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A Genome-wide Screen Identifies p97 as an Essential Regulator of DNA Damage-Dependent CDT1 Destruction

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SUMMARY

Several proteins, including the replication licensing factor CDT1 and the histone methyltransferase SET8, are targeted for proteolysis during DNA replication and repair by the E3 ubiquitin ligase CRL4^{CDT2}. CRL4^{CDT2} function is coupled to replication and repair because it only ubiquitinates substrates that associate with chromatin-bound PCNA. Here, we report a genome-wide siRNA screen that identifies multiple factors necessary for CDT1 destruction after UV irradiation. Among these, nucleotide excision repair factors promote CDT1 destruction due to a role in recruiting PCNA to damaged DNA. The COP9/Signalosome regulates CDT2 stability through CUL4 deneddylation. Finally, the p97 AAA^{+}-ATPase and its cofactor UFD1 are required for proteasome-dependent removal of ubiquitinated CDT1 and SET8 from chromatin and their subsequent degradation both in vivo and in a *Xenopus* egg extract system in vitro. This study provides insight into and a resource for the further exploration of pathways that promote timely degradation of chromatin-associated CRL4^{CDT2} substrates.

INTRODUCTION

Faithful segregation of the genome during mitosis is reliant upon precise DNA replication during the preceding S phase. An elaborate system of checks and balances has evolved in eukaryotic cells to ensure that genetic material is replicated exactly once in every cell cycle. A critical step in the initiation of DNA replication is the recruitment of the licensing factor CDT1 to origins of replication during G1 by the multisubunit origin recognition complex (ORC). CDT1 "licenses" origins by recruiting the MCM 2-7 helicase that unwinds double-stranded DNA at the start of S phase (reviewed in Arias and Walter, 2007). CDT1 is then destroyed upon initiation of replication in S phase. CDT1 activity is exquisitely controlled to prevent refiring of licensed origins (Arias and Walter, 2007). CDT1 expression in S phase leads to rereplication, aberrant chromosomal ploidy, genomic instability, and tumorigenesis (Jin et al., 2006; Lovejoy et al., 2006; Melixetian et al., 2004; Zhong et al., 2003).

Multiple mechanisms limit CDT1 activity during S phase. First, CDT1 protein abundance is regulated by cullin RING-based E3 ligases (CRLs) that ubiquitinate CDT1 and target it for proteolysis by the 26S proteasome. The best understood E3 for CDT1 is CUL4-DDB1^{CDT2} (CRL4^{CDT2}). Depletion of CRL4^{CDT2} in multiple model systems leads to stabilization of CDT1 in S phase (Arias and Walter, 2007). CRL4^{CDT2} also targets CDT1 in response to DNA damage (Higa et al., 2006; Hu et al., 2004; Jin et al., 2006). CRL4^{CDT2} substrates are ubiquitinated only when they are bound to PCNA via a degron that consists of a unique PCNA-interacting peptide (PIP-box) (Arias and Walter, 2006; Havens and Walter, 2009). Because substrates are only ubiquitinated when PCNA is bound to DNA, this prevents spurious proteolysis of substrates during G1 and couples substrate proteolysis to the onset of S phase (Arias and Walter, 2005). Second, in multicellular eukaryotes, residual CDT1 during S phase is bound to geminin, which inhibits CDT1's ability to load MCM2-7 onto origins of replication (Wohlschlegel et al., 2000). Third, SCF^{SKP2} has been proposed to promote CDT1 turnover via CDK2-dependent CDT1 phosphorylation during S phase in mammals (Nishitani et al., 2006). However, a CDT1 mutant that fails to bind SKP2 is degraded normally in S phase, and *Skp2*$^{-/-}$ fibroblasts do not show altered CDT1 protein abundance (Nishitani et al., 2006). Thus, CRL4^{CDT2} is thought to be the primary E3 ligase for control of CDT1 abundance both during the cell cycle and in response to DNA damage.

With the exception of PCNA and the E3 ligase, little is known concerning the molecular signals that initiate and sustain the CDT1 destruction pathway. Although DNA damage signaling through ATM/ATR is not required for CDT1 destruction, both CDT1 and CDT2 are phosphorylated in response to genotoxic stress (Higa et al., 2003; Matsuoka et al., 2007). The identity of the kinases that modify CDT1 and 2 and the significance of these phosphorylation events for CDT1 ubiquitination are unclear. Studies in *Xenopus* indicate that CDT1 ubiquitination on chromatin is not the rate-limiting step in its proteolysis, implying that there may be distinct regulatory steps that must occur following ubiquitination but before degradation (Arias and Walter, 2005). Importantly, it is unknown if and how the 26S

proteasome is able to access ubiquitinated CDT1 when it is embedded in a complex consisting of PCNA, the $CRL4^{CDT2}$ ligase, and chromatin. Recently, a number of $CRL4^{CDT2}$ substrates have been identified, including p21, SET8, XIC1, and E2F, that rely on PIP-box-dependent ubiquitination (Abbas et al., 2008, 2010; Centore et al., 2010; Jørgensen et al., 2011; Kim et al., 2008, 2010; Oda et al., 2010; Shibutani et al., 2008). It remains to be determined whether these and other $CRL4^{CDT2}$ substrates share additional mechanistic aspects of their destruction pathways.

To address these and related questions, we performed a genome-wide siRNA screen in human cells to identify proteins that regulate CDT1 proteolysis. To our knowledge, a screen for all the genes that orchestrate the turnover of a substrate via the ubiquitin proteasome system has not been reported. In addition, because many of the $CRL4^{CDT2}$ substrates share a unique ubiquitination paradigm, we expected that genes emerging from such a screen might have more general roles in the degradation of other $CRL4^{CDT2}$ targets.

In addition to components that are known to function in CDT1 turnover, including $CRL4^{CDT2}$ and the proteasome, we identified components that function upstream of CDT1 ubiquitination, including the nucleotide excision repair (NER) pathway and the COP9/signalosome (CSN). Among the strongest hits from the screen were the p97 AAA^{+}-ATPase and its cofactor UFD1 (Cdc48p and Ufd1p, respectively, in budding yeast). Cdc48p and the ubiquitin system were first linked when it was found that Cdc48p and Ufd1p are required for degradation of certain soluble ubiquitin-protein fusions in yeast (Ghislain et al., 1996). The best-understood function of p97 is in ER-associated degradation (ERAD) (Vembar and Brodsky, 2008). In this process, misfolded proteins in the ER lumen are extruded into the cytosol and ubiquitinated by ER membrane resident ligases. The ubiquitin binding domains in Ufd1p and members of the UBXD family of proteins in association with p97 bind the ubiquitinated substrate and facilitate extraction via the ER retro-translocation machinery. p97-mediated delivery to the proteasome is coupled to RAD23 function, which also binds ubiquitin chains and the proteasome (Richly et al., 2005). p97 has also been implicated in removal of proteins such as AURORA B kinase from chromatin to facilitate chromatin decondensation and nuclear envelope formation. However, in this case it is not clear whether AURORA B is delivered to the proteasome or recycled after deubiquitination (Ramadan et al., 2007). Recent work suggests a wider role for the p97 and UBXD family proteins in turnover of soluble HIF1α and the chromatin-associated RNA polymerase II protein Rpb1p (Alexandru et al., 2008; Verma et al., 2011).

Functional analysis of the p97 complex revealed an essential role in CDT1 destruction in response to DNA damage. p97, its ATPase activity, and its binding partner, UFD1, are critical for the extraction of ubiquitinated CDT1 from chromatin and subsequent degradation by the proteasome in vivo and in *Xenopus* egg extracts supplemented with damaged DNA. p97 is also required for SET8 degradation on chromatin. Hence, the mechanism we propose here has functional significance in the turnover of other $CRL4^{CDT2}$ substrates that are ubiquitinated and degraded on chromatin.

