Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis

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Replication licensing is carefully regulated to restrict replication to once in a cell cycle. In higher eukaryotes, regulation of the licensing factor Cdt1 by proteolysis and Geminin is essential to prevent re-replication. We show here that the N-terminal 100 amino acids of human Cdt1 are recognized for proteolysis by two distinct E3 ubiquitin ligases during S–G2 phases. Six highly conserved amino acids within the 10 first amino acids of Cdt1 are essential for DDB1-Cul4-mediated proteolysis. This region is also involved in proteolysis following DNA damage. The second E3 is SCF-Skp2, which recognizes the Cy-motif-mediated Cyclin E/A-cyclin-dependent kinase-phosphorylated region. Consistently, in HeLa cells cosilenced of Skp2 and Cul4, Cdt1 remained stable in S–G2 phases. The Cul4-containing E3 is active during ongoing replication, while SCF-Skp2 operates both in S and G2 phases. PCNA binds to Cdt1 through the six conserved N-terminal amino acids. PCNA is essential for Cul4- but not Skp2-directed degradation during DNA replication and following ultraviolet-irradiation. Our data unravel multiple distinct pathways regulating Cdt1 to block re-replication.

Introduction

Initiation of cell cycle events is controlled by the sequential activation and inactivation of Cyclin-dependent kinases

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(CDKs) (Nurse, 1994; Sherr, 1994). S-Cyclin/CDKs are required to initiate DNA replication, while M-Cyclin/CDKs are activated following completion of DNA replication to initiate mitosis. To ensure accurate transmission of the genetic information, it is essential that during each cell cycle no DNA segment is left unreplicated nor does it re-replicate before chromosome segregation occurs. The DNA replication licensing system regulates initiation of DNA replication and inhibition of re-replication by controlling the assembly of the pre-replicative complex (pre-RC) on origins of replication (Blow and Hodgson, 2002; Nishitani and Lygerou, 2004). The pre-RC is formed at the end of mitosis and in G1 phase in a stepwise process: association of Cdc6 and Cdt1 onto the origin recognition complex-bound origins leads to recruitment and loading of the hexameric MCM2–7 complex, which licenses origins for replication (Bell and Dutta, 2002; Blow and Dutta, 2005). Licensing is established in mammalian cells several hours before CDKs and the Dbf4-dependent kinase are activated to initiate replication. The MCM2–7 complex most likely acts as a replicative helicase, leaving the origin, and traveling ahead of the replication machinery (Aparicio *et al*, 1997; Ishimi, 1997; Labib and Diffley, 2001). Following firing, origins convert to the post-replicative state, and are not licensed again until the completion of cell division. CDKs, while necessary for initiation, inhibit pre-RC formation during S to G2/M by inactivating licensing factors through phosphorylation or direct association (Nguyen *et al*, 2001; Wuarin *et al*, 2002). For example, phosphorylation of licensing factors has been shown to lead to their degradation or nuclear exclusion (Drury *et al*, 1997; Jallepalli *et al*, 1997; Labib *et al*, 1999). In higher eukaryotes, Geminin, a specific inhibitor of Cdt1, accumulates from S phase. It binds to Cdt1 and inhibits MCM loading (McGarry and Kirschner, 1998; Wohlschlegel *et al*, 2000; Tada *et al*, 2001). Geminin and Cyclin B have a destruction box, and are degraded by the anaphase-promoting complex around anaphase of mitosis. Thus, origin licensing is restricted to the end of mitosis and G1 phase, when CDK activity is low and Geminin is absent (Diffley, 2004).

Control of the MCM2–7 loading factors, Cdc6 and Cdt1, is critical to prevent re-replication. High expression of Cdc18, the *Schizosaccharomyces pombe* Cdc6 homolog, induces massive over-replication in *S. pombe*, which is promoted by coexpression of Cdt1 (Nishitani and Nurse, 1995; Nishitani *et al*, 2000). In metazoans, regulation of Cdt1 is believed to be the major means through which re-replication is inhibited. High expression of Cdt1 in mammalian cells and *Drosophila* or addition of Cdt1 protein to G2 nuclei in *Xenopus* egg extracts induces re-replication (Vaziri *et al*, 2003; Thomer *et al*, 2004; Arias and Walter, 2005; Li and Blow, 2005; Maiorano *et al*, 2005; Yoshida *et al*, 2005). It was also shown that silencing of Geminin in human cells leads to re-replication (Melixetian *et al*, 2004; Zhu *et al*, 2004). Proteolytic control ensures that human Cdt1 is present only from late mitosis and in G1 phase. Its degradation is

promoted by ubiquitination in S phase independently of Geminin binding (Wohlschlegel *et al*, 2000; Nishitani *et al*, 2001, 2004; Xouri *et al*, 2004; Arias and Walter, 2005). Although SCF-Skp2 has been implicated in Cdt1 degradation, evidence against an involvement of SCF-Skp2 has also been reported. Cdt1 binds to and is phosphorylated by Cyclin E/CDK2 and Cyclin A/CDK2, which marks it for recognition by an Skp2-containing E3 ubiquitin ligase (Li *et al*, 2003; Liu *et al*, 2004; Nishitani *et al*, 2004; Sugimoto *et al*, 2004). However, a recent report showed that a mutant in the Cy-motif of Cdt1, which is refractory to Cyclin/CDK phosphorylation and Skp2 binding, is still degraded in S phase (Takeda *et al*, 2005). It was also noticed that Cdt1 does not accumulate in Skp2−/− mouse embryonic fibroblasts (MEFs) (Nakayama *et al*, 2004). On the other hand, Cdt1 is degraded by a Cul4 complex in *Caenorhabditis elegans* (Zhong *et al*, 2003) and proteolytic control of Cdt1 is crucial in this organism to block re-replication, since inactivation of Cul4 brings about massive re-replication. In addition, DNA damage such as ultraviolet (UV) radiation induces Cdt1 degradation through Cul4-mediated proteolysis in mammalian cells (Higa *et al*, 2003; Hu *et al*, 2004). However, Skp2-dependent Cdt1 degradation following UV-irradiation has also been reported (Kondo *et al*, 2004). To clarify the proteolytic control of Cdt1 during the cell cycle and following DNA damage in human cells, we performed a detailed domain analysis of human Cdt1. We found that Cdt1 is targeted for ubiquitination by two distinct E3 ubiquitin ligases, which are triggered to target Cdt1 through different pathways and recognize different parts of the N-terminal region of Cdt1.

Results

Cdt1 is degraded in the absence of Skp2 both after UV-irradiation and in S–G2 phase

Cdt1 is degraded promptly after the onset of S phase and upon DNA damage such as UV-irradiation (Wohlschlegel *et al*, 2000; Nishitani *et al*, 2001; Higa *et al*, 2003). To search for proteins involved in Cdt1 degradation, we analyzed proteins copurifying with Cdt1 from HeLa cells treated with the proteasome inhibitor MG132, and recovered Cyclin A and Skp2 (data not shown). We therefore wished to examine whether Skp2 is required for correct cell cycle proteolysis of Cdt1. In order to assess the cell cycle expression profile of Cdt1 in asynchronous populations, we employed double Immunofluorescence (IF) analysis for Cdt1 and Cyclin A. Our assay is based on the observation that in a normal cell cycle, Cdt1 is present exclusively in G1 cells, while Cyclin A is present from S phase to early M phase (Pines and Hunter, 1990; Nishitani *et al*, 2001). Thus, Cdt1-positive cells are not stained with Cyclin A and *vice versa* (Figure 1A, upper panel). Perturbations in cell cycle regulation of Cdt1 can therefore be directly assessed at the single-cell level in asynchronous populations by the appearance of Cdt1-Cyclin A double-positive cells. Following UV-irradiation, Cdt1 is undetectable in both Cyclin A-positive and -negative cells (Figure 1A, lower panel; UV). This sensitive assay allows quantitative assessment of the cell cycle degradation of Cdt1 and avoids the use of drugs for synchronization, which could themselves affect Cdt1 proteolysis.

