An Evolutionarily Conserved Function of Proliferating Cell Nuclear Antigen for Cdt1 Degradation by the Cul4-Ddb1 Ubiquitin Ligase in Response to DNA Damage

Received for publication, December 6, 2005, and in revised form, January 3, 2006
Published, JBC Papers in Press, January 3, 2006, D0110.1074/jcb-259464200
Jian Hu and Yue Xiong

From the Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, and Program in Molecular Biology and Biotechnology, University of North Carolina, Chapel Hill, North Carolina 27516-7295

The DNA replication licensing factor Cdt1 is degraded by the ubiquitin-proteasome pathway during S phase of the cell cycle, to ensure one round of DNA replication during each cell division and in response to DNA damage to halt DNA replication. Constitutive expression of Cdt1 causes DNA replication re-replication and is associated with the development of a subset of human non-small cell lung carcinomas. In mammalian cells, DNA damage-induced Cdt1 degradation is catalyzed by the Cul4-Ddb1-Roc1 E3 ubiquitin ligase. We report here that overexpression of the proliferating cell nuclear antigen (PCNA) inhibitory domain from the CDK inhibitors p21 and p57, but not the CDK-cyclin inhibitory domain, blocked Cdt1 degradation in cultured mammalian cells after UV irradiation. In vivo soluble Cdt1 and PCNA co-elute by gel filtration and associate with each other physically. Silencing PCNA in cultured mammalian cells or repression of pcr11 expression in fission yeast blocked Cdt1 degradation in response to DNA damage. Unexpectedly, deletion of Ddb1 in fission yeast cells also accumulated Cdt1 in the absence of DNA damage. We suggest that the Cul4-Ddb1 ligase evolved to ubiquitinate Cdt1 during normal cell growth as well as in response to DNA damage and a separate E3 ligase, possibly SCFSkp2, evolved to either share or take over the function of Cdt1 ubiquitination during normal cell growth and that PCNA is involved in mediating Cdt1 degradation by the Cul4-Ddb1 ligase in response to DNA damage.

Cdt1, first identified in the fission yeast Schizosaccharomyces pombe as a G1 START component Cdc10-dependent transcript whose loss-of-function prevents DNA replication (1), binds to the origin recognition complex with Cdc6 and origin recognition complex recruits the minichromosome maintenance 2–7 (MCM2–7) to assemble the prereplication complexes during G1, thereby controlling the initiation of DNA replication (2). Constitutive expression of Cdt1 alone in Caenorhabditis elegans, with Cdc6 in S. pombe or with Cdc6 and cyclin A-cdk2 in p53-deficient mammalian cells causes DNA re-replication (3–5). Constitutive expression of Cdt1 is also associated with the development of a subset of human non-small lung carcinomas (6), indicating the critical importance of regulating Cdt1 level for both initiating DNA replication and maintaining genome integrity.

In addition to Cdc10-dependent transcriptional regulation, at least four mechanisms have been proposed for controlling Cdt1 function at the protein level; Cdt1 is exported from the nucleus in S phase in the budding yeast Saccharomyces cerevisiae (7), is inhibited by the binding of geminin from S to M phase in metazoans (8), is degraded in human cell lines, possibly by the SCFSkp2 E3 ubiquitin ligase (9, 10), and is degraded in response to DNA damage and during normal C. elegans embryogenesis by the Cul4-Ddb1-Roc1 ubiquitin E3 ligase (3, 11, 12). Evolving multiple distinct mechanisms to negatively regulate the level of Cdt1 protein presumably functions to meet the needs of stopping DNA replication irreversibly at multiple stages of the cell cycle and development and rapidly in response to genotoxic insults.

