1 (Cdc10-dependent transcript 1) in S phase is a key mechanism that limits DNA replication to a single round per cell cycle in metazoans[1–6]. In *Xenopus* egg extracts, Cdt1 is destroyed on chromatin during DNA replication[1]. Here, we report that replication-dependent proteolysis of Cdt1 requires its interaction with proliferating cell nuclear antigen (PCNA), a homotrimeric processivity factor for DNA polymerases[7]. Cdt1 binds to PCNA through a consensus PCNA-interaction motif that is conserved in Cdt1 of all metazoans, and removal of PCNA from egg extracts inhibits replication-dependent Cdt1 destruction. Mutation of the PCNA-interaction motif yields a stabilized Cdt1 protein that induces re-replication. DDB1, a component of the Cul4 E3 ubiquitin ligase that mediates human Cdt1 proteolysis in response to DNA damage[8], is also required for replication-dependent Cdt1 destruction. Cdt1 and DDB1 interact in extracts, and DDB1 chromatin loading is dependent on the binding of Cdt1 to PCNA, which indicates that PCNA docking activates the pre-formed Cdt1–Cul4^{DDB1} ligase complex. Thus, PCNA functions as a platform for Cdt1 destruction, ensuring efficient and temporally restricted inactivation of a key cell-cycle regulator.

In eukaryotes, the MCM2–7 complex is recruited to origins of DNA replication in G1 phase by ORC, Cdc6 and Cdt1 (ref. 9). During S phase, MCM2–7 is activated by cyclin-dependent kinases (Cdks) and other proteins, resulting in origin unwinding and recruitment of processive DNA polymerases. The translocation of MCM2–7 away from the origin is thought to inactivate pre-replication complexes (pre-RCs), and re-initiation at the same origin cannot occur because *de novo* MCM2–7 loading is prohibited in S phase[10]. In metazoans, MCM2–7 re-loading in S phase is not allowed because Cdt1 is neutralized by two conserved mechanisms. First, Cdt1 is inactivated by geminin[11,12]. Second, Cdt1 is destroyed by ubiquitin-mediated proteolysis[1–6,13]. Interestingly, Cdt1 is also degraded after genotoxic stress[8,14,15].

To study Cdt1 destruction, we used a cell-free replication system in which sperm chromatin is added to a low-speed supernatant (LSS) of *Xenopus* egg cytoplasm. Pre-RCs assemble rapidly on the added sperm chromatin. Subsequently, a nuclear envelope forms and DNA replication initiates, leading to a single round of chromosome duplication[16]. Using this system, we previously showed that Cdt1 destruction is coupled to DNA replication via a mechanism that involves its polyubiquitylation on chromatin[1]. Cdt1 ubiquitylation requires DNA polymerase-α, as well as synthesis of a DNA primer. These data suggested that any factor downstream of primer synthesis[17] — including RFC, proliferating cell nuclear antigen (PCNA) or DNA polymerase-δ — might trigger Cdt1 destruction.

Proteins that interact with a structural motif of PCNA, called the interdomain connector loop, contain a PCNA-interaction protein motif[7] ('PIP' box; Fig. 1a). Strikingly, we discovered that the amino terminus of Cdt1 — which is essential for its S-phase destruction in frogs, humans and flies[2,4,18–20] — contains a PIP-box-like motif in all metazoans (Fig. 1a). Therefore, in all metazoans, Cdt1 might be destroyed in S phase by interacting with PCNA. Figure 1b (lane 3) shows that the N-terminal 25 amino acids of Cdt1 fused to glutathione S-transferase (GST) interacted efficiently with PCNA. Mutating the four consensus amino acids in the PIP box to alanine (Fig. 1a, bottom) yielded Cdt1(1–25)$^{\Delta PIP}$, which did not bind PCNA (Fig. 1b, lane 4). Recombinant full-length Cdt1 (rCdt1^{WT}) also interacted with PCNA, but a mutant of Cdt1 containing the same four alanine substitutions in the PIP box (rCdt1$^{\Delta PIP}$) bound less efficiently (Fig. 1c, compare lanes 3 and 4). Binding of rCdt1^{WT} to PCNA was sensitive to the addition of a 21-amino-acid synthetic peptide[21] derived from p21^{Cip}, which contains a consensus PIP box (Fig. 1c, compare lanes 3 and 5), whereas binding of rCdt1$^{\Delta PIP}$ was marginally affected (Fig. 1c, compare lanes 4 and 6). Therefore, the PIP box within Cdt1 mediates direct interaction of Cdt1 with PCNA.

To determine whether Cdt1 destruction requires PCNA, we used replication of M13 single-stranded DNA (ssDNA), as depletion of PCNA from LSS non-specifically compromised replication on sperm chromatin[21] (and data not shown). ssDNA replication requires only the events that are downstream of origin unwinding, including priming by DNA polymerase-α, loading of PCNA, and processive DNA synthesis by DNA polymerase-δ and/or -ε[21]. ssDNA replication is independent of nuclear-envelope assembly, and therefore occurs in a high-speed supernatant (HSS) of egg cytoplasm that lacks nuclear membranes.

[1]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA.
[2]Correspondence should be addressed to J.C.W. (e-mail: johannes_walter@hms.harvard.edu)

Received 31 August 2005; accepted 5 December 2005; published online 18 December 2005; DOI: 10.1038/ncb1346

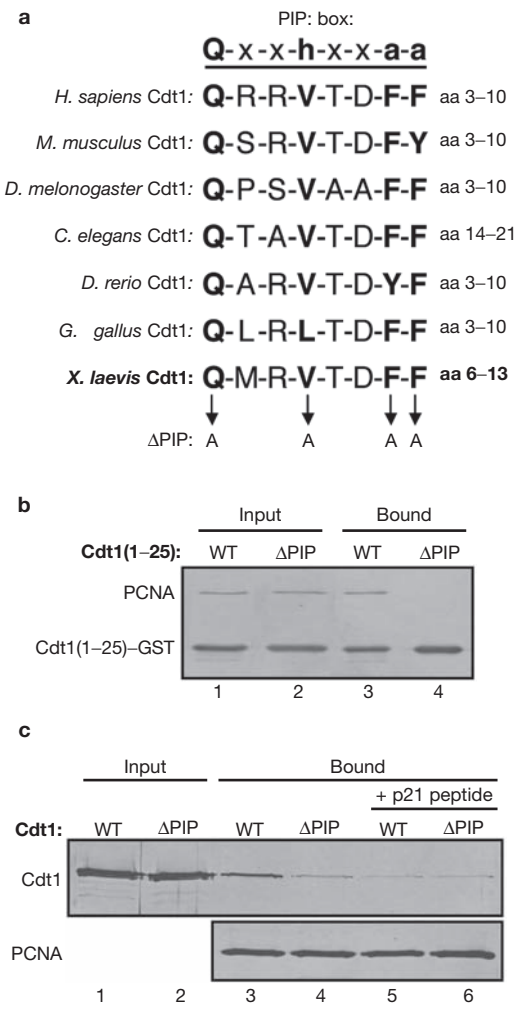


Figure 1 Cdt1 binds to PCNA via a conserved PIP box. (**a**) Sequence and location of the proliferating cell nuclear antigen (PCNA)-interaction protein (PIP) box in Ccd10-dependent transcript 1 (Cdt1) homologues. In the consensus PIP box motif, 'h' represents a hydrophobic amino acid (typically L, I or M), 'a' an aromatic (F or Y) and 'x' any amino acid. aa, amino acid. (**b**) Recombinant PCNA (rPCNA) was incubated with immobilized Cdt1(1–25) containing a wild-type (WT) or mutated PIP box (ΔPIP) fused to glutathione S-transferase (GST). Equal amounts of input and bound fractions were stained by Coomassie blue. (**c**) rPCNA was immobilized and mixed with $rCdt1^{WT}$ or $rCdt1^{\Delta PIP}$. Where indicated, 20 μM p21 peptide was included. Cdt1 input and PCNA-bound fractions were visualized by silver staining.