RESULTS

A Genome-wide siRNA Screen for Genes that Regulate Ubiquitin-Dependent Proteolysis of CDT1

To identify genes that regulate CDT1 turnover, we employed an immunofluorescence-based assay that measures the levels of endogenous nuclear CDT1 in untreated and UV-irradiated HeLa cells (Figures 1A, 1B, and S1). As CDT1 is degraded at the onset of S phase and is absent until the following G1, the assay measures CDT1 abundance in UV-treated G1 cells as well as inhibition of S phase turnover in undamaged cells. In this assay, CDT1 is undetectable within 10 min of UV irradiation, but depletion of components of the $CRL4^{CDT2}$ complex resulted in significant stabilization of CDT1, even 30 min after damage (Figures 1A and S1). To quantify CDT1 levels, we employed an algorithm that measured the number of CDT1-positive cells per field and the average CDT1 signal intensity/cell. Using this assay, an arrayed library of pooled siRNAs targeting ~21,000 genes (4 siRNAs/gene) was screened in triplicate using nontargeting and DDB1 siRNAs as negative and positive controls, respectively. We used the Z score metric (see Supplemental Experimental Procedures) to identify candidate genes in the CDT1 destruction pathway. The mean and standard deviation of Z scores were determined (Figure 1B and Table S1). Genes with a $Z \geq 2$ (~450) were deconvoluted and retested for CDT1 stabilization. From this analysis, ~35% of the genes from the primary screen were validated for the CDT1 stabilization phenotype (at least 2 of 4 siRNA duplexes causing stabilization) (Figure 1D and Table S1). Gene ontology analysis of candidate genes from the primary screen revealed enrichment for pathways involving ubiquitin, proteolysis, phosphorylation, DNA damage, and chromatin modifications, among other categories (Figure 1C).

All known facets of the CDT1 turnover pathway were identified in the primary screen, including (1) three components of the NEDD8-activating enzyme pathway that activates cullins with the ubiquitin-like protein NEDD8 (NAE1, UBC12 and NEDD8), (2) three subunits of the E3 ligase (DDB1, RBX1, and CDT2), and (3) 13 subunits of the 26S proteasome, including core and regulatory subunits (Figures 1E and 2A). We did not identify CUL4A/B from the screen, possibly due to redundancy between the two proteins. Many of these genes were among the strongest candidates to emerge from the screen, with 30%–90% CDT1-positive cells following UV treatment, and for those genes tested further, 90% had three or four siRNAs scoring in the deconvolution analysis (Table S1).

The NER Pathway Is Required to Promote CDT1 Destruction in Response to UV

In addition to the genes described above, the screen identified three genes known to participate in the repair of UV-induced photo-adducts: XPA, ERCC4 (XPF), and ERCC5 (XPG). Sequential recruitment of NER factors to UV lesions culminates in the excision of a ~30 bp fragment surrounding the lesion and loading of PCNA and polymerase δ for synthesis across the gap (Figure 1E) (Fousteri and Mullenders, 2008). Based on prior studies, the simplest interpretation of these results is that depletion of NER factors interferes with the repair events that normally culminate in the PCNA-dependent gap-filling reaction

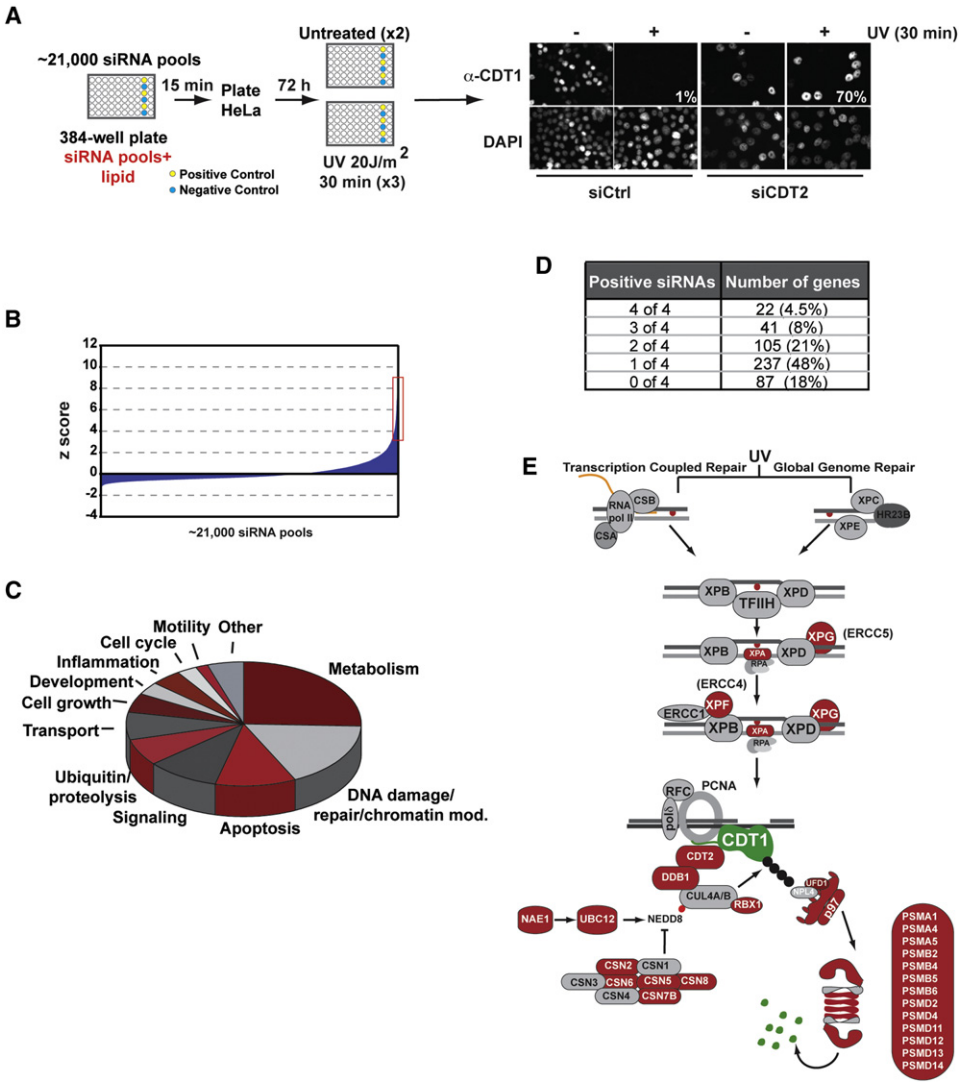

Figure 1. A Genome-wide siRNA Screen Identifies Genes Required for the DNA Damage-Dependent Turnover of CDT1

(A) Screen overview.

(B) Distribution of Z scores for genes tested in primary screen. Red box: genes with Z scores ≥ 3.

(C) Gene ontology-based classification of candidate genes from the primary screen.

(D) Results of deconvolution of siRNA pools from primary screen.

(E) Schematic representation of the pathway that signals CDT1 degradation. Red box: genes whose depletion led to a Z score ≥ 3. See Figure S1.

(Fousteri and Mullenders, 2008). Because CDT1 is only ubiquitinated in the context of chromatin-bound PCNA, the inability to load PCNA onto damaged DNA prevents CDT1 degradation by $CUL4^{CDT2}$. We tested this hypothesis by first depleting seven core genes in the NER pathway (ERCC1-5, XPA, and XPC) and determining the effects on UV-dependent CDT1 turnover. Depletion of ERCC1, 3, 4, 5, XPA, and XPC led to a significant increase in CDT1 abundance upon UV treatment with multiple siRNAs, paralleling the extent of depletion. Depletion of XPA and ERCC5 led to stabilization in a turnover experiment (Figures 2B–2E, S2A, and S2F).

To confirm that CDT1 stabilization was due to defects in PCNA loading, we asked if PCNA binding to chromatin was compromised in NER-depleted cells. As expected, PCNA was enriched in the chromatin fraction after UV irradiation of cells transfected with control siRNA. However, in ERCC4 or XPA-depleted cells, no such enrichment was observed (Figure S2C and data not shown). In addition, we used a micropore UV irradiation assay to create localized UV damage within nuclei (Moné et al., 2001), and the resulting DNA lesions were visualized using antibodies that recognize cyclobutane pyrimidine dimers (CPDs). We found strong colocalization between CPD and PCNA after UV treatment (Figure S2D). However, depletion of XPA resulted in a dramatic reduction in the number of PCNA-positive CPD foci, as expected (Figure S2E) (Miura and Sasaki, 1996). Importantly, NER factors appear to regulate CDT1 turnover only in

response to UV lesions, as depletion of these factors did not interfere with CDT1 turnover when cells were treated with ionizing irradiation (IR) (Figure S2B). Together, these results suggest that PCNA loading onto sites of UV damage by the NER pathway is important to promote CDT1 ubiquitination and turnover (Figure 1E). Because DNA-bound PCNA is essential for recruitment of many $CRL4^{CDT2}$ substrates, NER likely represents a general pathway to create the chromatin context that signals substrate ubiquitination.