The mechanisms underlying the ubiquitin-mediated degradation of Cdt1 remain incompletely understood. In particular, the identity and components of the Cdt1 E3 ubiquitin ligase are currently confusing. Two different E3 ligases, a Cul1-dependent SCFSkp2 E3 (10, 13) and a Cul4-Ddb1-dependent E3 (3, 11, 12), have been linked to Cdt1 degradation. Critically missing from the current understanding of these two ligases is promoting Cdt1 ubiquitination is the signal(s) that recruits Cdt1 to either ligase. We previously reported that Ddb1 preferentially associated with a slow migrating form of Cdt1, which likely corresponds to phosphorylated Cdt1 (12). The nature of this DNA damage induced Ddb1 kinase and whether Cdt1 phosphorylation is required for binding with Ddb1 is yet to be determined. Separately, a cyclin A-dependent CDK has been reported to promote Cdt1 phosphorylation and subsequent binding with the F-box protein Skp2 (13, 14), resulting in Cdt1 degradation during the normal cell cycle. Co-immunoprecipitation analysis showed that cells treated with the proteasome inhibitor MG132 prior to UV irradiation had increased Skp2-Cdt1 association (15), raising the possibility that both SCFSkp2 and Cul4-Ddb1 could mediate Cdt1 degradation following UV-induced DNA damage. In an effort to clarify the role of CDKs in regulating Cdt1 ubiquitination, we examined how CDK inhibitors may affect Cdt1 degradation during the DNA damage response. This investigation did not identify any evidence for the requirement of a CDK in regulating Cdt1 degradation in response to DNA damage but instead led to the unexpected finding that the proliferating cell nuclear antigen (PCNA) is required for Cdt1 degradation.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Culture, and Cell Transfection—Plasmids expressing human p21, p21<sup>PCNA</sup> (M148A/F151A), p27, p57QT(residues 142–198), and p57QT<sup>PCNA</sup> (L271A/F275A) were described previously (16), and the procedures for immunoprecipitation and immunoblotting were as described previously (12). All human cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in a 37 °C incubator with 5% CO<sub>2</sub>. Cell transfections were performed using a calcium phosphate buffer.

Antibodies, Proteins, Immunological Procedures, Mass Spectrometric Analysis, and Sequence Exclusion Chromatography—Antibodies to hemagglutinin (HA) (12CA5; Roche Applied Science, Mannheim, Germany), to protein A (Sigma), to PCNA (PC10, Santa Cruz Biotechnology, Santa Cruz, CA), to actin (NeoMarkers, Fremont, CA), and to p21, p27, and p57 (16, 17) were either purchased commercially or described previously. Rabbit polyclonal antibodies to Cdt4A, Db1, and Cdt1 have been described previously (12). To purify the Cdt1 complex, 10 150-μm plates of HEK 293T cells were transfected with pcDNA3-myc3-Cdt1 and lysed with a 0.5% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM Na<sub>2</sub>PO<sub>4</sub>). Lysates were incubated with affinity purified anti-Cdt1 (10 μg) antibody. Immunocomplexes were precipitated by protein A-agarose beads and then eluted by incubating with a molar excess of antigen peptide. Eluted immunocomplexes were resolved on an SDS-PAGE gel, stained with Coomassie Blue, and protein bands digested with trypsin before mass spectrometric analysis at the University of North Carolina-Duke Proteomics Core Facility. For size exclusion chromatography, HeLa cells were lysed in 0.5% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM Na<sub>2</sub>PO<sub>4</sub>) and fractionated on a Sepharose 200 gel filtration column. The fractions were resolved by SDS-PAGE before the levels of Cdt1 and PCNA were determined by immunoblotting.

RNA Interference—All siRNA oligonucleotides were synthesized with 3′-NH<sub>2</sub> overhangs by Dharmacon (Lafayette, CO) in a purified and annealed duplex form. The sequences targeting each human gene were as follows: Ddb1, 5′-CCCGUGUAGUAGCGGAAAAAC-3′ and PCNA, 5′-UCAAGGGACCUCUA-UCAACGA-3′. Opti-MEM medium (50 μl) was mixed with Lipofectamine 2000 according to the manufacturer’s instructions. siRNA transfections were performed in 10 μl volumes of siRNA complex. After 4 h, the siRNA transfections were washed with PBS and incubated for 48 h before harvesting.

