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Cdc6 Stability Is Regulated by the Huwe1 Ubiquitin Ligase after DNA Damage[D]

Jonathan R. Hall,* Evelyn Kow,[†‡] Kathleen R. Nevis,* Chiajung Karen Lu,[§] K. Scott Luce,* Qing Zhong,[§] and Jeanette Gowen Cook*

*Department of Biochemistry and Biophysics, School of Medicine and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599-7260; [†]Department of Molecular Genetics, Duke University Medical Center, Durham, NC 27710; and [§]Division of Biochemistry and Molecular Biology, Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720-3204

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The Cdc6 protein is an essential component of pre-replication complexes (preRCs), which assemble at origins of DNA replication during the G1 phase of the cell cycle. Previous studies have demonstrated that, in response to ionizing radiation, Cdc6 is ubiquitinated by the anaphase promoting complex (APC^{Cdh1}) in a p53-dependent manner. We find, however, that DNA damage caused by UV irradiation or DNA alkylation by methyl methane sulfonate (MMS) induces Cdc6 degradation independently of p53. We further demonstrate that Cdc6 degradation after these forms of DNA damage is also independent of cell cycle phase, Cdc6 phosphorylation of the known Cdk target residues, or the Cul4/DDB1 and APC^{Cdh1} ubiquitin E3 ligases. Instead Cdc6 directly binds a HECT-family ubiquitin E3 ligase, Huwe1 (also known as Mule, UreB1, ARF-BP1, Lasu1, and HectH9), and Huwe1 polyubiquitinates Cdc6 in vitro. Degradation of Cdc6 in UV-irradiated cells or in cells treated with MMS requires Huwe1 and is associated with release of Cdc6 from chromatin. Furthermore, yeast cells lacking the Huwe1 ortholog, Tom1, have a similar defect in Cdc6 degradation. Together, these findings demonstrate an important and conserved role for Huwe1 in regulating Cdc6 abundance after DNA damage.

INTRODUCTION

Duplication of large mammalian genomes requires that DNA replication initiate at thousands of chromosomal origins. In order for an origin to be competent for replication, it must first be bound by a multiprotein complex, the prereplication complex (preRC). PreRCs are constructed in a stepwise process through the chromatin binding of the origin recognition complex (ORC), which then recruits both the Cdc6 ATPase and Cdt1, two proteins that are required for the stable loading of the minichromosome maintenance complex (MCM). The Cdc6 and Cdt1-dependent loading of MCM complexes at origins licenses them for replication during the G1 phase of the cell cycle. Sufficient preRCs must be assembled during G1 to promote complete replication, but new preRCs must not assemble after S phase begins because relicensing of previously fired origins leads to rereplication and genome instability (Vaziri *et al.*, 2003; Melixetian *et al.*, 2004; Zhu *et al.*, 2004; Archambault *et al.*, 2005). For these reasons, preRC assembly is one of the most highly regulated events in the control of DNA replication. Cells restrict preRC assembly to the G1 period through a combination of overlapping mechanisms that regulate individual preRC components (reviewed in Bell and Dutta, 2002; Blow and Hodgson, 2002; Nishitani and Lygerou, 2002; Diffley, 2004; Machida *et al.*, 2005; Fujita, 2006).

Cdc6 is not only an essential factor for preRC construction, but it has also been implicated in the activation of the cell cycle checkpoint that prevents entry into mitosis while DNA replication is incomplete (Clay-Farrace *et al.*, 2003; Oehlmann *et al.*, 2004; Lau *et al.*, 2006). These observations suggest that Cdc6 functions not only during G1, but also in later cell cycle stages. Moreover, Cdc6 plays a role in setting the threshold for commitment to apoptosis (Pelizon *et al.*, 2002; Feng *et al.*, 2003; Yim *et al.*, 2003). These findings suggest that the regulation of Cdc6 has important consequences for multiple aspects of cell cycle control and cell fate determination. In addition, the high levels of Cdc6 protein observed in multiple cancers may contribute to cell cycle regulation defects and genome instability that consequently promote tumor progression (Williams *et al.*, 1998; Ohta *et al.*, 2001; Karakaidos *et al.*, 2004; Semple and Duncker, 2004).

Cdc6 is subject to multiple forms of regulation, including both transcriptional and posttranscriptional mechanisms. The human *cdc6* gene is regulated by the Rb-E2F transcriptional program that results in peak *cdc6* mRNA levels in late G1 phase (Hateboer *et al.*, 1998; Ohtani *et al.*, 1998; Yan *et al.*, 1998). The Cdc6 protein is degraded each cell cycle in early G1 as a consequence of ubiquitination by the Cdh1-activated form of the anaphase promoting complex (APC), a cell cycle–regulated ubiquitin E3 ligase (Petersen *et al.*, 2000). Ubiquitination of Cdc6 by APC^{Cdh1} is regulated not only by cell cycle–dependent fluctuations in APC activity, but also by phosphorylation of Cdc6 by cyclin-dependent kinases, most notably cyclin E/Cdk2. Cdk2-mediated phosphorylation blocks the association of Cdc6 with the Cdh1 protein, thus

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[‡] Present address: The Johns Hopkins Hospital, Room 100, Tower Building, Baltimore, MD 21287.

Address correspondence to: Jeanette Gowen Cook (jean_cook@med.unc.edu).

stabilizing Cdc6 when Cdk2 is active (Duursma and Agami, 2005; Mailand and Diffley, 2005).

Cdc6 is also ubiquitinated and degraded in response to DNA damage (Blanchard *et al.*, 2002; Duursma and Agami, 2005). Cells may eliminate Cdc6 to reduce the possibility of rereplicating DNA or to promote checkpoint functions that block mitosis with damaged DNA. One mechanism to ubiquitinate Cdc6 relies on inhibition of Cdk2 during a checkpoint response as a result of p53-mediated induction of the p21 Cdk inhibitor. Reduced Cdk2 activity destabilizes Cdc6 because it is no longer protected from ubiquitination by APC^{Cdh1} (Duursma and Agami, 2005). However, this pathway does not fully account for the degradation of Cdc6 after DNA damage. p53 is not required for Cdc6 degradation after UV irradiation or after treatment of cells with various DNA-alkylating agents such as adozelesin or methyl methane sulfonate (MMS; Blanchard *et al.*, 2002). Importantly, APC activity itself is inhibited during S phase and G2, times when it may be particularly important to regulate Cdc6 in order to prevent rereplication or to promote checkpoint activation. These observations implicate an as yet unidentified APC-independent pathway for degradation of Cdc6. We sought to determine this p53- and APC-independent mechanism of Cdc6 degradation, and we report here our finding that Cdc6 stability after DNA damage is controlled by a novel ubiquitin ligase, Huwe1.

MATERIALS AND METHODS

Cells and Treatments

HeLa, NHF1-hTERT (Heffernan *et al.*, 2002), and U-2OS cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (Sigma, St. Louis, MO). MMS, MG132 (Z-Leu-Leu-Leu-H), nocodazole, and cycloheximide (CHX) were purchased from Sigma (St. Louis, MO). UV irradiation was performed using a germicidal UV-C (254-nm) lamp (General Electric, Cleveland, OH) or alternatively by treatment of phosphate-buffered saline (PBS)-washed cells in a Stratalinker (Stratagene, La Jolla, CA). The endogenous Cdc6 locus in both the yeast strain BY4741 and its isogenic derivative *tom1::G418* (Invitrogen) was tagged at the 3′ end of the *cdc6* open reading frame with 13 tandem copies of the myc (9E10) epitope by the method of Longtine *et al.* (1998) to yield strains JCY200 (*wt*) and JCY201 (*tom1*Δ), respectively. The *tom1* deletion was confirmed by the temperature-sensitive growth at 37°C (data not shown).

Small Interfering RNA

Small interfering (siRNA) targeting Huwe1 (5′-GAGUUUGGAGUUUGUGAAGTT-3′), human Cdh1 (5′-UGUGAAGUCUCCCAGUCAGTT-3′), and the negative control green fluorescent protein (GFP; 5′-GGCUACGUCCAGGAGCGCACCTT-3′) were synthesized by Invitrogen and transfected at a final concentration of 100 nM using Dharmafect Reagent 1 (Dharmacon, Boulder, CO). DDB1, Cul4A, and Cul4B siRNA were described in Hu *et al.* (2004); geminin siRNA was described in Ballabeni *et al.* (2004).

