Cdt1, a protein essential in G1 for licensing of origins for DNA replication, is inhibited in S-phase, both by binding to geminin and degradation by proteasomes. Cdt1 is also degraded after DNA damage to stop licensing of new origins until after DNA repair. Phosphorylation of Cdt1 by cyclin-dependent kinases promotes its binding to SCF-Skp2 E3 ubiquitin ligase (9–12), but the Cdk2/Skp2-mediated pathway is not essential for the degradation of Cdt1. Here we show that the N terminus of Cdt1 contains a second degradation signal that is active after DNA damage and in S-phase and is dependent on the interaction of Cdt1 with proliferating cell nuclear antigen (PCNA) through a PCNA binding motif. The degradation involves N-terminal ubiquitination and requires Cul4 and Ddb1 proteins, components of an E3 ubiquitin ligase implicated in protein degradation by proteasomes (4). Overexpression of Cdt1 or down-regulation of geminin causes re-replication of DNA (5–8). DNA re-replication causes genomic instability, activation of checkpoint pathways, and eventually cell death or cell transformation (5). Therefore, Cdt1 is tightly regulated during the cell cycle to make sure that cells do not re-replicate their DNA. Phosphorylation of Cdt1 by Cdk2 promotes its binding to SCF-Skp2 E3 ubiquitin ligase (9–12), but the Cdk2/Skp2-mediated pathway is not essential for the degradation of Cdt1 in S-phase (10, 13).

Cdt1 is also targeted for degradation by ubiquitin ligases after DNA damage (14–16). In apparent conflict, SCF-Skp2 complex and Cul4/Ddb1 complex have both been reported to induce degradation of Cdt1 (14–16) but not in humans. In this report we identify the signal for the Skp2-independent degradation of Cdt1 and demonstrate that this signal operates through interaction of Cdt1 with PCNA and uses the Cul4/Ddb1 ubiquitin ligases. The redundancy of the two pathways in S-phase stands in contrast to the predominant use of the PCNA/Cul4/Ddb1 pathway after DNA damage.

In Vivo Ubiquitination

Plasmids and Mutagenesis—A 4× HA tag was cloned into the PMXpuro vector between EcoR1 and NotI, and then the NotI site was destroyed using a fill-in reaction followed by self-ligation. A linker was cloned into BamH1 and EcoR1 to generate a multcloning site with BamH1, EcoR1, and NotI. Full-length, 1–98, and deletion mutants of Cdt1 were cloned into this vector between EcoR1 and NotI, and then the NotI site was destroyed using a fill-in reaction followed by self-ligation to generate a multicloning site with BamH1, EcoR1, and NotI. Full-length, 1–98, and deletion mutants of Cdt1 were cloned into this vector between EcoR1 and NotI. GST tag was cloned in BamH1 and EcoR1 three times, each time destroying the BamH1 site and creating a new BamH1 site. The Cdt1 fragments were cloned into the multcloning site with BamH1, EcoR1, and NotI supplemented with 5% iron-supplemented calf serum. HCT116 cells were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were treated with 50 J/m2 of UV and 1 h later were lysed by directly adding 2× Laemmli sample buffer and sheared using a needle. Equal loading of proteins in immunoblots was ensured by comparing intensity of Ponceau S staining of lanes and intensity of background bands in immunoblots. To make recombinant virus, each construct was cloned into the PMXpuro vector (kindly provided by Dr. Kitamura at Tokyo University) and transfected to 293T cells with plasmids that express VSV-G envelope protein and viral gag and pol proteins (18). 48 h after transfection, the supernatant from the 293T cells was added to H1299 cells with 5 μg/ml of Polybrene, the mixture was incubated for 24 h, and then puromycin (3 ng/ml) was added to select infected cells stably expressing Cdt1 derivatives (19). A rabbit polyclonal antibody to Cdt1 was generated against the first 20 amino acids of Cdt1. To detect HA, 12CA5 monoclonal antibody or Y11 polyclonal antibody (Santa Cruz) was used.