To investigate if Cdt1 is proteolysed in the absence of Skp2, HeLa cells were transfected with siRNA specific for

Skp2 (Figure 1B). Western blotting (WB) for Skp2 and p27, a known target of Skp2, demonstrated the efficiency of the RNAi treatment. IF analysis showed that Skp2 protein was undetectable in a large proportion of siSkp2-treated cells (Supplementary Figure S1A). Cell cycle progression was not blocked due to accumulation of p27 in siSkp2-treated cells, as the percentage of BrdU-positive cells was similar in siSkp2 and control-treated cells (Supplementary Figure S1A). Total Cdt1 protein levels increased three-fold in Skp2-depleted cells in comparison to control cells (Figure 1Ba), as reported previously (Li *et al*, 2003). However in Skp2-silenced cells, Cdt1 was still absent in Cyclin A-positive cells (Figure 1Bb, upper panel) and BrdU-positive cells (Supplementary Figure S1B), indicating that cell cycle-specific proteolysis of Cdt1 was maintained. In addition, degradation of Cdt1 following DNA damage was unaffected by Skp2 depletion (Figure 1Bb, lower panel). This argues against Skp2 being essential for Cdt1 proteolysis during S phase and after UV-irradiation. We also observed that Cdt1 was degraded in Skp2−/− MEFs similar to wild-type cells (Figure 1C). When whole-cell extracts were immunoblotted, p27 protein levels were highly increased in Skp2−/− MEF, but the Cdt1 protein level remained similar to that in Skp2+/+ MEF (Figure 1Ca). Consistently, Cdt1 was detected only in a subpopulation of both MEF cultures by IF (data not shown). After UV-irradiation, Cdt1 was degraded in both cultures with similar kinetics (Figure 1Cb).

In addition to SCF-Skp2, the DDB1-Cul4 pathway has been implicated in Cdt1 degradation (Higa *et al*, 2003; Zhong *et al*, 2003; Hu *et al*, 2004). In order to investigate the requirement for DDB1 for Cdt1 proteolysis, siRNA for DDB1 was used. While UV-induced Cdt1 degradation was inhibited, as reported previously (Hu *et al*, 2004), Cdt1 was still correctly degraded in S–G2 cells (Supplementary Figure S2). These data indicate that neither Skp2 nor DDB1 are independently required for correct cell cycle-specific proteolysis of Cdt1.

Six conserved amino acids at the Cdt1 N-terminus are essential for degradation after UV, while this motif and the Cy-containing region are separately involved in S–G2 degradation

A fragment containing the N-terminal 1–189 amino acids of Cdt1 is degraded essentially the same as endogenous Cdt1 both in S–G2 phases (Nishitani *et al*, 2004) and after UV-irradiation (Supplementary Figure S3). To identify regions within the amino-terminus of Cdt1, which mediate proteolysis, a series of deletion constructs fused with 9myc-3NLS were made (Figure 2A) and cell lines stably expressing each construct to comparable levels were isolated (Figure 2B). In the control strain expressing 9myc-3NLS alone, the protein is stable in all cell cycle phases and after UV-irradiation (Supplementary Figure S4A). By assaying a series of constructs with progressively shorter Cdt1 N-terminal fragments (Figure 2C and Supplementary Figure S4), the N-terminal 28 amino acids were identified as sufficient to confer both UV-induced and S–G2-specific degradation. When the first 10 or 20 amino acids were deleted from the (1–101) N-terminal region of Cdt1 (construct (11–101) and (21–101)9myc-3NLS)), the protein became stable after UV-irradiation, while the S–G2 degradation was not affected (Figure 2D and Supplementary Figure S4D). When the N-terminus was further removed, the (38–101) protein became stable in
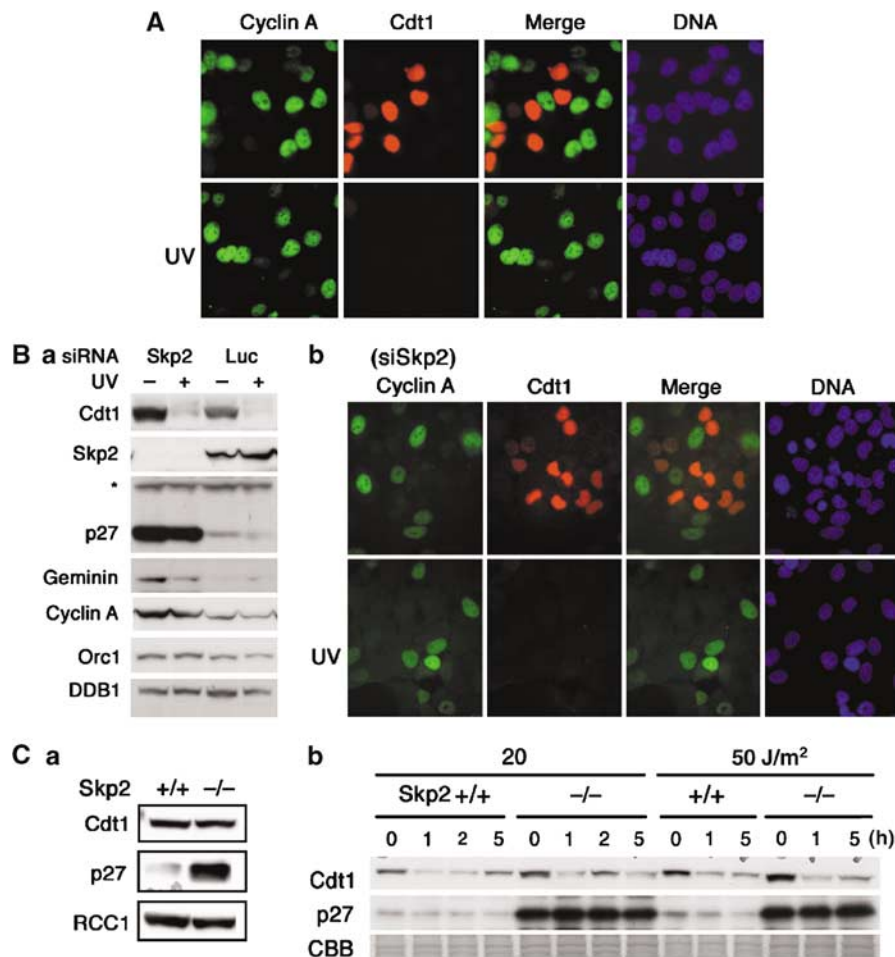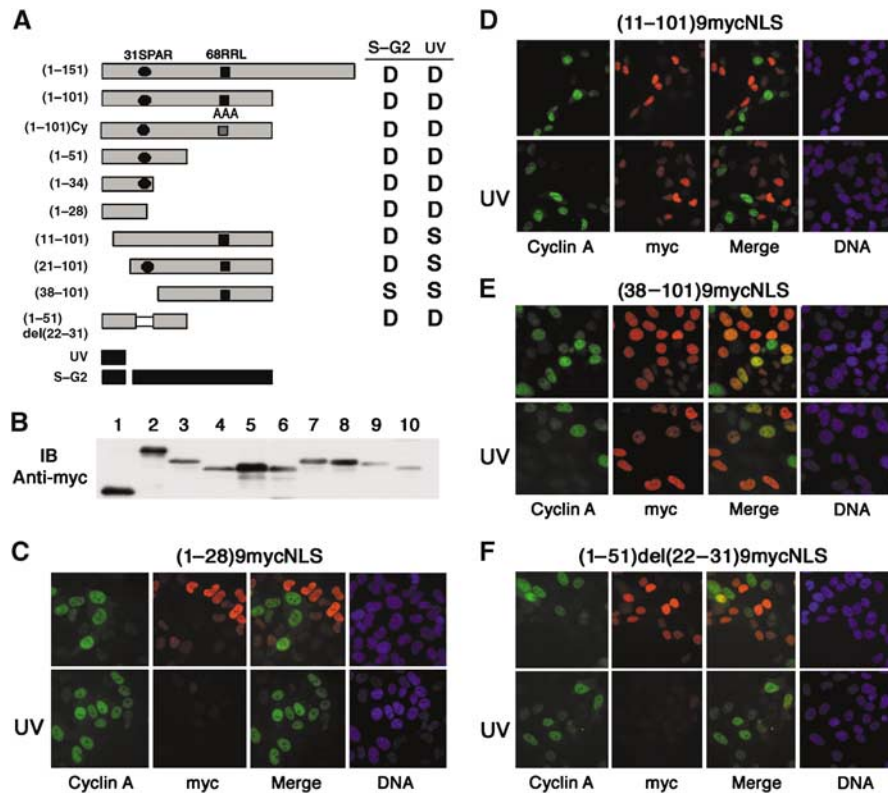
Figure 1 Cdt1 degradation in S–G2 phases and after UV-irradiation occurs in the absence of Skp2. (**A**) Double immunofluorescence analysis of HeLa cells with anti-Cyclin A and anti-Cdt1 antibodies. HeLa cells growing asynchronously (upper panel), or treated with UV (20 J/m^2, lower panel; UV) and then returned to culture for 1 h, were fixed and stained with the antibodies indicated. (**B**) Cdt1 is degraded in the absence of Skp2 in HeLa cells. Cells were transfected with siRNA for Skp2 or luciferase (Luc). At 48 h following transfection, half of the treated cells were irradiated with UV (20 J/m^2). After 1 h, cells were fixed for immunofluorescence as above or extracts prepared for immunoblotting with the indicated antibodies. The band marked with an asterisk on the p27 blot is a crossreacting band, which serves as a loading control. (**C**) Cdt1 degradation in Skp2−/− MEFs. (a) Cell extracts were prepared from Skp2+/+ and −/− MEFs and blotted with anti-Cdt1 and anti-p27 antibodies. RCC1 served as a loading control. (b) Asynchronous cultures of Skp2 +/+ and −/− MEFs were UV-irradiated as indicated, collected at the indicated times (in h), and Cdt1 and p27 protein levels analyzed. Total protein (CBB) served as a loading control.