Antibodies and Immunoblots

Anti-Cdc6 (sc-9964), Anti-Cdc6 (D-1), anti-cyclin A (C-19), anti-c-Myc (sc-40), anti-hemagglutinin (Y-11), anti-p53 (D01), anti-ScMcm2 (yN-19), anti-geminin (FL-209) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-cyclin B1 (V152) from Lab Vision (Fremont, CA), anti-Cdh1 (DH01) from Biomeda (Foster City, CA), anti-Orc2 from BD PharMingen (San Diego, CA), and anti-tubulin (DM1A) from Sigma. Phosphospecific antibodies to p53 phosphorylated on Ser15 and Chk2 phosphorylated on T69 were purchased from Cell Signaling Technologies (Beverly, MA). Anti-Huwe1 (anti-Lasu1, BL671) was purchased from Bethyl Laboratories (Montgomery, TX, for immunoprecipitations) or was the gift of S. Wing (McGill University) (Liu *et al.*, 2005; for immunoblots). Anti-Cul4 and anti-DDB1 antibodies were the gift of Y. Xiong (University of North Carolina at Chapel Hill) (Hu *et al.*, 2004); anti-yeast phosphoglycerate kinase (PGK) was a gift of H. Dohlman (University of North Carolina at Chapel Hill). Relative Cdc6 abundance was determined by densitometric analysis of light exposures of immunoblots using the ImageJ program (W. S. Rasband, ImageJ, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/, 1997–2006). The ratio of Cdc6 signal to tubulin signal (after background subtraction) is reported as the mean of two or more experiments.

Two-Hybrid Screen

The Gal4 DNA-binding domain was inserted at the carboxy terminus of human Cdc6 through a PCR strategy and expressed from the *GPD* promoter in plasmid p2U (gift of D. Picard, Université de Genève). Cotransformants of strain PJ69a (James *et al.*, 1996) with one of two cDNA fusion libraries (placental cDNA or thymus cDNA, Clontech, Palo Alto, CA) were selected on medium containing 3 mM 3-amino-1,2,4 triazole (Sigma). More than 8 million cotransformants of each library were screened; Huwe1 clones were identified from both libraries.

Plasmids and Viruses

A fragment encoding the carboxy-terminal 388 residues of Huwe1 ($Huwe1^{C}$; GenBank Accession BC063505) was subcloned into pDONOR221 (Invitrogen), followed by recombination into the glutathione *S*-transferase (GST) expression vector pDEST15 (Invitrogen). Adenovirus directing production of myc-epitope tagged Cdc6 has been described (Cook *et al.*, 2002). $Cdc6^{S3D}$ was constructed by site-directed mutagenesis of serines 54, 74, and 104 to aspartic acid in a construct that also bears a strong N-terminal nuclear localization signal (NLS) from SV40 T antigen. A $Cdc6^{S3D}$ mutant adenoviral construct without the NLS the mutation expresses a constitutively cytoplasmic protein that is stable in quiescent cells (Cook, unpublished observations). $Cdc6^{WT}$ bears the same NLS fusion.

In Vitro Protein-binding Assay

GST-$Huwe1^{C}$ was produced in BL21(DE3) purified on glutathione-Sepharose (GE Healthcare, Waukesha, WI) and incubated with cell lysates prepared in buffer 1 (50 mM HEPES, pH 7.2, 33 mM potassium acetate, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% Nonidet P-40, 10% glycerol, and protease and phosphatase inhibitors) essentially as described in Cook *et al.* (2004). GST-hCdc6 was produced by infection of SF21 insect cells with GST-hCdc6 baculovirus and purification of GST-hCdc6 was performed as described in Herbig *et al.* (1999).

Coimmunoprecipitation

HeLa cell nuclei were prepared by hypotonic lysis followed by brief sonication in S7 nuclease-containing buffer 1 supplemented with 1 mM $CaCl_2$. To aid Cdc6 solubility, NaCl was added to 250 mM for 5 min and then diluted to 45 mM. Portions of clarified lysate were reserved on ice, and the remainder was mixed with 3 μg of anti-Huwe1 (BL671) or normal rabbit serum at 4°C for 1 h. Lysates were centrifuged for 5 min at 13,000 × *g*, and supernatants were incubated with Protein A agarose (Roche, Indianapolis, IN) for an additional hour. Immune complexes were washed twice with buffer 1. Bound proteins were solubilized in 2× Huwe1 sample buffer (50 mM Tris, pH 6.8, 8 M urea, 75 mM dithiothreitol, 3% SDS, 0.05% Bromophenol blue) heated at 37°C for 5 min, and resolved by SDS-PAGE.

In Vitro Ubiquitination Assay

In a 10-μl reaction, 100 ng of purified recombinant GST-Cdc6 was incubated with an ATP-regenerating system (50 mM Tris, pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate, 3.5 U/ml creatine kinase), 10 μg ubiquitin, 10 ng human E1, 100 ng Ubch7, and Huwe1 as indicated at 37°C for 2 h in a manner similar to that described in Zhong *et al.* (2005). After terminating the reactions with SDS sample buffer, reaction products were fractionated by SDS-PAGE (6%) and analyzed by immunoblotting with anti-Cdc6 antibody (D-1, Santa Cruz) at 1:4000 dilution.

Chromatin fractionations were performed as described in Cook *et al.* (2002).

RESULTS

An APC-independent Mechanism for Cdc6 Degradation

To explore the regulation of Cdc6 after DNA damage, we treated an asynchronous population of HeLa cells with MMS or with UV irradiation. Similar to previous results (Blanchard *et al.*, 2002), both kinds of DNA damage resulted in significant loss of endogenous Cdc6 (Figure 1A, compare lanes 2 and 3 with lane 1), and the degradation was sensitive to proteasome inhibition by MG132 (Figure 1A, lanes 4–6). Under these conditions, we did not observe the caspase cleavage product of Cdc6 after DNA damage (data not shown); this cleavage normally occurs 12 h after induction of apoptosis (Pelizon *et al.*, 2002).

We next compared the kinetics of Cdc6 degradation in HeLa cells and in normal human fibroblasts (NHF; Figure 1B) to determine if differences in Cdc6 regulation might contribute—along with deregulated transcription—to the high levels of Cdc6 observed in cancer cells (Williams *et al.*,

