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Cdt1 proteolysis is promoted by dual PIP degrons and is modulated by PCNA ubiquitylation

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ABSTRACT

Cdt1 plays a critical role in DNA replication regulation by controlling licensing. In Metazoa, Cdt1 is regulated by CRL4^{Cdt2}-mediated ubiquitylation, which is triggered by DNA binding of proliferating cell nuclear antigen (PCNA). We show here that fission yeast Cdt1 interacts with PCNA *in vivo* and that DNA loading of PCNA is needed for Cdt1 proteolysis after DNA damage and in S phase. Activation of this pathway by ultraviolet (UV)-induced DNA damage requires upstream involvement of nucleotide excision repair or UVDE repair enzymes. Unexpectedly, two non-canonical PCNA-interacting peptide (PIP) motifs, which both have basic residues downstream, function redundantly in Cdt1 proteolysis. Finally, we show that poly-ubiquitylation of PCNA, which occurs after DNA damage, reduces Cdt1 proteolysis. This provides a mechanism for fine-tuning the activity of the CRL4^{Cdt2} pathway towards Cdt1, allowing Cdt1 proteolysis to be more efficient in S phase than after DNA damage.

INTRODUCTION

To maintain genome integrity, chromosomes must be replicated only once per cell cycle. An important regulatory step is the loading of the replicative helicase (Mcm2-7 complex) at replication origins, a process termed licensing or pre-replicative complex (pre-RC) formation. Licensing requires ORC, Cdt1 and Cdc6/18 and occurs only in late mitosis and G1, when cyclin-dependent kinase (CDK) activity is low (1). The transition to S phase occurs when Dbf4-dependent kinase (DDK) and CDK activity increase, which triggers the binding of additional replication factors, such as Cdc45, GINS and DNA polymerases, resulting in the onset of DNA replication (2).

Regulation of Cdt1 is one mechanism by which pre-RC formation is restricted to M and G1 phases (3). Deregulation of Cdt1 has severe consequences in metazoan cells, leading to DNA re-replication and genome instability (4–8). In addition, over-expression of Cdt1 has oncogenic potential (9) and has been observed in certain human cancers and cancer cell lines (10,11).

In fission yeast, Cdt1 is only synthesized in M-G1 (12,13). Once replication has initiated, Cdt1 is proteolyzed to ensure that no pre-RCs are assembled after initiation and the CRL4^{Cdt2} ubiquitin ligase has been implicated in this process in both Metazoa and fission yeast (14–17). In mammalian cells, a second SCF-Skp2 ubiquitin ligase, which operates in S and G2 phase in a CDK-dependent manner, functions in Cdt1 proteolysis (14,16,18,19). Additional mechanisms are used in eukaryotic cells to regulate Cdt1, including inhibition by geminin in Metazoa and CDK-dependent nuclear exclusion in budding yeast (20).

In addition to the S-phase degradation of Cdt1, proteolysis occurs after DNA damage via a CRL4^{Cdt2} pathway in Metazoa (16,21–24) and fission yeast (15). The function of Cdt1 turnover after DNA damage remains to be clarified (20).

Regarding activation of Cdt1 proteolysis, DNA-bound PCNA (proliferating cell nuclear antigen) has been shown to promote its CRL4^{Cdt2}-mediated ubiquitylation (14,16,21,25). PCNA is loaded onto DNA in S phase and during DNA repair, thus coordinating onset of these processes with Cdt1 proteolysis. Many other proteins are downregulated by this pathway, including p21 (26), Xic1 (27), CKI-I (28), DNA polymerase η (29) and E2F (30).

Many PCNA-binding proteins contain a PCNA-interacting peptide (PIP) motif (31), with the consensus sequence Q-x-x-h-x-x-a-a (where 'h' represents

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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hydrophobic and 'a' aromatic residues). Studies in *Xenopus* have shown that Cdt1 ubiquitylation occurs on chromatin in a process dependent on its interaction with PCNA (4,25), with the PCNA–Cdt1 complex then recruiting $CRL4^{Cdt2}$ (24,25). Interestingly, many PIP box-containing proteins are stable (32). Recent work showed that the degron of *Xenopus* Cdt1 contains a TD motif at positions 5 and 6 of the PIP box that confers high affinity to chromatin-bound PCNA and a positively charged amino acid 4 residues C-terminal to the PIP box that helps to recruit $CRL4^{Cdt2}$. When these sequence elements were transferred to Fen1, a stable PIP box protein, they induced $CRL4^{Cdt2}$-dependent proteolysis, indicating that these elements create a degron for $CRL4^{Cdt2}$ (21).

We address here the involvement of PCNA in fission yeast Cdt1 degradation in S phase and after DNA damage. In both situations, we found that Cdt1 turnover is dependent on DNA-bound PCNA and although fission yeast Cdt1 does not contain a canonical PIP box, we identify two variant PIP motifs which show sequence similarities to other targets of $CRL4^{Cdt2}$. Unexpectedly, we show that PCNA ubiquitylation, which occurs after DNA damage, can impede Cdt1 proteolysis, providing an additional mechanism to regulate the $CRL4^{Cdt2}$ pathway.

MATERIALS AND METHODS

Fission Yeast Methods

Strains used in this study are listed in Supplementary Table S1. Thiamine at 10 µg/ml was used to repress the *nmt1* promoter of the *pcn1* cold-sensitive allele. Standard genetic methods and flow cytometry were used as described previously (33,34).