MATERIALS AND METHODS

Cell Culture, Transfections, UV Treatment, Virus Infection, and Antibodies Used—293T cells and H1299 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% iron-supplemented calf serum. HCT116 cells were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were treated with 50 J/m2 of UV and 1 h later were lysed by directly adding 2× Laemmli sample buffer and sheared using a needle. Equal loading of proteins in immunoblots was ensured by comparing intensity of Ponceau S staining of lanes and intensity of background bands in immunoblots. To make recombinant virus, each construct was cloned into the PMXpuro vector (kindly provided by Dr. Kitamura at Tokyo University) and transfected to 293T cells with plasmids that express VSV-G envelope protein and viral gag and pol proteins (18). 48 h after transfection, the supernatant from the 293T cells was added to H1299 cells with 5 μg/ml of Polybrene, the mixture was incubated for 24 h, and then puromycin (3 ng/ml) was added to select infected cells stably expressing Cdt1 derivatives (19). A rabbit polyclonal antibody to Cdt1 was generated against the first 20 amino acids of Cdt1. To detect HA, 12CA5 monoclonal antibody or Y11 polyclonal antibody (Santa Cruz) was used.

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In Vivo Ubiquitination—1–98 or 1–98K(0) Cdt1 were transfected into 293T cells with or without a plasmid expressing Histag ubiquitin.
24 h later cells were treated with MG132 for 3 h followed by UV. 1 h later cells were lysed with lysis buffer (6 M guanidine-HCl 50 mM Tris-HCl, pH 8.0), sonicated until the lysate was no longer viscous and mixed with nickel-nitritotriacetic acid-agarose beads (Qiagen). Samples were rotated at room temperature for 2 h and then washed with lysis buffer and TI buffer (25 mM Tris-HCl, pH 6.8, 20 mM imidazole). 2× Laemmli sample buffer with 300 mM imidazole was added to the sample, and the mixture was boiled for 5 min and loaded onto gels. Ubiquitinated products were detected by anti HA monoclonal antibody (12CA5).

Interaction of Cdt1 and PCNA—For detecting the in vivo interaction, a plasmid expressing Cdt1 with a Myc epitope tag at the C terminus was transfected into 293T cells. The cells were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40 and freshly added protease inhibitor mix. The lysed cells were sonicated twice for 15 s to release chromatin-associated protein. For in vitro interaction, full-length Cdt1 or Cdt1 with a deletion of 20 N-terminal residues (Δ20) were transfected to 293T cells. 24 h later, non-chromatin associated Cdt1 was recovered by lysis in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40 and immunoprecipitated with the polyclonal HA antibody. Immunoprecipitated samples were mixed with bacterial lysate that had been induced to express recombinant PCNA for 1 h (21), washed with lysis buffer for four times, and then probed for PCNA and HA.

siRNA Transfection—The day before transfection, 2 × 10⁴ H1299 cells were plated in 6-well plates. RNA duplexes were transfected using Oligofectamine (Invitrogen) according to the manufacturer’s protocol. 160 pm RNA duplex was used for each transfection. The sequence of RNA oligonucleotide used are as follows: Cul4A, 5′-GAAACAUCCGAUCAGUACCUGUCUGUCAU-3′; Cul4B, 5′-AAGAAGGACCGUCUGUCAU-3′; DDB1, 5′-ACAGAGUGGCGAGAGCAUU-3′; and PCNA, 5′-GGGAAAGGCUUACCAUAGU-3′.

Cell-cycle Synchronization—To arrest cells in G2/M, cells were treated with 40 ng/ml nocodazole for 12 h, rounded cells were collected by shaking them off, washed three times, and re-plated. Cells were then harvested at the indicated time point. To arrest cells in G1, cells were treated with 10 μM mevastatin for 24 h. G1 arrest was confirmed by propidium iodide staining followed by flow cytometry.