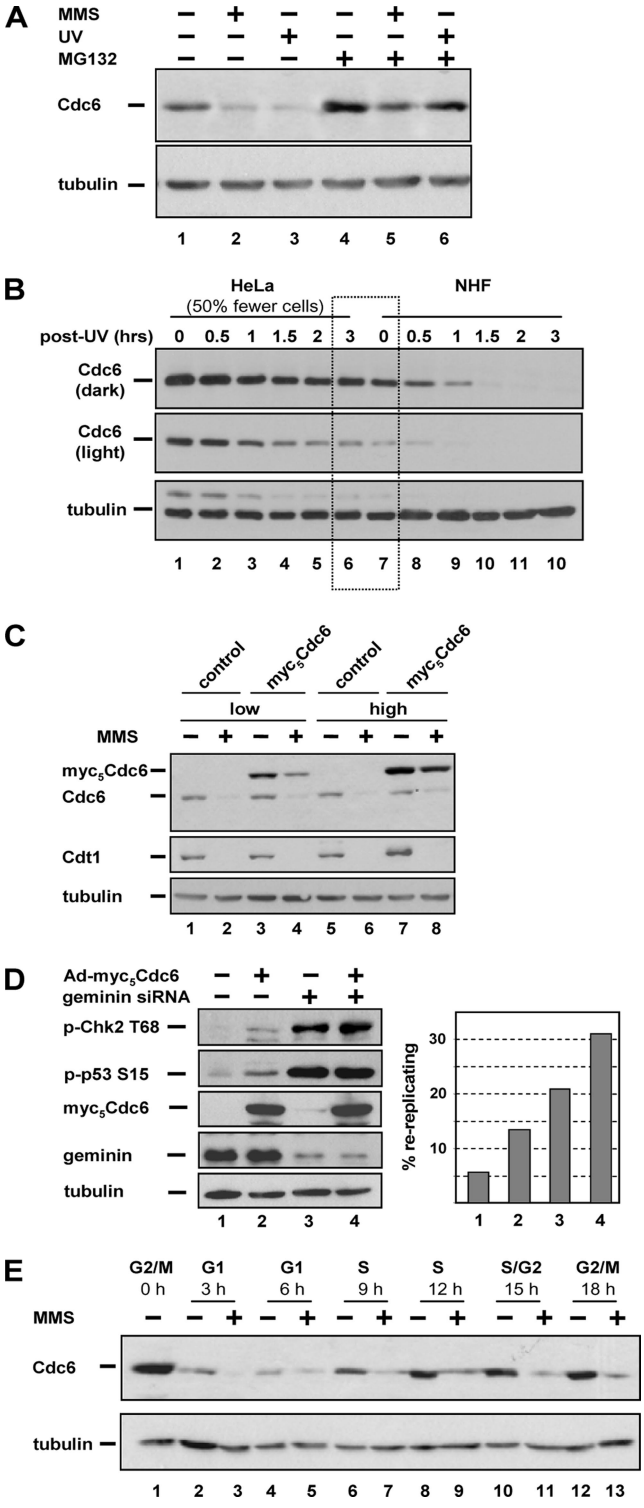

Figure 1. Cdc6 degradation after DNA damage depends on its abundance but not cell cycle phase. (A) Asynchronous HeLa cells were treated with 1 mM methyl methanesulfonate (MMS; lanes 2 and 5), with 30 J/m^2 UV light (lanes 3 and 6) and with 20 μM MG132 (lanes 4–6), or left untreated (lane 1) as indicated. Cells were harvested 3 h after treatment, and endogenous Cdc6 and tubulin levels were determined by immunoblotting. (B) Cells were UV irradiated as in A, and endogenous Cdc6 and tubulin were monitored in whole cell extracts of 60,000 HeLa or 120,000 NHF-hTert cells (normal human fibroblasts immortalized with telomerase) by immunoblotting. Both dark and light exposures of the Cdc6 signal are shown. (C) Asynchronous U-2OS cells were transduced with control virus or recombinant adenovirus producing Myc5-Cdc6 at a relative MOI of 3 (lanes 1–4) or 9 (lanes 5–8). Twenty-four hours after infection cells were treated with 1 mM MMS for 3 h. Endogenous and ectopic Cdc6, Cdt1, and tubulin were detected by immunoblotting. (D) HCT116 cells were transduced with control virus or Myc5-Cdc6 producing virus at a relative MOI of 25, then transfected with control siRNA or siRNA targeting geminin. Twenty-four hours after transfection, cells were synchronized in G2/M with 100 ng/ml nocodazole for 20 h. Arrested cells were collected and analyzed by immunoblotting for phosphorylated Chk2, phosphorylated p53, ectopic Cdc6, or endogenous geminin and tubulin (left) and by flow cytometry for cells with greater than 4N DNA content (see also Supplementary Figure S1). (E) U-2OS cells were synchronized at G2/M by treatment with 2 mM thymidine for 24 h followed 100 ng/ml nocodazole for 16 h. They were then collected by "mitotic shake-off" (lane 1) and replated. Cells were harvested at 3, 6, 9, 12, 15, and 18 h after release. One duplicate of each sample was treated with 1 mM MMS for 3 h before harvest (lanes 3, 5, 7, 9, 11, and 13).

1998; Ohta *et al.*, 2001; Karakaidos *et al.*, 2004). For this experiment, defined numbers of actively growing, subconfluent cells were analyzed for endogenous Cdc6 at various times after UV treatment. Whole cell extracts were prepared from 6×10^4 HeLas or 1.2×10^5 NHFs, in order to compare the relative amounts of Cdc6 remaining at each time after UV treatment. The overall rate of Cdc6 loss was similar in both cell lines (Figure 1B). However, HeLa cells consistently harbor at least 10-fold higher levels of Cdc6 per cell than NHFs (Figure 1B, compare lanes 1 and 7). Because of these high initial levels, even 3 h after DNA damage HeLa cells still contained at least as much Cdc6 as the *untreated* normal cells (Figure 1B, compare lanes 6 and 7). The amount of Cdc6 in naive normal cells is clearly enough to support a complete round of genomic DNA replication; thus cancer cells with high levels of Cdc6 have a relatively long period of abundant Cdc6 after DNA damage.

We specifically tested if Cdc6 overproduction could suppress DNA damage–induced Cdc6 degradation by overwhelming the capacity of the cells to target the excess protein. We infected U-2OS cells with either control adenovirus or virus-producing Cdc6 at either moderate doses or high doses and then treated cells with MMS to induce Cdc6 degradation. Both the ectopic and endogenous Cdc6 proteins were detected by immunoblot analysis. At the lower viral dose, ectopic Cdc6 accumulated to three times that of endogenous Cdc6 as estimated by densitometry (data not shown), and though it was reduced by DNA damage, some ectopic Cdc6 protein still remained (Figure 1C, lanes 3 and 4). Increasing the level of ectopic Cdc6 further to approximately six times that of endogenous Cdc6 significantly impaired the ability of these cells to degrade Cdc6 in the presence of MMS, but had no effect on the DNA damage–induced degradation of Cdt1 (Figure 1C, lanes 7 and 8).

To determine if high levels of Cdc6 can induce rereplication, we overproduced Cdc6 in cells arrested in G2. Extensive rereplication is associated with an activated cell cycle checkpoint characterized by phosphorylation of both the p53 tumor suppressor and the Chk2 protein kinase. These markers are strongly induced by depletion of geminin, a negative regulator of preRC assembly that inhibits Cdt1 (Zhu *et al.*, 2004; Figure 1D, lane 3). We infected HCT116 (p53+) cells with a high dose of Cdc6-producing adenovirus and held these cells in nocodazole for 20 h to block mitosis. Under these conditions, Cdc6 accumulated to ~12 times that of endogenous Cdc6 (data not shown). High levels of Cdc6

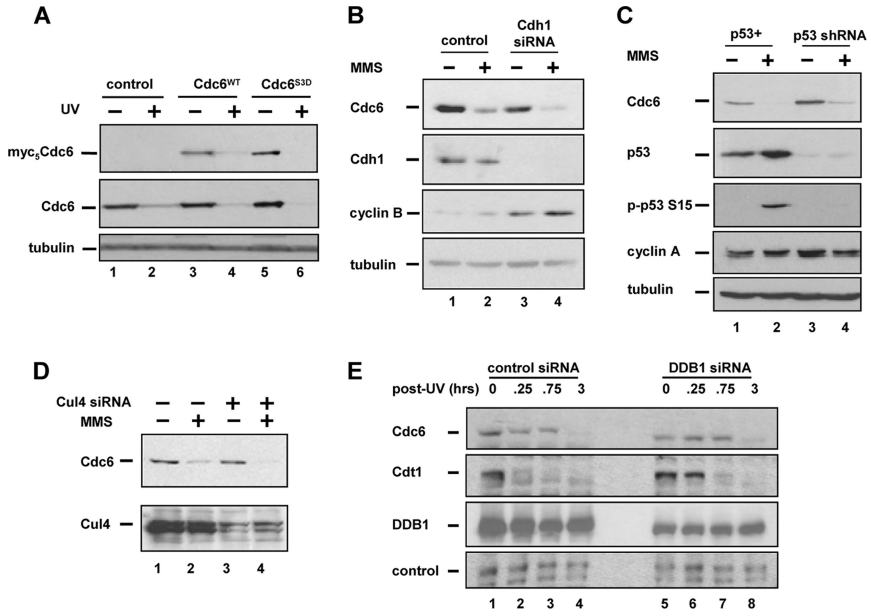

Figure 2. Cdc6 degradation after DNA damage is independent of Cdk phosphorylation, APC^{Cdh1}, p53 status, and Cul4/DDB1. (A) HeLa cells were infected with recombinant adenovirus producing GFP (control), myc-tagged Cdc6 (WT), or myc-tagged Cdc6 in which S54, S74, and S104 have been altered to aspartic acid. Viral doses were used that result in levels of ectopic Cdc6 that are lower than endogenous Cdc6 (the top panel is a longer exposure of the Cdc6 immunoblot than the middle panel). Cells were treated for 19 h with 3 mM thymidine to arrest in S phase before UV irradiation. Whole cell lysates were prepared 3 h after UV treatment, and both endogenous and ectopic Cdc6 and endogenous tubulin were detected by immunoblotting. (B) Asynchronous HeLa cells were treated with Cdh1 siRNA (lanes 3 and 4) or control siRNA (lanes 1 and 2) for 36 h. Three hours before harvest MMS was added to 1 mM (lanes 2 and 4). Endogenous Cdc6, Cdh1, cyclin B, and tubulin were analyzed by immunoblotting. (C) Normal human fibroblasts (immortalized with telomerase) or an isogenic derivative expressing a short hairpin RNA targeting p53 were treated with 1 mM MMS for 3 h and analyzed for endogenous Cdc6, p53, cyclin A, tubulin, and phosphorylated p53 (Ser 15) by immunoblotting. (D) HeLa cells were simultaneously treated with Cul4A and Cul4B siRNAs or control siRNA for 48 h followed by treatment with 1 mM MMS for 3 h. Endogenous Cul4A and Cdc6 were analyzed by immunoblotting. (E) Asynchronous HeLa cells were transfected with control or DDB1 siRNA for 48 h and then subjected to UV irradiation as in Figure 1A. Samples were collected at 0, 15, 45 min, and 3 h after irradiation, and endogenous Cdc6, Cdt1, and Ddb1 were analyzed by immunoblotting. (A nonspecific band is shown as a loading control.)

induced phosphorylation of both Chk2 and p53 (Figure 1D, compare lanes 1 and 2). Moreover, Cdc6 overproduction was sufficient to cause rereplication detectable as an increase in the number of cells with greater than 4N DNA content (Figure 1D and Supplementary Figure S1). Depletion of geminin caused robust rereplication as had been reported by others (Mihaylov *et al.*, 2002; Li and Blow, 2004; Melixetian *et al.*, 2004; Zhu *et al.*, 2004), and the combination of high levels of Cdc6 with geminin depletion increased the number of rereplicating cells even further (Figure 1D, numbers 3 and 4, and Supplementary Figure S4). We thus conclude that Cdc6 de-regulation can promote rereplication, and that the degradation of both Cdc6 and Cdt1 after DNA damage may be important for maintaining genome integrity.