Strain construction

To construct the PIP28 mutant strains, two partially complementary fragments for each mutation (XhoI-fragment and BamHI-fragment) were amplified using primers containing the mutations and a BamHI or XhoI restriction site (Supplementary Table SII); subsequently, the fragments were annealed and amplified. After DNA purification, the fragment was cloned into BamHI and XhoI digested pSMH2+, and integrated at the *cdt1*$^+$ locus after linearizing with SalI.

To construct the Cdt1–YFP strain, a *cdt1* promoter – *cdt1*–YFP (Pcdt1–cdt1–YFP) vector was used. The $PIP301^{A10}$ mutation (Figure 5A) was generated as described above, with primer pairs that contained the $PIP301^{A10}$ mutation and inserted into the Pcdt1–cdt1–YFP vector. The $PIP28^{A8}$ mutation (Figure 3A) was amplified from strain 2237 and inserted into Pcdt1–cdt1–YFP as an AfeI and XbaI restriction fragment. Plasmids were linearized by StuI digest and integrated at the *ura4* locus of strain 2270.

For C-terminal tagging of Cdt1 with the BiFC VC155 Venus half, a fragment consisting of the *cdt1* promoter plus the wild-type or mutant *cdt1* reading frame was amplified from plasmid DNA, using primers 947 and 948. This fragment was cloned into pFA6a-VC155-natMX6 (35) at the BamHI and PacI sites and the resulting plasmid was linearized by NsiI digestion and integrated as a second copy at the endogenous *cdt1* promoter.

For BiFC VN173 N-terminal tagging of *pcn1*, the VN173 fragment was amplified from the pFA6a-VN173-kanMX6 plasmid (35) using the primers 933 and 934. The PCR product was digested with PacI and AscI and cloned in pFA6a-kanMX6-P41nmt1-3HA (36) creating pFA6a-kanMX6-P41nmt1-VN173. Then, the *nmt1-41* promoter was extracted from the plasmid by digestion with BglII and PacI and substituted with the *pcn1* native promoter, amplified using the primers 937 and 938. Finally, *pcn1* was amplified with the primers 935 and 936, digested with BamHI and AscI and cloned in phase with VN173 producing pFA6a-kanMX6-Ppcn1-VN173-pcn1. This plasmid was integrated in the *pcn1* promoter locus of the genome. All BiFC strains were checked by western blotting using anti-GFP antibody (Rockland Immunochemicals, 600-103-215) to check that both Venus YFP halves were being expressed.

All plasmids and strains constructed were checked by sequencing. Primers used are shown in Supplementary Table S2.

Fluorescence microscopy

For analysis by fluorescence microscopy, cells were fixed in methanol/acetone and mounted in 1.2% low melting temperature agarose, containing 50 ng/ml DAPI. Images were collected using a Zeiss Axioplan microscope, coupled to a Hamamatsu ORCA ER camera; open source µManager software (37) was used to control the camera and microscope.

Cell-cycle synchronization and ultraviolet irradiation

Cells were arrested in G1 phase in EMM medium lacking NH_4Cl for 16 h at 25°C. *nda3-311* strains were grown at 32°C in rich medium and arrested in M phase by incubation for 4 h at 20°C. The *nda3-311 pcn1cs* strain was grown in EMM at 32°C and at the time of the shift to 20°C, thiamine was added to repress the *nmt1* promoter of the *pcn1* cold-sensitive allele. For UV exposure, cells were resuspended in water and irradiated with 100 J/m^2 (unless otherwise indicated) of 254 nm UV light in a 6-mm-deep stirred suspension at 20°C; the arrest was maintained unless otherwise indicated. For the *rfc1-44* strain, cells were grown at 25°C until mid-log phase and then were shifted to 37°C for 4 h before the irradiation. After irradiation, cells were incubated at the restrictive temperature.

Protein analysis

Protein extracts were made by trichloroacetic acid (TCA) extraction and analyzed by western blotting as described previously (16). TAP-tagged Cdt1 was detected with peroxidase–anti-peroxidase–soluble complex (P1291, Sigma). Cdt1–YFP was detected using anti-GFP antibody (catalogue number 11814460001, Roche) and α-tubulin was used as loading control and detected with antibody T5168 (Sigma). Western blotting was quantified