RESULTS AND DISCUSSION
To gain further insight into the signals necessary for the degradation of Cdt1, we started mapping the regions necessary for degradation after UV radiation. H1299 cells stably expressing Cdt1 were treated with 50/μm² UV. Full-length and 1–98 of Cdt1 were clearly degraded (Fig. 1A). We have demonstrated earlier that 1–98 of Cdt1 contains all the bands in the Western blot is shown as loading control (L.C.). Exo Cdt1: protein expressed from transfected plasmid. B, 1–18 is sufficient for degradation of Cdt1 by UV. N-terminal fragments of Cdt1 were C-terminally fused to 3xNLS, GST, and 4× HA tags, and expressed in H1299 cells. Stable cell lines were made and treated with UV, and degradation of proteins was examined as described in A. Endo Cdt1: endogenous Cdt1 expressed in cells. L.C and Exo Cdt1 are as described in A. 1–18 has been re-examined (with Endo Cdt1 control) in C, degradation of 1–18 is dependent on the proteasome pathway. Cells that stably express 1–18 with or without MG132 treatment for 3 h before exposure to UV. D, amino acids were deleted from the N terminus of full-length Cdt1 and examined for degradation. Deletion of 5 amino acids is sufficient for stabilization of Cdt1 after UV radiation. E, T7A that has Thr-7 substituted to alanine is stable after UV irradiation. Cdt1 was expressed as described in Fig. 1. Left panel: full-length Cdt1. Middle panel: full-length Cdt1 with T7A mutation. Right panel: 1–28 of Cdt1 with T7A mutation. The left and middle panels are from the same experiment and same gel. F, conserved sequence in the N-terminal part of Cdt1. G, PCNA binding sequence of other proteins in humans. Gln-3, Val-6, and Phe-8 are important features of a PCNA binding motif and well conserved between species. LigI, DNA ligase I, H, mutation of amino acids in the PCNA binding motif render Cdt1 refractory to degradation by UV. Lower panel: endogenous wild-type Cdt1 is degraded after radiation. The background band above the endogenous Cdt1 serves as loading control. Some of the exogenous Cdt1 peptides react to the anti-Cdt1 and are visible above the L.C band.
signals necessary for the degradation of Cdt1 (10, 13), and so further deletions were initiated on this fragment (data summarized in Fig. 2E). Because further truncation of 1–98 of Cdt1 deleted a nuclear localization signal (NLS, around residue 60) that is essential for degradation and made the protein too small to detect, we fused 3xNLS, GST, and 4×HA tags to the C terminus of every construct. Using this strategy, we found that 1–28Cdt1, 1–23Cdt1, and 1–18Cdt1 were degraded but 1–10Cdt1 was stable after UV irradiation, suggesting that residues 10–18 contributed to the UV degradation signal (Fig. 1B). The proteasome inhibitor, MG132, inhibited degradation of 1–18, suggesting that degradation of this portion of Cdt1 is dependent on the proteasome pathway (Fig. 1C).

Degradation of 1–28 and smaller portions of Cdt1 were, however, not as complete as that of full-length Cdt1 or 1–98 of Cdt1, suggesting that there may be a second signal for degradation in the 28–98 region, a point that we will return to later. When determining the N-terminal limit of the degradation signal, deletion of the first 5 amino acids of full-length Cdt1 (5) was sufficient to stabilize the protein after UV irradiation (Fig. 1D). Therefore the extreme N terminus of Cdt1 contained signals critical for UV-mediated degradation.

Because Cdt1 is phosphorylated after UV irradiation (14), the single potential phosphorylation site in 1–18 of Cdt1, Thr-7, was substituted to alanine (T7A). T7A (both full-length Cdt1 and as part of 1–28Cdt1) was resistant to UV-dependent degradation (Fig. 1E). An antibody specific for phospho-Thr-7, however, did not recognize Cdt1 after radiation. In vivo labeling of cells with [32P]orthophosphate also did not provide any evidence of phosphorylation of 1–28Cdt1 after UV irradiation. Mass spectrometry analysis did not pick up phosphorylation of Thr-7. These results suggested that the threonine residue itself (rather than its phosphorylation) was part of the degradation signal.