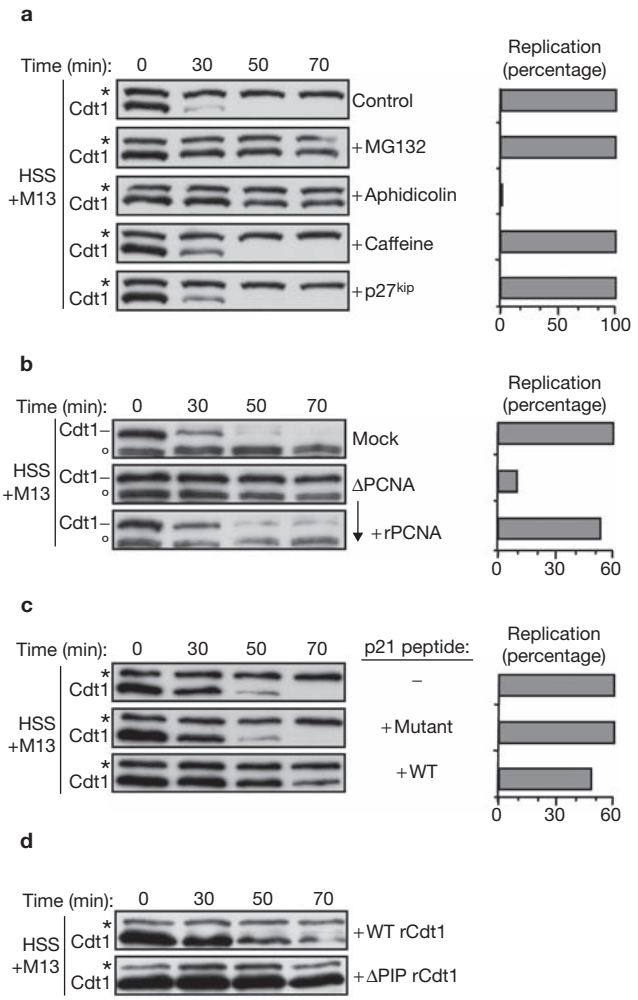


Figure 2 Replication of M13 ssDNA induces PCNA-dependent Cdt1 destruction. (**a**) M13 single-stranded DNA (ssDNA; 10 ng μl^{-1}) was incubated in high-speed supernatant (HSS) supplemented with MG132 (400 μM), aphidicolin (600 μM), caffeine (5 mM) or $p27^{Kip}$ (1 μM). At the indicated times, 1 μl of extract was blotted for Cdt1. *, Cross-reacting band serving as a loading control. The entire Cdt1 western blot is shown in Supplementary Information, Fig. S4. In panels **a**–**c**, the percentage of input DNA synthesized after 70 min is plotted. (**b**) M13 ssDNA was incubated in mock- or PCNA-depleted HSS and optionally supplemented with 6 μM recombinant PCNA (rPCNA). A different cross-reacting band (°) served as a loading control because the p21 peptide resin removes the band directly above Cdt1. (**c**) M13 ssDNA was incubated in HSS supplemented with 50 μM wild-type (WT) or mutant p21 peptide. (**d**) M13 ssDNA was incubated in Cdt1-depleted HSS supplemented with 40 nM $rCdt1^{WT}$ or $rCdt1^{\Delta PIP}$, as indicated.

Importantly, replication of ssDNA in HSS induced rapid Cdt1 destruction (Fig. 2a, control). As seen during the replication of sperm chromatin[1], destruction was sensitive to MG132 and aphidicolin, indicating that it involved the proteasome and DNA polymerase-α (Fig. 2a). Destruction was insensitive to caffeine, indicating that it did not involve checkpoint kinases (Fig. 2a). Finally, it did not require ORC (see Supplementary Information, Fig. S1a) or Cdk2 activity, as destruction was insensitive to $p27^{Kip}$ (Fig. 2a). Together, these experiments validate ssDNA replication as a model system for replication-dependent Cdt1 destruction, and demonstrate that this process does not require the interaction of Cdt1with ORC.

To test the requirement for PCNA in Cdt1 destruction, PCNA was depleted from HSS. The high concentration of PCNA in egg extracts precludes immunodepletion. However, >99% of PCNA is removed from HSS using p21 peptide resin[21] (and data not shown). In PCNA-depleted extract, ssDNA replication and Cdt1 destruction were significantly reduced, and both processes were rescued with 6 μM recombinant PCNA (rPCNA) (Fig. 2b), which is equivalent to the endogenous concentration of PCNA[21]. Addition of excess p21 peptide ($p21^{WT}$), but not a mutant peptide with a defective PIP box ($p21^{mutant}$), attenuated ssDNA-induced Cdt1 destruction (Fig. 2c), as expected if destruction requires interaction between the PIP box of Cdt1 and PCNA. Like endogenous Cdt1, $rCdt1^{WT}$ was destroyed when added to HSS containing ssDNA (Fig. 2d, upper panel). By contrast, $rCdt1^{\Delta PIP}$ was stable (Fig. 2d, lower panel). The data indicate that, during ssDNA replication, Cdt1 destruction requires a direct physical interaction between the PIP box of Cdt1 and PCNA.