Depletion of the CSN Destabilizes CDT2 and Inhibits CDT1 Turnover

The CSN complex contains the CSN5 protein, a zinc-isopeptidase that functions to remove NEDD8 from cullins, thereby rendering CRLs inactive (Cope and Deshaies, 2003). Thus, depletion of CSN components would be expected to result in hyper-neddylation of CRLs. As such, it was unanticipated that 5 of 8 CSN subunits emerged from our screen (Figures 1E and 2F and Table S1), given that hyper-neddylated $CRL4^{CDT2}$ would be expected a priori to maintain activity toward CDT1. We therefore examined the possibility that hyper-neddylation leads to loss of the CDT2 subunit of the $CRL4^{CDT2}$ complex via a process known as adaptor instability (Cope and Deshaies, 2006; Wee et al., 2005) (Figure 2I). CSN5 and 6 were depleted with multiple siRNAs, and the levels of CDT1 and CDT2 were monitored by immunoblotting. Strikingly, all siRNAs tested resulted in robust stabilization of CDT1, CUL4A hyper-neddylation, and a concomitant loss of CDT2 (Figures 2G–2I). Hence, CSN-mediated deneddylation of CUL4 plays a crucial role in maintaining steady-state abundance of CDT2.

p97 AAA^+-ATPase and Its Cofactor UFD1 Are Required for CDT1 Turnover

Among the genes displaying the strongest CDT1 stabilization in our screen (~60% CDT1-positive cells) was the AAA^+-ATPase p97. Depletion of UFD1, a p97 cofactor, also stabilized CDT1 after UV treatment in the screen, albeit to a slightly lesser extent (Table S1). Depletion of p97 via multiple siRNAs led to CDT1 stabilization that generally correlated with the depletion levels (Figures 3A and S3). CDT1 stabilization in p97-depleted cells occurred solely in the nucleus, ruling out an indirect effect through ERAD inhibition (Figure 3B).

As further validation, we generated a HeLa cell line that stably expressed near-endogenous levels of CDT1-GFP and confirmed that regulation occurred in a manner analogous to endogenous CDT1 (Figure S3). Depletion of p97 or UFD1 in these cells interfered with the turnover of CDT1-GFP after UV irradiation (Figure 3C). Although UFD1 functions as an obligate heterodimer with NPL4, NPL4 was not identified in the screen (Meyer et al., 2002). Nevertheless, we asked if depletion of NPL4 affected CDT1 destruction. We observed minimal CDT1-GFP stabilization in NPL4-depleted cells, indicating that p97 and UFD1 were primarily responsible for turnover of CDT1 (Figures 3C and 3F). While these results are consistent with recent studies suggesting differential requirements for UFD1 and NPL4 for specific substrates, we cannot completely rule out a role for NPL4 in CDT1 destruction (Beskow et al., 2009; Heo et al., 2010).

We rescued the p97 depletion phenotype by expressing Myc-p97 that was resistant to the most potent siRNA (si#6 in Figure S3). Transfection of HeLa cells or the CDT1-GFP line with the rescue construct in the background of p97 depletion reinstated UV-induced CDT1 degradation (Figures 3D and 3E). All subsequent studies involving p97 knockdown were carried out with the rescued siRNA.

Interestingly, depletion of p97 consistently led to stabilization of CDT1 in untreated cells (Figure 3A), implying that loss of p97 interfered with S phase turnover of CDT1. Hence, we asked if p97 depletion led to stabilization of CDT1 levels in S phase. However, p97 depletion (~72 hr) resulted in partial accumulation of cells at the G2/M phase of the cell cycle (data not shown). Hence, we were unable to determine conclusively if p97 was required for CDT1 turnover during S phase. To rule out that the stabilization of CDT1 after UV irradiation seen in p97-depleted cells was due to cell-cycle effects, we synchronized cells to the G1/S boundary (Figure 4A) and observed that loss of p97 or UFD1 in early S phase cells that were UV irradiated led to a delay in CDT1 destruction (Figures 4B–4F). The partial CDT1 stabilization observed in p97-depleted, G1/S synchronized cells is in part attributable to incomplete depletion of p97 in this experimental setting, as compared with asynchronous cells (Figure S3D). All subsequent experiments involving p97 knockdown were conducted in G1/S synchronized cells. Once again, loss of NPL4 resulted in very subtle CDT1 stabilization, consistent with our previous results (Figures 4D and 4F). Taken together, these data indicate a prominent role for p97-UFD1 in regulating CDT1 turnover in response to DNA damage.

p97 and UFD1 Function Downstream of CDT1 Recruitment to Chromatin

We next addressed how p97 impinges on the CDT1 turnover pathway. Based on the ability of p97 and UFD1 to bind ubiquitinated substrates, we reasoned that p97 complexes may function downstream of CDT1 ubiquitination on chromatin to extract CDT1 for delivery to the proteasome. We first examined the localization of CDT1 to chromatin under various conditions. In control samples, UV irradiation led to rapid loss of chromatin-bound CDT1 (Figure 5A), whereas depletion of CDT2 led to a dramatic accumulation of CDT1 on chromatin, presumably because CDT1 bound PCNA but was not degraded (Figure 5A). Indeed, PCNA depletion resulted in CDT1 stabilization, but only in the soluble fraction (Figure 5A). Consistent with a role for p97 and UFD1 in mobilizing CDT1 from chromatin after ubiquitination, their depletion resulted in the accumulation of CDT1 on chromatin in UV-treated cells (Figures 5B, lanes 7 and 8, and 5C lanes 3–6). Importantly, depletion of p97 did not interfere with recruitment of the CDT2 ligase to chromatin (Figure 5B). Depletion of PSMB5, a catalytic subunit of the proteasome, led to overt stabilization of CDT1 on chromatin, implying that removal of ubiquitinated CDT1 from chromatin is coupled to proteasomal degradation (Figure 5B, lane 6) (see Discussion). Interestingly, PSMB5 depletion also resulted in stabilization of a small fraction of CDT1 in the nucleoplasm, suggesting that p97 activity in these cells may be extracting CDT1 from chromatin (see below). We find that a fraction of p97-UFD1/NPL4 (hereafter referred to p97-UN) is found associated with chromatin and that total levels of the complex do not change on chromatin during the cell cycle (Figure S4). To test if p97 and UFD1 were involved in CDT1