Because persistent Cdc6 might contribute to genome instability we sought to understand the mechanism of DNA damage-induced Cdc6 degradation. To determine if Cdc6 degradation is affected by cell cycle stage, we synchronized U-2OS cells at G2/M by sequential treatment with thymidine and nocodazole followed by release from the arrest; cell cycle position was confirmed by flow cytometry (Supplementary Figure S2). In undamaged cells endogenous Cdc6 protein was low in G1 and accumulated throughout S phase and G2, as expected. Strikingly, DNA damage–induced Cdc6 degradation at every stage of the cell cycle despite the presumed inhibition of APC after the G1/S transition (Figure 1E).

APC-mediated Cdc6 degradation is blocked when Cdc6 is phosphorylated by Cdk2. Mutational alteration of the Cdk target sites to aspartic acid mimics phosphorylation and blocks interaction with APC (Duursma and Agami, 2005; Mailand and Diffley, 2005). Thus, if Cdc6 degradation after UV or MMS treatment occurred by promoting dephosphorylation of Cdc6 and sensitizing Cdc6 to APC, then these aspartic acid substitutions would prevent Cdc6 degradation. To test this possibility, we constructed adenoviral vectors to produce myc-epitope–tagged versions of both normal Cdc6 (WT) or Cdc6 in which the Cdk target sites, serines 54, 74, and 104, were altered to aspartic acid, $Cdc6^{S3D}$. Low doses of these viruses were used to infect HeLa cells such that the level of ectopic Cdc6 was less than endogenous Cdc6. UV irradiation caused the degradation of not only the endogenous Cdc6 (Figure 2A, lanes 1 and 2), but also the ectopically expressed Cdc6 (Figure 2A, lanes 3 and 4). Moreover, the S3D mutant showed equal susceptibility to UV-induced degradation (Figure 2A, lanes 5 and 6), indicating that phosphorylation at these serine residues does not control DNA damage-induced Cdc6 degradation.

Cdc6 ubiquitination by APC requires the targeting subunit, Cdh1 (Petersen *et al.*, 2000; Duursma and Agami, 2005). It was possible that during a DNA damage response, Cdc6 became more APC-sensitive by some mechanism other than dephosphorylation at the known Cdk sites. To definitively demonstrate that Cdc6 degradation can occur independently of APC^{Cdh1}, we eliminated Cdh1 in cells by siRNA transfection. Effective knockdown of Cdh1 was confirmed by immunoblot analysis and the resulting accumulation of cyclin B (Figure 2B). Despite the depletion of Cdh1, Cdc6 degradation after MMS treatment was unaffected (Figure 2B, compare lanes 2 and 4). APC-mediated ubiquitination of Cdc6 after ionizing radiation requires p53 (Duursma and Agami, 2005), but we observed no difference between normal human fibroblasts and isogenic cells deficient in p53 with regard to their ability to degrade Cdc6 after MMS treatment (Figure 2C), consistent with earlier findings in tumor cell lines lacking functional p53 (Blanchard *et al.*, 2002). Degradation of Cdc6 occurred despite the fact that naive p53-deficient cells have higher endogenous Cdc6 levels, presumably because of the combined effects of the p53-dependent deregulation of Cdks on both E2F-dependent *cdc6* transcription (Hateboer *et al.*, 1998; Ohtani *et al.*, 1998; Yan *et al.*, 1998) and APC-dependent Cdc6 protein stability during G1 (Duursma and Agami, 2005; Mailand and Diffley, 2005). We thus conclude that DNA dam-

age induces Cdc6 degradation regardless of cell cycle position, APC activity, or p53 status.

Like Cdc6, Cdt1 is ubiquitinated and degraded after DNA damage. Ubiquitination of Cdt1 under these conditions is dependent on the DDB1/Cul4 ubiquitin ligase (Higa *et al.*, 2003; Hu *et al.*, 2004); thus it was possible that Cul4 also ubiquitinated Cdc6 after DNA damage. However, cells treated with siRNAs targeting both Cul4A and Cul4B showed no difference in the degradation of Cdc6 after MMS treatment (Figure 2D, compare lanes 1 and 2 with lanes 3 and 4). Reduction of DDB1 by a similar siRNA strategy significantly delayed the normally rapid destruction of Cdt1 (Figure 2E, compare lanes 2 and 6), though the remaining DDB1 was capable of eliminating Cdt1 by later time points. (This hypomorphic phenotype is typical of DDB1-depleted cells; J. Hu and Y. Xiong, personal communication.) Importantly, UV-irradiated cells in which DDB1 is reduced by siRNA treatment degraded Cdc6 with the same kinetics as control cells (Figure 2E, compare lanes 1–4 with lanes 5–8). We thus hypothesized that an entirely different ubiquitin ligase plays an important role in Cdc6 ubiquitination and degradation in response to DNA damage.

Interaction of Cdc6 with Huwe1

We identified multiple isolates of a 4374 amino acid ubiquitin E3 ligase in a two-hybrid screen for proteins that interact with Cdc6 (Figure 3A). This enzyme is a member of the HECT family and can ubiquitinate the Mcl-1 anti-apoptotic protein, core histones, the c-myc transcription factor, and the p53 checkpoint mediator. These reports named the enzyme Mule (Warr *et al.*, 2005; Zhong *et al.*, 2005), Lasu1 (Liu *et al.*, 2005), HectH9 (Adhikary *et al.*, 2005), and ARF-BP1 (Chen *et al.*, 2005), respectively. We use the official gene name, HUWE1 for "HECT, UBA and WWE domain containing 1" (gene ID 10075) and refer to the protein as Huwe1. All of our two-hybrid isolates contain portions of the carboxy-terminal catalytic domain of Huwe1 (Figure 3C). Interestingly, this sequence is distinct from both the reported myc-interaction region of Huwe1 just amino-terminal to the HECT domain (Adhikary *et al.*, 2005) and the BH3 motif at amino acids 1972–1999, which binds Mcl-1 (Warr *et al.*, 2005; Zhong *et al.*, 2005). We constructed derivatives of the Cdc6 two-hybrid fusion in which increasing amounts of the amino terminal (noncatalytic) domain were deleted and tested them for interaction with the Huwe1 fusions. Removal of as many as the first 154 amino acids, but not the first 192, from Cdc6 had no effect on the two-hybrid interaction of Cdc6 with Huwe1, demonstrating that this region is dispensable for the Cdc6-Huwe1 interaction (Figure 3B). We note that the catalytic domain of Cdc6 is the most highly conserved domain and is included in the constructs that retain binding to the most highly conserved domain of Huwe1.

We confirmed that Cdc6 and Huwe1 interact biochemically. For this purpose, we produced a fusion of GST to the c-terminal domain of Huwe1 (amino acids 3987–4374 "GST-Huwe1^{C}") in *Escherichia coli*. Glutathione beads coated with GST or GST- Huwe1^{C} were incubated with a lysate of HeLa cells expressing epitope-tagged Cdc6 (myc_5-Cdc6). Under these conditions, Cdc6 bound to Huwe1, but not to GST control beads (Figure 3D, compare lanes 2 and 3). Furthermore, GST-Cdc6 produced in insect cells coprecipitates endogenous full-length Huwe1 from HeLa cell extracts (Figure 3E, compare lanes 2 and 3). To determine if these proteins can bind in the absence of other cellular proteins (human or yeast), we mixed purified recombinant Cdc6 with glutathione beads coated with purified recombinant GST-Huwe1^{C}. Cdc6 was specifically retained on these beads, indicating that these two proteins can interact directly (Figure 3F).

