Proteolysis of DNA Replication Licensing Factor Cdt1 in S-phase Is Performed Independently of Geminin through Its N-terminal Region*

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Licensing of replication origins is carefully regulated in a cell cycle to maintain genome integrity. Using an in vivo ubiquitination assay and temperature-sensitive cell lines we demonstrate that Cdt1 in mammalian cells is degraded through ubiquitin-dependent proteolysis in S-phase. siRNA experiments for Geminin indicate that Cdt1 is degraded in the absence of Geminin. The N terminus of Cdt1 is required for its nuclear localization, associates with cyclin A, but is dispensable for the association of Cdt1 with Geminin in cells. This region is responsible for proteolysis of Cdt1 in S-phase. On the other hand, the N terminus-truncated Cdt1 is stable in S-phase, and associates with the licensing inhibitor, Geminin. High level expression of this form of Cdt1 brings about cells having higher DNA content. Proteasome inhibitors stabilize Cdt1 in S-phase, and the protein is detected in the nucleus in a complex with Geminin. This form of Cdt1 associates with chromatin as tightly as that of G1-cells, when no Geminin is detected. Our data show that proteolysis and Geminin binding independently inactivate Cdt1 after the onset of S-phase to prevent re-replication.

Duplication of chromosomal DNA is a key event in the cell cycle. In eukaryotes, DNA replication initiates at several places along chromosomes, known as replication origins. Chromosomes must be replicated precisely once before they are separated into the two daughter cells at mitosis. Cyclin-dependent kinases (CDKs)† in a complex with their respective cyclin partners globally control the order of cell cycle events. Both initiation of replication and prevention of re-replication are under the control of CDKs. Initiation of replication is triggered by S-phase CDKs, while re-initiation is prevented by the action of both M-phase CDKs and S-phase CDKs, until CDK activity drops at the end of mitosis (1–3).

At the end of mitosis, when mitotic cyclins are degraded and CDK activity drops, origins are released from the restriction on replication and become licensed for replication by the formation of pre-replicative complexes (pre-RCs) (4–6). Initiator proteins essential for this process, ORC (origin recognition complex), Cdc6/18, Cdt1, and MCM (mini chromosome maintenance) proteins were identified through genetic and biochemical studies using mostly yeast and a Xenopus cell-free system as model systems. Current models postulate that licensing is established through the stepwise recruitment of initiator proteins on origins of replication to form the pre-RCs (7–11). ORC is bound to replication origins throughout the cell cycle at least in yeast, and acts as a landing pad for Cdc6/18 and Cdt1. These proteins in turn load MCM proteins on the chromatin, forming the pre-RCs. Recent studies show that additional factors, such as Yph1, Noc3, and Mcm10 are also required to establish and maintain licensing (12–14). Activation of S-phase CDK/cyclin complexes, together with the Cdc7-Dbx4 kinase, trigger the initiation of replication by unwinding the DNA and recruiting Cdc45 and replication enzymes (15–18). Phosphorylation of Shld2/Drc1 by S-CDKs facilitates this process (19, 20). The MCM proteins are proposed to have a role as replicative helicases (21–24).

Once replication is initiated, origin bound complexes are brought to a post-replicative state (post-RC) and origins are prevented from becoming re-licensed until chromosome separation is completed. An important role for the mitotic CDK in preventing re-licensing is demonstrated in yeast and mammalian cells, where its inactivation results in re-replication of chromosomal DNA (3, 25–27). Many of the initiator proteins are targets of CDKs. Phosphorylation on these proteins results in their inhibition by, for example, degradation or re-localization to the cytoplasm. Different organisms appear to have developed variations on the modes of regulation of initiator proteins. Cdc18 of Schizosaccharomyces pombe and Cdc6 of Saccharomyces cerevisiae are phosphorylated by CDKs and recognized by the SCF ubiquitin-degradation system (28–30). MCM proteins in S. cerevisiae are excluded from the nucleus after S-phase in a phosphorylation-dependent manner, while phosphorylation of Cdc6/18 by cyclin A in mammalian cells has been shown to target it to the cytoplasm (31–35). Cdt1 of S. cerevisiae is exported to the cytoplasm, while mammalian Cdt1 is degraded after initiation (36–38). In mammalian cells, the Orc1 subunit of ORC is specifically degraded after entry into S-phase (39, 40). Thus, eukaryotic cells have developed a redundant system to ensure that DNA replication takes place only once per cell cycle. If such controls fail, genomic instability may occur. For example, overexpression of Cdc18 in S. pombe brings about overreplication of the genome and this is enhanced when putative CDK phosphorylation sites on Cdc18 are mutated to non-phosphorylatable amino acids, while S. cer-
Cdt1 fragment into p3xFLAG-myc-CMV-26 (Sigma) using primers GG-
pFlag-hCdt1-myc was constructed by PCR amplifying the NotI-XbaI
vector was previously described (36). pcDNA3xHA-hCdt1 and
agment of pCMV-hCdt1 (161-end) NLS with a SphI-NotI fragment from
pCMV-hCdt1 (161-end) was constructed by replacing the SphI-NotI fr-
CATGTGGCGAGAAGGC and GGGGCGGCCGCCAGCCCCTCCTCAG-
CGTG. To construct pCMV-hCdt1 (161-end) NLSmyc, the corresponding
AGTGCCCAGGATGC and GGGGCGGCCGCCAGCCCCTCCTCAGCA-
AGTG, and cloned into pcDNA3.1
for NLS-hCdt1 (139-end) was amplified by PCR using primers; CCCC-
A
NLS-hCdt1 (139-end) (shown as myc
/H9004

These observations suggest that controlling Cdt1 is particu-
larly important for genome stability. Cdt1 was recently re-
ported to have an oncogenic potential when ectopically ex-
pressed in cells (47).