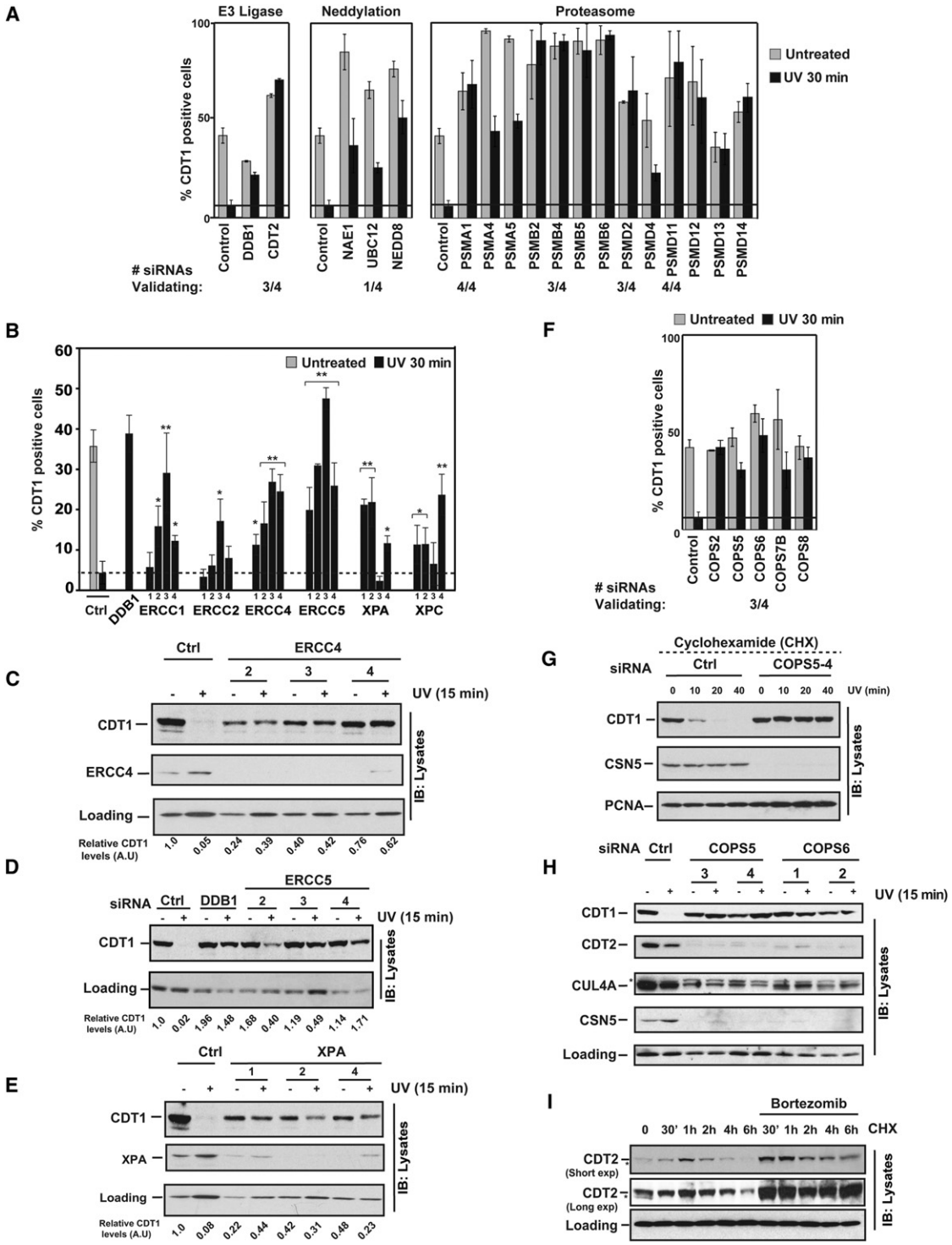

Figure 2. Identification of Elements Upstream of CDT1 Ubiquitination on DNA

(A) Unbiased identification of known elements in CDT1 degradation from the siRNA screen. Error bars represent standard deviation from triplicate observations.

(B–E) Depletion of NER genes results in stabilization of CDT1 after DNA damage. In (B), genes in the NER pathway were depleted with four siRNAs per gene, UV treated and stained for CDT1. Percent CDT1 positive cells is shown. Error bars represent standard deviation from triplicate observations (*$p \leq 0.05$ and **$p \leq 0.01$, respectively, determined by Student's t test). Shown in (C)–(E) is an immunoblot of HeLa cell extracts depleted of NER genes and probed for CDT1 post-UV treatment. CDT1 levels were quantified and normalized to loading control.

(F and G) Loss of CSN5/6 leads to CDT1 stabilization in response to UV treatment. Error bars represent standard deviation from triplicate observations.

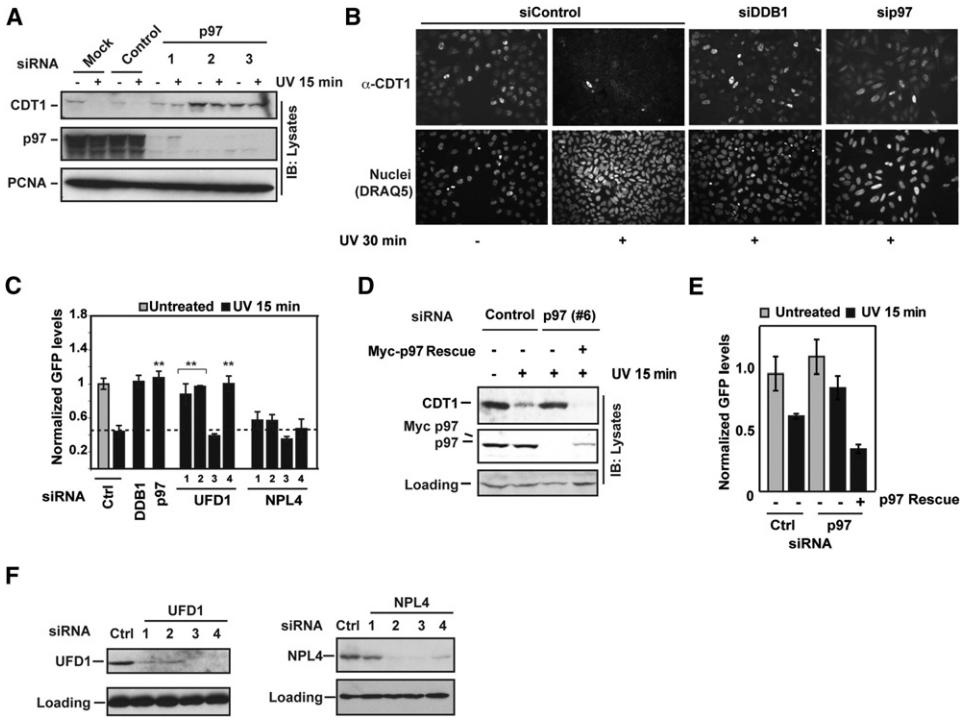

Figure 3. The p97 AAA$^+$-ATPase and Its Cofactor UFD1 Are Required for the Destruction of CDT1

(A–C) Depletion of the p97 complex leads to CDT1 stabilization in response to UV damage. UV-irradiated cell extracts from p97-depleted samples were probed for CDT1 levels (A). Loss of p97 leads to stabilization of CDT1 in the nucleus (B). p97-UN were depleted with multiple siRNAs in the CDT1-GFP HeLa line (C). GFP levels were measured using flow cytometry. Error bars represent standard deviation from triplicate observations (*$p \leq 0.05$ and **$p \leq 0.01$, respectively, determined by Student's t test).

(D and E) Transfection of siRNA-resistant p97 cDNA rescues UV-dependent CDT1 destruction in HeLa cells (D) and in the CDT1-GFP stable cell line (E). Error bars represent standard deviation from triplicate observations.

(F) Extent of depletion of UFD1 and NPL4. See Figure S3.

turnover in response to other forms of DNA damage, we treated cells with ionizing radiation. Loss of p97 or UFD1 led to CDT1 stabilization on chromatin in response to IR, suggesting a general regulation of CDT1 by this complex (Figure 5D).

The ATPase Activity of p97 Is Required for CDT1 Turnover

The ability of p97 to hydrolyze ATP is central to its biochemical function as a "segregase" to extract substrates from multiprotein complexes. We sought to determine if the catalytic activity of p97 was required for CDT1 turnover. Each p97 monomer has three domains connected by conserved linkers. An N-terminal ubiquitin-binding domain is followed by two AAA$^+$-ATPase domains (D1 and D2) that harbor conserved residues required for the binding (Walker A) and hydrolysis (Walker B) of ATP (Figure 5E) (DeLaBarre et al., 2006).

We asked if p97 Walker A/B mutants behaved in a dominant-negative manner when expressed in cells. We modified a fluorescence-based in-cell assay used previously to measure ERAD impairment by p97 mutants (DeLaBarre et al., 2006). The CDT1-GFP reporter line was transfected with Myc-p97 wild-type, Walker A (K251A, K524A), or Walker B (E305Q, E578Q) constructs. Transfected cells were detectable due to an IRES-CD4 cell surface marker in the p97 expression cassette. Dual color flow cytometry measured levels of CDT1-GFP in the p97 (CD4$^+$)-transfected population. The p97 D2 mutants (K524A or E578Q) caused significant stabilization of CDT1 after UV irradiation compared to cells expressing wild-type p97 (Figure 5E). D1 mutants did not interfere with CDT1 degradation to the same extent, consistent with the current understanding of the relative contributions of the D1 and D2 domains in ATP hydrolysis (data not shown). We also performed rescue experiments with the D2 mutants in the background of p97 depletion. HeLa cells depleted of p97 were transfected with siRNA-resistant wild-type p97 or D2 mutants. As before, the CDT1 stabilization defect on chromatin was significantly rescued by the p97 wild-type rescue construct. In contrast, the E578Q mutant failed to restore CDT1 turnover by UV, and CDT1 accumulated on chromatin (Figure 5F). We saw weaker CDT1 stabilization with the K524A mutant; however, prior studies have reported that expression of this mutant is toxic to

(H) Loss of CSN5/6 leads to CUL4 hyper-neddylation and loss of CDT2. Loss of CSN5 also destabilizes CSN6.

(I) CDT2 half-life measurement. *: nonphosphorylated CDT2. The CDT2 antibody more efficiently recognizes the phosphorylated form of the protein, giving rise to the apparent increase in CDT2 levels at 1 hr. See Figure S2.