S–G2 phases, as well as after UV-irradiation (Figure 2E). The results are summarized in Figure 2A (right side). These data show that the first 10 amino acids of Cdt1 are essential for degradation after UV-irradiation. For S–G2 degradation, two alternative explanations were possible: since both the 1–28 and 21–101 regions exhibited correct cell cycle proteolysis, which was lost in the 38–101 construct, either a region between amino acids 21–28 of Cdt1 was essential and sufficient to confer cell cycle-specific proteolysis, or redundant elements were present within the N-terminus. In order to discriminate between the two possibilities, a deletion construct of 1–51 lacking amino acids 22–31 was constructed. This protein was degraded correctly both after UV-irradiation and in S–G2 phase (Figure 2F). Taken together, the mutation analysis leads to the hypothesis that two regions, one within the first 20 amino acids of Cdt1 and a second one in the 21–101 region, are separately involved in S–G2 proteolysis.

In order to pinpoint the amino acids required for cell cycle proteolysis, we scanned the amino-terminus of Cdt1 for known motifs and phylogenetically conserved amino acids.

Upon comparison of the N-terminal regions of human, mouse and *Xenopus* Cdt1 proteins, six conserved amino acids were detected within the first 10 amino acids of Cdt1 (Figure 3, referred to hereafter as the QXRVTDF-motif). To investigate if these amino acids are important, the six amino acids were changed to alanines in the (1–101) construct, to generate construct A6(1–101) 9myc-3NLS. The Cy-motif, present in amino acids 68RRL70 of Cdt1, previously shown to be required for CDK/Cyclin association and phosphorylation (Liu *et al*, 2004; Sugimoto *et al*, 2004) could be involved in cell cycle proteolysis, and was therefore mutated to alanines to generate construct Cy(1–101). Stable cell lines expressing Cy(1–101), A6(1–101) and the double mutant A6Cy(1–101) to comparable levels were isolated (Figure 3B and C). The Cy(1–101) mutant was correctly proteolysed both in S–G2 and following UV damage (Figure 3D). The A6(1–101) remained stable after UV-irradiation, demonstrating that the six conserved amino acids are essential for degradation after DNA damage, while it was still degraded in S–G2 phase (Figure 3E). Strikingly, the double mutant A6Cy(1–101)

Figure 2 N-terminal domain analysis of Cdt1. (**A**) N-terminal constructs fused to 9myc3NLS. The results of the degradation assay for each construct during S–G2 phases and after UV are summarized on the right (D, degraded or S, stable). At the bottom, predicted regions required for proteolysis are shown as black bars. (**B**) Western blot analysis of stable cell lines, using an anti-myc antibody. Lane 1: 9myc3NLS only; lane 2: (1–151); lane 3: (1–101); lane 4: (1–51); lane 5: (1–34); lane 6: (1–28); lane 7: (11–101); lane 8: (21–101); lane 9: (38–101); and lane 10: (1–51) deleted of (22–31). (**C**) Asynchronously growing stable (1–28)9mycNLS cells were stained with anti-Cyclin A and anti-myc antibodies in the absence of UV treatment (upper panel) or 1 h after UV treatment (lower panel; UV). (**D–F**) Asynchronous populations of each cell line indicated were examined as in (C).
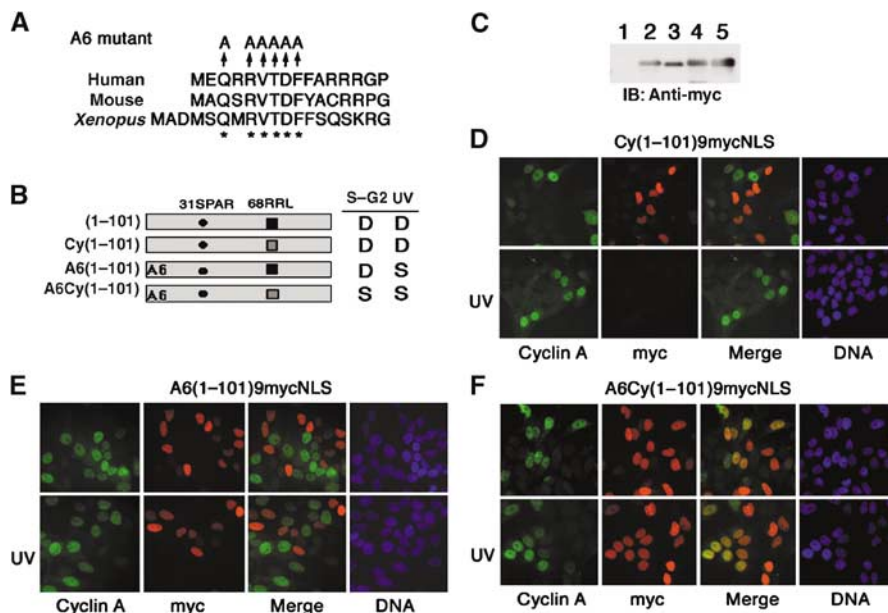
Figure 3 Two domains in the N-terminus are involved in degradation. (**A**) Alignment of N-terminal amino acids of human, mouse and *Xenopus* Cdt1. The six conserved amino acids were mutated to alanine to generate mutant A6. (**B**) (1–101) constructs with the indicated mutations that were fused with 9mycNLS. Their stability (D, degraded or S, stable) in S–G2 phases (S–G2) or after UV irradiation (UV) is summarized on the right. (**C**) Stable cell lines. Whole-cell extracts prepared from each cell line were blotted with anti-myc antibody (lane1: HeLa cell; lane 2: (1–101); lane 3: Cy(1–101); lane 4: A6(1–101); lane 5: A6Cy(1–101). (**D–F**) Each stable cell line indicated was stained with anti-Cyclin A and anti-myc antibodies in the absence of UV treatment (upper panel) or after UV irradiation (lower panel, UV) as indicated.

became stable both in S–G2 and after UV-irradiation (Figure 3F). Consistently, when the A6 mutation was introduced into the (1–28)9myc-3NLS construct, which was degraded in both cases (Figure 2C), the A6(1–28) 9myc-3NLS became stable in both situations (Supplementary Figure S4E).

We conclude that six phylogenetically conserved amino acids within the first 10 amino acids of Cdt1 mediate both the UV-induced and S–G2 degradation, while the Cy-dependent region is specific for S–G2 degradation.