We are interested to understand how Cdt1 regulation is
achieved during the cell cycle and to gain insight into the
function of Geminin in the cell. For the majority of the mam-
mal cell cycle, Geminin accumulates when Cdt1 levels are
low (36), suggesting that inhibition of Cdt1 by Geminin may act
redundantly to its proteolysis, that Geminin binding to Cdt1
may promote its degradation, or that Geminin may have a role
in addition to Cdt1 inhibition. Here, we show that Cdt1 is
degraded through a ubiquitin-proteasome pathway that does
not require the presence of Geminin.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa, COS7, 293T, tsBN75, and ts41 cell lines were
cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine
serum. tsBN75 and ts41 cell lines were grown at the permissive tem-
perature of 39°C, or at the restrictive temperature of 40°C. Synchron-
ization of HeLa cells in S-phase by a double thymidine method or in
M-phase, were performed as described (36). For transient transfections
in COS7 cells, LipofectAMINE (Invitrogen) was used, and in 293T cells,
TransIT-293 (Mirus) was used. Proteasome inhibitors (MG-132, PS-I
from Calbiochem and Peptide Institute) were used at 20 μM. For analy-
sis of DNA content by flow cytometry, synchronized cells, or re-repli-
cating ts41 cells were fixed in 50% methanol, treated with RNase A (500
μg/ml) for 1 h and stained with propidium iodide (20 μg/ml), and
measured on FACScan (BD Biosciences).

Plasmids—To express proteins in mammalian cells fused with nine
copies of the Myc epitope (9×myc) at the N terminus, a fragment encod-
ing 9 copies of the Myc epitope (9×myc) at the N terminus, a fragment encod-
ing 9×myc-hCdt1 was previously described (36). pcDNA3.1 (Invitrogen),
myc-hCdt1 (shown as mycCdt1 in Fig. 6A) was constructed by
cloning the Sall-HindIII hCdt1 fragment into pcDNA3.1-9×myc at
HindIII site. pcDNA3-1-9×myc-hCdt1 (1753-450) (shown as mycCdt1
in Fig. 6A) was made by cutting out the internal EcoIII fragment from
the pcDNA3-1-9×myc-hCdt1. To construct pcDNA3-1-9×myc
-NLS-hCdt1 (139-end) (shown as mycCdt1 in Fig. 6A), a fragment encod-
ing for NLS-hCdt1 (139-end) was amplified by PCR using primers:
GGCTCGAGATGGATATCCCAAAAAAGAAAAGAAAAAGTCGCTGA-
AGTTGCGAGAAGGC and GGGGCGGCCGCCAGCCCCTCCTCAGCTG.

Stabilization of hCdt1 in Cells That Have Defects in Ubiquitin-
dependent Proteolysis—Our previous work suggested that
hCdt1 is targeted for proteolysis after S-phase onset (36). Consis-
tent with ubiquitin-dependent proteolysis, multi-ubiquiti-

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**Fig. 1.** The ubiquitin-proteasome pathway is required for Cdt1 degradation during S-phase. A, Cdt1 was examined in ts41 cells deficient in Cdc53/Cullin function. ts41 cells were cultured in the presence of hydroxyurea at 32 °C (lanes 1–4) or 40 °C (lanes 6–8) for 13, 17, and 21 h, or incubated first at 32 °C for 17 h and then at 40 °C for 4 h (lane 5). For lanes 9–12, an asynchronous culture of ts41 cells was shifted to 40 °C for the hours indicated. Cell extracts were made at the indicated time points, and Western blotting performed to assess the levels of Cdt1, Cdc6/18, Mcm3, and Geminin proteins. B and C, re-replicating ts41 cells. After temperature shift to 40 °C, cells were collected at the indicated time points, stained with propidium iodide (PI), and visualized by microscopy (phase contrast) (B) or DNA content examined by flow cytometry (C).

nated hCdt1 species were readily detected in anti-hCdt1 immunoprecipitates from cells co-transfected with plasmids expressing hCdt1 and ubiquitin (data not shown). To investigate this further, we used two cell lines that have temperature-sensitive defects in genes essential for ubiquitin-dependent proteolysis: tsBN75 and ts41. tsBN75 is derived from cell line BHK and has a temperature-sensitive mutation in the ubiquitin-activating enzyme E1 (50). Cdt1 was barely detected in BHK cells arrested in S-phase, but was stabilized when the E1 enzyme was inactivated at the restrictive temperature of 40 °C in tsBN75 cells (data not shown). ts41 cells, derived from a Chinese hamster cell line, have a temperature-sensitive defect in the NEDD8 conjugation pathway, which is essential for the function of the Cullin-Cdc53 family of E3 ubiquitin ligases, such as SCF (48, 49). The level of Cdt1 decreased as cells were arrested in S-phase with hydroxyurea at the permissive temperature of 32 °C (Fig. 1A, lanes 1–4), as we previously reported for HeLa cells (36). When cultured at the non-permissive temperature of 40 °C, a significant increase in the levels of Cdt1 was observed (Fig. 1A, lanes 6–8). The same level of accumulation was observed when cells were first cultured at 32 °C and then shifted to 40 °C for the last 4 h of HU treatment (Fig. 1A, lane 5). In this cell line, a retarded migrating form of Cdt1 was notably detected, probably due to a high extent of phosphorylation of Cdt1 (see later). Similarly to HeLa cells, both Cdc6/18 and Geminin levels were high in S-phase, regardless of the incubation temperature, while Mcm3 levels were constant during the treatments.