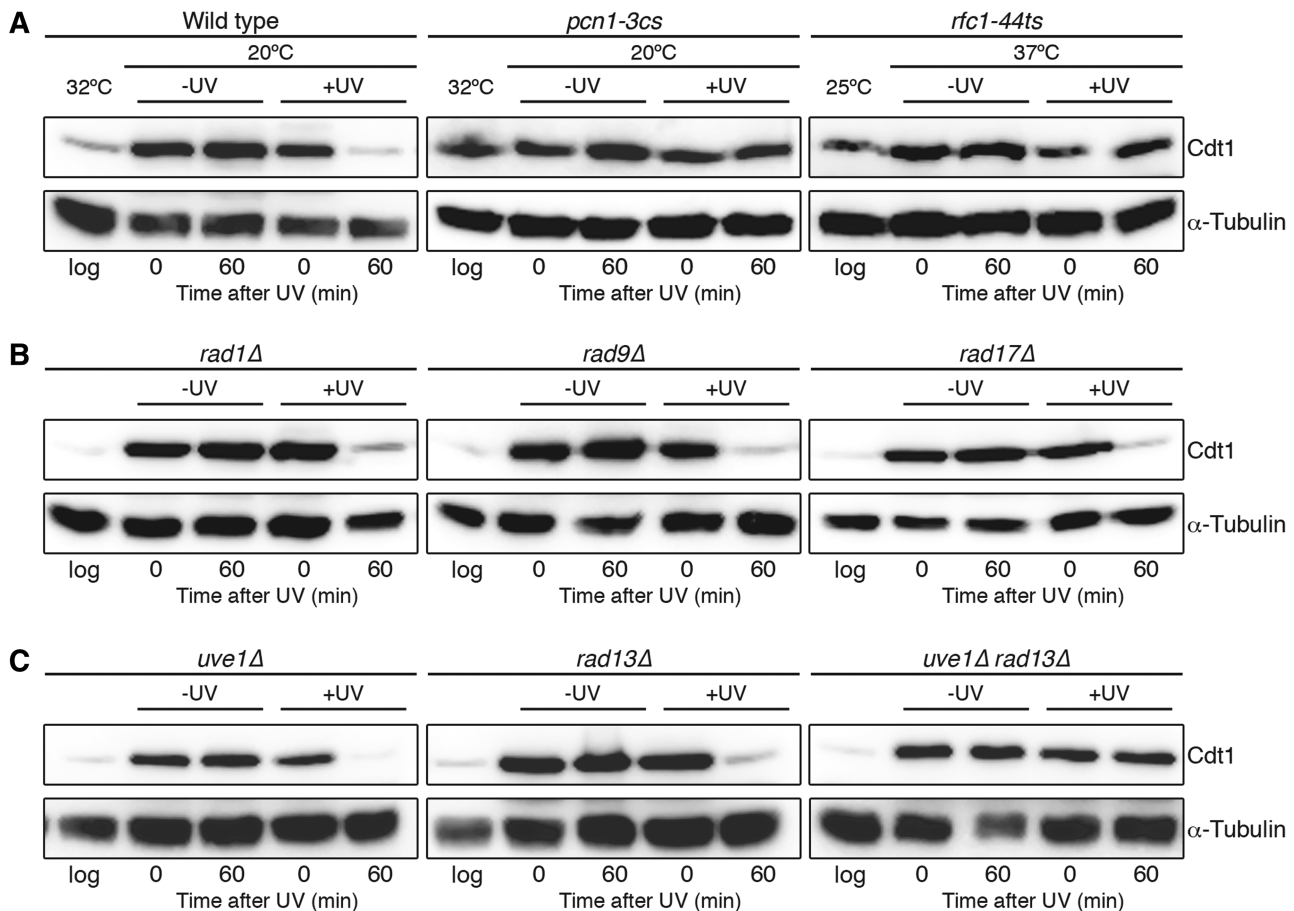

Figure 1. Chromatin-bound PCNA and activity of NER or UVER pathways are required for Cdt1 proteolysis after UV-induced damage. Panels show western blot analysis of Cdt1–TAP levels in cell extracts prepared after UV irradiation (100 J/m^2) or mock irradiation. All strains used (except the *rfc1-44* strain) contain the *nda3-311* allele and were arrested in mitosis by incubation at 20°C for 4 h, UV irradiated and kept at the restrictive temperature. The *rfc1-44* strain was grown at 25°C, shifted to 37°C for 4 h to inactivate the RFC loader and then irradiated and maintained at the restrictive temperature. (**A**) Cdt1 levels in a wild-type strain (2069), after inactivation of PCNA in a *pcn1cs* strain (2225) or after RFC loader inactivation in an *rfc1-44ts* mutant (1737). (**B**) Cdt1 levels when the 9-1-1 complex has been inactivated in a *rad1Δ* mutant (2083), a *rad9Δ* mutant (2081), or in absence of Rad17-RFC loader activity in a *rad17Δ* mutant (2084). (**C**) Cdt1 levels when NER (*rad13Δ* mutant, 2077), UVER (*uve1Δ* mutant, 2079) or both pathways for excision of photoproducts (*uve1Δ rad13Δ* mutant, 1954) were blocked.

using Alpha Innotech software, normalizing each Cdt1 value from the α-tubulin loading control.

RESULTS

Cdt1 proteolysis after DNA damage is dependent on chromatin-bound PCNA

Previous results from our group showed that DNA damage induces Cdt1 proteolysis in *Schizosaccharomyces pombe* through a pathway dependent on Cdt2 and Ddb1 (15). To analyse whether this proteolysis is dependent on PCNA, cells where PCNA was inactivated were subjected to UV (UV-C) irradiation and Cdt1 levels were determined by western blotting. PCNA was inactivated using a conditional allele in prometaphase-arrested (*nda3-311*) cells. Inactivation of PCNA resulted in stabilization of Cdt1 after UV-induced DNA damage compared to control cells (Figure 1A). To determine if DNA-associated PCNA is required for Cdt1 proteolysis, we inactivated the replication factor C (RFC) clamp loader complex using an *rfc1-44ts* mutant and tested whether this affects the change in Cdt1 levels after UV irradiation. Inactivation of Rfc1 resulted in stabilization of Cdt1 after DNA damage, similar to the result seen when PCNA was downregulated (Figure 1A).