### Two E3 ligases are involved in Cdt1 degradation

Our mutational analysis indicated that two redundant pathways could confer S–G2-specific proteolysis of Cdt1, one requiring the first 10 amino acids of Cdt1 and a second Cy-motif dependant. In order to determine which E3 ligase(s) was responsible for each pathway, we combined cell lines expressing mutated forms of Cdt1 with inactivation of putative E3 ligases. SCF-Skp2 and DDB1-Cul4, which we showed above not to be independently required for Cdt1 cell cycle proteolysis, were good candidates for mediating the two pathways. Initial experiments using Cdt1 N-terminal fragments showed that siRNA-mediated silencing of Skp2 led to stabilization of the A6(1–101) mutant in S–G2 cells

(Supplementary Figure S5A), while silencing of Cul4A and B led to stabilization of the (1–34)9myc-3NLS fragment, which lacks the Cy-motif, both following DNA damage and in S–G2 (Supplementary Figure S5B). These experiments led to the hypothesis that Skp2 may mediate the Cy-motif-dependent pathway, while Cul4A/B may mediate the QXRVTDF-motif-dependent pathway.

In order to further investigate this hypothesis, we introduced the A6 and Cy mutations independently in the context of the full-length Cdt1. Since the Cy mutant had a defect in nuclear import (data not shown), three copies of the SV40 NLS together with a myc tag was fused to the C-terminus of all constructs, as illustrated in Figure 4A. Stable cell lines were isolated, which express each of these mutants to levels similar to endogenous Cdt1 (Figure 4B). WT-Cdt1-3NLSmyc was degraded correctly both after UV-irradiation and in S–G2, indicating that addition of 3NLSmyc at the C-terminus did not affect Cdt1 proteolysis (Supplementary Figure S5C). The full-length A6-Cdt1-3NLSmyc (A6-Cdt1) was stable after UV-irradiation (data not shown), but was degraded in S–G2 (Figure 4C; siLuc). As shown in Figure 4C, silencing of Skp2, but not of Cul4, by RNAi led to stabilization of A6-Cdt1 in S–G2, as marked by the appearance of Cdt1/Cyclin A
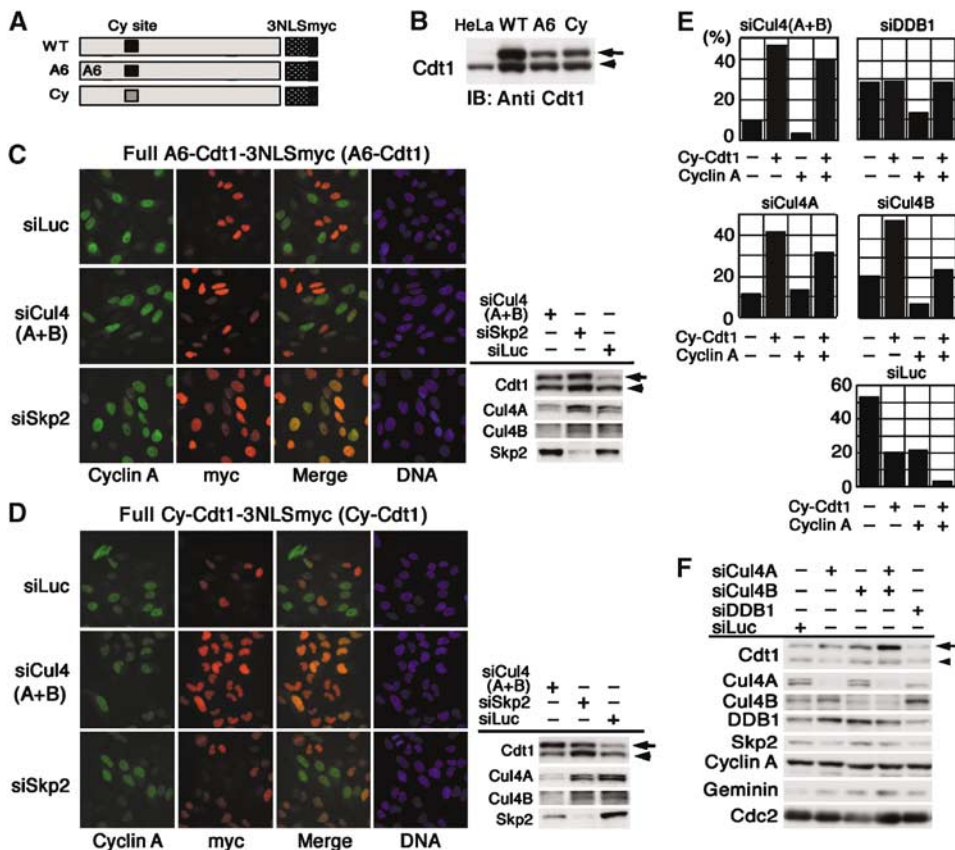
Figure 4 Two regions are recognized by two E3 ubiquitin ligases. (**A**) Full-size Cdt1 constructs fused with 3NLSmyc; full WT-Cdt1- (WT), A6-Cdt1- (A6) and Cy-Cdt1-3NLSmyc (Cy). (**B**) Stable lines, lane1: HeLa; lane 2: WT; lane 3: A6; and lane 4: Cy. Whole-cell extracts were prepared from each stable cell line and immunoblotted with anti-Cdt1 antibodies. (**C**) Stabilization of full A6-Cdt1-3NLSmyc after Skp2 silencing. Cells were transfected with the indicated siRNA, and costained with anti-Cyclin A and anti-myc antibodies (left) or extracts were prepared to blot with the indicated antibodies (right). (**D**) Stabilization of full Cy-Cdt1-3NLSmyc after Cul4 (A + B) silencing. Cells were treated as in (C). (**E**, **F**). Stabilization of full Cy-Cdt1-3NLSmyc (Cy-Cdt1) after silencing of DDB1 or Cul4. Cells were transfected with the indicated siRNAs, and treated for immunofluorescence or Western blotting (WB) with the antibodies for indicated proteins (F). Cells stained (+) or not stained (−) for Cy-Cdt1 and Cyclin A were counted, and frequency is shown (%) (E). In all Western blots, the arrowheads indicate endogenous Cdt1, while arrows indicate the full-sized Cdt1 constructs fused with 3NLSmyc.
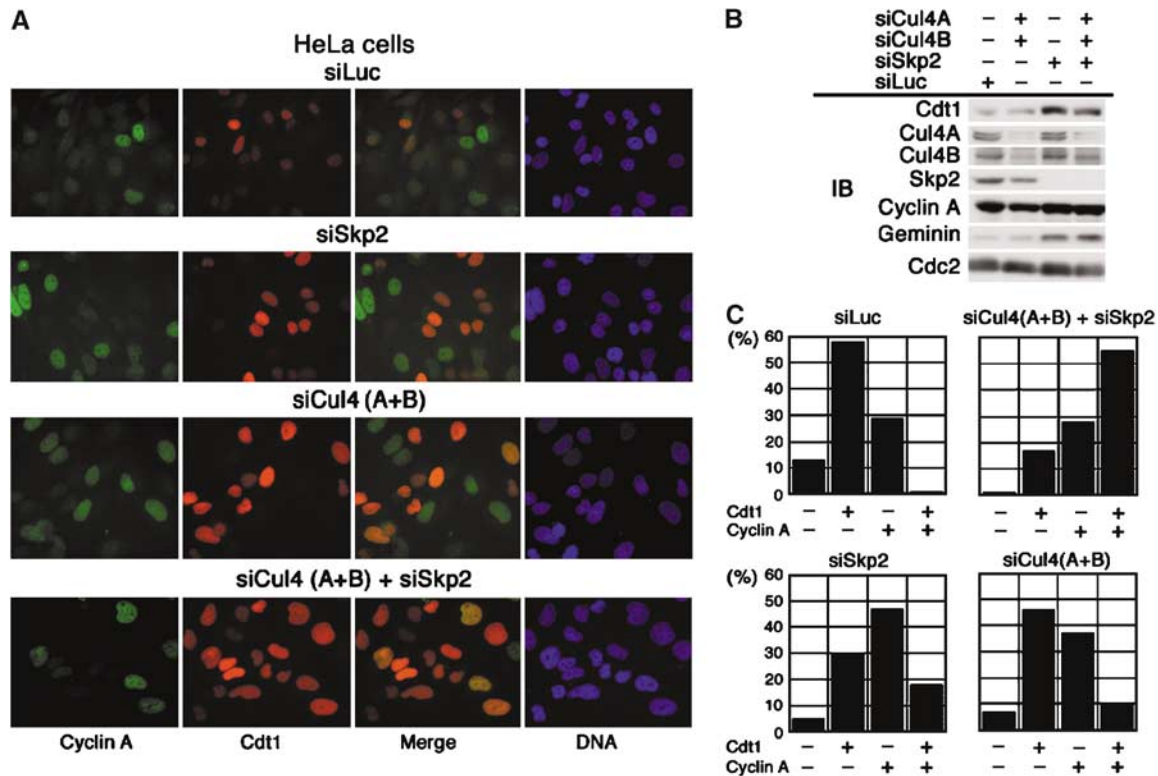
Figure 5 Stabilization of Cdt1 in S–G2 phases after cosilencing of Skp2 and Cul4. HeLa cells were transfected with the indicated siRNAs and examined by double immunofluorescence with anti-Cyclin A and anti-Cdt1 antibodies (**A**) or Western blotting (WB) with indicated antibodies (**B**). (**C**) Quantification of immunofluorescence images shown in (A). Percentage of cells stained (+) or not stained (−) for Cdt1 and Cyclin A is shown.