ts41 cells show a dramatic phenotype at the restrictive temperature. ts41 cells cultured at 40 °C start re-replicating their chromosomal DNA (51). Since high level expression of cdc18 and cdt1 induces re-replication in fission yeast, it was interesting to investigate the protein levels of replication initiator proteins during the course of re-replication of ts41 cells. As shown in Fig. 1A, lanes 9–12, both Cdc6/18 and Cdt1 levels were high when cells were re-replicating. On the contrary, Geminin level showed only a moderate increase. Re-replication of ts41 cells during the time course of the experiment is evident both by microscopy (Fig. 1B) and by FACS analysis (Fig. 1C). The FACS profile of re-replicating ts41 cells indicates a continuous increase in DNA content.

Cell Cycle-dependent Accumulation of Cdt1 and Orc1—Orc1, the largest subunit of the Origin Recognition Complex, was recently shown to be degraded after the initiation of DNA replication (39, 40), similar to Cdt1. We therefore wished to compare the accumulation of Cdt1 and Orc1 during the cell cycle using synchronized cell cultures of HeLa cells. In a synchronized cell culture made by double thymidine block and release, both Cdt1 and Orc1 levels were low during S-phase, and accumulated with rather similar kinetics as cells entered into the following G1-phase (Fig. 2A). In cells arrested in mitosis by treatment with nucodazole, Orc1 was not detectable, while hCdt1 was present in a hypermodified form, consistent with our previous observations (36). Upon release from the nucodazole block, Cdt1 was quickly converted to the unmodified form while Orc1 accumulated with slightly delayed kinetics during early G1, and decreased in S-phase somewhat later than Cdt1 (Fig. 2B). These differences in accumulation timing between Cdt1 and Orc1 suggest that the corresponding regulatory mechanisms may not be identical.

**hCdt1 Stabilized by Proteasome Inhibitor Treatment Is Located in the Nucleus in a Complex with Geminin**—To gain an
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insight into the regulation of Cdt1 during S-phase, we wished to examine the behavior of hCdt1 stabilized in S-phase cells by treatment with proteasome inhibitors. Firstly, the subcellular localization of Cdt1 and Geminin was examined by immunofluorescence, in HeLa cells arrested in early S-phase by a double thymidine block and then further cultured for 3 h in the block with or without proteasome inhibitors (PS-INH). Consistent with Western blot analysis, Cdt1 was not detected in S-phase arrested cells in the absence of proteasome inhibitors. Geminin staining was evident in the nucleus and excluded from the nucleoli (Fig. 3A, left), similar to our previous findings for asynchronously growing cells (36). Upon proteasome inhibitor treatment of S-phase arrested cells, Cdt1 was stabilized and located in the nucleus, but excluded from the nucleoli, in a similar fashion with Geminin (Fig. 3A, right). This Cdt1 staining contrasts to our previous finding that hCdt1 antibodies stain both the nucleoplasm and the nucleoli in G1 cells from an asynchronous population (36). In cells traversing S-phase following release from a double thymidine block (early S (0–5) and late S (3–6)), Cdt1 stabilized by proteasome inhibitor treatment was similarly excluded from the nucleoli (Fig. 3B). This difference in localization is not due to the proteasome inhibitor treatment per se, since in a population of G1 cells, Cdt1 stained the whole nuclear region in the presence or absence of proteasome inhibitors. It is conceivable that Geminin binding to Cdt1 during S-phase would change the localization of Cdt1 in the nucleus. We estimate that ~5–10 × 10^4 molecules of Cdt1 protein are present in a G1 or a proteasome inhibitor-treated S-phase HeLa cell and that the number of Geminin molecules in an S-phase cell is equal to or twice the number of Cdt1 molecules in a G1 cell (data not shown).

We next determined whether proteasome-inhibitor stabilized Cdt1 could be detected in a complex with Geminin in S-phase arrested cells. Whole cell extracts were prepared from cells first arrested in S-phase by a double thymidine block and further incubated for 3 h in the block in the presence of proteasome inhibitors. Cdt1 and Geminin proteins were immunoprecipitated by specific antibodies to each protein. As a control, normal rabbit IgG was used. As shown in Fig. 3, C–1, Geminin was co-precipitated by anti-hCdt1 antibodies and Cdt1 by anti-Geminin antibodies, indicating that they formed a complex.