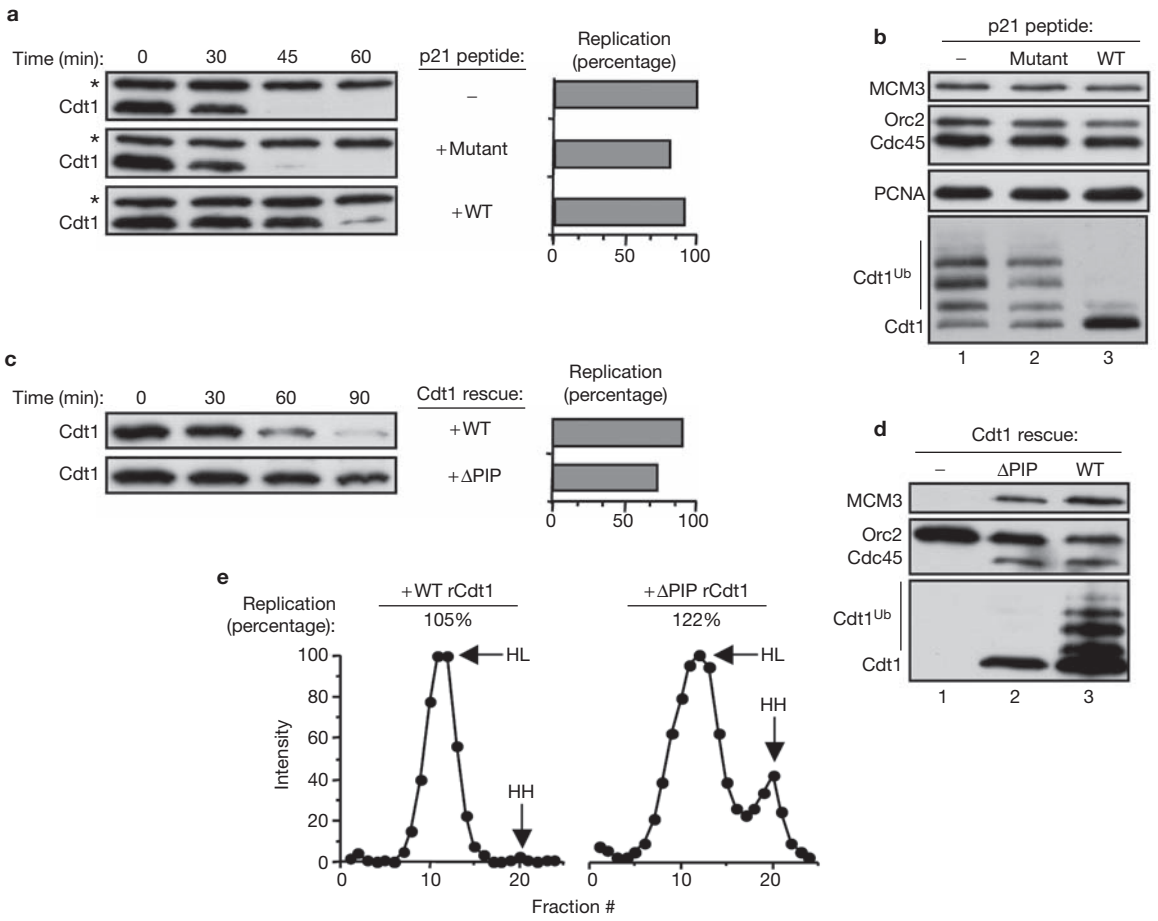


Figure 3 PCNA-dependent Cdt1 destruction in LSS limits DNA replication to a single round. (**a**, **b**) Sperm chromatin (3,000 per µl) was incubated in low-speed supernatant (LSS) supplemented with 50 µM mutant or wild-type (WT) p21 peptide. (**a**) Cdt1 stability was analysed as in Fig. 2a, and the percentage of input DNA that was replicated after 90 min is plotted. The asterisk indicates a cross-reacting band serving as a loading control. (**b**) Extracts described in (**a**) were additionally supplemented with 250 µM methylated ubiquitin. Chromatin was isolated after 60 min and blotted for the indicated proteins. (**c**, **d**) Sperm chromatin was added to Cdt1-depleted LSS supplemented with 50 nM $rCdt1^{WT}$ or $rCdt1^{\Delta PIP}$. (**c**) Reactions were analysed as in (**a**). (**d**) As in (**b**), except that chromatin was isolated after 80 min. (**e**) Sperm chromatin (2,000 per µl) was incubated in LSS containing 120 nM $rCdt1^{WT}$ or $rCdt1^{\Delta PIP}$ in the presence of [α–^{32}P]dATP and BrdU. After 180 min, replication products were fractionated on a CsCl gradient[1]. HH, heavy-heavy DNA, 1.8 g ml^{-1}; HL, heavy-light DNA, 1.75 g ml^{-1}. The percentage of input DNA that was replicated after 180 min is indicated. Data shown is representative of six independent experiments.

To address whether PCNA-dependent Cdt1 destruction limits replication to once per cell cycle, we first sought to verify that this mechanism functions during the replication of sperm chromatin in LSS. Addition of $p21^{mutant}$ peptide to LSS had no effect on Cdt1 destruction, whereas $p21^{WT}$ stabilized Cdt1 (Fig. 3a). To examine the effect of p21 peptides on Cdt1 ubiquitylation, we exploited the fact that ubiquitylated Cdt1 is detected on replicating chromatin in extracts that had been supplemented with methylated ubiquitin, which is conjugated to targets but is deficient in polyubiquitin chain formation[1]. Cdt1 ubiquitylation on chromatin was severely inhibited by $p21^{WT}$, but was modestly affected by $p21^{mutant}$ (Fig. 3b). We next added $rCdt1^{\Delta PIP}$ to Cdt1-depleted LSS and found that it was more stable than $rCdt1^{WT}$ (Fig. 3c) and, unlike $rCdt1^{WT}$, it was not ubiquitylated detectably on chromatin (Fig. 3d). These results indicate that, in the context of chromosomal DNA replication, Cdt1 must interact with PCNA to be destroyed. Consistent with a Cdt1–PCNA interaction, the appearance of ubiquitylated Cdt1 on chromatin occurred with similar kinetics as the loading of PCNA (Fig. 4e, below).

Geminin inhibition of Cdt1 and Cdt1 destruction are each sufficient to prevent re-replication in egg extracts[1–3,5]. To address whether PCNA-dependent Cdt1 destruction limits re-replication, we added $rCdt1^{WT}$ or $rCdt1^{\Delta PIP}$ to LSS at concentrations that exceeded the endogenous concentration of geminin, and measured re-replication using density-substitution analysis[1]. $rCdt1^{WT}$ did not induce re-replication, as seen by the absence of 'Heavy-Heavy' DNA, whereas $rCdt1^{\Delta PIP}$ induced significant re-replication (Fig. 3e). Therefore, PCNA-dependent Cdt1 proteolysis is sufficient to functionally neutralize Cdt1 in S phase.

We found that, before replication initiates, only ~5% of Cdt1 is bound to pre-RCs (see Supplementary Information, Fig. S2a), yet the vast majority of Cdt1 is destroyed in a replication- and PCNA-dependent fashion[1] (Figs 2b, d, 3c). An important question is, therefore, whether the large pool of soluble Cdt1 is destroyed by interacting with chromatin-bound PCNA, nucleoplasmic PCNA or both. Our observation that Cdt1 is ubiquitylated on chromatin in a manner dependent on its PIP box (Fig. 3d) strongly indicates that chromatin-bound PCNA can facilitate Cdt1 destruction. To investigate what fraction of Cdt1 is ubiquitylated on chromatin, we