double-positive cells. Consistently, A6-Cdt1 levels increased dramatically in Skp2-silenced cells on Western blot (Figure 4C). When the SCF-component Cul1 was silenced, A6-Cdt1, but not Cy-Cdt1, became stable in most Cyclin A-positive cells (Supplementary Figure S6). This shows that SCF-Skp2 is required for the degradation of A6-Cdt1 and is therefore likely to be the mediator of the Cy-dependent pathway leading to S–G2 degradation of Cdt1.

In order to assess the involvement of DDB1-Cul4 in S–G2 degradation of Cdt1, the cell line expressing full Cy-Cdt1-3NLSmyc (Cy-Cdt1) was subjected to siRNA treatment. In this case, silencing of Cul4(A + B), but not of Skp2, led to stabilization of Cy-Cdt1 in S–G2 (Figure 4D). On WB, an increase in total Cy-Cdt1 levels was apparent following silencing of Cul4(A + B), as compared to siLuc-treated cells. Such an increase was not detected for the endogenous Cdt1 (Figure 4D and F). When DDB1 was silenced, the number of Cdt1/Cyclin A double-positive cells increased (Figure 4E and F). As compared to when Cul4A and Cul4B were silenced individually, when both Cul4A and Cul4B were silenced together, Cy-Cdt1 was expressed in the majority of cells in the population, whether Cyclin A positive or negative (Figure 4E and representative images in Figure 4D). Taken together, our data argue that DDB1-Cul4 mediates the QXRVTDF-motif-dependent proteolysis pathway, while Skp2 mediates the Cy-motif-dependent pathway.

Finally, in order to address whether Skp2 and DDB1-Cul4 are the major E3 ligases required for proteolysis of endogenous Cdt1, or additional components could mediate S–G2 degradation of Cdt1 in their absence, endogenous Cdt1 levels were examined in HeLa cells following siRNA treatment for these factors. In control siLuc-treated cells, less than 3% of Cyclin A-positive/Cdt1-positive cells were detected. In the population of Skp2- or Cul4(A + B)-silenced HeLa cells, 20–30% of Cyclin A-positive cells showed Cdt1 signal (Figure 5A and C), while when Skp2 and Cul4(A + B) were cosilenced, approximately 70% of Cyclin A-positive cells became positive for Cdt1, showing that cell cycle-specific proteolysis of Cdt1 was severely abrogated. We noticed that, while DNA content was not significantly increased in Skp2 and Cul4(A + B) cosilenced cells, they had larger nuclei (Supplementary Figure S7). We conclude that SCF-Skp2 and DDB1-Cul4 are major mediators of the cell cycle-specific proteolysis of Cdt1.

The Cul4-containing complex is active only in S phase, while SCF-Skp2 during both S and G2 phases

Cdt1 is degraded in Cyclin A-positive S- and G2-phase cells. To address more precisely during which phases each E3 is active, Cdt1 proteins were examined in cells arrested at early S phase by thymidine–aphidicolin (S–0 h), washed and released for 2 h (S–2 h) into S phase, and for 7 h, when most cells are in G2 (as shown by flow-cytometry analysis). Full WT-Cdt1-3NLSmyc was undetectable in S–0 h, S–2 h and G2 samples (Figure 6), similar to the endogenous Cdt1 protein in HeLa cells (Nishitani *et al*, 2001). Full A6-Cdt1-3NLSmyc was similarly degraded, indicating that SCF-Skp2 targets Cdt1 both in S and G2 phases. Strikingly, however, full Cy-Cdt1-3NLSmyc was stable in the G2 population. In addition, we noticed that in aphidicolin-arrested cells (S–0 h), 25.1% of Cyclin A-positive cells were stained, although weakly, for Cy-Cdt1. The signal was reduced upon release, as replication resumed. These observations suggest that the Cul4-contain-
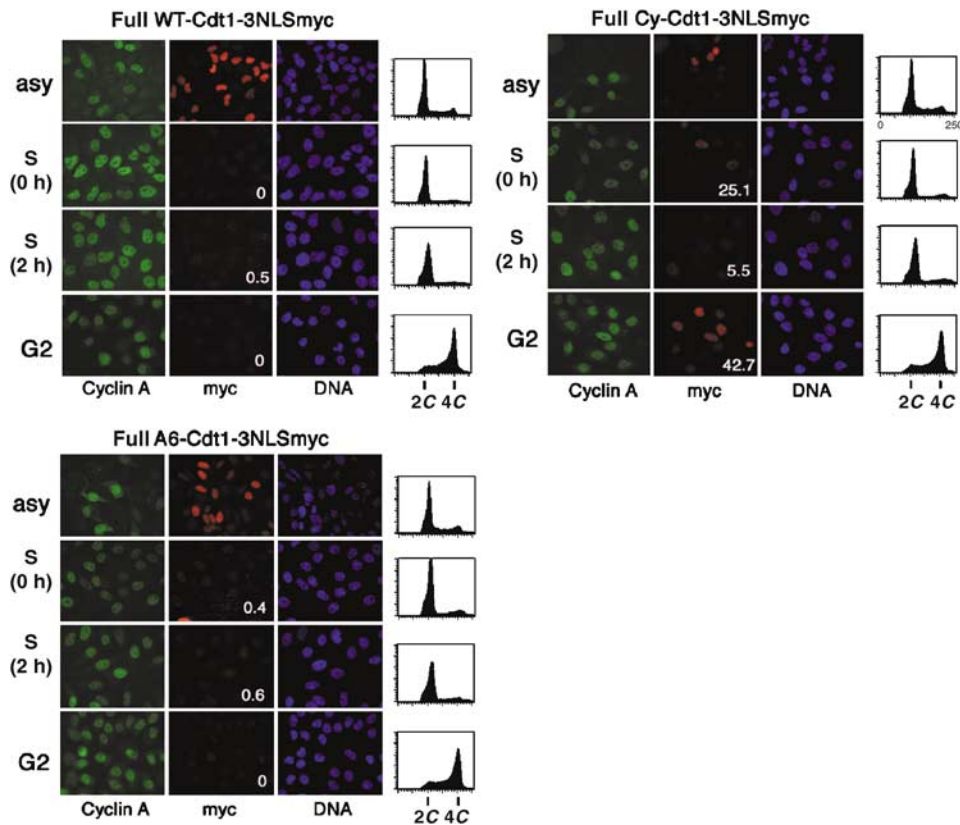
Figure 6 Cul4 E3 is active only in S phase, while SCF-Skp2 both in S and G2 phases. Cell lines stably expressing the indicated constructs were synchronized in early S phase by thymidine–aphidicolin (S–0 h), released for 2 h (S–2 h) or for 7 h in the presence of nocodazole (G2), and examined by double staining with anti-Cyclin A and anti-myc antibodies. Percentage of cells double positive for Cyclin A and Cdt1 is shown (%). Synchronization of each culture was examined by flow cytometry (small panels).

ing E3 operates only in cells actively replicating DNA, and not during the G2 phase.