Cdt1 is normally unstable in S-phase and co-expression of Cdt1 and Geminin is likely to be restricted to a short period around the G1/S-boundary. We previously reported that Cdt1 and Geminin co-exist in nocodazole-arrested mitotic HeLa cells (36). Thus, we investigated whether a complex of Cdt1 with Geminin could also be detected in such a situation. Whole cell extracts were prepared from cells arrested in mitosis with nocodazole, and immunoprecipitation was carried out with anti-Cdt1, anti-Geminin, and normal IgG as a control. Complex formation was confirmed by co-precipitation with either antibody (Fig. 3, C-2, lanes 4–6). In mitotic cell extracts, Cdt1 was detected as a slower migrating form, and so was half of Geminin (Fig. 3, C-2, lanes 1–3). This mobility change is most likely caused by phosphorylation, since upon λ-phosphatase treatment, Cdt1 exhibited a mobility similar to that of Cdt1 extracted from asynchronously growing cells (Fig. 3, C-2, lanes 7 and 8). Cdt1 also migrated slightly slower in proteasome inhibitor-treated S-phase cells than in asynchronously growing cells, a mobility shift also likely due to phosphorylation, as it was modified by treatment with λ-phosphatase (Fig. 3, C-3).

Cdt1 Associates with Chromatin in the Presence of Geminin—Geminin’s function during the cell cycle remains unknown. In a Xenopus oocyte-based in vitro licensing assay, recombinant Geminin has been shown to inhibit the chromatin loading of MCM proteins by binding to Cdt1 (38, 45). Geminin could perform this role by interfering with the chromatin association of Cdt1. A recent report showed that Geminin inhibits the ability of Cdt1 to bind to DNA in vitro (52). We were interested to investigate the chromatin association of Cdt1 and Geminin during the mammalian cell cycle and address whether Geminin affects Cdt1 chromatin association in S-phase. HeLa cells at various cell cycle stages were lysed with 0.1% Triton X-100 in a buffer containing 50 mM NaCl (WCE) and separated into soluble (S50) and insoluble (P50) fractions. The P50 fraction was further treated with 200 mM NaCl and the insoluble chromatin and/or nuclear matrix-associated fraction (P200) was obtained. Each preparation was blotted with antibodies for several licensing factors, as indicated in Fig. 4, while α-tubulin served as a control. The sample in lane 1 was prepared from cells in G1-phase (released from a nocodazole arrest for 7 h). Samples in lanes 2 and 3 were prepared from cells arrested in early S-phase by a double thymidine block and further cultured in the block in the presence or absence of the proteasome inhibitor MG-132 respectively, while in lane 4 DNA replication was allowed during the MG-132 treatment by washing out the thymidine. Cells in lanes 5–8 were released from a double thymidine block for 0, 3, 6, and 10 h, and thus in early S (lane 5), middle S (lane 6), G2/M (lane 7) or G1-phase of next cell cycle (lane 8), respectively (data not shown, see flow cytometry in Fig. 2A).

Total protein levels of licensing factors during the cell cycle (WCE) are consistent with previous observations. Cdt1 levels are high in G1 (lanes 1 and 8) and low in S and G2 (lanes 3 and 5–7) whereas they are stabilized in S-phase cells by proteasome inhibitor treatment (lanes 2 and 4). In contrast, Geminin levels are low in G1 and high in S and G2 cells. Mcm3 and Orc2 protein levels remain mostly constant during the cell cycle and Cdc6/18 protein levels are reduced in G2/mitosis and G1 (lanes 1, 7, and 8).

In G1 cells, about half of Cdt1 was detected in the chromatin enriched fraction P50 (lanes 1 and 8, compare S50 to P50), indicating that a significant fraction of Cdt1 associates with chromatin in G1. In these cells, Geminin was undetectable. When Cdt1 chromatin association was compared between cells in G1 and MG-132-treated S-phase cells, which express Geminin to high levels, Cdt1 remained to the same extent in the P50 and P200 fractions (compare lanes 2 and 4 with lanes 1 and 8) suggesting that the accumulation of Geminin in S-phase does not prevent Cdt1 chromatin association. In contrast, while a small fraction of Geminin was found associated with chromatin in S and G2 cells, which express Cdt1 to low levels (lanes 3 and 5–7), more Geminin was detected in the chromatin-enriched P50 fraction in MG-132-treated S-phase cells, which accumulate Cdt1 (lanes 2 and 4). While this observation could be due to the proteasome inhibitor treatment, it is intriguing to specu-
late that Geminin may have a higher affinity for chromatin when bound to Cdt1.

Long exposure of the Cdt1 immunoblotting revealed a ladder of high molecular weight bands in MG-132-treated S-phase cells in lanes 2 and 4, which may correspond to ubiquitinated forms of Cdt1 (WCE). More of these forms were recovered in the P50 fraction as opposed to the S50 fraction, suggesting that Cdt1 ubiquitination may preferentially occur on the chromatin.

Blotting with Mcm3 antibodies validates our fractionation method. Mcm3 is primarily recovered in the chromatin-enriched fractions in G1 and S-phase cells and becomes more soluble as DNA replication is completed (increase in Mcm3 amount in S50 in lanes 6 and 7). Orc2 and Cdc6/18 are detected mostly in the chromatin-enriched fractions throughout the cell cycle. Interestingly, more Orc2 was detected in fraction P200 in the presence of Cdt1 than when Cdt1 is absent (lanes 1, 2, 4, and 8 versus lanes 3, 5, 6, and 7). A similar tendency was observed in the case of Cdc6/18, while the chromatin affinity of Mcm3 did not show any correlation with the amount of Cdt1. We conclude that the accumulation of Geminin during S-phase does not affect the ability of proteasome inhibitor stabilized Cdt1 to be bound to chromatin.