In addition to RFC, other RFC-like complexes have been identified and demonstrated to possess clamp loading activity. One of these is the Rad17-RFC loader, which loads the Rad9-Rad1-Hus1 (911) complex onto sites of DNA damage. The 911 complex shows structural similarity to PCNA and it has been proposed to substitute for PCNA during cellular stress, although it does not complement PCNA function during normal replication (38). Although previous results showed that Cdt1 proteolysis after DNA damage was independent of some checkpoint proteins (15), this complex had not been examined. Cdt1 proteolysis after DNA damage was still observed (Figure 1B) in 911 and *rad17* mutants, thus this complex is not required for this process.

To identify steps upstream of PCNA loading required for Cdt1 proteolysis after DNA damage, we inactivated

early stages in two pathways for excision of photoproducts: nucleotide excision repair (NER) and an alternative pathway dependent on Uve1 (UVER) (39). Rad13 incises 3′ to damaged DNA in NER (40), while Uve1 makes an incision 5′ to the lesion in UVER (41,42) and these events are required for the generation of single-stranded DNA that is subsequently filled in by repair polymerases (39). We measured Cdt1 levels in mitotically arrested cells after UV irradiation in single *uve1Δ* or *rad13Δ* mutants and also in a *uve1Δ rad13Δ* double mutant where both pathways are inactivated. As shown in Figure 1C, in the single mutants Cdt1 levels decreased after irradiation while levels of the protein remained high in the double mutant. Thus, either pathway can activate Cdt1 degradation after DNA damage.

S-phase Cdt1 regulation requires DNA-associated PCNA

To determine if there is a common mechanism for Cdt1 proteolysis after DNA damage and in S phase, we analysed whether PCNA is required for Cdt1 turnover during S phase. As before, Cdt1 levels were analysed in a strain carrying a conditional *pcn1* allele. After PCNA inactivation, Cdt1 levels increased suggesting that PCNA function is required (Figure 2A). The effect of RFC inactivation on Cdt1 was analysed by western blotting and fluorescence microscopy. Inactivation of Rfc1 caused a dramatic increase in the percentage of cells with nuclear Cdt1–YFP, and in the intensity of Cdt1–YFP fluorescence, compared to wild type (Figure 2B). This result correlated with western blotting analysis (Figure 2C). Cells were arrested in G1, then released from the block at 37°C to inactivate Rfc1; Cdt1 levels increased 1 h after release and remained high in contrast to the pattern shown in wild-type cells where Cdt1 decreased during S phase. To see if this was due to an indirect effect of blocking cells in S phase, we analysed Cdt1 levels in other S phase mutants. Neither a *pol2ts* (DNA polymerase ε) nor a *cdc17-M75* mutant (DNA ligase) showed stabilization of Cdt1 after the inactivation of these enzymes (Figure 2D). Although the *pol2ts* mutation might be expected also to block PCNA association with DNA by virtue of blocking replication, the mutant is quite leaky and some replication does occur (Supplementary Figure S1). Taken together, these results show that mutations in PCNA or RFC are not stabilizing Cdt1 by an indirect effect in blocking replication and that DNA-associated PCNA promotes Cdt1 ubiquitylation by CRL4^{Cdt2} allowing its subsequent proteolysis.

Interaction between metazoan PCNA and Cdt1 has been detected *in vitro* but direct demonstration of this interaction *in vivo* has not been achieved. We addressed this by bimolecular fluorescence complementation (BiFC) (35,43), where PCNA and Cdt1 were tagged with N- and C-terminal domains of Venus-YFP, respectively, and are expressed from their native promoters. No fluorescence was seen when the tagged proteins were expressed separately (data not shown), or in a *cdt2*$^+$ background (Figure 2E, upper panels). However, when co-expressed in a *cdt2Δ* background to stabilize Cdt1, nuclear YFP fluorescence was seen in all the cells, irrespective of cell-cycle stage, with little variation in the intensity of YFP fluorescence (Figure 2E, lower panels). This indicates that the Cdt1 and PCNA interaction occurs *in vivo* and can be detected provided the rapid turnover of Cdt1 is blocked by inactivating CRL4^{Cdt2}. We also conclude that the interaction between Cdt1 and PCNA does not require Cdt2, as reported in *Xenopus* (21). PCNA is predominantly associated with chromatin in S phase, so the fact that YFP fluorescence can be detected at all stages of the cell cycle may indicate that the Cdt1–PCNA interaction can occur off chromatin.

An N terminal motif is important for Cdt1 proteolysis after DNA damage

In metazoan Cdt1, a PIP motif is located near the N-terminus and there is a partial match to this motif in fission yeast beginning at amino acid 28 (PIP28, Figure 3A). In addition, Havens and Walter (2009) showed that the *Xenopus* Cdt1 degron requires a basic amino acid 4 residues downstream of the PIP box which recruits CRL4^{Cdt2} to the Cdt1–PCNA complex. This basic amino acid is also found 4 residues downstream of fission yeast PIP28 (Figure 3A) in a positively charged region. We analysed Cdt1 levels after DNA damage in two strains, one where PIP28 was mutated and partially deleted (PIP28$^{\Delta}$, Figure 3A) and another where key residues in the PIP box and positively charged region were substituted by alanine (PIP28^{A8}, Figure 3A). In both cases, partial stabilization of Cdt1 after DNA damage was seen (PIP28$^{\Delta}$ and PIP28^{A8}, Figure 3B).