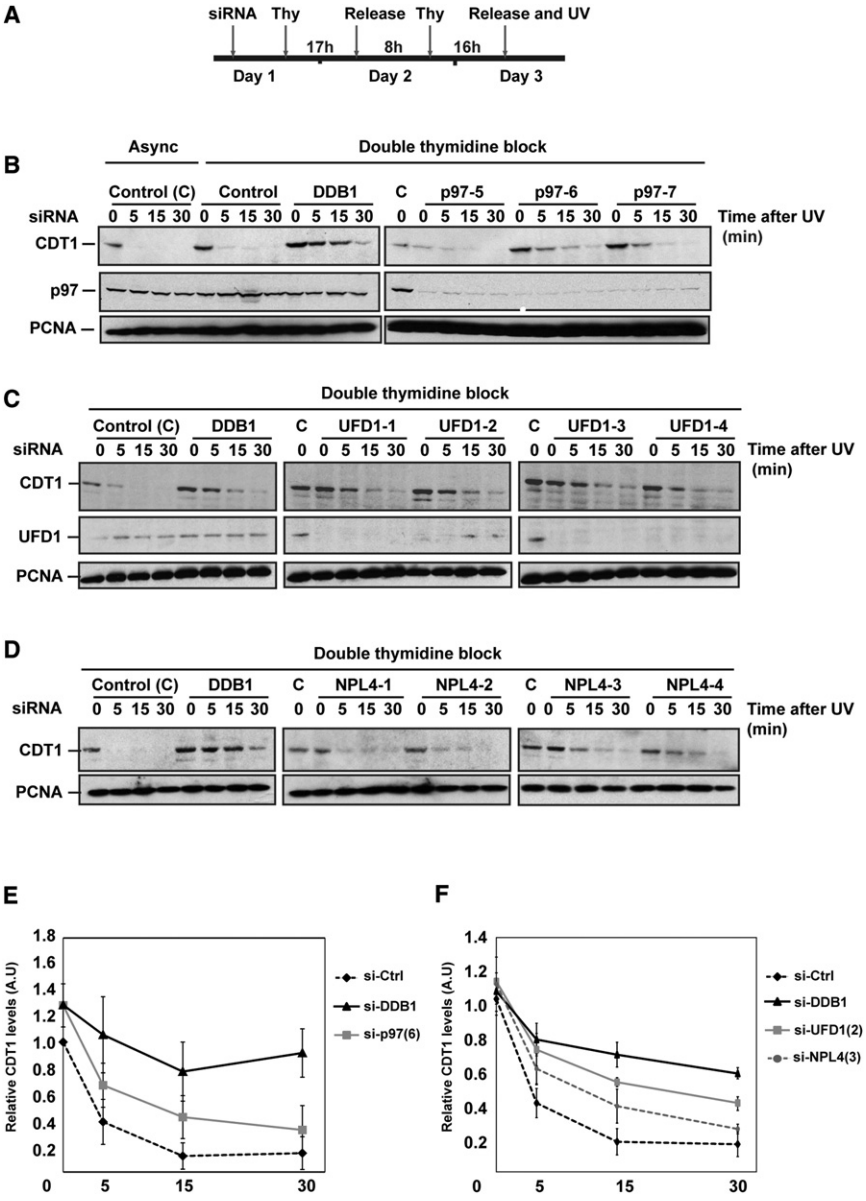

Figure 4. Depletion of p97 Stabilizes CDT1 in G1/S-Arrested, UV-Treated Cells

(A) Synchronization protocol to arrest cells at the G1/S transition using double thymidine block.

(B–D) HeLa cells were transfected with siRNAs targeting p97 (B), UFD1 (C), NPL4 (D), or DDB1 as a control and arrested at the G1/S transition. Upon release into S phase, cells were UV treated and extracts from the indicated time points were immunoblotted.

(E and F) Quantitation of CDT1 levels with the indicated siRNAs. Error bars represent standard deviation from triplicate observations.

cells (DeLaBarre et al., 2006). In our assay this was exacerbated by p97 depletion. Hence, the weaker CDT1 stabilization phenotype we observe with this mutant may be partially attributable to cellular toxicity. Together, these results indicate that ATP hydrolysis by p97 plays a critical role in CDT1 turnover downstream of chromatin recruitment.

p97 Is Required for the Turnover of the CRL4^{CDT2} Substrate SET8

The data thus far are consistent with a role for p97-UN downstream of CDT1 recruitment to chromatin-bound PCNA. We next examined if p97 participates in the destruction of another CRL4^{CDT2} target, the histone methyltransferase SET8. SET8 is degraded on chromatin in a PCNA-dependent manner after UV irradiation (Figure 5G). In p97-depleted cells, we observed stabilization of SET8 on chromatin after UV irradiation (Figure 5H). Strikingly, in the absence of p97, much of the SET8 migrated more slowly upon SDS-PAGE, consistent with polyubiquitination (Figure 5H). Expression of siRNA-resistant p97 in p97-depleted cells reinstated SET8 degradation after UV treatment, while expression of the D2 mutants did not (Figure 5H and data not shown). These results indicate that p97 complexes function in the turnover of multiple targets of the CRL4^{CDT2} pathway downstream of their ubiquitination. Thus far, we have been unable to detect CDT1 polyubiquitination on chromatin in the absence of p97, likely reflecting robust deubiquitinating activity that does not affect SET8 to the same extent.

p97 Functions on In Vitro Assembled DNA Templates to Promote Degradation of Ubiquitinated CDT1

To explore the mechanism by which the p97 complex regulates CDT1 proteolysis by the 26S proteasome, we turned to a biochemically tractable system employing high-speed supernatants (HSS) from *Xenopus* egg extracts (Arias and Walter, 2006). Addition of an immobilized methyl methanesulfonate-treated DNA (MMS-DNA) template to HSS triggers PCNA loading, CDT1 ubiquitination by CRL4^{CDT2}, and its subsequent degradation by the proteasome (Figure 6A). In addition, steady-state levels of ubiquitinated CDT1 can be observed upon purification of immobilized MMS-DNA from extracts (Havens and Walter, 2009) (Figure 6A).

We first asked if p97 was required for DNA damage-dependent degradation of CDT1 in HSS. p97 was depleted from egg extracts with a validated p97 antibody (Heubes and Stemmann, 2007). The high concentration of p97 in egg extract (~3 μM) precluded complete depletion; we estimate that about 3%–5% remained in our Δp97 extract (Figure 6C). Depletion of p97 resulted in almost complete codepletion of UFD1 and NPL4