Cdt1 Is Degraded in the Absence of Geminin in S-phase—Geminin starts to accumulate in the cell cycle when Cdt1 disappears (36), which could indicate that Geminin binding to Cdt1 may promote its degradation. To address this question, we used siRNA to repress Geminin production. When asynchronously growing HeLa cells were transfected with siRNAs specific for Geminin (Gem1 and Gem2), Geminin protein levels decreased at 48 h post-transfection, while a control double-stranded RNA (dsRNA for GFP) had no effect. Cdt1 protein levels were unaffected by repression of Geminin production (Fig. 5A). Next, we examined protein levels during S-phase. To arrest cells in S-phase, hydroxyurea was added at 28 h post-transfection and cells cultured for another 18 h (Fig. 5B). Most of the cells were arrested at early S-phase as analyzed by flow cytometry.
Control of Licensing Factor Cdt1 by Proteolysis

Since depletion of Geminin in Xenopus embryos causes Chk1 activation and subsequent G2 arrest, we examined if checkpoint kinases were activated in Geminin-silenced cells using antibodies specific to phospho-Chk1(Ser-345), and phospho-Chk2(Thr-68). Neither the activation of these kinases was observed nor the indication of DNA damage as revealed by immunoblotting with anti-γH2AX antibody (Fig. 5E). HU, aphidicolin (Aphi), and UV-treated cells were included as positive controls. The flow cytometry analysis showed no significant change in cell cycle profile, when Geminin was silenced (Fig. 5C).

The N Terminus of Cdt1 Has an NLS and Associates with Cyclin A but Not with Geminin—Cyclin-dependent kinases have a central role in regulating once per cell cycle replication and have been implicated in the phosphorylation dependent proteolysis of licensing factors, including the yeast Cdc6/18. Cyclin A is believed to play a crucial role in preventing re-replication in higher eukaryotes (53, 54).

Cdt1 has consensus sites for cyclin binding and phosphorylation by CDKs in its N terminus. We therefore examined if Cdt1 could interact with cyclins in extracts of HeLa cells. Cdt1

Fig. 4. Chromatin binding assay showing that Geminin binding to Cdt1 does not inhibit its chromatin association. HeLa cells were lysed in a buffer containing 0.1% Triton X-100, supplemented with 50 mM NaCl (WCE) and separated into soluble (S50) and insoluble (P50) fractions by centrifugation. The P50 fraction was re-suspended in a same buffer with 200 mM NaCl, and insoluble fraction (P200) was obtained after spin-down. Cell cultures were as follows: lanes 1–3, cells released from nocodazole arrest for 7 h; lanes 2 and 3, S-phase cells treated with or without proteasome inhibitor as described in the legend to Fig. 3; lanes 4–8, S-phase cells released from arrest and cultured in the presence of proteasome inhibitor; lanes 5–8, S-phase cells released from arrest for 0, 3, 6, and 10 h, respectively. Each fraction was immunoblotted with the indicated antibodies.

Fig. 5. Geminin is not required for Cdt1 degradation in S-phase. A, Geminin silencing. HeLa cells were transfected with siRNAs for Geminin (Gem1 and Gem2), or control RNA for GFP. 48 h post-transfection, cell extracts were prepared and protein levels of Geminin, Cdt1, and Cdc2 analyzed. B, HeLa cells were transfected with siRNAs for Geminin (Gem1 and Gem2), control RNA for GFP, scramble RNA (Scr) or without RNA (w/o RNA). Cells were treated with 2.5 mM hydroxyurea at 28 h after transfection, cultured for 18 h in the presence of hydroxyurea, and collected. The levels of Geminin, Cdt1, and Cdc2 as a loading control were analyzed by Western blotting. For comparison, cell extract from asynchronously growing cells was included (asy). C, DNA content of cells treated as in A and B was analyzed by flow cytometry. D, HeLa cells were treated as in B, but incubated with MG132 or Me2SO for the last 3 h of HU treatment. Gem2(AM) is a dsRNA made by an in vitro siRNA construction kit. E, the same extracts as in panel A were blotted with antibodies specific to phosphorylated Chk1, Chk2, and H2AX together with extracts from asynchronously growing HeLa cells, or cells treated with HU, aphidicolin (Aphi), or UV.
The N terminus of Cdt1 has NLS activity, binds to cyclin A, but is not involved in Geminin binding. A, series of Cdt1 constructs. The black regions indicate the putative coiled-coil regions. B, Cdt1-cyclin A interaction. B-1, HeLa cells (lane 1) and a HeLa cell line stably expressing Flag-Cdt1-myc (lane 2) were blotted with anti Cdt1 antibody. B-2, Cdt1 was immuno-precipitated with FLAG antibody (Flag) or control IgGs (cont) from the Flag-Cdt1-myc expressing HeLa cell lines, and blotted with cyclin A, B, and E antibodies. 10% of input extract (ext) was run along with the immunoprecipitates. B-3, 293T cells were transfected with plasmids expressing the Cdt1 constructs indicated on top (plasmids), immunoprecipitated with either anti-Myc or anti-HA antibodies as indicated (IP-Ab, second row), the immunoprecipitates (IP) were separated by SDS-PAGE along with 10% of the corresponding total extract (ext) and immunoblotted with anti-cyclin A (αCycA) and anti-Myc or anti-HA antibodies as indicated. C, Cdt1-Geminin interaction. C-1, COS7 cells were co-transfected with T7-Geminin and with Myc-tagged Cdt1 constructs, or a Myc-tagged Cdt1 as control. T7-Geminin was immunoprecipitated using anti-T7 antibodies and blotted with anti-Myc to analyze the co-precipitation of Cdt1. 5% of the input extract was run along with the immunoprecipitates to show the expression level of each protein. C-2, T7-Geminin was co-expressed with full-length hCdt1 (lanes 1, 5, and 9), hCdt1(141-end)NLSmyc (lanes 2, 6, and 10), hCdt1(161-end)NLSmyc (lanes 3, 7, and 11) or hCdt1(161-end)NLSmyc (lanes 4, 8, and 12), and immunoprecipitations were carried out with anti-T7 antibody (lanes 5–8) or anti-HA antibody as control (lanes 9–12). Immunoprecipitates were separated by SDS-PAGE, together with 10% of each input extract (lanes 1–4) and immunoblotted with anti-Cdt1 and anti-HA antibodies. C-3, following co-expression of T7-Geminin with 3×HA(1–189)/Cdt1 or 3×HA full-length Cdt1 and immunoprecipitation with anti-T7 antibodies, immunoprecipitates and 10% of the corresponding extracts were immunoblotted with anti-HA antibodies. The asterisk indicates a band cross-reacting with anti-HA. D, full-length hCdt1, hCdt1(161-end) or hCdt1(161-end)/NLSmyc were transfected into 293T cells, and stained with anti-hCdt1 antibodies.