Finally, we tested if Huwe1 and Cdc6 associate when they are expressed at their endogenous levels. To determine if Huwe1 and Cdc6 interact during a DNA damage response, we irradiated cells with UV (or left them untreated) and then added MG132 to block the degradation of Cdc6. We prepared nuclear lysates from HeLa cells and released Cdc6 from chromatin with nuclease. These extracts were subjected to immunoprecipitation with anti-Huwe1 antibodies and then probed for endogenous Cdc6. Cdc6 was found in Huwe1 immunoprecipitates both in the absence and in the presence of DNA damage (Figure 3G, lanes 5 and 6).

If Huwe1 is the ubiquitin ligase that controls Cdc6 degradation, then Huwe1 should be able to ubiquitinate Cdc6. We therefore tested the ability of full-length (492 kDa) recombinant Huwe1 purified from insect cells (Zhong *et al.*, 2005) to polyubiquitinate recombinant Cdc6 in vitro. We incubated purified GST-Cdc6 with Huwe1 in the presence of E1 and E2 enzymes and ubiquitin under conditions similar to those initially defined for ubiquitination of the Mcl-1 protein by Huwe1 (a.k.a. Mule; Zhong *et al.*, 2005). Substantial polyubiquitination of Cdc6 was detected by immunoblot analysis of the reaction products, and the ubiquitination was dependent on the concentration of Huwe1 (Figure 3H, compare lanes 2–4). These results suggest that Huwe1 binds Cdc6 for the purpose of catalyzing Cdc6 ubiquitination and that Huwe1 may play a role in Cdc6 stability in cells.

Huwe1-dependent Regulation of Cdc6 Stability in Cells

Huwe1 is not induced by DNA damage (Figures 3G and 4, A–E), suggesting that some mechanism other than enhanced expression must operate to promote Cdc6 ubiquitination after DNA damage. To explore how Cdc6 becomes unstable after DNA damage, we tested if Cdc6 chromatin association is affected by DNA damage. HeLa cells were UV irradiated and harvested at different times after UV treatment up to 3 h. Additional cells were simultaneously treated with UV irradiation and MG132 to block Cdc6 degradation. Nonionic detergent insoluble pellets were prepared from these cells, and DNA-bound proteins were released from these pellets with micrococcal nuclease. Proteins released by nuclease digestion were defined as the chromatin-bound proteins, and their abundance was compared with their overall abundance in portions of the starting whole cell lysates. In naive HeLa cells, Cdc6 is readily detected in chromatin fractions (Figure 4A, lanes 1 and 8). Within 30 min after UV treatment, the amount of Cdc6 remaining on chromatin was substantially reduced (Figure 4A, compare lanes 8 and 9), even though at this early time point the overall amount of Cdc6 in the whole cell lysate was still largely unchanged (Figure 4A, compare lanes 1 and 2). Most strikingly, when Cdc6 degradation was blocked with MG132, the overall level of Cdc6 protein remained unchanged, as expected (Figure 4A, compare lanes 1 and 7), but the amount of chromatin bound Cdc6 was almost undetectable (Figure 4A, compare lanes 8 and 14). Importantly, Huwe1 itself is not appreciably associated with chromatin either before or after DNA damage (Figure 4A, lanes 8–14). These data suggest that Cdc6 is released from chromatin after DNA damage into the soluble fraction where it can associate with the soluble Huwe1. This mechanism of induced interaction was not detectable in the coimmunoprecipitation experiments in Figure 3G because Cdc6 required solubilization by nuclease digestion of the lysates before immunoprecipitation.

To test if Huwe1 is required for the degradation of Cdc6 in cells, we designed siRNA molecules to target the Huwe1