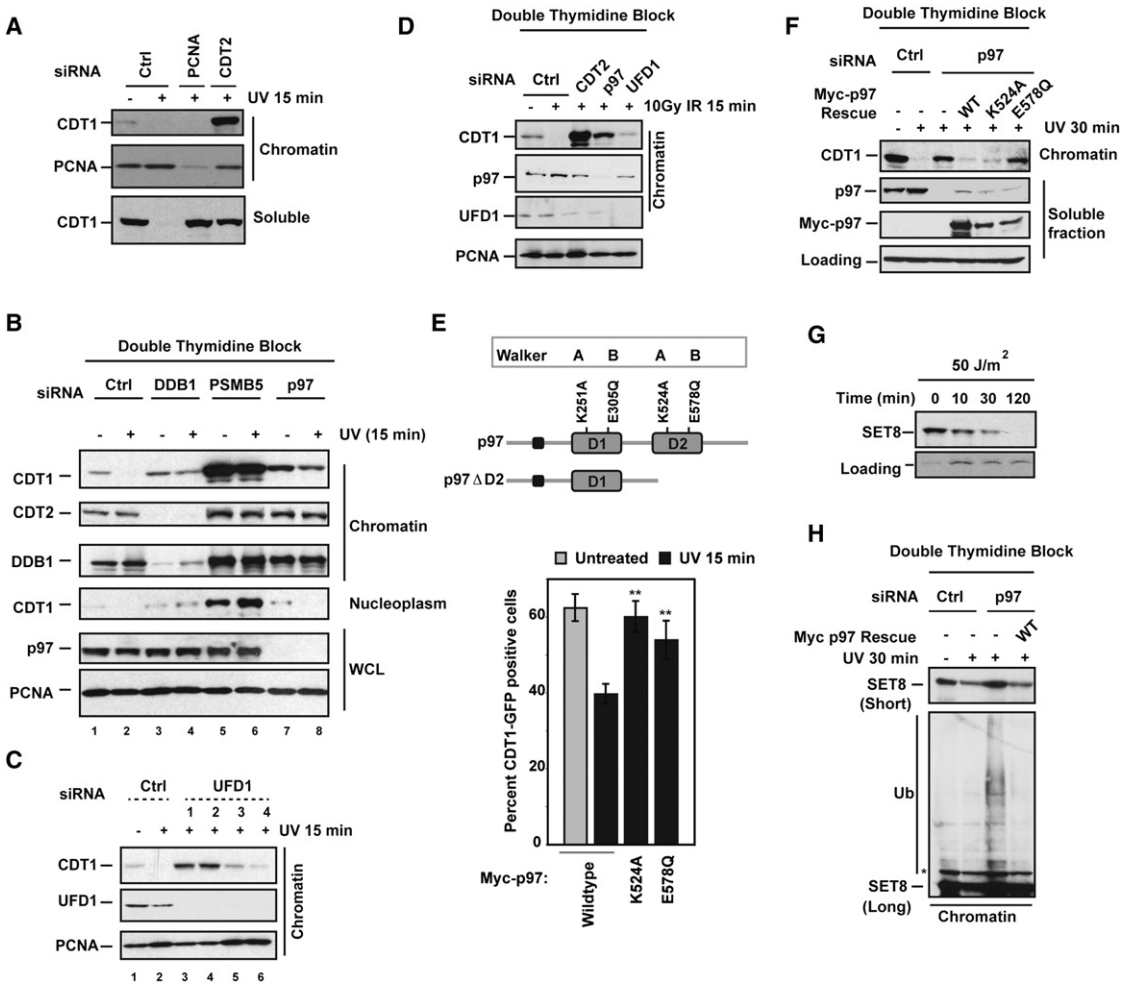

Figure 5. ATPase Activity of p97 Promotes Removal of CDT1 from Chromatin in Response to DNA Damage

(A) Requirement of PCNA in loading CDT1 on chromatin after DNA damage.

(B and C) HeLa cells were transfected with the indicated siRNAs, irradiated, and fractionated. Levels of chromatin-bound CDT1 and p97 complexes were determined by immunoblotting the indicated fractions.

(D) p97 and UFD1 are required for CDT1 turnover in response to IR.

(E) The catalytic activity of p97 is required for CDT1 destruction. Upper panel: schematic of p97 domains, mutants, and deletion constructs. Wild-type or catalytically inactive p97 was transfected into CDT1-GFP cells and UV treated. CDT1-GFP was measured by flow cytometry. Error bars represent standard deviation from triplicate observations. **$p \leq 0.05$, determined by Student's t test.

(F) p97 D2 mutants fail to rescue CDT1 degradation by UV.

(G) Time course of SET8 degradation by UV.

(H) p97 is required for SET8 degradation by UV. The samples used in (E) were probed for SET8. * nonspecific band.

(Figure 6C). In p97-depleted HSS supplemented with MMS-DNA, CDT1 was not degraded after 30 min, while its levels decreased substantially in mock-depleted extracts (Figure 6C, compare lanes 1–4 to lanes 5–8). To ensure that the CDT1 stabilization observed was specific to p97-UN depletion, recombinant human p97-UN purified from insect cells (Figure 6B) was added back to depleted HSS (~150 nM purified complex). p97-UN addition to depleted HSS restored CDT1 degradation, whereas a mutant that lacks the D2 domain (p97ΔD2-UN) did not (Figure 6C, lanes 9–12 and 13–16).

To test if p97 functions downstream of CDT1 ubiquitination, we developed an HSS-based approach in which turnover of CDT1 on purified MMS-DNA could be monitored (Figure 6A). Briefly, the fraction of CDT1 that is ubiquitinated on MMS DNA can be recovered upon centrifugation of HSS through a sucrose cushion to remove soluble proteins. Proteomic analysis of purified MMS-DNA revealed the presence of 20 of 28 proteasome subunits, p97, UFD1, and PCNA (Table S3), suggesting that the degradation machinery may be available for use in CDT1 turnover. Consistent with this, CDT1-ubiquitin conjugates decreased over time on MMS-DNA in an ATP-dependent manner (Figure 6D, lanes 1–4, and data not shown). However, when p97-depleted HSS was employed, the rate of conjugate loss was substantially reduced (Figure 6D, lanes 5–8).

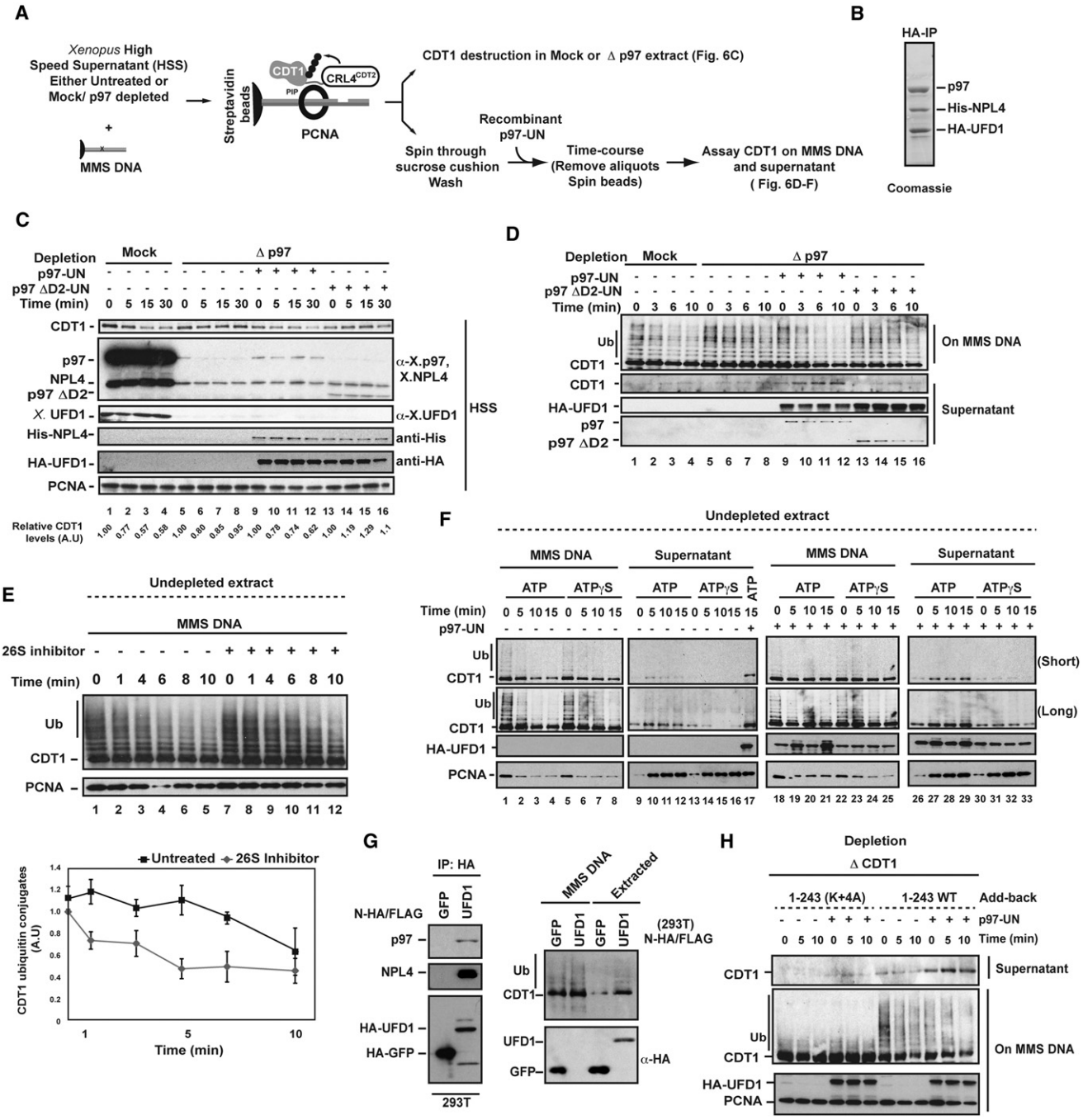

Figure 6. p97-UN and the 26S Proteasome Function on Damaged DNA to Degrade CDT1

(A) Schematic of CDT1 turnover assays in *Xenopus* egg extracts.

(B) Coomassie of Sf9-derived human p97-UN.