We therefore focused on the sequence features near the T7 and discovered that there were a few amino acids in the N terminus of Cdt1 conserved between species (Fig. 1F), and together they resemble a potential PCNA binding motif (22) (Fig. 1G). The most important feature of a PCNA binding motif is glutamine (Gln-3), a hydrophobic residue (Val-6), and two aromatic residues (Phe-9 and Phe-10). To determine the significance of the PCNA interaction motif, we mutated some of these amino acids to alanine and examined degradation of the mutants by UV (Fig. 1H). Cdt1 is completely stable when Phe-9 was...
substituted to alanine. Point mutation of Gln-3, Arg-5, or Val-6 also rendered Cdt1 partially refractory to degradation by UV. These results suggest that the conserved sequence near the N terminus of Cdt1 is critical for UV-dependent degradation.

Next we checked whether the N-terminal degradation signal of Cdt1 was active in S-phase (Fig. 2A; summarized in Fig. 2E). Cells were released from a nocodazole-induced M-phase block, and degradation of Cdt1 was examined by immunoblotting at different time points. Because the Skp2-dependent degradation signal was dependent on phosphorylation of T29, we eliminated that pathway by focusing on 1–28Cdt1. Both 1–28Cdt1 and 1–18Cdt1 were clearly degraded in S-phase, whereas 1–10Cdt1 was stable. So the C-terminal limit of the degradation signal was between residues 10 and 18. Deletions from the N terminus indicated that 5–28Cdt1 was totally stable in S-phase (Fig. 2B), indicating that the N-terminal limit of the degradation signal was between residues 1 and 5. These results suggested that the 1–18 region of Cdt1, required for degradation after UV, is also capable of mediating degradation in S-phase. To determine whether the Skp2-dependent degradation signal downstream from residue 28 plays any role in the S-phase degradation of Cdt1, we evaluated the effect of deleting the N-terminal 5 amino acids in full-length Cdt1 (Δ5Cdt1). In contrast to 5–28Cdt1, Δ5Cdt1 was still degraded in S-phase (Fig. 2C), suggesting that residues downstream from 28 played a redundant role in Cdt1 degradation during S-phase. The F-box protein, Skp2, associates with Cdt1 when Thr-29 is phosphorylated, but full-length Cdt1 is still degraded in S-phase when the Skp2 binding site is disrupted either by a T29A mutation that removes the site of phosphorylation by cdks2 or by a Cy mutation that eliminates interaction with the cyclin and inhibits phosphorylation on Thr-29 (10). Cdt1 with mutation of either Cy motif or Thr-29 (T29A) was degraded in S-phase, whereas 1–10Cdt1 was stable. So the N-terminal region and the Skp2 binding region of Cdt1 constitute independent and redundant signals for degradation in S-phase.

1–18 of Cdt1 does not have any lysine residue but is still degraded by a proteasome-dependent pathway (Fig. 1C). Although ubiquitin is usually attached to the ε-NH₂ groups of internal lysine residues, occasionally proteins with no internal lysines or mutations in all internal lysine residues were still ubiquitinated and degraded in a proteasome-dependent pathway (23, 24). In some cases, the attachment of ubiquitin to the free α-NH₂ group at the N terminus has been shown by mass spectrometry (25). Because there is no lysine residue in residues 1–18 of Cdt1, we wondered whether the degradation of Cdt1 by UV is dependent on N-terminal ubiquitination. Because 1–18Cdt1 has a tag with internal lysine residues that may serve as receptors for ubiquitin, we made 1–98Cdt1 without any lysine residue and fused a 4× HA tag that has no lysine residue to the C terminus (1–98K(0)). 1–98K(0) was still degraded by UV irradiation, and the degradation was inhibited by MG132 treatment (Fig. 3A). Since adding large tags to the N terminus has been reported to stabilize proteins by inhibiting N-terminal ubiquitination (26), we added His₉ and 4× HA tags to the N terminus of Cdt1 (HHACdt1). HHACdt1 is partially resistant to UV-induced degradation (Fig. 3B). To confirm that 1–98K(0) Cdt1 was indeed being ubiquitinated in vivo, His₉-ubiquitin was co-expressed with wild-type and K(0) versions of 1–98 Cdt1 (Fig. 3C). In the presence of His₉-ubiquitin, 1–98 wt Cdt1 produces slower moving forms that are pulled down on nickel resin, indicative of protein with attached polyubiquitin chains. Even in the absence of His₉-ubiquitin, the nickel resin pulls down a few high molecular weight forms of 1–98K(0) (Fig. 3C). Despite these background bands, two to four additional slower moving isoforms of 1–98K(0) are seen when His₉-ubiquitin is present, indicating that polyubiquitin chains are attached to 1–98K(0) Cdt1 despite the absence of any internal lysine residues. 1–98K(0) is therefore degraded by UV in a proteasome-dependent reaction, this degradation is blocked by a bulky adduct attached to the N terminus, and the protein is polyubiquitinated despite the absence of internal lysine residues. These results suggest that, although internal lysine residues in Cdt1 may be ubiquitinated, they are not essential for the proteasome-dependent degradation of Cdt1 because of ubiquitination at the N terminus. This is similar to the N-terminal ubiquitination reported for other proteins (26).