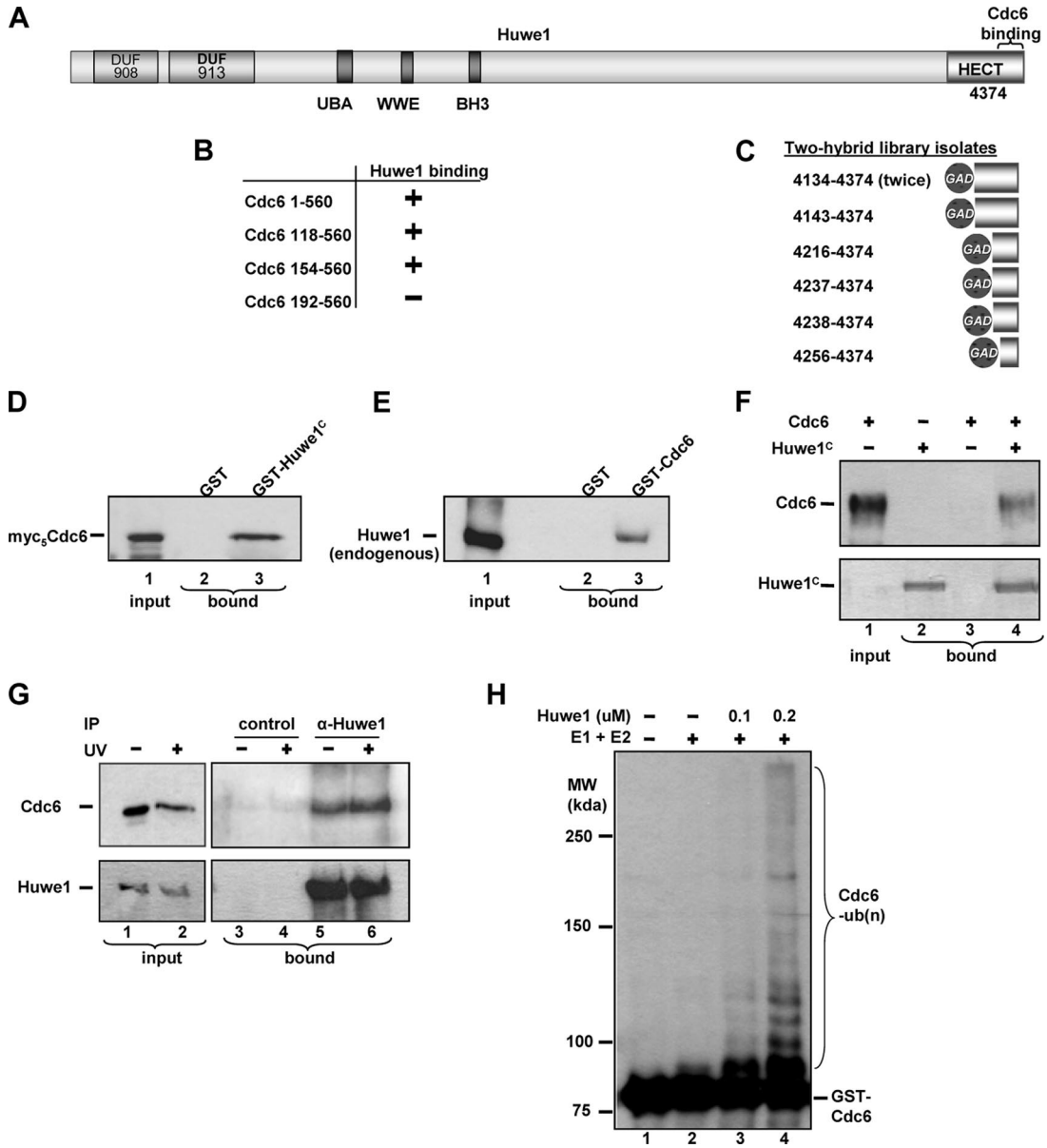

Figure 3. The ubiquitin E3 ligase, Huwe1, binds Cdc6 and catalyzes its polyubiquitination. (A) A schematic of the full-length Huwe1 protein (also known as Mule, Lasu1, ARF-BP1, UreB1, and HectH9) is provided: DUF908 and DUF913, conserved domains of unknown function; UBA, ubiquitin association domain; WWE motif, conserved putative protein interaction motif; BH3, Bcl-2 homology region 3; HECT, homologous to E6-AP carboxy terminus (catalytic domain). (B) Gal4 DNA-binding domain fusions to the indicated Cdc6 sequences were expressed in a yeast reporter strain (PJ69a) with the carboxy-terminal 240 residues of Huwe1 fused to the Gal4 transcriptional activation domain. Interaction was scored relative to growth of the empty vector control on selective medium containing 30 mM 3-amino-1,2,4-triazole. (C) A schematic of independent isolates of two-hybrid fusions to Huwe1 from Cdc6 interaction screens is shown. The amino acids included in each library clone are given; the longest clone was isolated twice. (D) The catalytic domain of Huwe1 (Huwe1^C) was produced as a GST fusion in *E. coli* and incubated with a lysate of HeLa cells infected with adenovirus directing production of Cdc6 fused to five copies of the myc epitope tag (myc$_5$-Cdc6). Ectopic Cdc6 in portions of the input (5%) and in the fractions bound to glutathione beads was detected with anti-myc antibody. (E) GST and GST-Cdc6 were produced in insect cells from a baculoviral vector, isolated on glutathione beads, and incubated with a HeLa cell lysate. Endogenous Huwe1 in portions of the input (5%) and in the bound fractions was detected with anti-Huwe1 antibody. (F) Cdc6 purified as a GST fusion from insect cells and cleaved from GST with thrombin was incubated with glutathione beads in the presence of purified GST-Huwe1^C. Cdc6 in the bound (lanes 2–4) and input (10%, lane 1) fractions was detected with anti-Cdc6 antibody; GST- Huwe1^C was detected by Coomassie staining. (G) Nuclear extracts of HeLa cells treated with MG132 for 3 h (lanes 1, 3, and 5) or with MG132 for 3 h after 30 J of UV irradiation (lanes 2, 4, and 6) were digested with micrococcal nuclease to release Cdc6 from chromatin and then subjected to immunoprecipitation with normal rabbit serum (lanes 3 and 4) or anti-Huwe1 antibody (lanes 5 and 6). Endogenous Cdc6 and Huwe1 in the input (1%, lanes 1 and 2) and bound fractions (lanes 3–6) were detected by immunoblotting. (H) Purified GST-Cdc6 (100 ng) was incubated with ubiquitin, E1, and E2 enzymes plus the indicated concentrations of purified Huwe1 for 2 h as described in Materials and Methods. The reaction products were resolved by 6% SDS-PAGE and immunoblotted with anti-Cdc6 antibody.

mRNA. Cells transfected with Huwe1 siRNA showed significant knockdown of endogenous Huwe1 protein after 48 h (Figure 4B, middle panel, compare lanes 1–3 with lanes 4–6). Asynchronous cells transfected with control siRNA

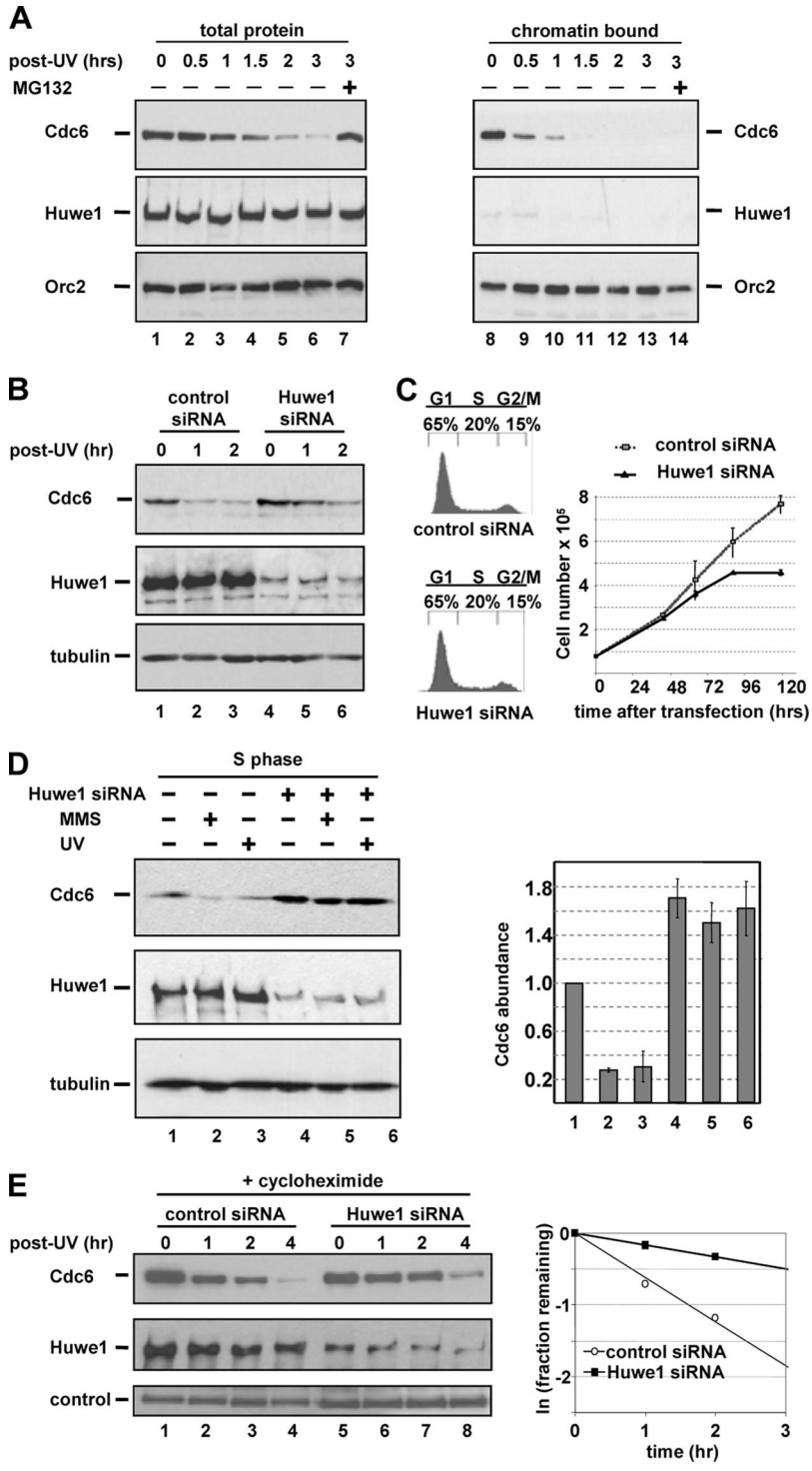

Figure 4. Huwe1 is required for Cdc6 degradation after DNA damage. (A) Asynchronous HeLa cells were irradiated with UV and harvested at the indicated time points. The sample harvested 3 h after UV treatment was duplicated and treated with MG132 immediately after irradiation. Cells were fractionated into whole cell lysates and chromatin bound fractions and probed for endogenous Cdc6, Huwe1, and Orc2. (B) Asynchronous HeLa cells were transfected with Huwe1 siRNA (lanes 4–6) or control siRNA (lanes 1–3) for 48 h. Cells were irradiated with UV as in Figure 1A, and endogenous Cdc6, Huwe1, and tubulin were detected by immunoblotting. (C) Left, HeLa cells treated with control siRNA or Huwe1 siRNA were fixed and stained with propidium iodide for analysis by flow cytometry 41 h after transfection. Right, cell numbers after transfection with control siRNA or Huwe1 siRNA were measured in duplicate cultures counted on 5 consecutive days. (D) HeLa cells were transfected with control siRNA (lanes 1–3) or Huwe1 siRNA (lanes 4–6) for 18 h followed by addition of thymidine for 18 h, for a total of 36 h in siRNA. Cells were treated with 1 mM MMS or 30 J/m^2 UV 3 h before harvest in the continued presence of thymidine. Relative Cdc6 abundance was determined by densitometry and normalized to tubulin with the amount of Cdc6 in the first lane arbitrarily set to 1. (E) Left, HeLa cells were transfected with control siRNA or Huwe1 siRNA and blocked in S phase with thymidine as in Figure 4D. Cells were subjected to UV irradiation, and cycloheximide was immediately added to10 μg/ml in the continued presence of thymidine. Samples were collected at 0, 1, 2, and 4 h after UV treatment. Right, semilog plot of the initial rate of Cdc6 decay. The average half-life of Cdc6 in control-treated cells is 1.2 ($\pm$0.18) h, and in Huwe1 siRNA-treated cells is 4.7 ($\pm$0.8) h.

rapidly degraded Cdc6 after DNA damage induced by UV, but cells with reduced Huwe1 did so considerably less efficiently (Figure 4B, top panel, compare lanes 1–3 with lanes 4–6). This result is similar to the effects of DDB1 knockdown on Cdt1 stability after DNA damage (Figure 2E) in that Huwe1-depleted cells are hypomorphic for Cdc6 degradation.

If reduction of Huwe1 induced a delay or arrest in G2, then Cdc6 levels might simply have been higher as a consequence of an indirect cell cycle effect. However, duplicate cultures treated with Huwe1 siRNA and analyzed by flow cytometry showed no differences in cell cycle distributions (Figure 4C). When we cultured cells lacking Huwe1 for an additional 3 days, we noted a significant proliferation defect, in keeping with reports by others (Adhikary *et al.*, 2005; Figure 4C), but we observed no apparent changes in cell cycle distribution compared with control cultures even at 5 days after transfection (data not shown). The growth arrest is not associated with robust changes in Cdc6 (data not shown), but may be explained by deregulation of other

Huwe1 substrates such as c-myc and Mcl-1 (Adhikary *et al.*, 2005; Chen *et al.*, 2005; Warr *et al.*, 2005; Zhong *et al.*, 2005). We thus conclude that the increased Cdc6 in UV-treated cells as a consequence of the loss of Huwe1 is not a reflection of a cell cycle arrest, but rather a more direct effect on steady-state Cdc6 abundance.