protein was immunoprecipitated from a strain stably expressing Flag-Cdt1-myc (Fig 6, B-1) with FLAG antibody and control IgGs, and blotted with antibodies against different cyclins. Cyclin A, but neither cyclin B nor cyclin E were detected in the FLAG antibody immunoprecipitates (Fig. 6, B-2).

To localize the region of Cdt1 responsible for cyclin A binding in cells, we constructed a series of deletion constructs of Cdt1 (Fig. 6A) and tested their ability to bind to cyclin A after transfection (Fig. 6, B-3). Western blotting shows that cyclin A binding to Cdt1 is lost when the N-terminal 139 amino acids of Cdt1 are deleted (∆N), whereas a fragment of Cdt1 from amino acid 1 to 189 (HA(1–189)) is sufficient to confer binding to cyclin A. In a second set of experiments, each construct was tested for Geminin binding in cells after co-transfection together with T7-tagged Geminin. Complex formation was examined by co-precipitation with anti-T7 antibodies (Fig. 6, C-1–3). The construct lacking the N terminus up to amino acid 160, with (Cdt1(161-end)/NLSmyc) or without (Cdt1(161-end)) the SV40 NLS fused to its C terminus, efficiently co-precipitated with Geminin (Fig. 6, C-2). In contrast, N-terminal constructs able to confer cyclin A binding, failed to efficiently bind to Geminin (Fig. 6, C-3). The region between amino acids 120–150 of Cdt1 is estimated to form a coiled-coil, a structure often involved in protein-protein interactions. This region has however been deleted from construct Cdt1(161-end), which efficiently binds to Geminin. Our in vitro data are consistent with recent two-hybrid and in vitro data, which localize the Geminin interaction domain to amino acids 177–380 of mouse Cdt1 (52). We therefore conclude that the N terminus of Cdt1 is required for the interaction of Cdt1 with cyclin A and is dispensable for Geminin interaction.

As shown in Fig. 6D, full-length Cdt1 expressed in 293T cells is located in the nucleus, similar to endogenous Cdt1. In contrast, Cdt1(161-end), which lacks the N terminus, is located in the cytoplasm. Addition of the SV40 NLS to this construct recovered its nuclear localization, indicating that the first 160
amino acids of Cdt1 contain a nuclear localization signal. A putative bipartite NLS is located between amino acids 48 and 71 of Cdt1. Consistently, the N terminus of Cdt1 (amino acids 1–172) is able to direct lacZ to the nucleus when fused to it (data not shown).

The N Terminus of Cdt1 Is Responsible for Its Cell Cycle-dependent Proteolysis—To determine which region of Cdt1 is responsible for its degradation during S-phase, we made cell lines stably expressing the N-terminal 1–189 amino acid of Cdt1 fused with the triple HA tag (3×HA1–189), and a strain expressing the N-terminal-truncated Cdt1(161-end) with NLSmyc construct (Fig. 7A). As shown in Fig. 6D, truncation of the N terminus results in defects in nuclear localization, use of a construct bearing the SV40-NLS was therefore important to ensure that both mutant proteins would be correctly localized to the nucleus. Protein levels in both cell lines were examined using synchronized cell cultures made by release from nocodazole arrest. Cells enter S-phase around 10–13 h post-release (see Fig. 2). 3×HA1–189/Cdt1 protein levels peaked around 9 h and then declined as cells entered S-phase with a timing similar to the endogenous Cdt1 protein (Fig. 7B). In contrast, Cdt1(161-end)/NLSmyc protein remained stable in S-phase (Fig. 7C). Thus, the N-terminal region of Cdt1 is required and sufficient to confer instability during S-phase. The Cdt1(161-end) without the NLS is also stable in S-phase (data not shown).