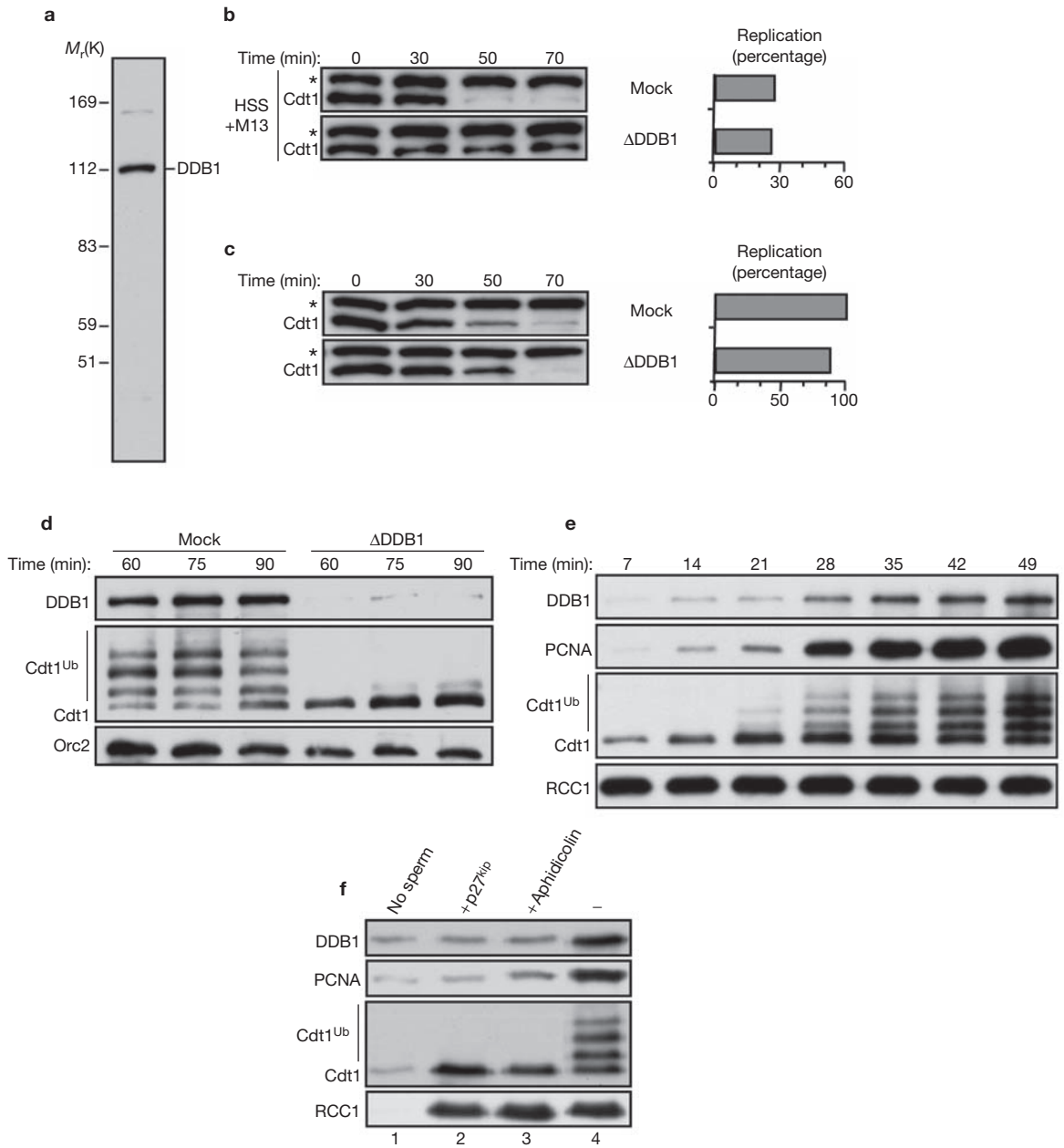


Figure 4 DDB1 is required for Cdt1 destruction. (**a**) One µl of *Xenopus* nuclear extract[32] was blotted with DDB1 antibody. M_r(K), relative molecular mass. (**b**) M13 single-stranded DNA (ssDNA) was incubated in mock or DDB1-depleted high-speed supernatant (HSS) and analysed as in Fig. 2a. (**c**, **d**) Sperm chromatin was incubated in mock or DDB1-depleted low-speed supernatant (LSS). (**c**) Reactions were analysed as in Fig. 2a, and the percentage of input DNA that was replicated at 60 min is plotted. The asterisk indicates a cross-reacting band serving as a loading control. (**d**) Extract was further supplemented with methylated ubiquitin. Chromatin was recovered at the indicated times and blotted. (**e**) Sperm chromatin was incubated in LSS containing methylated ubiquitin, isolated at the indicated times and blotted. (**f**) LSS was optionally supplemented with 1 µM $p27^{Kip}$ or 300 µM aphidicolin in the presence (lanes 2–4) or absence (lane 1) of sperm. Chromatin was recovered after 50 min and blotted.

added sperm chromatin to a nucleus-free DNA-replication system (see Supplementary Information, Fig. S1b), which supports Cdt1 destruction[1] and allows for the rapid separation of chromatin from nucleoplasm. In the presence of methylated ubiquitin, replication-dependent ubiquitylation of Cdt1 was detected in the total extract (see Supplementary Information, Fig. S2b, compare lanes 1 and 3). Importantly, when sperm chromatin was removed from the soluble replication extract by centrifugation, most of the ubiquitylated Cdt1 was co-depleted (see Supplementary Information, Fig. S2b, compare lanes 1 and 2) and recovered in the chromatin pellet (see Supplementary Information, Fig. S2c), indicating that Cdt1 ubiquitylation occurs predominantly on chromatin. If PCNA were localized exclusively to chromatin, it might explain the restriction of Cdt1 ubiquitylation to chromatin. However, only ~1% of PCNA was bound to chromatin in early S phase (see Supplementary Information, Fig. S2d); as expected given its high endogenous concentration (~6 µM). The large pools of soluble Cdt1 and PCNA, the restriction of Cdt1 ubiquitylation to chromatin and the dependence of this process on PCNA imply that only chromatin-bound PCNA supports Cdt1 ubiquitylation.

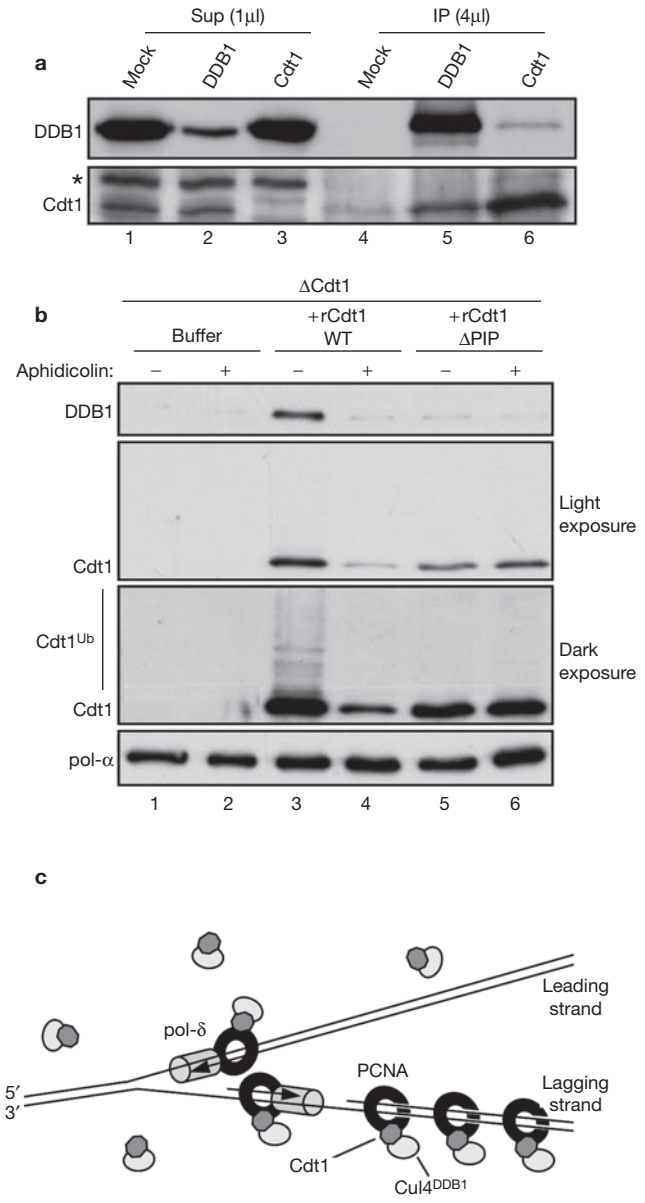


Figure 5 DDB1 is recruited to chromatin by Cdt1. (**a**) DDB1, Cdt1 or control antibodies were used for immunoprecipitation from high-speed supernatant (HSS) and 1 µl of supernatant extract (Sup) (lanes 1–3) or immunoprecipitated (IP) proteins from 4 µl of extract (lanes 4–6) were blotted. The asterisk indicates a cross-reacting band serving as a loading control. (**b**) Cdt1-depleted HSS was supplemented with 40 nM rCdt1^{WT} (lanes 3–4), rCdt1$^{\Delta PIP}$ (lanes 5–6) or buffer (lanes 1–2), and single-stranded DNA (ssDNA) coupled to magnetic beads for 15 min and bound proteins were isolated and blotted. Where indicated, 300 µM aphidicolin was included. pol-α, DNA polymerase-α; WT, wild type. (**c**) Model: Chromatin-bound proliferating cell nuclear antigen (PCNA) mediates replication-dependent Cdt1 ubiquitylation by Cul4^{DDB1}.

The question arises as to why PCNA is used to trigger Cdt1 destruction. Interestingly, it has been proposed that PCNA persists on DNA after Okazaki-fragment maturation and, therefore, accumulates to high levels on the lagging strand[22]. Consistent with this model, we determined that the ratio of PCNA trimers to replication forks (as measured by the number of chromatin-bound Cdc45 molecules[23]) increased progressively during S phase, reaching ~13:1 (see Supplementary Information, Fig. S2e). Assuming that ~10,000 origins (20,000 replication forks) are active at any moment in S phase, and that ~10 trimers of PCNA are present per fork at steady-state levels, then 200,000 molecules of Cdt1 could be marked for simultaneous destruction.