Because disruption of the PCNA binding motif stabilized Cdt1 after UV irradiation, we first examined whether PCNA interacts with Cdt1. When full-length Cdt1 was transfected to 293T cells, immunoprecipitation of PCNA co-immunoprecipitated Cdt1 and this co-immunoprecipitated Cdt1 was degraded by UV (Fig. 4A). The proteasome inhibitor MG132 not only inhibited the degradation of the Cdt1 but also promoted the progressive accumulation of Cdt1 associated with PCNA suggesting the continued recruitment of Cdt1 to PCNA in the presence of DNA damage. To assay the interaction in vitro, Cdt1 was recovered independently of cellular PCNA by a different lysis procedure and mixed with recombinant PCNA in vitro. PCNA specifically associated with full-length
FIGURE 4. PCNA, Cul4, and DDB1 are involved in the degradation of Cdt1 after UV radiation. A, extracts from cells expressing Cdt1-Myc were immunoprecipitated with anti-PCNA antibody as indicated and immunoblotted for Cdt1 or PCNA. Cells were harvested at the indicated minutes after UV irradiation. **Left panel**: UV radiation causes a degradation of total and PCNA-bound Cdt1. **Right panel**: PCNA bound Cdt1 is stabilized and accumulates progressively upon UV irradiation of MG132-treated cells. B, full-length Cdt1, but not Δ20 Cdt1, interacts with PCNA in vitro. Cdt1-HA immobilized in an immunoprecipitate was mixed with recombinant PCNA and proteins associated with the beads detected by immunoblotting. FL: full-length Cdt1. Δ20: Cdt1 with a deletion of 20 amino acids from the N terminus. C, siRNA against PCNA or DDB1 stabilizes full-length Cdt1 after UV irradiation. H1299 cells were transfected with siRNA duplex and 78 h later cells were treated with UV and harvested. Degradation of endogenous Cdt1 was determined by blotting with anti-Cdt1. Knockdown of PCNA and DDB1 was confirmed by immunoblotting. GL2: negative control siRNA duplex against grasshopper luciferase. D, degradation of 1–98K(0) Cdt1 or of 1–18Cdt1 is dependent on Cul4, DDB1, and PCNA. H1299 cells that express either mutant were transfected with siRNA duplex targeting PCNA, DDB1, and combination of both Cul4A and Cul4B. Cells were treated with UV after 78 h of transfection, and degradation of mutants was examined by blotting with anti-HA. L.C.: nonspecific bands that serve as loading control. E, decrease of PCNA, DDB1, Cul4A, and Cul4B after siRNA transfection in the experiments in Fig. 4D. F, Cdt1 degradation after irradiation is independent of S-phase or DNA replication. HCT116 cells were treated with either Me2SO or 10 μM mevastatin and the cell lysates were immunoblotted for Cdt1 (left panel). β-Actin was used as the loading control (L.C.). Cell-cycle arrest was confirmed by propidium iodide fluorescence-activated cell sorting (right panel).
Cdt1 but not Δ20, deleted in the N-terminal PCNA interacting motif (Fig. 4B). In a parallel study the interaction of *Xenopus* PCNA with *Xenopus* Cdt1 was shown to be dependent on the PCNA-interacting peptide of Cdt1 (27). Specifically, the interaction of *Xenopus* Cdt1 with *Xenopus* PCNA was competitively inhibited by a wild-type PCNA-interacting peptide from the cdk inhibitor p21. In addition, point mutations in the PCNA interacting motif of *Xenopus* Cdt1 disrupted its interaction with PCNA. Therefore, the interaction of Cdt1 with PCNA appears to be mediated by the conserved PCNA interacting peptide at the N terminus.