High Expression of the Stable, N terminus-truncated Form of Cdt1 Brings about Cells with a Higher DNA Content—Cdt1 is strictly regulated during the cell cycle so as to be present only during G1 phase, and its ectopic expression together with cdc6/18 can bring about re-replication in fission yeast (10). We showed here that the N-terminal 189 amino acids of Cdt1 are required and sufficient to confer cyclin A binding and cell cycle-specific degradation and that a mutant Cdt1 lacking amino acids 2–160 is stable during the cell cycle. We therefore wished to examine if such a stable Cdt1 form would be more potent in inducing untimely replication. To overexpress Cdt1 and its mutant forms and to select transfected cells, we used an IRES-nlsGFP vector that produces a single mRNA encoding both the protein of interest and NLS-GFP and therefore permits identification of transfected cells through detection of GFP. Each construct was transfected into 293T cells, and 48 h post-transfection DNA content was monitored for total cells and GFP-positive (region R1) and -negative (region R2) cells. We speculate that Cdt1 was overexpressed more than 50 times the levels seen in normal cells. As shown in Fig. 8A, cells possessing higher than 4C DNA content were induced when transfected with N terminus truncated forms of Cdt1, but not with vector alone, suggesting re-replication in these cells. Full-size Cdt1 was less effective. An increase in DNA content was not observed when Cdc6 was transfected (data not shown). Time course analysis of the increase in DNA content observed by expression of the (161-end)/NLSmyc Cdt1 mutant form is shown in Fig. 8B. We therefore conclude that a mutant form of Cdt1, which fails to be correctly proteolysed during S-phase is able to promote an increase in DNA content more efficiently than wild-type Cdt1.

Interestingly, the levels of phosphorylated Chk1 and Chk2 are markedly increased in cells overexpressing full-length Cdt1, (141-end)/NLSmyc, or (161-end)/NLSmyc Cdt1 constructs (Fig. 8C), indicating that checkpoints sensing DNA replication defects or DNA damage are indeed activated in these cells.

DISCUSSION

Once replication is initiated, re-replication of the genome is prevented by blocking the licensing of replicated chromosomes until chromosome separation is completed. This is performed by inactivating key initiator proteins after replication starts. Cdt1, a central regulator of licensing, accumulates only during the G1 phase, when licensing is legitimate. Here we present evidence that Cdt1 is degraded through a ubiquitin-dependent pathway in S-phase. Firstly, we used an in vivo ubiquitination assay to demonstrate that Cdt1 is multiply ubiquitinated (data not shown). Secondly, when the ubiquitin-dependent proteolysis pathway is inactivated in the cell lines tsBN75 (temperature-sensitive mutant for the ubiquitin-activating enzyme E1, data not shown) or ts41 (temperature-sensitive mutant defective in NEDD8 conjugation of the Cdc53/Cullins), Cdt1 is stabilized in S-phase. In fission yeast, both Cdc18 and Cdt1 disappear in S-phase, but in mammalian cells only Cdt1 does. Cdc6/18 levels remain constant after initiation. Instead, in mammalian cells the Orc1 subunit of ORC is subjected to proteolysis that may require the SCF ubiquitin ligase, Skp1-Cullin1 and Skp2 as its F box component (39). Since Cdt1 is stabilized in ts41 cells, it is likely that Cdt1 is also ubiquitinated by the SCF family of E3 ligases. A recent report indeed implicates Skp2 in regulating Cdt1 degradation during S-phase in human cells (55), while Cul-4, a member of the Cdc53/Cullin family, has been implicated in Cdt1 degradation in Caenorhabditis elegans (56). Cell cycle specific recognition of substrates by SCF family members is mediated by regulated phosphorylation of the substrates. Cyclin A is believed to be central in controlling once per cell cycle replication both in Drosophila and mammalian cells (53, 54). Orc1 is phosphorylated by cyclin A for degradation, while phosphorylated Cdc6/18 is exported to cytoplasm in mammalian cells. We show that Cdt1 can form a complex with cyclin A, and the N-terminal region of Cdt1 is essential and sufficient both for cyclin A binding and for S-phase-specific proteolysis. Consistently, Cdt1 has a sequence in the N terminus similar to the Cy motif, a cyclin-CDK binding site, that is also found in Cdc6/18 (36). Thus, it is conceivable that Cdt1 phosphorylation by cyclin A-CDK complexes targets Cdt1 for degradation in S-phase. In that respect, the N termi-
nus of Cdt1 was shown to be directly or indirectly required for Skp2 association (55), while recent data show that cyclin A-CDK phosphorylates Cdt1, and this leads to an association with Skp2 and proteolysis (57). A mutant form of Cdt1 that is defective in cyclin A and Skp2 association is, however, still degraded in S-phase to ~25% of levels seen in asynchronously growing cells (57). In contrast, the N-terminally truncated Cdt1 (161-end)NLS is fully resistant to proteolysis (this study), suggesting that there might be additional mechanisms that recognize the N terminus for degradation independently of cyclin A binding. Indeed, Cullin4 containing complexes have been implicated in Cdt1 proteolysis following -irradiation, and it is conceivable that such complexes may also be implicated in Cdt1 proteolysis during the cell cycle (58). The observation that Cdt1 is degraded somewhat earlier than Orc1 after the onset of S-phase (Fig. 2) may reflect a difference in the mechanisms of proteolysis between them. Analyzing the stability of Cdt1 in Skp2 knockout cell lines may be informative for clarifying essential components in the pathway that targets Cdt1 for degradation. Cdt1 stabilized by proteasome inhibitors in S-phase is modified by both ubiquitination and phosphorylation. Interestingly, ubiquitinated forms of Cdt1 are recovered preferentially in a chromatin enriched fraction. This suggests that Cdt1 may be degraded on chromatin. Degradation of human Orc1 is suggested to occur on chromatin (39). A localized presence of ubiquitin ligase on the replication origin would facilitate the degradation of Cdt1 and Orc1 associated with origin sequences.