To analyse the relative importance of the PIP box and the positively charged region, we analysed Cdt1 levels after DNA damage when either the PIP box (PIP28^{A2} and PIP28^{A5}, Figure 3A) or the positively charged region was mutated (PIP28^{RKs}, Figure 3A). Mutating several amino acids in the PIP box had a more dramatic effect on Cdt1 stabilization than just mutating the conserved Q and L residues (compare PIP28^{A5} or PIP28^{A8} with PIP28^{A2} Figure 3B). Mutation of all positively charged residues (+1 to +9) also stabilized Cdt1 after DNA damage (Figure 3B, PIP28^{RKs}) but, unlike in *Xenopus*, a single substitution of the basic +4 residue downstream of the PIP box did not affect Cdt1 degradation (Figure 3A, B, PIP28^{A+4}).

Cdt1 nuclear localization was not altered by these mutations (Supplementary Figure S2), thus the effect on Cdt1 levels is not an indirect consequence of loss of nuclear localization. These results indicate that a PIP motif in combination with basic downstream residues is important for Cdt1 degradation after DNA damage, but the requirements are subtly different from those identified in *Xenopus*.

Consistent with the observation that some reduction in Cdt1 levels occurs in all of the PIP28 mutants after DNA damage, higher molecular weight forms of Cdt1 were revealed when westerns were over-exposed, indicative of ubiquitylation, but these were not seen in a *cdt2Δ* mutant, or in mutants defective in PCNA or RFC (Supplementary Figure S3). This implies that while the PIP28 sequence is