PCNA binds to the N-terminus of Cdt1 and is involved in the Cul4-dependent degradation pathway

Our data so far indicated that Cdt1 is targeted for proteolysis by a Cul4 complex, when replication is ongoing. What would trigger Cul4 to target Cdt1, however, was not clear. In *Escherichia coli*, the DNA polymerase III sliding clamp has been shown to inactivate the initiator protein DnaA, thereby blocking re-initiation (Katayama *et al*, 1998). By a combination of Cdt1 affinity chromatography and mass spectrometric analysis, we identified PCNA, the eukaryotic sliding clamp, as a candidate for a novel Cdt1-binding protein (details of this work will be published elsewhere). The specific binding of PCNA to Cdt1 was confirmed in an *in vitro* pull-down assay showing that PCNA in the HeLa nuclear extract binds to GST-Cdt1, but not to GST alone (Figure 7A). Geminin serves as a positive control in this assay. This finding prompted us to speculate that PCNA may be involved in inactivation of Cdt1 during DNA replication. In order to address this, we first investigated whether PCNA binding is dependent on the QXRVTDF-motif. Full-sized WT-, A6-, Cy- and A6Cy-Cdt1-3NLSmyc were transfected into 293T cells and immunoprecipitated. PCNA was detected in WT-Cdt1 precipitate, and at higher amount in Cy-Cdt1 precipitates, but not in vector-transfected cell extract (Figure 7B). The association was lost in A6- and A6Cy mutants. These data show that PCNA interacts with Cdt1 dependent on the QXRVTDF-motif.

Secondly, PCNA expression was silenced and Cdt1 degradation examined in HeLa cells. When PCNA was silenced, three- to four-fold increase of Cdt1 was observed (Figure 7C). This was not primarily due to G1 arrest, because Cyclin A-positive cells were detected equally to siLuc-treated cells (Figure 7D and E). Costaining with Cyclin A showed that Cdt1 was correctly degraded in S–G2 cells. However, Cdt1 remained stable after UV-irradiation, indicating that PCNA is involved in Cul4-mediated Cdt1 degradation following DNA damage (Figure 7D). Next, PCNA was silenced in full Cy-Cdt1-3NLSmyc stable cell line, whose degradation in S phase is dependent on Cul4. As shown in Figure 7E, Cy-Cdt1 became stable in Cyclin A-positive cells, as well as following DNA damage (data not shown). We conclude that PCNA binds to Cdt1 through the QXRVTDF-motif, and that the QXRVTDF-motif-dependent degradation pathway, which is mediated by Cul4, requires PCNA binding. Taken together, these data suggest that PCNA may bridge Cdt1 to the Cul4 ubiquitin ligase complex.

Silencing of Geminin in HeLa cells having extra copies of Cdt1 induces re-replication

In addition to proteolysis, Cdt1 is inactivated by Geminin binding. Silencing of Geminin was shown to induce re-replication in several cell lines (Melixetian *et al*, 2004; Zhu *et al*, 2004), but not in HeLa cells (Kulartz and Knippers, 2004; Nishitani *et al*, 2004). This might be due to cell type-specific responses, Cdt1-Geminin protein amount ratios and/ or activity of proteolysis in the different cell lines. Therefore,
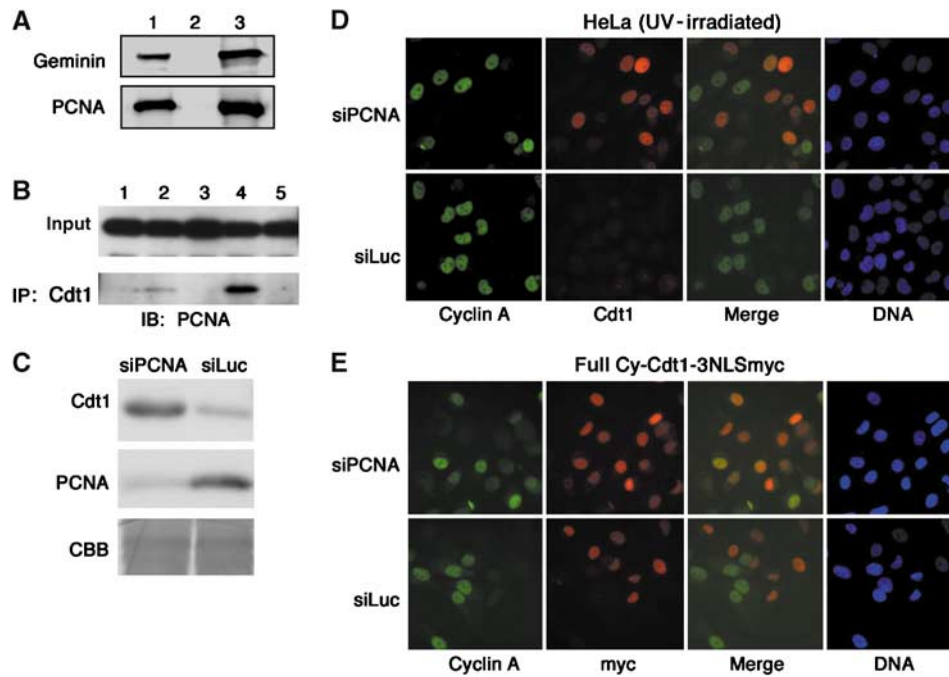
Figure 7 PCNA is involved in the Cul4-directed proteolysis pathway. (**A**) PCNA binds to GST-Cdt1, but not to GST. HeLa cell nuclear extract (lane 1) was mixed with GST (lane 2) or GST-Cdt1 (lane 3), and the precipitates were blotted with anti-PCNA and anti-Geminin antibodies. 15% of input was applied. (**B**) PCNA binds to the N-terminus of Cdt1. 293T cells were transfected with vector (lane 1), full size of WT- (lane 2), A6- (lane 3), Cy- (lane 4) or A6Cy- (lane 5) Cdt1-3NLSmyc expression plasmid, and cell extracts were immunoprecipitated with anti-Cdt1 antibodies. Five percent of input and immunoprecipitates (IP) were blotted with anti-PCNA antibodies. (**C**) Silencing of PCNA. HeLa cells were transfected twice with siRNA for PCNA or luciferase with a 24 h interval, collected 72 h after the first transfection and total cell extract was blotted for Cdt1 and PCNA. Total protein staining (CBB) is shown as a loading control. (**D**) Cdt1 remains stable after UV irradiation in HeLa cells treated with siPCNA. HeLa cells treated with siRNAs as above were UV-irradiated and fixed 1 h later for double staining with anti-Cyclin A and anti-Cdt1 antibodies. (**E**) Silencing of PCNA results in stabilization of full Cy-Cdt1-3NLSmyc in Cyclin A-positive cells. The stable cell line was transfected with siRNAs as above, and fixed for double staining with anti-Cyclin A and anti-myc antibodies.
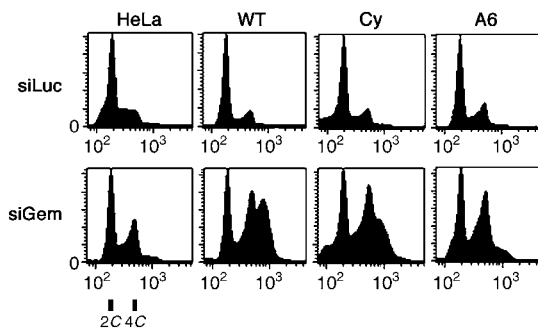
Figure 8 Knocking down of Geminin in full WT- and Cy-Cdt1-3NLSmyc stable cell lines induces re-replication. HeLa cells and stable cell lines expressing full size of WT-, Cy- or A6-Cdt1-3NLSmyc (WT, Cy and A6, respectively) were transfected with siRNA for Geminin (siGem) or luciferase. At 72 h post-transfection, cells were collected, and DNA content analyzed by flow cytometry.

we tried Geminin silencing in HeLa cells stably expressing wild-type and mutant forms of Cdt1-3NLSmyc. In the parental HeLa cell line 72 h post-transfection with Geminin siRNA, cells with $4C$ DNA content increased, but cells with higher than $4C$ were not detected. In contrast, in full-size WT-Cdt1- or Cy-Cdt1-3NLSmyc-expressing stable cell lines, cells with more than $4C$ content accumulated (Figure 8), suggesting that Cdt1 protein levels within the cell are critical for induction of re-replication upon Geminin depletion. In the full A6-Cdt1-expressing cell culture, however, such cells were

not efficiently detected, suggesting that this mutant, which does not interact with PCNA, may be less functional.