Cul4^{DDB1} is an E3 ubiquitin ligase in which Cul4 serves as the scaffold protein and DDB1 is thought to function as the specificity factor[24]. In worms, S-phase Cdt1 destruction requires Cul4 (ref. 6) and, in humans, Cul4 and DDB1 are required for Cdt1 destruction after DNA damage[8,14]. To test whether replication-dependent Cdt1 destruction requires DDB1, we raised antibodies against a fragment of *Xenopus* DDB1 (see Supplementary Information, Fig. S3a), which recognized a single band of the expected molecular weight in extracts (Fig. 4a). Immunodepletion of ~93% of DDB1 (see Supplementary Information, Fig. S3b) inhibited Cdt1 destruction in response to ssDNA replication in HSS (Fig. 4b). A similar degree of DDB1 depletion from LSS did not significantly affect Cdt1 destruction that was induced by sperm chromatin (Fig. 4c), perhaps due to sequestration of the remaining DDB1 within nuclei. However, DDB1 depletion from LSS did significantly inhibit the ubiquitylation of Cdt1 on chromatin (Fig. 4d). The greater effect of DDB1 depletion on Cdt1 ubiquitylation (Fig. 4d) versus destruction (Fig. 4c) is consistent with our previous observation that ubiquitylation is not rate-limiting for Cdt1 destruction[1]. Strikingly, DDB1 bound to chromatin, and the kinetics closely correlated with the binding of PCNA and the appearance of ubiquitylated Cdt1 in the presence (Fig. 4e) or absence (data not shown) of methylated ubiquitin. Moreover, like PCNA loading and Cdt1 ubiquitylation, DDB1 recruitment to chromatin required replication initiation and primer synthesis (Fig. 4f). Together, the results indicate that DDB1 is required for replication-dependent Cdt1 proteolysis. In addition, the data indicate that DDB1 localizes to chromatin in a PCNA-dependent fashion, although it contains no recognizable PIP box. The dependence of Cdt1 destruction on Cul4 and the binding of DDB1 to Cul4 in human cells[8,14] predict that there should be replication-dependent chromatin localization of Cul4, but the absence of suitable antibodies currently precludes a direct test of this idea.

To further explore the mechanism of DDB1-mediated destruction of Cdt1, we asked whether the two proteins interact in the absence of chromatin. Figure 5a shows that ~30% of Cdt1 was co-immunoprecipitated with DDB1 (compare lanes 2 and 5, lower panel). Cdt1 antibodies also consistently co-precipitated DDB1 above background levels (Fig. 5a, compare lanes 4 and 6, upper panel). By contrast, we did not detect complexes of PCNA and Cdt1 in *Xenopus* egg extracts (data not shown). We next addressed whether DDB1 binding to chromatin is Cdt1-dependent. To circumvent the complication that Cdt1 is required to stimulate DNA replication on sperm chromatin, and therefore plays an indirect role in recruiting DDB1, we used ssDNA, which supports DNA replication and Cdt1 destruction independently of pre-RC formation (see Supplementary Information, Fig. S1a). When ssDNA was immobilized on magnetic beads, it triggered Cdt1 destruction (data not shown). Importantly, in Cdt1-depleted HSS, DDB1 recruitment to ssDNA was completely dependent on the addition of rCdt1^{WT} (Fig. 5b, compare lanes 1 and 3), and it was sensitive to aphidicolin (Fig. 5b, compare lanes 3 and 4). Moreover, rCdt1$^{\Delta PIP}$ did not support DDB1 recruitment (Fig. 5b, compare lanes 3 and 5). Therefore, DDB1 binding to chromatin depends on the interaction of Cdt1 with PCNA. Additionally, Cdt1 loading (Fig. 5b, Cdt1 'Light') and ubiquitylation (Fig. 5b, Cdt1 'Dark') on ssDNA was aphidicolin-sensitive (compare lanes 3 and 4) and PIP-box-dependent

(compare lanes 3 and 5), indicating that Cdt1 recruitment to chromatin and its ubiquitylation are both PCNA-dependent. Unfortunately, PCNA depletion from HSS non-specifically inactivated the extract for Cdt1 ubiquitylation on immobilized ssDNA. Nevertheless, the data strongly indicate that a complex of Cdt1 and DDB1 is directly recruited to chromatin via PCNA.

Our data support a model in which chromatin-bound PCNA functions as a platform that facilitates DDB1-mediated ubiquitylation of Cdt1 during S phase (Fig. 5c) — a mechanism that is sufficient to block detectable re-replication. Cdt1 and DDB1 seem to load onto chromatin as a complex, as Cdt1 and DDB1 interact in the absence of chromatin, and because DDB1 binding to DNA is Cdt1-dependent. Direct binding of Cdt1 to PCNA on chromatin is supported by the fact that Cdt1 ubiquitylation occurs predominantly on chromatin (see Supplementary Information, Fig. S2b), with the same kinetics as PCNA loading (Fig. 4e), and in a manner that is dependent on the Cdt1 PIP box (Fig. 3d) and on DNA-replication initiation[1]. Moreover, Cdt1 binds to ssDNA templates in a replication- and PIP-box-dependent fashion (Fig. 5b). The presence of Cdt1 at the replication elongation complex in *Drosophila* follicle cells is also consistent with our model[25]. It is, at present, unclear why the complex of Cdt1 and DDB1 does not bind to soluble PCNA in extracts. PCNA might undergo a conformational change or modification after loading onto chromatin, which allows efficient Cdt1 binding, an idea that is consistent with the observation that Fen1 binds to different regions of PCNA depending on whether PCNA is soluble or DNA-bound[26]. Whatever the mechanism, the dependence of Cdt1 ubiquitylation on chromatin-bound PCNA insures that this process is coupled to S phase.

PCNA-dependent Cdt1 destruction seems to be highly conserved. First, all metazoans contain a PIP box at the N terminus of Cdt1, a region that is essential for Cdt1 destruction in humans, frogs and flies[2,4,18–20]. Second, recent experiments in flies show that Cdt1 destruction in S phase is dependent on replication[27]. Third, in humans, Cdt1 and PCNA can interact, and small deletions of the PIP box stabilize the protein in S phase (A. Dutta, personal communication). Recently, it was found in *Xenopus* egg extracts that the destruction of recombinant Xic1, a p27-like Cdk2 inhibitor, is also PCNA-dependent[28], raising the possibility that other proteins are normally degraded by this pathway.

Together with previous results, our data indicate that Cdt1 can be destroyed in S phase by two pathways that involve different E3 ubiquitin ligases. Whereas SCF^{Skp2}-mediated destruction of Cdt1 is dependent on direct phosphorylation of Cdt1 by Cdk2 (ref. 19), $Cul4^{DDB1}$-mediated destruction is dependent on Cdt1 binding to PCNA (this paper). In human cells, the two mechanisms seem to be redundant[20,29]. However, in other metazoans, the replication-dependent $Cul4^{DDB1}$ pathway seems to predominate. In support of this, mutation of the PIP box in *Xenopus* Cdt1 is sufficient to stabilize Cdt1 during S phase (this paper) and, in flies, mutations that affect initiation of DNA replication stabilize Cdt1 (ref. 27). Finally, loss of Cul4 in worms is sufficient to inhibit Cdt1 destruction and induce over-replication[6].

Interestingly, *Escherichia coli* uses a similar strategy to prevent re-replication: the binding of Hda protein to β, the prokaryotic equivalent of PCNA, leads to inactivation of the replication-initiator protein DnaA[30]. This example of convergent evolution suggests that processivity factors are ideally suited for the negative regulation of replication re-initiation in S phase. □

METHODS

Egg extract and immunological methods. Preparation of HSS, LSS, nucleoplasmic extract (NPE), chromatin isolations, replication assays, immunodepletions and density-substitution experiments were performed as described previously[1]. For immunoprecipitations, 1 volume of HSS was diluted with 3 volumes of ELB salts (2.5 mM $MgCl_2$, 50 mM KCl, 10 mM HEPES, pH 7.7) containing 10 mM EDTA, and the diluted extract was mixed with 0.2 volumes of Protein A sepharose (GE Healthcare, Piscataway, NJ) coupled with 10 mg ml^{-1} immunoglobulin G for 60 min at 4°C. The supernatant was recovered and beads were washed three times with ELB salts + 10 mM EDTA and resuspended in sample buffer. To recover total and soluble fractions during replication, replication was initiated in HSS and NPE as described previously[1] and reactions were stopped by dilution into ELB + 0.6% Triton-X-100, layered over a sucrose cushion and the chromatin was pelleted by centrifugation. 'Total' fractions were removed from the top of the sucrose cushion immediately before, and 'soluble' fractions immediately after, centrifugation. The p21 peptide[21] (CKRRQTSMTDFYHSKRRAIAS) was synthesized by Invitrogen (Carlsbad, CA); mutant peptide (CKRRATSATDAAHSKRRAIAS) was synthesized by the Biopolymers Laboratory (Harvard Medical School, Boston, MA). To deplete PCNA, peptides were coupled to Sulfo-link coupling gel (Pierce, Rockford, IL) at 1.8 mg ml^{-1} resin according to the manufacturer's instructions. One volume of HSS was mixed with 0.8 volumes resin for 80 min and the HSS was recovered by centrifugation through a Nitex membrane. M13 ssDNA was obtained from New England Biolabs (Beverly, MA). Antibodies against Cdt1, MCM3, Orc2, Cdc45, RCC1 and PCNA were used as described previously[1]. Cdt1 was immunoprecipitated with a peptide antibody raised against the carboxy-terminal 20 amino acids of Cdt1. Densitometry was performed on a Gel Doc system (Bio-Rad, Hercules, CA).