Two-thirds of the asynchronous Huwe1-depleted cells in Figure 4B were in G1 (Figure 4C). Presumably these cells contained active APC, which may have contributed to Cdc6 ubiquitination in the absence of Huwe1. To focus specifically on the APC-independent degradation of Cdc6, we arrested cells in S phase by treatment with thymidine. In thymidine-arrested cells in the absence of DNA damage, Cdc6 is stable because APC in inactive. This assertion is supported by the observation that treatment of S phase cells with the proteasome inhibitor MG132 had no effect on Cdc6 abundance (Petersen *et al.*, 2000 and Figure S3A). In response to DNA damage, however, S phase cells degraded Cdc6 after UV or MMS treatment (Figure 4D, compares lanes 2 and 3 to lane 1). In marked contrast, we observed much less degradation of Cdc6 after DNA damage caused by either UV or MMS in Huwe1-depleted cells (Figure 4D, compare lanes 5 and 6 to lane 4). Reduction of Huwe1 with a different siRNA molecule had the same effect (Supplementary Figure S3B).

It was possible that Huwe1 regulated Cdc6 abundance by indirectly controlling Cdc6 transcription or translation. We occasionally (though not consistently) observed an increase in Cdc6 abundance after Huwe1 depletion even in the absence of exogenous damage (for instance, Figure 4D, compare lanes 1 and 4). The fact that Cdc6 is not normally ubiquitinated in S phase–arrested cells suggests that Huwe1 depletion may have affected Cdc6 levels before the arrest so that they arrived in S phase with more Cdc6. It is also possible that endogenous sources of DNA damage may restrict Cdc6 abundance through Huwe1. Alternatively Huwe1 may regulate Cdc6 synthesis indirectly through transcriptional mechanisms involving other Huwe1 substrates such as c-myc or p53. To distinguish effects of Huwe1-depletion on Cdc6 stability from effects on Cdc6 synthesis, we treated control cells or Huwe1-depleted cells with cycloheximide (CHX) to block new protein synthesis. We monitored the loss of Cdc6 after treatment of thymidine-blocked HeLa cells with both UV irradiation and CHX. In CHX-treated cells, Cdc6 was degraded after UV irradiation (Figure 4E, lanes 1–4), demonstrating that upregulation of UV-induced genes is not required for Cdc6 degradation after DNA damage. Depletion of Huwe1 had no effect on the degradation of Cdt1 (Supplementary Figure S4A) or on the accumulation of p53 (Supplementary Figure S4B), indicating that Huwe1 depletion does not interfere with the DNA damage response. Importantly, Cdc6 persisted in UV-irradiated Huwe1-deficient cells even in the presence of CHX (Figure 4E, compare lanes 2–4 with lanes 6–8). This result demonstrates that Huwe1 is required for Cdc6 degradation per se and that any potential indirect effects of Huwe1 that might have occurred through transcriptional regulation of *cdc6* or other genes cannot account for the persistence of Cdc6 after DNA damage. The defect in Cdc6 degradation was most dramatic in the first hours after irradiation; the remaining Huwe1 was presumably capable of supporting some Cdc6 degradation over the full course of 4 h, though even at this late time point, more Cdc6 persists in Huwe1-deficient cells than in control cells (Figure 4E). During the initial 2 h after UV treatment the half-life of Cdc6 increased from 1.2 h in control cells to 4.7 h in cells with reduced Huwe1 (Figure 4E, compare lanes 4 and 8). Therefore, Huwe1 is required for Cdc6 degradation after DNA damage at times when APC is inactive.

Conservation of Huwe1-dependent Regulation of Cdc6

Huwe1 is an evolutionarily conserved ubiquitin ligase; similar sequences containing not only a C-terminal catalytic domain, but also the two N-terminal conserved domains of unknown function (DUF908 and DUF913, Figure 3A) not present in other HECT family ligases are identifiable in the genomes of plants, flies, worms, and yeast. In the budding yeast *Saccharomyces cerevisiae*, the Huwe1 ortholog is Tom1 for temperature-dependent organization in mitotic nucleus (distinct from the human Tom1 protein, target of Myb1). Yeast Tom1, like Huwe1, has been proposed to have multiple nuclear substrates, and *tom1* mutants are temperature-sensitive for growth at 37°C (Saleh *et al.*, 1998; Utsugi *et al.*, 1999). To test if the Huwe1-dependent degradation of Cdc6 is similarly conserved in budding yeast, we constructed isogenic wild-type and *tom1* deletion strains in which the endogenous *cdc6* gene has been modified to include 13 tandem copies of the myc epitope. Cultures of these strains were grown at 30°C (the permissive temperature), and then treated with MMS. Wild-type yeast cells degraded Cdc6-myc within 60 min, but showed no loss of endogenous yeast Mcm2 or PGK (Figure 5A, lanes 1–3). Deletion of the Huwe1 ortholog, *tom1*, resulted in somewhat increased Cdc6 in

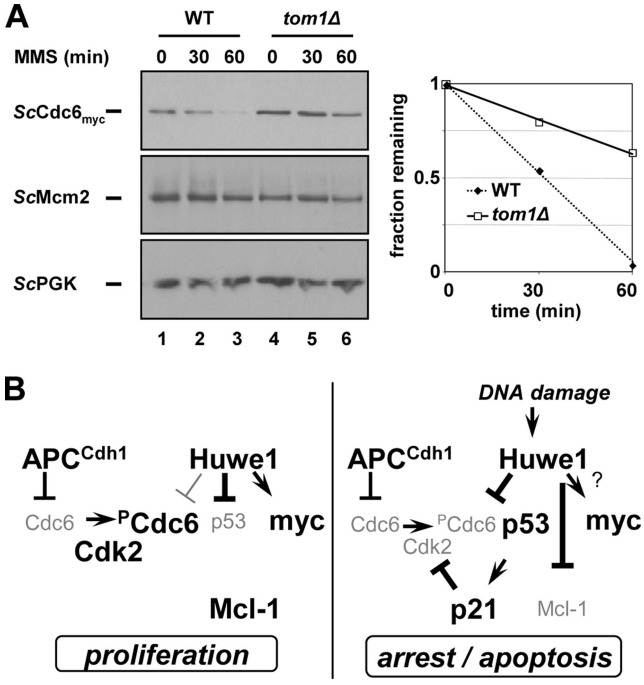

Figure 5. Conservation of Huwe1 regulation of Cdc6. (A) Yeast strains JCY200 WT and JCY201 tom1Δ in which the endogenous Cdc6 locus bears an in-frame insertion of 13 myc epitopes at the 3′ end of the Cdc6 open reading frame were grown in YPD and treated with 0.05% (vol/vol) MMS. Time points were collected following the addition of MMS, and Cdc6 was detected by immunoblotting with the anti-myc antibody; endogenous Mcm2 and phosphoglycerate kinase immunoblots are shown as controls. (B) Model: Cdc6 degradation by APC and Huwe1 after DNA damage contributes to the balance between cell growth and apoptosis. Under normal cell growth conditions, Huwe1 activates c-myc and induces p53 degradation. Cdc6 stability is primarily controlled by APC as cells transit mitosis. In response to DNA damage, p53 is stabilized, but Mcl-1 and Cdc6 are ubiquitinated and degraded. See text for discussion.

naive cells, but importantly, significantly less Cdc6-myc degradation in the presence of MMS (Figure 5A, lanes 4–6).

At the nonpermissive temperature (37°C), *tom1* mutants display a G2/M arrest accompanied by defects in protein synthesis (Utsugi *et al.*, 1999). To rule out effects on Cdc6 abundance due to potential cell cycle changes that might have manifested even at the permissive temperature, we monitored the budding of both wild-type and *tom1* strains grown at 30°C. We found no differences in the relative numbers of unbudded, small-budded, or large-budded cells, and we observed only minor differences in the overall growth rate at 30°C (data not shown). Given these observations, plus the fact that yeast Cdc6 is most abundant in G1 rather than G2 (Piatti *et al.*, 1995; Drury *et al.*, 1997), it seems unlikely that the enhanced levels of Cdc6 were due to roles of Tom1 in G2/M progression or protein synthesis. These results indicate that Tom1 is required for degradation of yeast Cdc6 after DNA damage and suggest that the regulation of Cdc6 abundance by Huwe1-type ubiquitin ligases is conserved.