The important role of proteasome-dependent proteolysis, especially that mediated by the Cdc53/Cullin family of ubiquitin ligases, to prevent re-replication has been demonstrated in several cases. In C. elegans, inactivation of Cul-4 causes massive re-replication with an increased level of Cdt1 (56). The fission yeast Pop1 protein is a F-box component of the SCF complex. In pop1 mutants, Cdc18 accumulated and an increase in ploidy was observed (59). Similarly, in mouse, knock-out of Skp2 results in polyploidy in some tissues (60). When cultured at high temperature, ts41 cells undergo re-replication. We show that similar to Cdt1, Cdc6/Cdc18 protein levels also increase in ts41 cells undergoing re-replication, while Geminin does not accumulate to a similar extend. The DNA content in ts41 cells increases continuously, like that of fission yeast cells overexpressing cdc18 alone or co-expressed with cdt1. Thus, in mammalian cells as well as in yeast, the stabilization of key licensing regulators may cause re-replication.

Recently, it was shown that overexpression of Cdt1 and Cdc6 induces re-replication in p53-deficient mammalian cells (61). Indeed, we show that ectopic expression of Cdt1 and two N-terminally deleted Cdt1 mutants in 293T cells leads to activation of the checkpoint kinases Chk1 and Chk2 and an increase in DNA content. Interestingly, N-terminally deleted Cdt1 is more potent at inducing an increase in DNA content than full length Cdt1 when over-expressed in 293T cells. This form of Cdt1 is unable to bind to cyclin A and is stabilized in S-phase. This raises the interesting possibility that defects in Cdt1 regulation may predispose cells to genomic instability.

Geminin has been shown to inhibit licensing in Xenopus egg extracts and to bind stably to Cdt1 (38, 45). Inactivation of
Geminin in Drosophila leads to rereplication (54, 62). However, Geminin and Cdt1 are only co-expressed for brief windows during the mammalian cell cycle (during early S and mitosis) raising the question of when and how Geminin acts. Since Geminin accumulates as Cdt1 protein levels decrease, Geminin binding to Cdt1 could target it for degradation. Our data however demonstrate that this is unlikely to be the case: Cdt1 was degraded in S-phase even when Geminin expression was inhibited by siRNA. In addition, the N terminus of Cdt1, which is not able to bind to Geminin, can still be degraded in S-phase. Recent in vitro binding studies suggest that Geminin binding to Cdt1 inhibits its DNA binding activity (52). We show, however, that Cdt1 stabilized by proteasome inhibitor treatment in early S-phase cells, when Geminin levels are high, localizes to the nucleus in a complex with Geminin and associates with chromatin as tightly as Cdt1 in G1-phase, when Geminin is undetectable. In S and G2, Geminin may only be present as a failsafe mechanism. When cells experience a stress such as DNA damage or heat shock, CDK activity and/or the proteasome pathway might be temporarily inactivated and Cdt1 might escape from degradation. In such a situation, Geminin would bind to and inhibit Cdt1. Geminin’s essential role might be to inhibit premature licensing during mitosis, as previously suggested for the meiosis II of Xenopus (45). Indeed, we show that Geminin and both accumulate in a mitotic block, are both modified by phosphorylation and form a complex. It is however conceivable that Geminin in S and G2 cells may have a role in addition to Cdt1 inhibition and binding to Cdt1 may serve to target Geminin to the nucleus. It is interesting in that respect that Geminin antisense oligo treatment of Xenopus embryos results in a checkpoint-dependent cell cycle arrest at G2 (63).

A homologue of Geminin has not yet been identified in yeast. If indeed Geminin does not exist in unicellular eukaryotes, it may not be intrinsically essential for a single cell to proliferate. However, Geminin appears to be essential for the completion of the cell cycle in Drosophila and Xenopus. Recent reports show an oncogenic activity of Cdt1 and re-replication in cells having high amounts of Cdt1 (47, 56, 61) while we show here that ectopic expression of a stable mutant of Cdt1 can induce an increase in DNA content. These data, taken together showing that controlling Cdt1 is especially important in higher eukaryotes, and thus, having two independent systems to completely inhibit Cdt1 may be essential in order to ensure a complete block over re-replication.

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