* This work was supported by National Institutes of Health Grant GM607113 (to Y.X.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence and reprint requests should be addressed: 22-012 Lineberger Cancer Center, University of North Carolina, Chapel Hill, NC 27516-7295. Tel: 919-962-2142; Fax: 919-966-8799; E-mail: yxiong@email.unc.edu.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
The p57KIP2 CDK inhibitor also contains a C-terminal domain, often referred and its ability to block Cdt1 degradation following UV irradiation (Fig. 1B). Although this finding is consistent with the notion that CDK may cause Cdt1 phosphorylation and thereby promote its degradation in response to DNA damage, surprisingly, overexpression of p57 had no detectable effect on Cdt1 degradation in the same experiment. Both p21 and p27 have indistinguishable CDK substrate specificity in their N termini. The major difference between these two CDK inhibitors lies in their C-terminal regions; while p21 contains a PCNA binding and inhibitory domain in its C-terminal region, the C-terminal sequence of p27 plays roles in regulating the phosphorylation of p27 and stability but has no PCNA binding activity. This prompted us to determine which region in p21, the N-terminal CDK-cyclin binding or C-terminal PCNA binding sequence, is required for inhibiting Cdt1 degradation following UV irradiation.

The C-terminal PCNA binding domain in p21 has been extensively characterized and residues required for the binding of p21 to PCNA have been identified (20–23). Combined mutations of Met148 and Phe151 to Ala in p21 (referred to as p21ΔPCNA) completely disrupted its binding with PCNA (22, 23) and its ability to block Cdt1 degradation following UV irradiation (Fig. 1B). The p57ΔCDK CDK inhibitor also contains a C-terminal domain, often referred to as the QT domain, that shares sequence homology with the C-terminal PCNA binding domain of p21 and binds with and inhibits the function of PCNA (16). To further confirm the role of PCNA in regulating Cdt1 degradation, we immunoprecipitated the p57QT domain and examined Cdt1 degradation following UV irradiation. Overexpression of the p57QT peptide, but not a L271A/E275A mutant peptide (referred to as p57QTΔPCNA) that cannot bind with PCNA (16), blocked Cdt1 degradation after UV irradiation (Fig. 1C), demonstrating that inhibition of PCNA blocked Cdt1 degradation in response to DNA damage. These results also indicate that CDKs that are inhibited by p21 and p27 (CDK1–CDK6) are unlikely to be required for DNA damage-induced Cdt1 degradation. In the absence of DNA damage, overexpression of both p21 and p27 increased the steady-state level of Cdt1 protein, particularly the slower migrating form (Fig. 1A). This observation suggests that either a p21/p27-sensitive CDK(s) is involved in regulating Cdt1 stability during normal cell growth, or the Cdt1 increased resulted indirectly from the G1 cell cycle arrest caused by p21 or p27 overexpression. Our subsequent study was focused on the control of Cdt1 stability during the DNA damage response.

To obtain further evidence corroborating the function of PCNA in controlling Cdt1 degradation, we examined the distribution of these two proteins by gel filtration. Size exclusion chromatography analyses indicated that soluble (non-chromatin-associated) PCNA and Cdt1 co-eluted in a fraction at molecular mass of less than 158 kDa that is consistent with the formation in vivo of a complex containing PCNA, Cdt1 (Fig. 2A). Cu4A, on the other hand, is present in high molecular mass fractions and is not detected in the low molecular mass fractions that contain both Cdt1 and PCNA. The patterns of co-elution of Cdt1 and PCNA and mutually exclusive distribution of PCNA-Cdt1 from Cu4A are consistent with the possibility that PCNA is involved in promoting Cdt1 ubiquitination and subsequent degradation of Cdt1 by the Cu4A ligase.