Protein purification. The *Cdt1* gene carrying alanine substitutions in the PIP box (Fig. 1a) was generated by polymerase chain reaction (PCR)-based mutagenesis and cloned into pDONR201 (Invitrogen). $Cdt1^{WT}$ and $Cdt1^{\Delta PIP}$ were purified from insect cells as previously described, and the tag was removed[1]. To construct Cdt1(1–25)–GST, PCR amplification of GST with primers to incorporate the N-terminal 25 amino acids of Cdt1 onto the N terminus and a strep tag onto the C terminus of GST was performed, and the product was cloned into pETDuet-1 (Novagen/EMD, San Diego, CA) via *Nco*I and *Xho*I. Cdt1(1–25) was tagged at the C terminus because N-terminal tags inhibited binding to PCNA, probably due to obstruction of the PCNA-interaction motif. Proteins were purified from *E. coli* over StrepTactin resin (IBA, St. Louis, MO), according to the manufacturer's instructions. rPCNA wild type was kindly provided by J. Pascal and T. Ellenberger (Harvard Medical School). The XDDB1 homologue (EST sequence TC288730) was identified by a BLAST search against hDDB1 (93% identical). A C-terminal fragment containing amino acids 708–1141 of XDDB1 was cloned into *Eco*RI and *Xho*I sites in pETDuet-1, expressed in *E. coli*, purified from inclusion bodies under denaturing conditions and injected into rabbits. The resulting anti-serum was affinity-purified over an NHS-activated Hi-Trap HP column (GE Healthcare) coupled to $XDDB1^{708–1141}$. Affinity-purified XDDB1 antibody was used for all DDB1 western blots, except for those shown in Supplementary Information, Fig. S3a, in which crude serum was used. His-tagged rCdc45 was prepared as described previously[31].

***In vitro* binding assays.** For binding of rPCNA to immobilized N-terminal fragments of Cdt1 (wild type or ΔPIP), rPCNA (6 μM) was added to Cdt1(1–25)–GST that had been immobilized on StrepTactin resin (IBA) (12 μM final) in ELB salts + 1 mM DTT. Reactions were incubated for 30 min at 22°C, washed extensively with ELB salts + 0.1% Triton-X-100, and bound fractions were eluted with StrepTactin elution buffer (Sigma, St. Louis, MO). For binding between full-length rCdt1 and immobilized rPCNA, rCdt1 (wild type or ΔPIP) (0.5 μM final) was added to rPCNA coupled to amino-linked coupling gel (Pierce) (6 μM final) in 50 mM Tris (pH 8.0), 0.2 M NaCl and 0.5% Triton-X-100. After incubation for 30 min at 22°C, the resin was washed extensively with the same buffer, and bound proteins were eluted with SDS sample buffer. In reactions that included p21 peptide competitor, the PCNA resin was pre-blocked with 0.4 mM p21 peptide for 2 h at 4°C, unbound peptide was removed by washing, and the resin was used as described above, except that the binding reaction contained an additional 20 μM p21 peptide.

Single-stranded DNA-binding assay. A 2-kb fragment from bacteriophage M13mp18 was PCR-amplified[26], with one of the primers containing a single biotin at its 5′ end and the PCR product was coupled to M-280 Streptavidin Dynabeads (Dynal/Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. To generate ssDNA, beads were washed twice with 0.1 M NaOH to remove the non-biotinylated DNA strand, and the resin was stored in ELB salts. For ssDNA binding assays, resin was added to HSS at a final concentration of 3 ng ssDNA μl^{-1} extract. Reactions were further supplemented with biotin-coated Dynabeads at a final concentration of 8 mg ml^{-1} to aid in visualization of the pellet during isolation steps. A total of 0.5% Triton-X-100 was then added to reduce non-specific binding, and reactions were incubated on a rotating wheel to prevent sedimentation. Samples were processed essentially as described previously for chromatin isolation[1]. Briefly, 10 µl reactions were diluted sixfold with ELB + 0.6% Triton-X-100, layered over a sucrose cushion, centrifuged and all but ~40 µl of the sucrose cushion was aspirated. The beads were then completely resuspended in 200 µl ELB, centrifuged and aspirated completely before addition of sample buffer.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We thank T. Takahashi for numerous helpful suggestions, and W. Harper and D. Finley for insightful comments on the manuscript. We also thank C. Cvetic and T. Prokhorova for preparation of the Orc2 and Cdt1 antisera, and J. Pascal and T. Ellenberger for providing recombinant PCNA. E.E.A is an Howard Hughes Medical Institute pre-doctoral fellow. This work was supported by an American Cancer Society grant (106201) to J.C.W.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturecellbiology/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Arias, E. E. & Walter, J. C. Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in *Xenopus* egg extracts. *Genes Dev.* **19,** 114–126 (2005).
2. Li, A. & Blow, J. J. Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in *Xenopus*. *EMBO J.* **24,** 395–404 (2005).
3. Maiorano, D., Krasinska, L., Lutzmann, M. & Mechali, M. Recombinant Cdt1 induces rereplication of G2 nuclei in *Xenopus* egg extracts. *Curr. Biol.* **15,** 146–153 (2005).
4. Thomer, M., May, N. R., Aggarwal, B. D., Kwok, G. & Calvi, B. R. *Drosophila* double-parked is sufficient to induce re-replication during development and is regulated by cyclin E/CDK2. *Development* **131,** 4807–4818 (2004).
5. Yoshida, K., Takisawa, H. & Kubota, Y. Intrinsic nuclear import activity of geminin is essential to prevent re-initiation of DNA replication in *Xenopus* eggs. *Genes Cells* **10,** 63–73 (2005).
6. Zhong, W., Feng, H., Santiago, F. E. & Kipreos, E. T. CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing. *Nature* **423,** 885–889 (2003).
7. Maga, G. & Hubscher, U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J. Cell Sci.* **116,** 3051–3060 (2003).
8. Hu, J., McCall, C. M., Ohta, T. & Xiong, Y. Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage. *Nature Cell Biol.* **6,** 1003–1009 (2004).
9. Dutta, A. & Bell, S. P. Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell. Dev. Biol.* **13,** 293–332 (1997).
10. Diffley, J. F. Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells. *Genes Dev.* **10,** 2819–2830 (1996).
11. Tada, S., Li, A., Maiorano, D., Mechali, M. & Blow, J. J. Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nature Cell Biol.* **3,** 107–113 (2001).
12. Wohlschlegel, J. A. *et al.* Inhibition of eukaryotic DNA replication by geminin binding to cdt1. *Science* **290,** 2309–2312 (2000).
13. Nishitani, H., Taraviras, S., Lygerou, Z. & Nishimoto, T. The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. *J. Biol. Chem.* **276,** 44905–44911 (2001).
14. Higa, L. A., Mihaylov, I. S., Banks, D. P., Zheng, J. & Zhang, H. Radiation-mediated proteolysis of CDT1 by CUL4-ROC1 and CSN complexes constitutes a new checkpoint. *Nature Cell Biol.* **5,** 1008–1015 (2003).
15. Kondo, T. *et al.* Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex. *J. Biol. Chem.* **279,** 27315–27319 (2004).
16. Arias, E. E. & Walter, J. C. Initiation of DNA replication in xenopus egg extracts. *Front. Biosci.* **9,** 3029–3045 (2004).
17. Hubscher, U., Maga, G. & Spadari, S. Eukaryotic DNA polymerases. *Annu. Rev. Biochem.* **71,** 133–163 (2002).
18. Nishitani, H., Lygerou, Z. & Nishimoto, T. Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region. *J. Biol. Chem.* **279,** 30807–30816 (2004).
19. Li, X., Zhao, Q., Liao, R., Sun, P. & Wu, X. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *J. Biol. Chem.* **278,** 30854–30858 (2003).
20. Takeda, D. Y., Parvin, J. D. & Dutta, A. Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase. *J. Biol. Chem.* **280,** 23416–23423 (2005).
21. Mattock, H. *et al.* Use of peptides from p21 (Waf1/Cip1) to investigate PCNA function in *Xenopus* egg extracts. *Exp. Cell Res.* **265,** 242–251 (2001).
22. Shibahara, K. & Stillman, B. Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* **96,** 575–585 (1999).
23. Edwards, M. C. *et al.* MCM2-7 complexes bind chromatin in a distributed pattern surrounding ORC in *Xenopus* egg extracts. *J. Biol. Chem.* **277,** 33049–33057 (2002).
24. Petroski, M. D. & Deshaies, R. J. Function and regulation of cullin-RING ubiquitin ligases. *Natuer Rev. Mol. Cell Biol.* **6,** 9–20 (2005).
25. Claycomb, J. M., MacAlpine, D. M., Evans, J. G., Bell, S. P. & Orr-Weaver, T. L. Visualization of replication initiation and elongation in *Drosophila*. *J. Cell Biol.* **159,** 225–236 (2002).
26. Gomes, X. V. & Burgers, P. M. Two modes of FEN1 binding to PCNA regulated by DNA. *EMBO J.* **19,** 3811–3821 (2000).
27. May, N. R., Thomer, M., Murnen, K. F. & Calvi, B. R. The origin binding protein Double parked, and its inhibitor geminin, increase in response to replication stress. *J. Cell Sci.* **108,** 4207–4717 (2005).
28. Chuang, L. C. & Yew, P. R. Proliferating cell nuclear antigen recruits cyclin-dependent kinase inhibitor Xic1 to DNA and couples its proteolysis to DNA polymerase switching. *J. Biol. Chem.* **280,** 35299–35309 (2005).
29. Liu, E., Li, X., Yan, F., Zhao, Q. & Wu, X. Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. *J. Biol. Chem.* **279,** 17283–17288 (2004).
30. Su'etsugu, M., Shimuta, T. R., Ishida, T., Kawakami, H. & Katayama, T. Protein associations in DnaA-ATP hydrolysis mediated by the Hda-replicase clamp complex. *J. Biol. Chem.* **280,** 6528–6536 (2005).
31. Mimura, S., Masuda, T., Matsui, T. & Takisawa, H. Central role for cdc45 in establishing an initiation complex of DNA replication in *Xenopus* egg extracts. *Genes Cells* **5,** 439–452 (2000).
32. Walter, J., Sun, L. & Newport, J. Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell* **1,** 519–529 (1998).