DISCUSSION

Taken together, the results presented here indicate that Cdc6 ubiquitination induced by both UV irradiation and by DNA alkylation can be carried out by the Huwe1 enzyme. This study complements previous work on the cell cycle– and ionizing radiation–induced ubiquitination of Cdc6 carried out by APC^{Cdh1}(Petersen *et al.*, 2000; Duursma and Agami, 2005; Mailand and Diffley, 2005) The demonstration of two independent ubiquitin ligases for Cdc6 is another example of a common theme in cell cycle regulation, namely multiple overlapping regulatory mechanisms. Presumably these multiple pathways evolved to ensure tight control over essential processes under a variety of conditions.

DNA damage sufficient to induce Cdk2 inhibition can accelerate Cdc6 degradation by inhibiting the protective phosphorylation at Ser54, but this mechanism can only operate when APC is active (Duursma and Agami, 2005). On the other hand, we find that Huwe1 ubiquitination of Cdc6 can occur in S phase, even when Cdc6 has been altered to mimic phosphorylation by Cdk2. Therefore, like Cdt1, which associates with two different ubiquitin ligase complexes, Skp2 and Cul4, (Li *et al.*, 2003; Kondo *et al.*, 2004; Liu *et al.*, 2004; Sugimoto *et al.*, 2004; Takeda *et al.*, 2005; Hu and Xiong, 2006; Senga *et al.*, 2006), Cdc6 is regulated by both APC and Huwe1. Because the human genome contains many thousands of potential origins, these different control pathways may be required to prevent relicensing at even a small percentage of origins. Multiple mechanisms may also need to be in place to adequately respond to a variety of cellular insults.

DNA damage activates an intracellular signaling pathway that culminates in cell cycle checkpoint arrest through inhibition of Cdks (reviewed in Abraham, 2001; Sancar *et al.*, 2004). If the damage occurs during S phase or G2 and is subsequently repaired (instead of inducing apoptosis), then cells could recover with new preRCs on the already duplicated DNA. Even though geminin is not degraded after DNA damage (Higa *et al.*, 2003 and our unpublished observations), the persistence of geminin during a checkpoint response is not sufficient to block substantial preRC reassembly. This assertion is supported by previous findings that direct inhibition of Cdk activity during G2, either pharmacologically or genetically, permits reloading of MCM proteins onto chromatin even in the presence of geminin (Bates *et al.*, 1998; Ballabeni *et al.*, 2004; Zhu *et al.*, 2005). Thus, there is a risk of genome instability if preRCs are permitted to assemble while Cdk activity is inhibited. Presumably to guard against this threat, both Cdc6 and Cdt1 are actively degraded after DNA damage, and we suggest that the degradation during S phase and G2 is particularly important.

The Huwe1-dependent mechanism of Cdc6 degradation after DNA damage caused by UV radiation or MMS is distinct from the APC^{Cdh1}-dependent mechanism of Cdc6 degradation caused by ionizing radiation (IR). Substantial IR-induced Cdc6 degradation is only observed in p53-proficient cells and requires induction of p21 expression (Duursma and Agami, 2005). In contrast, Cdc6 degradation induced by UV radiation, MMS, or adozelesin (another DNA alkylating compound) occurs equally well in both p53-proficient and p53-deficient cells (Figure 2C and Blanchard *et al.*, 2002). The cellular response to IR is primarily mediated by the ATM and Chk2 kinases that are stimulated by double-strand DNA breaks. On the other hand, most other forms of DNA damage, including UV and MMS-induced damage, primarily trigger activation of the ATR and Chk1 kinases. These two kinase cascades are related but distinct in both the signals that trigger their activation and in their primary substrates. It is possible that differences in the kinase pathways that are activated by DNA damage account for the different mechanisms of Cdc6 degradation. In support of a contribution of ATR/ATM checkpoint kinases to Cdc6 degradation, we observe partial stabilization of Cdc6 after DNA damage in the presence of caffeine (Supplementary Figure S5).

Cdc6 is substantially overproduced in a wide variety of cancer cell types. This overproduction results in a longer time needed to eliminate Cdc6 protein from cancer cells experiencing DNA damage than from normal cells, leaving a longer window of opportunity for these cells to assemble preRCs. The abundant Cdc6 that arises from near ubiquitous deregulation of the E2F-RB transcriptional program in cancers could contribute to the observed genomic instability associated with transformation. We note that most cancer cells also have a disrupted p53 pathway that could blunt the APC-mediated mechanism of Cdc6 degradation. Thus Huwe1, which is also highly expressed in multiple tumor cell lines (Chen *et al.*, 2005; Yoon *et al.*, 2005), may represent the primary means of degrading Cdc6 in p53-null cells and may be particularly important in the response to chemotherapies that damage DNA.

Huwe1-depletion in the absence of DNA damage did not result in overt rereplication (data not shown). If the purpose of Cdc6 degradation after DNA damage is to prevent rereplication, then it is likely that in order to observe significant rereplication in these short-term assays, not only would Cdc6 degradation need to be blocked, and perhaps Cdt1 degradation, but Cdk activity would need to be maintained in order to permit origin firing. We suggest however that over the course of many cell cycles the degradation of both Cdc6 and Cdt1 when cells encounter DNA damage contributes to maintaining strict regulation of preRC assembly or to promoting appropriate checkpoint and apoptotic responses. Consistent with this model, modest overproduction of Cdt1 has minimal effects on the growth properties or genome stability in cultured mouse cells, but those cells have a higher propensity for tumorigenesis in vivo (Arentson *et al.*, 2002). We predict that stabilization of Cdc6 would have a similar phenotype, particularly in sensitized backgrounds or in cells subjected to repeated rounds of sublethal DNA damage.

Although most of the other known targets of Huwe1 (p53, Mcl-1, and c-myc) are specific to metazoan species, Huwe1-

dependent regulation of Cdc6 may be ubiquitous in eukaryotes, because both human and yeast cells rely on this conserved enzyme for Cdc6 degradation. Cdc6 is certainly not the only target of Huwe1; Huwe1 is a very large protein with multiple protein–protein interaction domains for binding other proteins (Adhikary *et al.*, 2005; Chen *et al.*, 2005; Warr *et al.*, 2005; Zhong *et al.*, 2005). We propose the following model to accommodate what is currently understood concerning the regulation of human Cdc6 after DNA damage in the context of other recently identified Huwe1 substrates (illustrated in Figure 5B): During normal cell growth, Huwe1 ubiquitinates p53 to induce p53 degradation (Chen *et al.*, 2005) and ubiquitinates and activates c-myc (Adhikary *et al.*, 2005). Huwe1 ubiquitinates the anti-apoptotic Mcl-1 protein but its access to Mcl-1 is not obvious until cells are exposed to DNA damage (Zhong *et al.*, 2005, and Zhong, unpublished observations). Hence, under normal growth conditions cells have active c-myc transcription, low p53, high Mcl-1, and cell cycle–regulated Cdc6 levels that are controlled by a combination of E2F-dependent transcription and APC-mediated degradation, and this combination supports robust proliferation (Figure 5B, left). In response to DNA damage Huwe1 ubiquitinates Cdc6 and Mcl-1 to induce their degradation, but no longer ubiquitinates p53, resulting in low Cdc6 and Mcl-1 with stabilized p53, thus promoting cell cycle arrest, apoptosis, and inhibition of new preRC assembly (Figure 5B, right).

Given the role of Huwe1 in the regulation of Cdc6, p53, c-myc, and Mcl-1, one might speculate that Huwe1 activity is regulated by DNA damage signals. Nevertheless we do not detect changes in Huwe1 protein levels after DNA damage (Figures 3G and 4, A, B, D, and E) or during cell cycle progression (Supplementary Figure S2). Instead, we find that Cdc6 is lost from the chromatin fraction after DNA damage and may have greater access to the soluble Huwe1 under these conditions. The mechanism by which Cdc6 is released from chromatin is not yet known, but clearly does not involve degradation of Orc2 (Figure 4A). It is tempting to speculate that one or more of the DNA damage signaling kinases regulates the chromatin association of Cdc6. This study prompts significant questions regarding the intracellular context of the Huwe1-Cdc6 interaction and the upstream regulators of Huwe1 and/or Cdc6 during a DNA damage response. The regulation of Cdc6 by Huwe1 is likely to be complex, and its elucidation is an important future goal.

Note added in proof. While this manuscript was in review, Matsuoka *et al.* (2007) isolated Huwe1 in a screen for proteins phosphorylated by the ATR and ATM kinases in response to DNA damage.

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