Using a newly generated Cdt1 antibody (12), we immunopurified Cdt1 complexes from an Nonidet P-40-soluble extract and examined them by Coomasie blue staining (Fig. 2B). Cdt1-interacting proteins were identified as the polypeptides that were competed off by a molar excess of antigen peptide. In addition to a 60-kDa band that was identified as Cdt1 (see below), the only prominent polypeptides visualized were several bands clustered at a molecular mass of around 35 kDa. Mass spectrometry analyses identified, in addition to the 60-kDa Cdt1, two proteins: geminin and PCNA (Fig. 2C). The close migration of geminin and PCNA prevented us from determining the relative stoichiometric ratio of Cdt1 and these two proteins. Overexpression of p21 reduced the PCNA-Cdt1 association (Fig. 2D), suggesting that one possible mechanism by which p21 stabilizes Cdt1 is to hinder PCNA-Cdt1 association.

FIGURE 1. Inhibition of PCNA prevents the degradation of Cdt1 in response to UV. HEK 293T cells were transfected with plasmids expressing p21 and p27 (A), a p21 mutant deficient in binding with PCNA (p21ΔPCNA) (B), and the PCNA binding domain of p57 (HA-p57QT) or a mutant of p57QT deficient in PCNA binding (HA-p57QTΔPCNA) (C). Cells were UV-irradiated (50 J/m2) 24 h after transfection and then lysed 30 min later. The steady-state levels of Cdt1 as well as p21, p27, p57, Ddb1, and actin proteins were determined by direct immunoblotting.

This result, however, needs to be interpreted cautiously as the possibility remains that p21 could also impede CTD1-PCNA association by inhibiting CDK-mediated Cdt1 modification.

To seek in vivo evidence supporting a role of PCNA in Cdt1 degradation, we first performed RNAi-based experiments in cultured mammalian cells to determine how silencing the expression of PCNA affects the steady-state level of Cdt1 protein. Consistent with our previously published data (12), knocking down Ddb1 by siRNA accumulated Cdt1 in UV-treated HeLa cells (Fig. 3A). Notably, reduction of the steady-state level of PCNA by siRNA accumulated Cdt1 following UV irradiation as well (Fig. 3A, comparing lanes 1 and 3) but did not increase Cdt1 protein level in non-irradiated cells (comparing lanes 2 and 4). The partial effect of PCNA RNAi on degradation of Cdt1 is likely due to incomplete depletion of PCNA protein.

For further in vivo experiments, we utilized fission yeast, which have conserved PCNA, Cdt1, and the Cul4-Ddb1 E3 ligase. We constructed fission yeast strains expressing TAP-tagged Cdt1 under the control of its native promoters in both Ddb1Δ and Ddb1ΔΔ backgrounds. The steady-state level of TAP-Cdt1 is much higher in Ddb1Δ than in Ddb1ΔΔ cells (Fig. 3B, comparing lanes 1 and 3). This result provides the first evidence that in fission yeast Ddb1Δ is also involved in controlling Cdt1 degradation as in mammalian cells and that a Ddb1Δ-mediated E3 ligase in fission yeast cells also controls Cdt1 degradation during normal cell growth in the absence of DNA damage. We then examined the level of Cdt1 in TAP-Cdt1, Ddb1Δ and TAP-Cdt1, Ddb1ΔΔ cells sustaining either inhibition of DNA replication or DNA damage. Unexpectedly, unlike DNA damage (see below), inhibition of DNA replication by treatment of cells...
with hydroxyurea (HU) increased, rather than decreased, the steady-state level of Cdt1-TAP (Fig. 3B, comparing lanes 1 and 2). To confirm this result, we constructed yeast strains expressing TAP-tagged Spd1 (Spd1-TAP) under the control of its native promoters in both Ddb1\(^{+}\) and Ddb1\(^{−}\) backgrounds. Spd1 is an inhibitor of ribonucleotide reductase and is also targeted for degradation by the Cul4-Ddb1 ligase both during S phase and in response to DNA damage in \(S\). pombe (24–26). Consistent with previous reports, inhibition of DNA replication caused complete degradation of Spd1 and deletion of Ddb1 blocked HU-induced Spd1 degradation (Fig. 3C, comparing lanes 1 and 3). We also noted that during the normal cell cycle, the steady-state level of Spd1 is higher in Ddb1\(^{+}\) cells than in Ddb1\(^{−}\) cells (Fig. 3C, comparing lanes 2 and 4), confirming that Ddb1 in fission yeast cells is involved in degradation of Spd1 both during normal cell cycle and when DNA replication is blocked. HU inhibits ribonucleotide reductase, and thus dNTP production, therefore arresting the DNA replication fork. Our results reveal a major difference between Cdt1 and Spd1 degradation during the normal cell cycle. We speculate that during \(S\) phase, Spd1 and Cdt1 are degraded before and after replication fork formation, respectively, and an additional factor(s) may contribute differently to the ubiquitination of these two proteins by the same Ddb1-mediated E3 ligase.