As a complementary functional assay, the ability of WT-Cdt1 and the mutants constructed in this study to induce over-replication when transfected into 293T cells was assessed. Overexpression of full WT-Cdt1-3NLSmyc led to the appearance of cells with large nuclei of $4C$–$8C$ DNA content (Supplementary Figure S8) consistent with previous studies (Vaziri *et al*, 2003; Nishitani *et al*, 2004). Re-replication was enhanced with the full Cy-Cdt1. In contrast, full A6-Cdt1 was less effective. Combining A6 to the Cy mutant (A6Cy-Cdt1) reduced the ability of full Cy-Cdt1 to induce re-replication.

Discussion

Cells employ multiple pathways to prevent re-replication in a single cell cycle. In human cells, Cdt1 is ubiquitinated and degraded in S phase. Here, we demonstrate that human Cdt1 degradation in S phase is performed separately by two E3 ubiquitin ligases, which recognize different parts of the Cdt1 protein within the N-terminal 100-amino-acid region. One is an F-box protein Skp2-containing SCF complex, and the other is a DDB1-Cul4-containing complex. SCF-Skp2 plays an important role during late G1 to S–G2 phases of the cell cycle (Vodermaier, 2004). Cdt1 has been previously shown to be recognized by SCF-Skp2 following phosphorylation by Cyclin E/A-CDK (Liu *et al*, 2004; Sugimoto *et al*, 2004). The Cy-motif 68RRL acts as a docking site for Cyclin A-CDK. The amino-acid sequence around 31SPARPALR shows a high similarity to

187TPKKPGLR of p27, a well-known substrate of Skp2 (underlined are consensus CDK phosphorylation sites), and a recent report suggests that the 29T of Cdt1 closely located to 31SP is a phosphorylation target (Takeda *et al*, 2005). The DDB1-Cul4 complex requires at most the first 28 amino acids to target Cdt1 for proteolysis after UV-irradiation and in S phase. Six amino acids conserved between human, mouse and *Xenopus* Cdt1 within the first 10 amino acids (the QXRVTDF-motif) are essential for this recognition. The mouse Cdt1 also has both 3QXRVTDF-motif and Cy-motif (65RRL), and thus may be degraded by the two pathways described here.

In *Xenopus* egg extracts and *Drosophila*, Cdt1 was shown to be degraded by a CDK-independent, but replication-coupled pathway (Arias and Walter, 2005; May *et al*, 2005). Since the (1–28) region does not bind to Cyclin A nor does it contain consensus CDK phosphorylation sites, the Cul4-mediated Cdt1 proteolysis is likely to be independent of CDK activity. Thus, it is possible that the Cul4-mediated pathway described here corresponds to the replication-coupled pathway. Indeed, our data indicate that Cul4-mediated proteolysis requires ongoing DNA replication, as it appears at least partly defective when DNA replication is inhibited by aphidicolin and in G2 phase. More strikingly, we show that the QXRVTDF-motif is required for association of the eukaryotic sliding clamp PCNA with Cdt1, and that PCNA is required for Cul4-dependent Cdt1 proteolysis. While revising our manuscript, a similar observation was reported by J Walter's group, who identified the N-terminal region as containing a consensus PCNA interaction protein box (PIP), a motif present in several PCNA-interacting proteins (Arias and Walter, 2006). This PIP box fully overlaps with the QXRVTDF-motif identified in this study. In *E. coli*, a system known as RIDA (Regulatory Inactivation of DnaA) ensures inactivation of the initiator protein DnaA by association with the DNA polymerase III β-sliding clamp subunit following initiation of DNA replication (Katayama *et al*, 1998; Su'etsugu *et al*, 2005). The system we describe here is highly reminiscent of the *E. coli* system, suggesting that in eukaryotes and prokaryotes alike, the sliding clamp may be used to couple replication to the inactivation of initiator proteins. Previous reports suggested that DDB1 directly binds to Cdt1 for ubiquitination *in vitro* (Hu *et al*, 2004). Our data suggest that PCNA binds to Cdt1 and links it to the DDB1-Cul4 complex. In the *E. coli* RIDA system, DnaA was inactivated by the sliding clamp and Hda protein only after these were loaded on the initiated DNA. Cdt1 may be similarly recognized both by PCNA and by DDB1 in order to be ubiquitinated. PCNA may therefore bridge DDB1-Cul4 and Cdt1 on chromatin. Consistently, we observed ubiquitinated form of Cdt1 in the chromatin fraction (Nishitani *et al*, 2004).

Our results indicate that Cul4 targets Cdt1 in G1 phase only following DNA damage, while in S phase Cdt1 is targeted in the absence of an external trigger. Is PCNA loading enough for Cdt1 degradation both during repair processes following DNA damage in G1 and DNA replication in S phase? It is possible that Cdt1 is modified for degradation after UV-irradiation and in S phase. This is however unlikely, since Cdt1 prepared without UV treatment was ubiquitinated by Cul4 complexes *in vitro* (Higa *et al*, 2003). The Cul4 E3 may be activated after DNA damage and in S phase. For example, a DDB2-containing DDB1-Cul4 complex is activated by

removal of the CSN complex after UV-irradiation (Groisman *et al*, 2003). The DDB1-Cul4-mediated Cdt1 degradation in S phase appears to be independent of checkpoint gene products, since full Cy-Cdt1, whose degradation is dependent on DDB1-Cul4, was degraded in the presence of caffeine that inhibits ATM/ATR (data not shown). When full-size Cy-Cdt1 was overexpressed, it induced re-replication more efficiently than WT-Cdt1 did, while the A6 mutant was less effective. The A6-Cy double mutant was similarly defective to induce re-replication when overexpressed, while Geminin silencing did not induce re-replication in A6-Cdt1 stables (Figure 8 and Supplementary Figure S8). The N-terminal domain, although required for degradation, may have an additional role for promoting initiation. Further biochemical analysis will be required to assess the positive and negative functions of the various motifs present within the Cdt1 N-terminus.

Taken together, our results demonstrate that Cdt1 in human cells is inactivated by multiple pathways after the onset of S phase, which act independently of each other. These are illustrated in Figure 9. Geminin appears from S-phase onset, when Cdt1 proteolysis starts (Wohlschlegel *et al*, 2000; Nishitani *et al*, 2001). We have shown previously that Cdt1 is degraded in S phase independently of Geminin (Nishitani *et al*, 2004). A similar result was reported in the *Xenopus* extract system (Arias and Walter, 2005). Each eukaryote may have evolved to use some or all of these pathways, and to different extents, depending on species and cell line. For example a Geminin homologue in *C. elegans* was recently reported to block Cdt1 function, but re-replication was not observed after Geminin silencing (Yanagi *et al*, 2005). Rather, inactivation of Cul4 induces accumulation of Cdt1 and re-replication in this organism (Zhong *et al*, 2003). Similarly, in *Xenopus*, depletion of Geminin does not induce re-replication (McGarry and Kirschner, 1998). In several human cell lines, on the contrary, re-replication was observed after Geminin expression was silenced. HeLa cells cannot re-replicate, however, when Geminin is silenced. We show here that re-replication can be induced in HeLa cells expressing additional Cdt1 constructs. The ratio of Cdt1/Geminin and/or the levels of proteolysis activity may account for such a
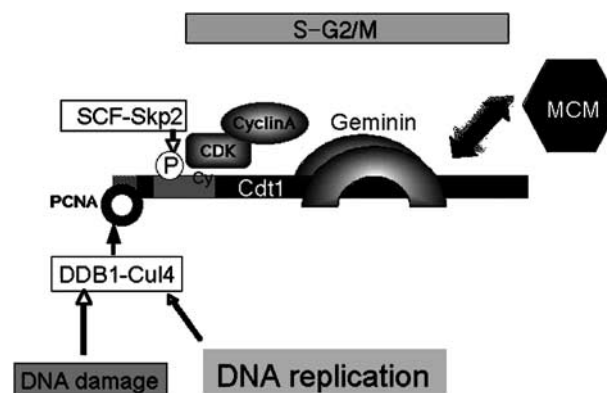
Figure 9 A model showing that Cdt1 is inactivated by multiple pathways during the cell cycle. During S–G2 and early M phases, a dimer of Geminin binds to Cdt1 and blocks interaction with the MCM complex. CDK-Cyclins phosphorylate Cdt1 and target it for SCF-Skp2-directed ubiquitination followed by degradation. During DNA replication, chromatin-loaded PCNA associates with the N-terminus of Cdt1 and promotes DDB1-Cul4-directed ubiquitination of Cdt1. This process also acts when DNA is damaged in G1 phase, probably through replication coupled repair processes.