(C) MMS-DNA was incubated with mock or p97-depleted extracts. Sf9-derived human p97-UN or p97ΔD2-UN (150 nM) was added back to depleted extract (compared with ~3 μM p97 for undepleted extracts). Note that the immunoblot signal for endogenous and recombinant p97 complexes cannot be compared as the antibodies used here against *Xenopus* p97 do not react as efficiently with the human protein. CDT1 destruction over time in total extract is shown. CDT1 levels were quantified. Each lane was normalized to time 0 for the relevant set.

(D) MMS-DNA was incubated with mock or Δp97 extracts and purified. Sf9-purified p97-UN or p97ΔD2-UN was added to purified MMS-DNA. Aliquots were withdrawn over time and centrifuged, and the levels of CDT1 on MMS-DNA and in the supernatant were determined.

(E) MMS-DNA was incubated with HSS, purified, and pretreated with proteasome inhibitors (bortezomib and epoxomycin) for 5 min. Reactions were then started and aliquots withdrawn and centrifuged; the level of CDT1 on DNA was determined by immunoblotting. Ubiquitin conjugates on CDT1 were quantified using ImageJ and normalized to unmodified CDT1. Error bars represent standard deviation from triplicate observations.

Importantly, addition of p97-UN to MMS-DNA complexes isolated from Δp97 HSS resulted in loss of ubiquitinated CDT1 from DNA, whereas in samples where the p97ΔD2-UN mutant was added, ubiquitinated CDT1 persisted on chromatin (Figure 6D, lanes 9–12 and 13–16).

To examine the contribution of the proteasome, CDT1 turnover reactions were performed on purified MMS-DNA in the presence of proteasome inhibitors. MMS-DNA was incubated with HSS, purified, and then treated with proteasome inhibitors to inactivate the chymotrypsin-like proteolytic sites in the 20S core particle prior to initiation of turnover by ATP addition (Figure 6E). Proteasome inhibition markedly delayed the loss of polyubiquitin chains on CDT1 (Figure 6E, lanes 7–12).

These studies indicate that the catalytic activity of p97 is required in vitro for turnover of CDT1 downstream of its ubiquitination on damaged DNA.

p97-UN Extracts CDT1 from Chromatin in an ATP-Dependent Manner In Vitro

The proposed "segregase" activity of p97 led us to ask whether p97 might promote removal of CDT1 from MMS-DNA independently of degradation. To examine this possibility, supernatants from CDT1 turnover assays on purified MMS-DNA were removed, and the levels of CDT1 on MMS-DNA and in the supernatant were determined by immunoblotting. In samples containing buffer only, CDT1 remained on MMS-DNA, and trace amounts were recovered in the extracted supernatant fraction (Figure 6F, lanes 1–4, 9–12). Addition of p97-UN reproducibly resulted in the release of ~20% of the CDT1 into the supernatant within 5 min (Figure 6F, lanes 26–29). However, when the reaction was carried out in the presence of a nonhydrolyzable ATP analog (ATPγS), p97 did not extract CDT1 (Figure 6F, lanes 30–33). In contrast, PCNA and RPA dissociated from MMS-DNA in a p97-UN- and ATP-independent manner (Figure 6F and data not shown). The p97 ΔD2-UN mutant was unable to promote extraction compared to wild-type p97 complexes (Figure 6D, compare CDT1 supernatant fraction in lanes 9–12 to 13–16). We also performed CDT1 extraction assays using p97-UN complexes purified from a stable HA-UFD1 293T cell line (Figure 6G). The mammalian p97-UN complex extracted CDT1 from MMS-DNA, whereas HA-GFP, used as a control, did not (Figure 6H). Finally, we asked if p97-dependent extraction of CDT1 on MMS-treated DNA required CDT1 ubiquitination, thereby reflecting the presumed role of ubiquitin binding in the recognition of CDT1 by the p97 complex. We depleted endogenous CDT1 from HSS and added back either wild-type CDT1 or a PIP-box mutant that interacts with PCNA but fails to recruit CDT2 and is no longer ubiquitinated (CDT1 K + 4A) (Havens and Walter, 2009). While p97-UN efficiently extracted wild-type CDT1 from MMS-DNA, it was unable to extract the K + 4A mutant (Figure 6H). As the small amount of CDT1 released from DNA made it difficult to observe its ubiquitinated forms, we performed extraction assays in the presence of the deubiquitinating enzyme inhibitor ubiquitin aldehyde (Figure S5A). Under these conditions, ubiquitin conjugates on extracted CDT1 were more apparent. Given the presence of robust deubiquitinating activity on MMS DNA (discerned by incorporation of HA-ubiquitin vinylsulfone into MMS-DNA-bound *Xenopus* proteins) (Figure S5B), it is likely that removal of ubiquitin during the assay is responsible for the apparent enrichment in unmodified CDT1 in the soluble fraction.

Taken together, our studies suggest that the ATPase activity of p97 is required for CDT1 extraction from protein-DNA complexes for proteasomal destruction.

DISCUSSION

A Genome-wide siRNA Screen Uncovers Sequential Steps in the CDT1 Destruction Pathway

The majority of studies examining proteasomal turnover of ubiquitinated proteins focus on two central steps: the ubiquitination event itself and degradation by the proteasome. Typically, the ubiquitination event is regulated by the generation of a degron on the substrate, which serves as a binding site for a cognate E3. However, ubiquitination and turnover events take place in an extended biological network, wherein an upstream regulatory pathway several steps removed from the degron-producing event may be responsible for determining the timing of ubiquitination. An example is the ubiquitination of the Sic1p Cdk inhibitor in budding yeast, where a threshold of G1 Cdk activity is required to mark phosphodegrons on Sic1 for recognition by SCF^{Cdc4p} (Bai et al., 1996). The activity and abundance of G1 cyclins is in turn regulated by transcriptional circuits that are linked to multiple sensors of nutrient availability. Moreover, there may be regulatory inputs into the ubiquitination machinery that function independently of degron-producing signals. Finally, it is becoming clear that there are diverse pathways that may be used to deliver ubiquitinated proteins to the proteasome. A systematic unbiased approach is therefore required for the complete understanding of the pathways that impinge on turnover of a given protein. We applied such an approach to identify genes required for DNA damage-dependent turnover of CDT1.

Our screen identified more than 150 genes whose depletion led to significant stabilization of CDT1 in response to UV (Figure 1). In addition to known components of the $CRL4^{CDT2}$ pathway and the proteasome, we also identified candidate upstream and downstream regulatory components. In particular, we demonstrate that the NER machinery is required for CDT1 turnover by regulating PCNA recruitment to chromatin (Figure 2). We provide evidence that CDT2 abundance is exquisitely sensitive to CSN-mediated cycles of CUL4 neddylation and deneddylation. Finally, as discussed below, our screen also

(F) *Xenopus* egg extract was incubated with MMS-DNA, and the beads were recovered and incubated with buffer alone or p97-UN in the presence of ATP or ATPγS. The beads (MMS-DNA) and supernatants (Extracted) were probed for the indicated proteins. For direct comparison of p97 extraction versus buffer alone, lane 17 is identical to lane 29.

(G) The mammalian p97-UN complex or control GFP was isolated from 293T cells stably expressing HA-FLAG-UFD1 or GFP. MMS-DNA (processed as in F) was incubated with 293T-derived p97-UN or GFP for 15 min. CDT1 levels were determined by immunoblotting.

(H) HSS was depleted of CDT1 and reconstituted with a CDT1(1-243), either wild-type or a PIP-box mutant (K + 4A). Samples were processed as in (F).

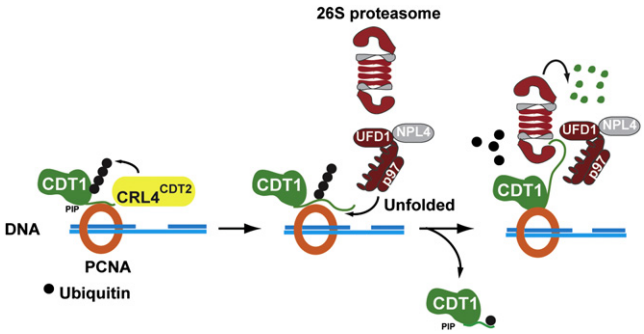

Figure 7. A Model for p97-Mediated Degradation of CDT1 on Damaged DNA
See text for details.

identified components of the p97 complex. Thus far, only a small fraction of the proteins identified with multiple siRNAs in our screen have been mechanistically examined for their roles on CDT1 turnover. Moreover, a number of known components in the pathway were identified with single siRNAs causing CDT1 stabilization (Table S1). Thus, the screen provides a useful resource for further decoding of additional pathways important for damage-dependent turnover of $CRL4^{CDT2}$ targets.