We note that in ddb1\(^{−}\) cells, Cdt1 accumulated to a much higher level (comparing lanes 1 and 3 of Fig. 3B or lanes 6 and 8 in Fig. 3D) than Spd1 (comparing lanes 2 and 4 of Fig. 3C), indicating that although Ddb1 is required for the degradation of both proteins, the level of these two proteins is regulated by an additional mechanism(s). We postulate that there may exist a feedback regulatory mechanism to repress the expression of Spd1, but not Cdt1, in ddb1\(^{−}\) cells during each cycle. As a result, Cdt1 continuously accumulates to a very high level that, although not inhibiting cell growth, would contribute to deregulated DNA replication and thus genomic instability.

Treatment of cells with methylmethane sulfonate (MMS), a DNA damage reagent, caused Cdt1 degradation in Ddb1\(^{+}\) cells (Fig. 3D, comparing lanes 3 and 4 or lanes 5 and 6) but had no effect on the steady-state level of Cdt1 in ddb1\(^{−}\) cells (lanes 7 and 8), indicating that DNA damage-induced, Ddb1-mediated Cdt1 degradation had evolved as early as in yeast cells. To investigate whether PCNA is required for Cdt1 degradation in response to DNA damage in \(S\). pombe, we generated a strain in which PCNA encoding gene \(pcn1\) expression is under the control of the thiamine-repressible \(nmt81\) promoter and \(TAP\) is tagged after the Cdt1 gene (\(nmt81\)-\(pcn1\), Cdt1-TAP). Repression of \(pcn1\) expression by thiamine addition was confirmed by direct blotting with an antibody to CDT1 (left panel). The steady-state levels of Cdt1 and PCNA protein were determined by direct immunoblotting.

With hydroxyurea (HU) increased, rather than decreased, the steady-state level of Cdt1-TAP (Fig. 3B, comparing lanes 1 and 2). To confirm this result, we constructed yeast strains expressing TAP-tagged Spd1 (Spd1-TAP) under the control of its native promoters in both Ddb1\(^{+}\) and Ddb1\(^{−}\) backgrounds. Spd1 is an inhibitor of ribonucleotide reductase and is also targeted for degradation by the Cul4-Ddb1 ligase both during S phase and in response to DNA damage in \(S\). pombe (24–26). Consistent with previous reports, inhibition of DNA replication caused complete degradation of Spd1 and deletion of Ddb1 blocked HU-induced Spd1 degradation (Fig. 3C, comparing lanes 1 and 3). We also noted that during the normal cell cycle, the steady-state level of Spd1 is higher in Ddb1\(^{+}\) cells than in Ddb1\(^{−}\) cells (Fig. 3C, comparing lanes 2 and 4), confirming that Ddb1 in fission yeast cells is involved in degradation of Spd1 both during normal cell cycle and when DNA replication is blocked. HU inhibits ribonucleotide reductase, and thus dNTP production, therefore arresting the DNA replication fork. Our results reveal a major difference between Cdt1 and Spd1 degradation during the normal cell cycle. We speculate that during S phase, Spd1 and Cdt1 are degraded before and after replication fork formation, respectively, and an additional factor(s) may contribute differently to the ubiquitination of these two proteins by the same Ddb1-mediated E3 ligase.