To determine whether PCNA was required for the degradation of Cdt1, we depleted PCNA by siRNA and checked Cdt1 degradation after UV treatment. PCNA was clearly decreased by siRNA (Fig. 4C). The degradation of endogenous Cdt1 was affected by silencing PCNA, consistent with the notion that the PCNA-dependent mode of degradation is critical after UV (Fig. 4C). 1–18Cdt1 or 1–98K(0) Cdt1 lack internal lysine residues used by the SCF-Skp2 pathway for ubiquitination. Silencing of PCNA also inhibited the degradation of these derivatives of Cdt1 after UV (Fig. 4, D and E), consistent with the hypothesis that the PCNA-dependent pathway of degradation can be mediated by N-terminal ubiquitination.

One concern after PCNA siRNA is that inhibition of DNA replication might activate checkpoint pathways similar to what we have reported after ORC2 depletion (28), and the cells might arrest in G1 with low Cdk2 kinase activity. If the Cdk2/Skp2-dependent pathway was critical for Cdt1 degradation after UV, then such a G1 arrest might appear to stabilize Cdt1. To address this issue we wished to demonstrate that Cdt1 was degraded by UV even if cells are arrested in G1 with low Cdk2 kinase activity. HCT116 cells can be arrested in G1 with low Cdk2 kinase activity using the hydroxy methyl glutaryl-CoA reductase inhibitor, meva-statim (29). The propidium iodide-stained fluorescence-activated cell sorting analysis showed that the cells were indeed arrested mostly in G1 (Fig. 4F, right). Despite this arrest in G1, Cdt1 was still degraded after UV damage (Fig. 4F, left) suggesting this degradation pathway is independent of Cdk2 activity, and that the stabilization seen after PCNA depletion cannot be ascribed to a G1 arrest.

The PCNA-dependent, N-terminal ubiquitination-mediated degradation of a substrate is unusual. No E3 ligase has been specifically implicated in N-terminal ubiquitination of proteins. Cul4A, Cul4B, and DDB1, however, have been reported to be involved in Cdt1 degradation by UV. As reported earlier (14–16), DDB1 is co-immunoprecipitated with Cdt1 from cell extracts (not shown) and silencing DDB1 made full-length Cdt1 resistant to degradation after radiation (Fig. 4B). We therefore wondered whether Cul4 and DDB1 might be the E3 used by the PCNA-dependent N-terminal degradation signal. By silencing either Cul4 (A and -B) or DDB1, both 1–98K(0) Cdt1 and 1–18Cdt1 became resistant to degradation by UV (Fig. 4C). Therefore, Cul4, DDB1, and PCNA are involved in N-terminal ubiquitination of Cdt1, although they could also contribute to the ubiquitination of internal lysines.

We have shown that the N terminus of Cdt1 regulates Cdt1 degradation after UV damage and in S-phase. PCNA is involved in the UV-induced degradation of Cdt1 possibly by interacting with the N-terminal residues of Cdt1. How PCNA promotes the degradation of Cdt1 is unknown. Because DDB1 recruits Cdt1 to Cul4 (14–16), PCNA might stabilize the interaction, activate the catalytic function of Cul4, protect the Cdt1 from de-ubiquitination, or target the ubiquitinated Cdt1 to the proteasome. In *Xenopus*, Cdt1 is ubiquitinated on the chromatin in S-phase, so if Cdt1 degradation by UV also takes place on chromatin, it is tempting to speculate that PCNA facilitates the ubiquitination by bringing the Cdt1–DDB1–Cul4 complex to chromatin. Because PCNA is mono-ubiquitinated upon DNA damage (30), it is also possible that modified PCNA specifically interacts with Cdt1–DDB1–Cul4 complex.