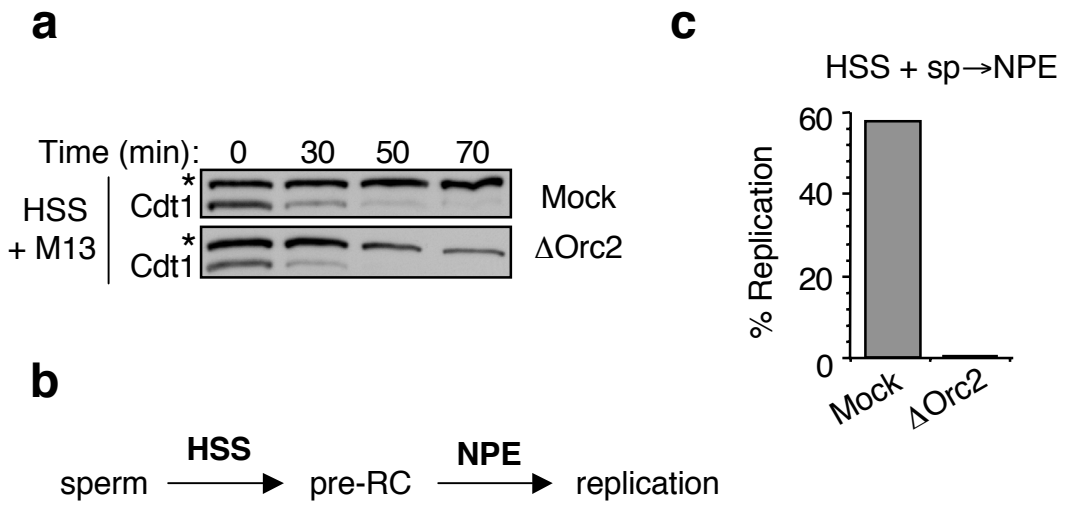


Fig. S1: M13 ssDNA-induced destruction of Cdt1 does not require ORC.

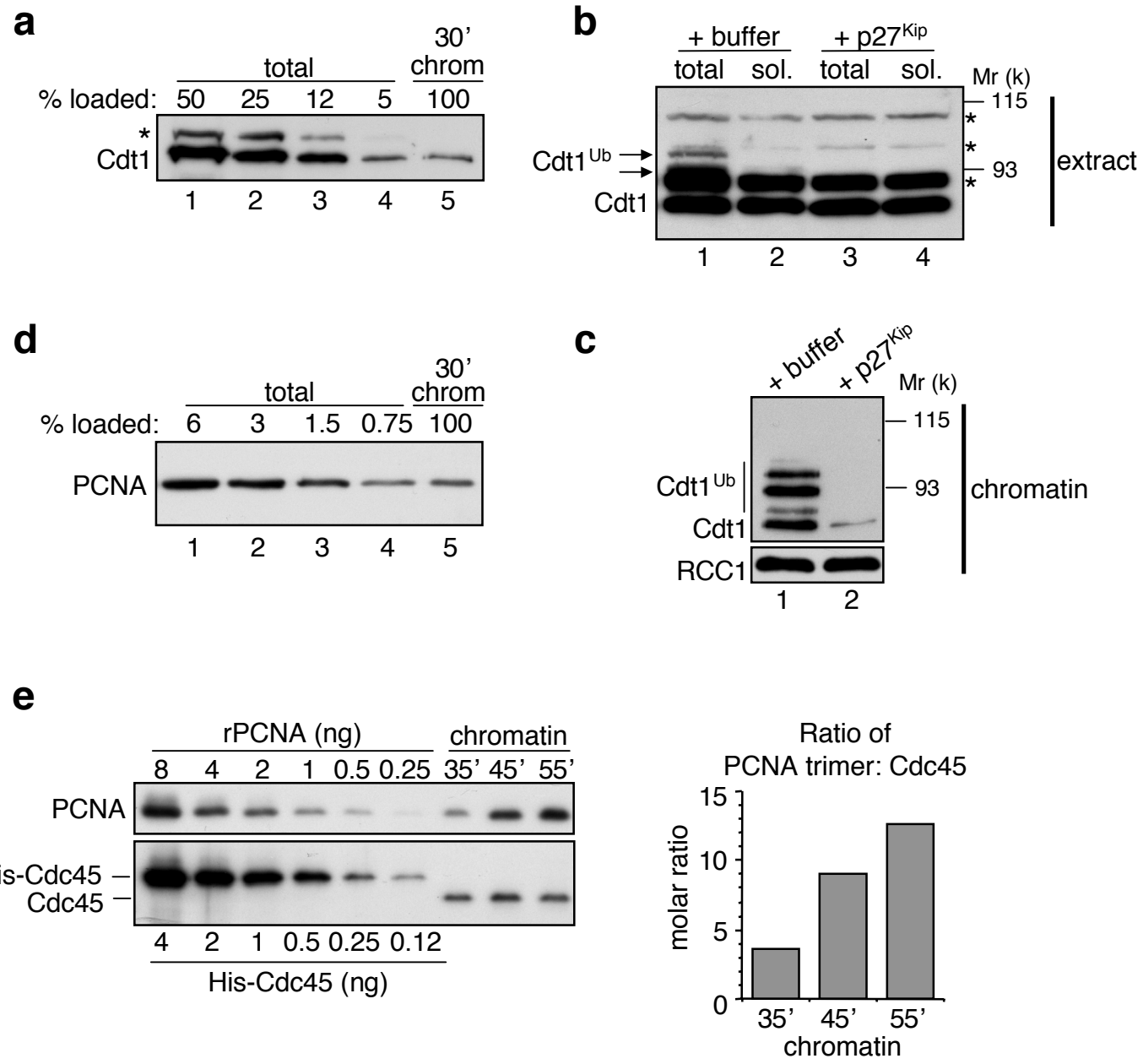
S1a: M13 ssDNA was incubated in mock- or Orc2-depleted HSS and reactions were analyzed as in Figure 2a. *, cross-reacting band serving as a loading control.