We note that in ddb1\(^{−}\) cells, Cdt1 accumulated to a much higher level (comparing lanes 1 and 3 of Fig. 3B or lanes 6 and 8 in Fig. 3D) than Spd1 (comparing lanes 2 and 4 of Fig. 3C), indicating that although Ddb1 is required for the degradation of both proteins, the level of these two proteins is regulated by an additional mechanism(s). We postulate that there may exist a feedback regulatory mechanism to repress the expression of Spd1, but not Cdt1, in ddb1\(^{−}\) cells during each cycle. As a result, Cdt1 continuously accumulates to a very high level that, although not inhibiting cell growth, would contribute to deregulated DNA replication and thus genomic instability.

Treatment of cells with methylmethane sulfonate (MMS), a DNA damage reagent, caused Cdt1 degradation in Ddb1\(^{+}\) cells (Fig. 3D, comparing lanes 3 and 4 or lanes 5 and 6) but had no effect on the steady-state level of Cdt1 in ddb1\(^{−}\) cells (lanes 7 and 8), indicating that DNA damage-induced, Ddb1-mediated Cdt1 degradation had evolved as early as in yeast cells. To investigate whether PCNA is required for Cdt1 degradation in response to DNA damage in \(S\). pombe, we generated a strain in which PCNA encoding gene \(pcn1\) expression is under the control of the thiamine-repressible \(nmt81\) promoter and \(TAP\) is tagged after the Cdt1 gene (\(nmt81\)-\(pcn1\), Cdt1-TAP). Repression of \(pcn1\) expression by thiamine addition was confirmed by direct blotting with an antibody to CDT1 (left panel). The steady-state levels of Cdt1 and PCNA protein were determined by direct immunoblotting.
null cells. This is likely because depletion of PCNA arrests the cell cycle and thus prevents further Cdt1 accumulation, while Ddb1 null cells continue to proliferate and accumulate Cdt1.

In this study, we present three separate lines of evidence that collectively support a role of PCNA in mediating Cdt1 degradation by Cul4-Ddb1 in response to DNA damage. First, overexpression of the PCNA inhibitory domain of either p21 or p57, but not the CDC7-cyclin inhibitory domain, blocked Cdt1 degradation in UV-irradiated cells. Second, soluble PCNA and Cdt1 co-exist in the same fractions and PCNA and Cdt1 physically associate with each other in vivo. Third, knocking down PCNA in cultured mammalian cells and repression of PCNA expression in fission yeast both block Cdt1 degradation following DNA damage. Together with the findings that Ddb1 is required for DNA damage-induced Cdt1 degradation in mammalian cells (12) and in fission yeast (Fig. 3D), we suggest that PCNA-dependent Cdt1 degradation by Cul4-Ddb1 ligase in response to DNA damage evolved quite early and has been conserved during evolution.