CDT1 and SET8 Turnover Requires p97 Activity

p97 was among the strongest candidate genes identified in our screen, producing a level of CDT1 stabilization that was equal to that observed with DDB1 depletion. The p97 cofactor UFD1 was also required for efficient CDT1 turnover following UV irradiation. Depletion of p97, UFD1, or PSMB5 led to accumulation of CDT1 on chromatin after UV, suggesting that loss of these components blocks removal of CDT1 from chromatin. However, the extent of accumulation of CDT1 on chromatin upon p97 depletion was not as high as that observed with PSMB5, suggesting that there may be a p97-independent pathway for removal of a portion of CDT1 from chromatin, or that residual p97 on chromatin due to incomplete depletion promoted CDT1 release. The role of p97 was extended to another $CRL4^{CDT2}$ substrate, SET8, which is ubiquitinated on chromatin in a PCNA-dependent manner. In the absence of p97, ubiquitinated SET8 accumulated on chromatin.

The cellular abundance of p97 and its ability to associate with a cohort of ubiquitin-binding cofactors in different cellular compartments places it ideally at the interface of ubiquitination and degradation by the proteasome (Richly et al., 2005). However, few substrates of this complex have been identified outside of ERAD. While we did not identify any additional p97 cofactors in our genome-wide screen, we cannot exclude their involvement in turnover of CDT2 substrates due to either genetic redundancy or incomplete depletion. Indeed, it is conceivable that distinct substrates on chromatin use a variety of p97 cofactors to achieve coupling to the proteasome. For example, Ubx4p/Ubx5p have been implicated specifically in Rpb1p turnover on chromatin (Verma et al., 2011). Endogenous p97 complexes also contain ubiquitin ligase activity via association with UFD2 proteins. In yeast, Ufd2p has been shown to extend ubiquitin chains on p97 targets, potentially providing additional ubiquitin molecules for higher-affinity capture by the p97 complex or the proteasome (Koegl et al., 1999). It remains to be seen if such chain-extending ubiquitin ligases contribute to turnover of $CRL4^{CDT2}$ targets that are coupled to p97 complexes.

Models for p97- and Proteasome-Dependent Degradation of Ubiquitinated CDT1 in a Protein-DNA Complex

A central unanswered question in the ubiquitin field is how ubiquitinated proteins within higher order complexes are presented to the proteasome. The 19S proteasome regulatory complex contains ATP-dependent unfolding activity, which acts upon ubiquitinated substrates and feeds them to the proteasome core (Finley, 2009). A key step in this process is the recognition of flexible termini or unstructured elements on the ubiquitinated protein, by proteasome unfoldases (Lee et al., 2001; Prakash et al., 2009). However, it appears that in certain contexts, ubiquitinated proteins cannot be properly delivered to the proteasome without the assistance of the p97 segregase, perhaps because they are embedded in complexes that mask determinants recognized by the proteasomal unfoldase (Beskow et al., 2009). Alternatively, recent work on Rpb1p suggests that ubiquitinated substrates may be able to engage the proteasome in the absence of p97/Cdc48p but that Cdc48p activity is nevertheless required for substrate to be competent for degradation (Verma et al., 2011). One possibility is that p97 provides local unfolding of the substrate, thereby producing an unstructured polypeptide that can be recognized productively by the 19S regulatory particle for delivery to the 20S core. Thus, a requirement for p97 may be particularly relevant in the context of highly compact and topologically constrained chromatin structures, which may limit proteasomal access.

Our studies support a role for p97 in coupling CDT1 extraction/unfolding to proteasome delivery and degradation. The sites of ubiquitination on CDT1 reside in the N-terminal domain between residues 1 and 243 (Havens and Walter, 2009), which is tightly associated with PCNA on DNA. Given the ubiquitin-binding capacity of p97 complexes, the simplest model is that recruitment of p97 to ubiquitinated CDT1 facilitates ATP-dependent unfolding of CDT1, followed by its release from PCNA and subsequent capture by, or facilitated transfer to, the proteasome (Figure 7). We provide five findings consistent with this model: (1) turnover of CDT1 in vivo required the ATPase activity of p97 in addition to the ubiquitin binding cofactor UFD1 (Figures 3–5); (2) in the absence of p97, CDT1 and SET8 accumulate on chromatin (Figure 5); (3) in *Xenopus* egg extracts, depletion of p97 resulted in defective CDT1 degradation and extraction, which was rescued by wild-type p97 complexes but not mutants that lacked the D2 ATPase domain (Figure 6); (4) p97-mediated extraction of CDT1 from damaged DNA required CDT1 ubiquitination (Figure 6); and (5) proteomic analysis of MMS-DNA-associated proteins from *Xenopus* egg extracts revealed the presence of proteasome and p97 complex subunits (Table S3). A fraction of p97-UN is associated with chromatin in resting cells, yet DNA damage can induce active recruitment of p97 to sites of DNA damage (K. Ramadan, personal communication). In this setting, p97 may be capable of interacting productively with the proteasome to promote degradation of ubiquitinated

CDT1. These findings are consistent with previous reports of proteasome association with chromatin under basal and stimulus-specific conditions (Muratani and Tansey, 2003). The behavior of CDT1-ubiquitin conjugates on damaged DNA is consistent with a requirement for both p97 and the proteasome in CDT1 degradation. Precisely how p97 and the proteasome work together remains to be explored. In HeLa cells depleted of the proteasome subunit PSMB5, the majority of CDT1 is stabilized on chromatin, but we also observed release of a fraction of CDT1 into the soluble pool (Figure 5B). In contrast, release of CDT1 into the soluble pool was not seen upon depletion of either DDB1 or p97, suggesting a role for both ubiquitination and extraction in CDT1 removal from chromatin. In vitro, p97 promoted degradation of CDT1 and promoted release of a small fraction of CDT1 from chromatin in a manner that required not only ATP hydrolysis by p97 but also CDT1 ubiquitination (Figures 6F, 6G, and 7). The simplest explanation is that while CDT1 degradation normally involves a coupling between the activity of p97 and the proteasome, p97 nevertheless has the capacity to at least partially remove CDT1 from chromatin when proteasome activity is inhibited or when coupling is inefficient, as may occur in the in vitro setting. Further biochemical reconstitution studies are required to fully understand the determinants for p97 and proteasomal-dependent degradation of $CRL4^{CDT2}$ substrates.

This study provides insight into the complexity of the molecular circuitry necessary for DNA damage-dependent turnover of CDT1 and SET8. We envision that these systems may be important for turnover of other $CRL4^{CDT2}$ targets. Moreover, this work provides an experimental paradigm for systematic elucidation of pathways that control signal-dependent turnover of other ubiquitination substrates.

EXPERIMENTAL PROCEDURES

For detailed experimental methods, see Supplemental Experimental Procedures.

siRNA Screen and Analysis

HeLa cells were reverse transfected in 384-well plates with an arrayed library of ~21,000 siRNAs in a pooled format. Seventy-two hours posttransfection, duplicate plates were untreated and triplicate plates were treated with 20 J/m^2 UV for 30 min. Cells were fixed and stained for endogenous CDT1 and total nuclei. Plates were imaged for CDT1 intensity and the percent of CDT1-positive cells. Candidate genes were identified using Z scores based on the percent CDT1-positive cells. Genes with $z \geq 2$ were deconvolved using the above assay and considered to validate if 2 of 4 siRNAs resulted in CDT1 stabilization post-UV. See Table S1.

Cell Culture, Fractionation, Protein Purification, and Flow Cytometry

HeLa cells were fractionated into soluble and chromatin-enriched nucleosomes. Sf9 cells were infected with HA-UFD1, His-NPL4 (to maintain stability of UFD1), and p97 virus, and the complex was purified with anti-HA resin (Sigma). Commercial antibodies and siRNA duplexes are detailed in Supplemental Experimental Procedures and Table S2, respectively. Flow cytometry was performed on a FACS ARIA.

Xenopus laevis Depletion and Chromatin Spin-Down Assays

High-speed egg extracts were prepared as described (Havens and Walter, 2009) and depleted of p97 (Heubes and Stemmann, 2007). MMS-DNA coupled to Dynal beads (Invitrogen) was added to extract supplemented with ATP and 250 μM ubiquitin and incubated for 10 min. The beads were washed and centrifuged through a sucrose cushion and incubated with Sf9 purified proteins at 30°C for time course experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2011.06.036.

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