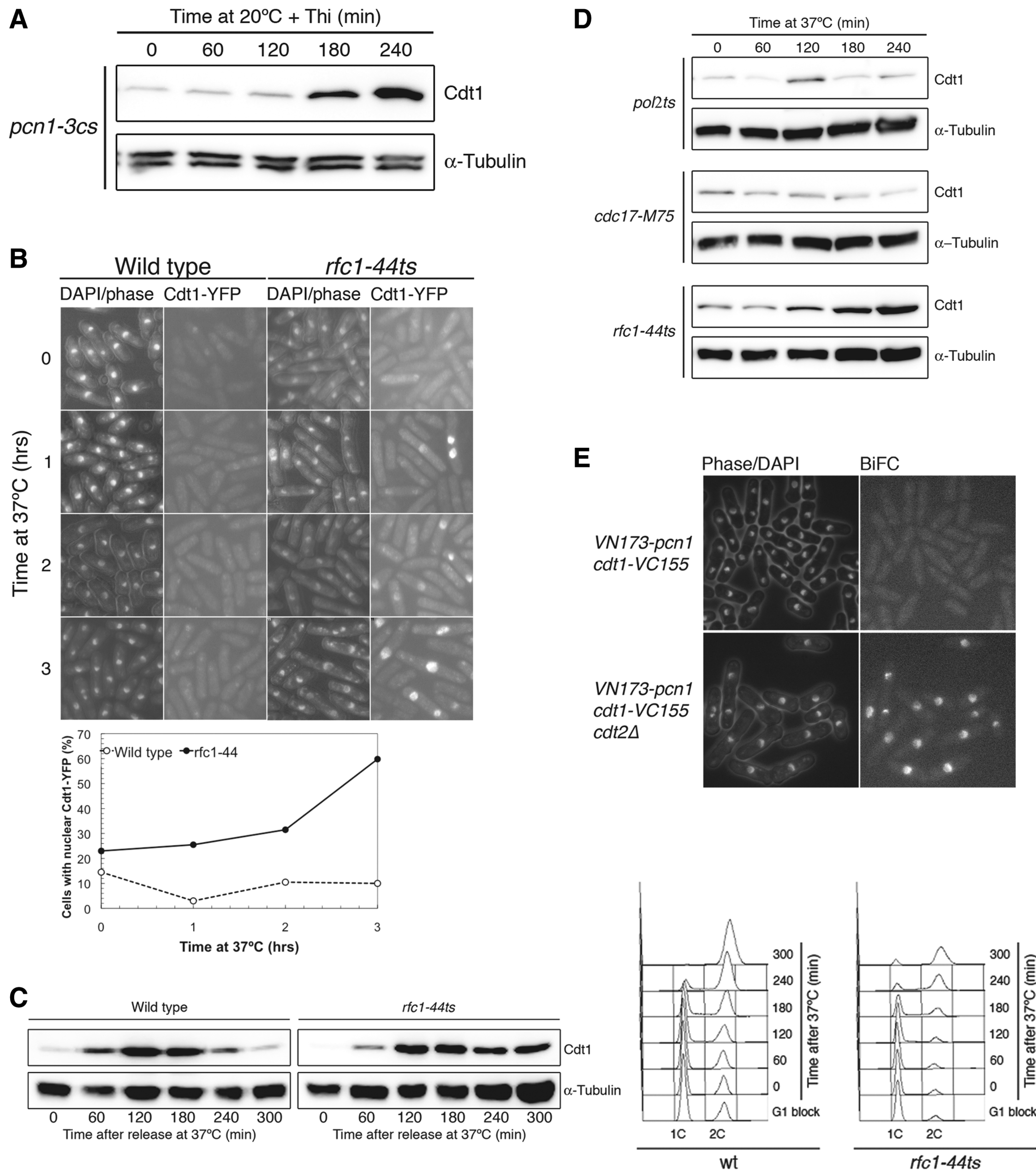

Figure 2. Chromatin-bound PCNA is required for Cdt1 proteolysis in S phase. (**A**) Western blot analysis showing Cdt1-TAP levels in a *pcn1Δ* strain where a cold sensitive *pcn1-3* allele is expressed under the *nmt1* promoter in a pREP81 plasmid (strain 2224). Cells were grown at the permissive temperature in minimal media, shifted to 20°C and 10 µg/ml thiamine was added to inactivate PCNA and block expression of the gene. (**B**) Cdt1-yellow fluorescent protein (YFP) imaged in a wild-type strain (1856) and in an *rfc1-44ts* mutant (1852) after incubation at 37°C (top panel); lower panel shows percentage of these cells showing nuclear Cdt1–YFP. (**C**) Cdt1–TAP levels in wild type (1540) and *rfc1-44ts* (1737) nitrogen-starved G1 arrested cells released into the cell cycle at 37°C (left-hand panels) and flow-cytometric analysis of the same cells (right-hand panels). Note that the *rfc1-44ts* mutant is leaky, and does not show a tight S phase after the release. (**D**) Cdt1-TAP levels in *pol2ts* (2217), *cdc17-M75* (2218) and *rfc1-44ts* (1737) cells after incubation at restrictive temperature. Flow cytometric analysis of DNA contents of cells in this experiment is shown in Supplementary Figure S1. (**E**) Bimolecular fluorescence complementation (BiFC) of *VN173-pcn1/cdt1-VC155* cells in a wild-type background (upper panels) (2600) and in a *cdt2Δ* background (lower panels) (2601). Both PCNA and Cdt1 constructs are expressed from native promoters. Cells shown are fixed but similar results were obtained with live cells.

important, its inactivation does not completely block Cdt1 ubiquitylation.

The PIP degron in the N-terminus of Cdt1 is not essential for its S-phase degradation

Previous work suggests a common mechanism for Cdt1 degradation both in S phase and after DNA damage. To examine whether the putative PIP element of Cdt1 is required for its turnover in S phase, the PIP28 mutants described in Figure 3A were arrested in mitosis and then cells were released into the cell cycle by shifting to the permissive temperature of 32°C (Figure 4A). On releasing wild-type cells from the mitotic block, Cdt1 levels remained high for 30 min and then decreased (Figure 4B,