Our study also raises three new questions. First, what is the exact biochemical role of PCNA in promoting Cdt1 degradation? We previously reported that Ddb1 can directly bind to Cdt1 (12), suggesting that PCNA is not required for recruiting Cdt1 to the Cul4-Ddb1 ligase. Thus far, our in vitro immunoprecipitation-based Cdt1 ubiquitination assay has neither identified a significant amount of PCNA nor revealed a role of PCNA in Cdt1 ubiquitination by the Cul4-Ddb1 ligase. One factor obviously missing from these assays is subcellular localization. In particular, given that all four proteins, Cdt1, PCNA, Ddb1, and Cul4, are associated with chromatin, it is likely that Cdt1 ubiquitination may occur in situ at the site of damaged DNA on chromatin and that PCNA plays a critical role in bringing about in situ Cdt1 ubiquitination, a function that is not possible to be detected in the current in vitro assay or by reconstitution. In fact, in Xenopus, Cdt1 is degraded during the course of DNA replication on chromatin (27). Whether this replication-dependent Cdt1 degrada-

tion on chromatin is catalyzed by the Cul4-Ddb1 ligase or regulated by CDK remains to be determined.

The second issue concerns the role of Ddb1, a factor that was initially identified as a component of a damaged DNA binding activity and has long been associated with DNA repair processes, during normal cell growth in the absence of DNA damage. Surprisingly, depletion of Ddb1 in fission yeast cells accumulated Cdt1 as well as Spd1 in non-damaged cells (Fig. 3, B–D), revealing a function of Ddb1 in controlling normal cell growth. It remains to be deter-
mmined whether this function of Ddb1 is also conserved in higher eukaryotes.

Related to this issue is the role of Skp2 in Cdt1 degradation. Accumulating evidence has suggested that another E3 ligase, possibly SCDP1/Skp2, is involved in causing Cdt1 ubiquitination and degradation in mammalian cells (10, 13, 14). Consistent with the presence of multiple E3 for Cdt1 degradation, knocking down Ddb1, while accumulating abundant Ddb1 following DNA damage, was not sufficient to accumulate Cdt1 during normal cell growth (11, 12). We have thus far obtained no evidence in fission yeast that would suggest the involvement of another E3 ligase in promoting Cdt1 ubiquitination. Depletion of Ddb1 accumulated Cdt1 to a very high level (Fig. 3D), arguing that if there exists another E3 ligase in fission yeast that controls Cdt1 ubiquitination, its activity must be very minor in comparison with that of Ddb1. We suggest that the Cul4-Ddb1 ligase evolved early to ubiquitinate Cdt1 both during normal cell growth as well as in response to DNA damage. Later in evolution, a separate E3 ligase evolved to either share or take over the function of Cdt1 ubiquitination during normal cell growth, but DNA damage-induced Cdt1 degradation continues to be carried out by the Cul4-Ddb1 ligase.

Acknowledgments—We thank Dr. Teresa Wang for providing S. pombe strains, Chad McCall for reading the manuscript, and other members of the Xiong laboratory for discussion throughout this work.