S1b: Schematic of DNA replication in the soluble, nucleus-free system[1]. Sperm chromatin is incubated in HSS to form pre-RCs, followed by addition of a highly concentrated nucleoplasmic extract (NPE) which supports origin firing from pre-RCs assembled in HSS.

S1c: As ORC is not required for ssDNA replication[2], we tested whether ORC was functionally depleted from HSS by testing the ability of the mock- and Orc2-depleted HSS from Fig. S1a to support sperm chromatin replication. HSS was incubated with sperm chromatin (3,000/μL final) for 30', followed by addition of NPE. The percentage of input DNA replicated after 60' is plotted.

1. Walter, J., Sun, L. & Newport, J. Regulated chromosomal DNA replication in the absence of a nucleus. *Mol Cell* **1**, 519-29 (1998).
2. Carpenter, P. B., Mueller, P. R. & Dunphy, W. G. Role for a Xenopus Orc2-related protein in controlling DNA replication. *Nature* **379**, 357-60 (1996).

Walter_Supplementary Figure S1



Walter_Supplementary Figure S2

Figure S2: Cdt1 is ubiquitinated predominantly on chromatin.

S2a, d: Sperm chromatin was incubated in LSS for 30' with 1 μM $p27^{Kip}$, to prevent replication initiation (a), or with no inhibitor (d), isolated, and blotted. 100% loading is the equivalent of 4 μL of extract (lanes 1-4) or of the chromatin-bound protein from 4 μL of extract (lane 5).

S2b: Replication was initiated in the nucleus free-replication system[1] by incubation of sperm chromatin (3,000/μL final) in HSS followed by addition of NPE in the presence of 250 μM methylated ubiquitin and 70 nM $rCdt1^{WT}$ in the presence (lanes 3-4) or absence (lanes 1-2) of 1 μM $p27^{Kip}$ for 20 minutes. Samples were withdrawn before (lanes 1 and 3) or immediately after (lanes 2 and 4) centrifugation to remove the chromatin and blotted for Cdt1. *, cross-reacting band. Arrows indicate ubiquitinated forms of Cdt1.

S2c: The chromatin removed from samples 1 and 3 in (b) was blotted as indicated.

S2e: Sperm chromatin was incubated in LSS for the indicated times and blotted next to the indicated amounts of rPCNA or his-tagged rCdc45. The amount of PCNA and Cdc45 on chromatin was calculated at each time point by densitometry, and the molar ratio of PCNA trimers to Cdc45 is plotted. The experiment was performed 4 times, with a representative data set shown.

1. Walter, J., Sun, L. & Newport, J. Regulated chromosomal DNA replication in the absence of a nucleus. *Mol Cell* **1**, 519-29 (1998).

Walter_Supplementary Figure S2

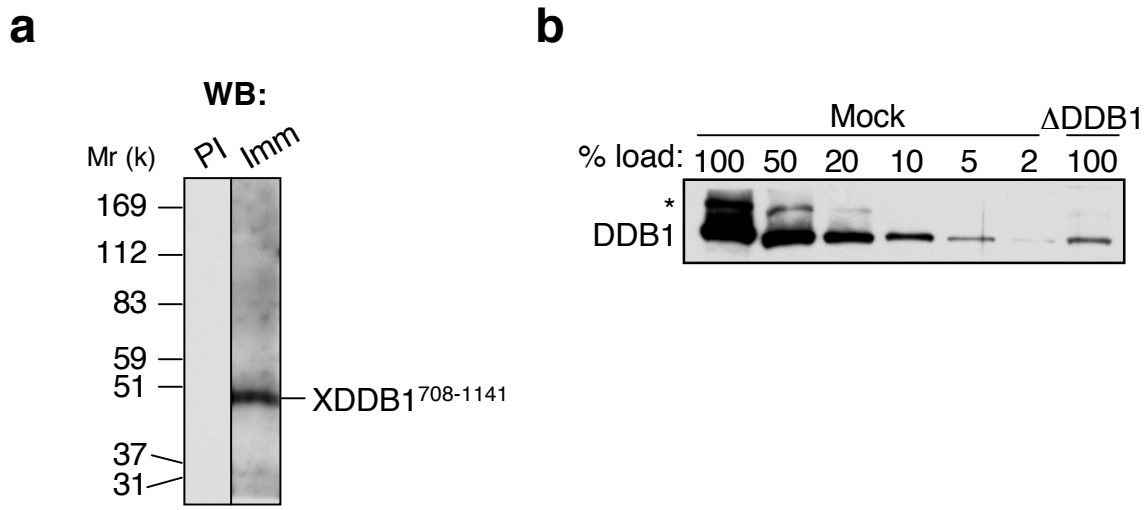


Fig. S3: Characterization of α-XDDB1 serum.

S3a: 0.25 ng of XDDB1$^{708\text{-}1141}$ was probed with a 1:2,000 dilution of pre-immune (PI) or immune serum (Imm). Images are of a 10-second film exposure.

S3b: DDB1 depletion from LSS. Mock- or DDB1-depleted LSS was blotted for DDB1. 100% load equals 1 μL LSS. *, cross-reacting band.

Walter_Supplementary Figure S3

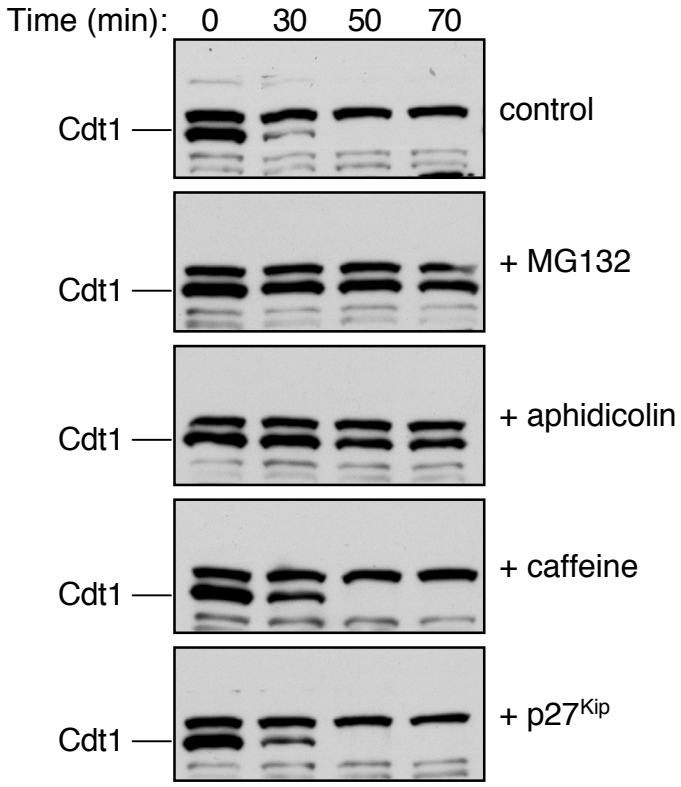


Fig. S4: Shown are the full Cdt1 Western blots from Figure 2A spanning a region of ~50-90 kD. The position of Cdt1 in each panel is indicated.

Walter_Supplementary Figure S4