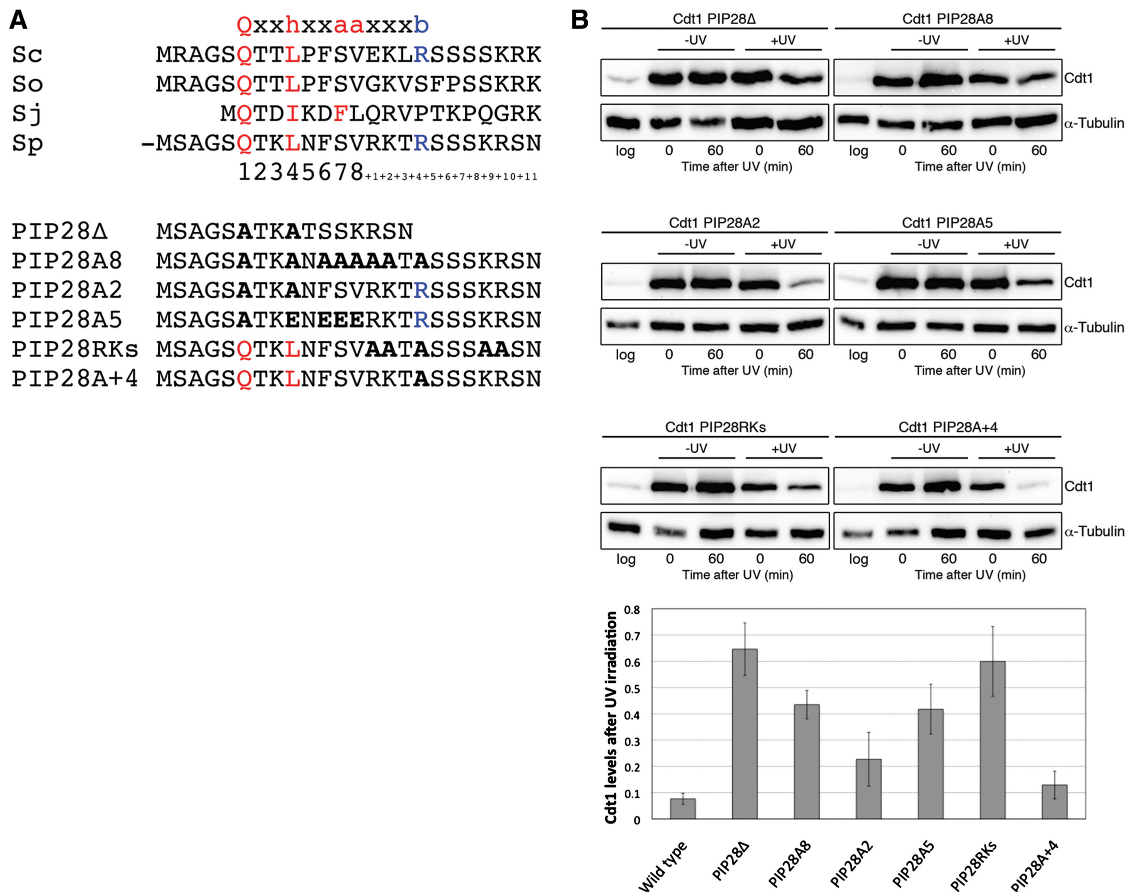

Figure 3. A PIP degron in Cdt1 is required for its proteolysis after UV irradiation. (**A**) Alignment of N-terminal Cdt1 PIP boxes in different *Schizosaccharomyces* species (Sc = *S. cryophila*, So = *S. octosporus*, Sj = *S. japonicus* and Sp = *S. pombe;* the first methionine indicated for the Sp sequence is position 23) and the sequences of *S. pombe* PIP box mutants used in (**B**). In Cdt1 PIP28^{A2} (1959) and Cdt1 PIP28^{A5} (2043) only elements of the PIP box are mutated, while positively charged amino acids downstream of the PIP box are changed in strains PIP28^{RKs} (2407) and PIP28^{A+4} (2390); both regions are mutated in PIP28$^{\Delta}$ (2238) and the PIP28^{A8} (2237). Canonical PIP box amino acids are shown in red, and numbering shows the location with respect to the first Q residue. Downstream amino acids are numbered +1 etc.; a positively charged residue at position +4 is shown in blue and changed amino acids are shown in bold. (B) Upper panels show western analysis of Cdt1-TAP levels in *nda3-311* mitotically arrested cells after UV irradiation (see Figure 1 for experimental details). Lower panel shows quantification of Cdt1 levels after UV irradiation. The Cdt1 level shown is the ratio of the +UV 60 min Cdt1 level relative to the $t = 0$ sample after normalizing to the tubulin loading control. Error bars show SD.

Wt Cdt1, left-hand panel), which corresponds to the time of S phase as judged by flow cytometric analysis (data not shown). Cdt2 levels were also analysed; Cdt2 is present in mitotically arrested cells (Supplementary Figure S4), and levels peak around the time of S phase as previously reported (44). Surprisingly, proteolysis of Cdt1 occurred in S phase in all of the PIP28 mutants with kinetics similar to the wild-type control (Figure 4B, left-hand panel). Since the previous experiments were carried out with cells arrested at 20°C, we controlled for the effect of temperature on DNA damage-mediated proteolysis by UV irradiating mitotically arrested cells and then releasing into the cell cycle at 32°C (Figure 4A). After DNA damage, onset of S phase is delayed about 1 h, starting around 90 min after release (45,46). Most of the PIP28 mutants nevertheless still showed relatively high Cdt1 levels (Figure 4B, right-hand panel), similar to the pattern seen in cells at the lower temperature where the mitotic arrest was maintained (Figure 4B, central panel). This shows that, while inactivation of the PIP28 partially stabilizes Cdt1 levels after DNA damage, it is not essential for S-phase-mediated proteolysis.

A second putative PIP box motif at position 301 is involved in the cell-cycle regulation of Cdt1

Since the S-phase regulation of Cdt1 is dependent on chromatin-bound PCNA and the CRL4^{Cdt2} ubiquitin ligase, one interpretation of the phenotype of the PIP28 mutants is that there is a second PCNA interacting region in Cdt1. Interestingly, there is a partial match to the PIP consensus, starting at position 301 of the protein, which also has positively charged residues downstream of the motif. This PIP301 motif is conserved within the *Schizosaccharomyces* genus (Figure 5A) but not within Metazoa.

To analyse the involvement of this region in the S-phase regulation of Cdt1, this PIP301 motif was mutated (to generate PIP301^{A10}, Figure 5A) and analysed in combination with either a wild-type N-terminus or with the PIP28^{A8} mutation. Although the PIP301 motif is not conserved in