REFERENCES

1. Hofmann, J. F., and Beach, D. (1994) EMBO J. 13, 425–434
2. Bell, S. P., and Dutta, A. (2002) Annu. Rev. Biochem. 71, 333–374
3. Zhong, W., Feng, H., Santiago, F. E., and Kuroe, E. T. (2003) Nature 423, 885–889
4. Yanow, S. K., Lygerou, Z., and Nurse, P. (2001) EMBO J. 20, 4648–4656
5. Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S., and Dutta, A. (2003) Mol. Cell 11, 979–1008
6. Karakaidos, P., Taraviras, S., Vassilou, L. V., Zacharatos, P., Kastrinakis, N. G., Kougioumtzoglou, D., Kouloukoussa, M., Nishitani, H., Papavassiliou, A. G., Lygerou, Z., and Gorgoulis, V. G. (2004) Am. J. Pathol. 165, 1351–1365
7. Tanaka, S., and Dilley, J. F. (2002) Nat. Cell Biol. 4, 198–207
8. Wohlschlegel, J. A., Dwyer, B. T., Bhat, S. K., Cvetic, C., Walter, J. C., and Dutta, A. (2000) Science 290, 2309–2312
9. Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000) Nature 404, 625–628
10. Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003) J. Biol. Chem. 278, 30854–30858
11. Higa, L. A., Mihaylov, I. S., Banks, D. P., Zheng, J., and Zhang, H. (2003) Nat. Cell Biol. 5, 1008–1015
12. Hu, J., McCall, C. M., Ohto, T., and Xiong, Y. (2004) Nat. Cell Biol. 6, 1003–1009
13. Sugimoto, N., Tatsuno, Y., Tsurumi, T., Matsukage, A., Kiyono, T., Nishitani, H., and Fujita, M. (2004) J. Biol. Chem. 279, 19691–19697
14. Liu, E., Li, X., Yan, F., Zhao, Q., and Wu, X. (2004) J. Biol. Chem. 279, 17283–17288
15. Kondo, T., Kobayashi, M., Tanaka, I., Yokoyama, A., Sazuki, S., Kato, N., Onozawa, M., Chiba, K., Hashino, S., Immamura, M., Minami, Y., Minaminono, N., and Asaka, M. (2004) J. Biol. Chem. 279, 27315–27319
16. Watanabe, H., Pan, Z.-Q., Schreiber-Agus, N., DePinho, R. A., Hurwitzi, J., and Xiong, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1392–1397
17. Franklin, D. S., and Xiong, Y. (1996) Mol. Cell. Biol. 17, 1587–1599
18. Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., III, Steever, A. B., Wach, A., Philippsen, P., and Frangopoulos, J. J. (1998) Yeast 14, 943–951
19. Arroyo, M. P., and Wang, T. S. (1999) Methods 18, 335–348
20. Chen, J., Jackson, P. K., Kirschner, M. W., and Dutta, A. (1995) Nature 374, 386–388
21. Luo, Y., Hurwitzi, J., and Massague, J. (1995) Nature 375, 159–161
22. Nakashima, M., Roberts-Rye, S., Pereira-Smith, O. M., and Smith, J. R. (1995) J. Biol. Chem. 270, 17060–17063
23. Warbrick, E., Lane, D. P., Glover, D. M., and Cox, L. S. (1995)Curr. Biol. 5, 275–282
24. Liu, C., Powell, K. A., Munday, K., Wu, L., Carr, A. M., and Caspary, T. (2003) Genes Dev. 17, 1130–1140
25. Bondar, T., Pononmarev, A., and Raychaudhuri, P. (2004) J. Biol. Chem. 279, 9937–9943
26. Holmgren, C., Fleck, O., Hansen, H. A., Liu, C., Slaby, R., Carr, A. M., and Nielsen, O. (2005) Genes Dev. 19, 853–862
27. Arias, E. E., and Walter, J. C. (2005) Genes Dev. 19, 114–126

FIGURE 3. PCNA is required for Cdt1 degradation in response to DNA damage. A, logarithmically growing HeLa cells were transfected with siRNA oligonucleotides silencing Ddb1 and PCNA. Cells were UV-irradiated (50 j/m2) 48 h after transfection and then lysed after 30 min. The steady-state levels of Cdt1, as well as Ddb1, PCNA, and actin, were determined by direct immunoblotting. B and C, S. pombe strains Cdt1-TAP and Cdt1-DDB1, ddb1Δ or spd1Δ and spd1-TAP and spd1-DDB1, ddb1Δ were treated with 20 μM HU for 4 h or untreated. Yeast cells were collected and lysed with acid-washed glass beads. Steady-state levels of Cdt1-TAP and spd1-TAP proteins were determined by direct immunoblotting. D, S. pombe strains nmd81-Pcn1, Cdt1-TAP was treated with 150 μM thiamine for 12 h and then treated with 0.1% MMS for 3 h. S. pombe strains Cdt1-TAP and Cdt1-DDB1, ddb1Δ were treated with 0.1% MMS for 3 h or untreated. Yeast were collected and lysed with acid-washed glass beads. Steady-state levels of Cdt1-TAP and PCNA were determined by direct immunoblotting.