This study resolves the many conflicting reports of mechanisms of degradation of Cdt1. A number of reports suggested that Cy motif mediated phosphorylation of Cdt1 targets it to the SCF-Skp2 complex for ubiquitination and degradation, and yet, at least in animal cells and in *Xenopus* egg extracts, elimination (or absence) of the Skp2 interaction did not abrogate the degradation of Cdt1 in S-phase. On the other hand, Cul4 has been implicated in the degradation of Cdt1 in *C. elegans* and with DDB1 in the degradation of mammalian Cdt1 after DNA damage but not in S-phase. Our results suggest that there are at least two independent pathways for the degradation of Cdt1: a Cdk2/Skp2-dependent pathway that uses Thr-29 and ubiquitination of internal lysines and a PCNA/DDB1/Cul4-dependent pathway that uses an N-terminal PCNA-interacting motif and N-terminal ubiquitination (Fig. 2F). Both these pathways are independently capable of degrading mammalian Cdt1 in S-phase, whereas the PCNA-dependent pathway is critical for degradation after DNA damage. A direct physical interaction between PCNA and Cdt1 is required for Cdt1 destruction during DNA replication in *Xenopus* egg extracts (27). Our results suggest that the PCNA-dependent ubiquitination and degradation of Cdt1 is not an isolated process in *Xenopus* egg extracts but is seen in somatic cells in other species and utilized to degrade Cdt1 after DNA damage.

N-terminal ubiquitination is a controversial process but an increasing number of proteins are reported to be degraded by this pathway. That 1–98K(0) Cdt1 is degraded by UV in a proteasome-dependent manner and is ubiquitinated *in vivo* and that a large tag on the N terminus inhibits Cdt1 degradation, strongly suggest that N-terminal ubiquitination is critical for Cdt1 degradation. So far, proteins such as MyoD, LMP1 (from Epstein-Barr virus), LMP2A, E7 (from human papilloma-virus), p21, and Id2 have been reported to be N-terminally ubiquitinated (26). Of these, p21 has a PCNA-interacting motif at the C terminus (31, 32) so that PCNA may be essential for the N-terminal ubiquitination of this protein. Indeed a recent report implicates PCNA in the degradation of recombinant Xic1 (a p21 homolog) in *Xenopus* egg extracts, suggesting that this PCNA-dependent ubiquitination machinery may be important for other proteins (33). It is not clear whether there are E3 ligases that are specific for N-terminal ubiquitination versus internal ubiquitination, but our results suggest that at least the Cul4–DDB1 complex is capable of promoting ubiquitination at the N terminus, because they are essential for the degradation of 1–18Cdt1 and 1–98K(0) Cdt1, proteins that do not have an internal lysine but are still degraded by a proteasome-dependent pathway.

In summary, our results indicate that PCNA, the ring-shaped clamp that is central for coordinating the action of a number of proteins involved in DNA replication, DNA damage repair, and chromatin assembly, also plays a critical role in the degradation of Cdt1. Degradation of Cdt1 is critical to ensure that licensing is restricted to once per cell cycle, and so it is interesting that two redundant E3 ubiquitin ligase pathways, Cdk2–Skp2 and PCNA–Cul4, are used to achieve this end. This is reminiscent of the redundant use of Mdm2 and E6AP for the ubiquitination of the tumor suppressor protein p53 and suggests that such redundancy in use of E3 ligases against specific substrates may be more widespread than currently suspected.
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DNA: Replication, Repair, and Recombination:
PCNA Is a Cofactor for Cdt1 Degradation by CUL4/DDB1-mediated N-terminal Ubiquitination

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