difference in cell line response to Geminin silencing. In contrast to *C. elegans*, we show here that re-replication was not induced after Cul4 silencing in HeLa cells. This is likely to be at least partly due to efficient Cdt1 degradation by the SCF-Skp2 pathway. Actually, following Skp2 silencing in HeLa cells, Cdt1 protein levels increased more than three-fold, while this was not apparent when Cul4 was silenced (Figure 5B). This suggests that SCF-Skp2 is a major degradation pathway in this cell line. Our data furthermore suggest that, while both Cul4 and Skp2 E3 ligases can target Cdt1 for proteolysis during S phase, the two proteolytic systems are not entirely redundant: Cul4 is required for Cdt1 proteolysis following DNA damage, while Skp2 is required for Cdt1 proteolysis in G2. Since the Cul4 system is active only during DNA replication, higher eukaryotes may have acquired an SCF-Skp2 system to ensure that Cdt1 is absent in G2 phase. During rapid cell divisions after fertilization that lack measurable G2 phase, the SCF-Skp2 system may not be required; however, at later stages of development when cells have an extended G2 phase, SCF-Skp2 pathway may become essential. Consistently, overexpression of Cy-mutated Cdt1 in 293T cells induced a higher extent of re-replication than WT-Cdt1 (Supplementary Figure 8). Multiple pathways may be required to ensure that Cdt1 levels will remain accurately regulated in the face of the various conditions experienced by higher eukaryotic cells. Further studies will unravel the molecular pathways, which direct the cell cycle-specific regulation of Cdt1 and the function of these pathways during normal and abnormal cell division.

Materials and methods

Cell culture

HeLa and 293T cells were culture in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. MEF cells were culture as described (Nakayama *et al*, 2004). For synchronization of HeLa cells in early S phase, cells were treated with thymidine (2 mM), released and treated with aphidicolin (5 μg/ml). Nocodazole was used at 0.1 μg/ml. UV-irradiation was carried out at 20–50 J/m^2 using a Stratalinker. For transient transfection into 293T cells, Trans-IT293 (Mirus) was used. To isolate stably expressing HeLa cell lines, cells were transfected with plasmids containing Neo genes by Trans-ITL1 (Mirus), and selected in a medium containing 400 μg/ml G418. siRNAs were transfected with Oligofectamine (Invitrogen). For analysis of DNA content, flow cytometry was carried out as described (Nishitani *et al*, 2004).

Plasmids

To express N-terminus of Cdt1 as a 9mycNLS-fused protein, 9myc-encoding fragment was amplified by PCR using primers: primer-1, GGGCTCGAGGGTTCACGGGGGATCCTCTAGAG and primer-2, GGGGCGGCCGCCGAGGAGGATCCGTTCAAGTC using cDNA3.1-9myc, cut with *Xho*I–*Not*I, and cloned into the pCMV/myc/NLS plasmid, resulting in pCMV-9myc3NLSmyc. A series of N-terminus constructs of Cdt1 were amplified using the following primer sets, cut with *Nco*I–*Xho*I, and cloned into pCMV-9myc3NLSmyc, to construct, for example, pCMV-(1–151) 9myc3NLSmyc, pCMV-(1–101) 9myc3NLSmyc and so on. For amplification of (1–151), primer-3 GGGGTCGACCCATGGAGCAGCGCCGCGTCAC and primer-4 GGGCTCGAGGCAGGACTCCCCAGCATCCTG; (1–101), primer-3 and primer-

5 GGGCTCGAGGGATTTCTTTATCTTCTGGCC; (1–51), primer-3 and primer-6 GGGCTCGAGGGCGCGCTTGCGGCTGCCACTG; (1–34), primer-3 and primer-7 GGGCTCGAGCCTGGCGGGGCTGGGGGTGCG; (1–28), primer-3 and primer-8 GGGCTCGAGGCGGCAGGCCAGCTTGGGCGG; (11–101), primer-9 GGGCCATGGCGCGCCGCCGCCCCGGGCCC and primer-5; (21–101), primer-10 GGGCCATGGCGCCGCCCAAGCTGGCCTGC and primer-5; and (38–101), primer- GGGCCATGGCAGTCCGCGCCCCGGCCTCC and primer-5. The Cy mutant was described in Sugimoto *et al* (2004). The A6 mutant was constructed by PCR amplification using a primer CCGCCATGGAGGCGCGCGCCGCTGCCGCAGCCTTCGCGCGCCGCCGCCCCGG. The deletion mutant of amino acids (22–31) was carried out using an *in vitro* mutagenesis kit (Takara) using an oligonucleotide CCCGGGCCCCCCGCATCGCTAGCCCCGCCAGGCCCGCACTC.

Antibodies, WB and immunofluorescence

For WB, total whole-cell lysates were prepared by lysing cell pellets directly in SDS–PAGE buffer. For IF, HeLa cells were fixed in 3.8% paraformaldehyde in PBS for 10 min, permeabilized in 0.25% Triton X-100 in PBS and stained with indicated antibodies as described. For double staining, Alexa-488-conjugated anti-mouse and Alexa-592-conjugated anti-rabbit antibodies were used as secondary antibodies with Hoechst 33258 to visualize DNA. Antibodies used were as follows: Cdt1 (rabbit as described (Nishitani *et al*, 2001), used at 1:2000 dilution for WB and 1:250 for IF), Cyclin A (Neomarkers Ab-6, Santa Cruz H-432; 1:100 for IF) and myc (rabbit antibodies, Santa Cruz; 1:200 for IF). Other antibodies used for WB were anti-Geminin (Xouri *et al*, 2004; 1:1000), anti-Orc1 (a gift from Dr Stillman; 1:1000), anti-Cyclin A (rabbit, lab stock; 1:2000), anti-Cdc2 (rabbit, lab stock; 1:5000), anti-DDB1 (Santa Cruz; 1:1000), anti-Skp2 (Santa Cruz H-435; 1:1000), anti-PCNA (MBL; 1:2000), anti-p27 (Santa Cruz F-8; 1:1000), anti-RCC1 (lab stock; 1:3000), anti-Cul4A (Rockland; 1:1000) and anti-Cul4B (Santa Cruz; 1:2000).

RNAi experiment

The following dsRNA were made by Dharmacon, and transfected at 100 μM using Oligofectamine: Geminin, CUGGCAGAAGUAGCAGAAC; Skp2, GCAUGUACAGGUGGCUGUU; DDB1, GGACCUGCUGUUUAUCUUG; Cul4A, GAACUUCCGAGACAGACCU; Cul4B, AAGCCUAAAUUACCAGAAA; and PCNA, CGGUGACACUCAGUAUGUC. For Cul4, PCNA and DDB1 silencing, cells were transfected twice with a 24 h interval, and analyzed 72 h after the first transfection.

GST-Cdt1 pull-down assay

HeLa cells (1 l) were first extracted with mCSK buffer (Fujita *et al*, 1997), and the remnant nuclear pellet was resuspended in 5 ml mCSK buffer containing 0.5 M NaCl and protease inhibitors. After centrifugation, the soluble nuclear fraction was filtrated. GST-Cdt1 or GST (1 mg) was bound to glutathione beads, and the beads were mixed with the 0.5 ml nuclear extracts diluted with buffer A (25 mM Tris–HCl, pH 7.4, 0.01% NP-40, 1 mM DTT, 0.5 mM PMSF, 10% glycerol). After washing three times with buffer A containing 0.15 M NaCl, the bound proteins were eluted with 20 mM glutathione and analyzed by immunoblotting.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

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