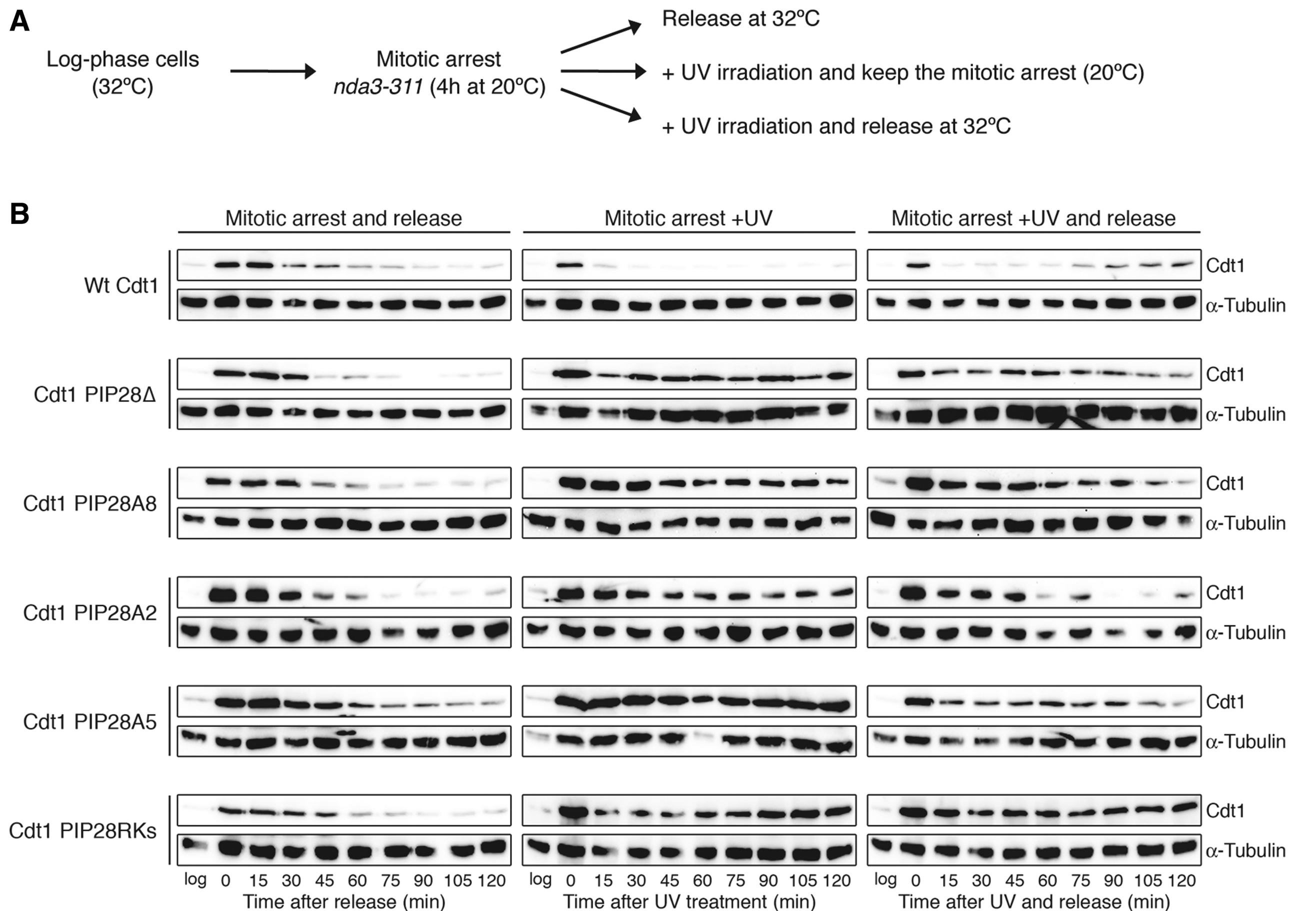

Figure 4. N-terminal PIP28 degron of Cdt1 is not essential for its proteolysis in S phase. (**A**) Scheme of experiment. (**B**) Cdt1-TAP levels in cell extracts from PIP28 mutants shown in Figure 3A following the experimental procedure shown in (A).

Saccharomyces cerevisiae, nearby conserved residues of the protein are essential for Cdt1 function (47); therefore, the mutated Cdt1 was introduced as a second copy, in a $cdt1^+$ wild-type background.

Wild-type Cdt1-YFP is visible only in the nuclei of some binucleate (late M/G1) cells (Figure 5B). Strikingly, in the double PIP28^{A8} PIP301^{A10} mutant, Cdt1 is detected in cells at all stages of the cell cycle, indicating that its S-phase proteolysis has been inhibited. In contrast, the single PIP301^{A10} and PIP28^{A8} mutants are similar to wild type, although the latter shows a slight increase in the number of uninucleate (G2) cells with nuclear Cdt1, suggesting that the PIP28 degron is more efficient than PIP301 in promoting S-phase proteolysis.

These mutants were also analysed by western blotting extracts from mitotically arrested cells that were released in synchrony into the cell cycle. Cdt1-YFP levels in the single PIP28^{A8} and PIP301^{A10} strains declined in S phase similar to wild-type cells [Figure 5C; S phase is around 30 min after release from flow cytometric analysis (data not shown)]. Similar to the result seen by fluorescence microscopy, the PIP28^{A8} mutant appears to be slightly more impaired in Cdt1 proteolysis (Figure 5C, compare 30–45 min). However, Cdt1–YFP levels were stable when the double PIP28^{A8} PIP301^{A10} mutant cells carried out S phase. Thus, both PIP28 and the PIP301 motifs appear to play a redundant role in the regulation of Cdt1 during S phase, although PIP28 may be a more efficient degron. The log phase level of Cdt1 in the double PIP mutant appears low in Figure 5C and D, in contrast to results obtained by fluorescence microscopy, although clear stabilization of Cdt1 is seen when a higher antibody concentration is used for detection (Supplementary Figure S5).

We also analysed the effects of the PIP301^{A10} mutation on Cdt1 proteolysis after UV irradiation. The reduction in Cdt1 levels was similar to that seen with the wild-type protein and unlike the situation seen with mutation of the PIP28 motif, which resulted in Cdt1 stabilization (Figure 5D). However, Cdt1 is more stable after DNA damage in the double PIP28^{A8} PIP301^{A10} mutant than either the single PIP28^{A8} or PIP301^{A10} mutants. Thus, both PIP motifs appear to contribute to DNA damage-mediated proteolysis, although mutation of the PIP28 motif has a more dramatic phenotype than mutation of the PIP301 sequence.

Finally, we used BiFC to determine whether the Cdt1 stabilization in the PIP28^{A8} PIP301^{A10} mutant was due to the lack of interaction between PCNA and the mutated Cdt1, as expected. Consistent with the effects of PIP

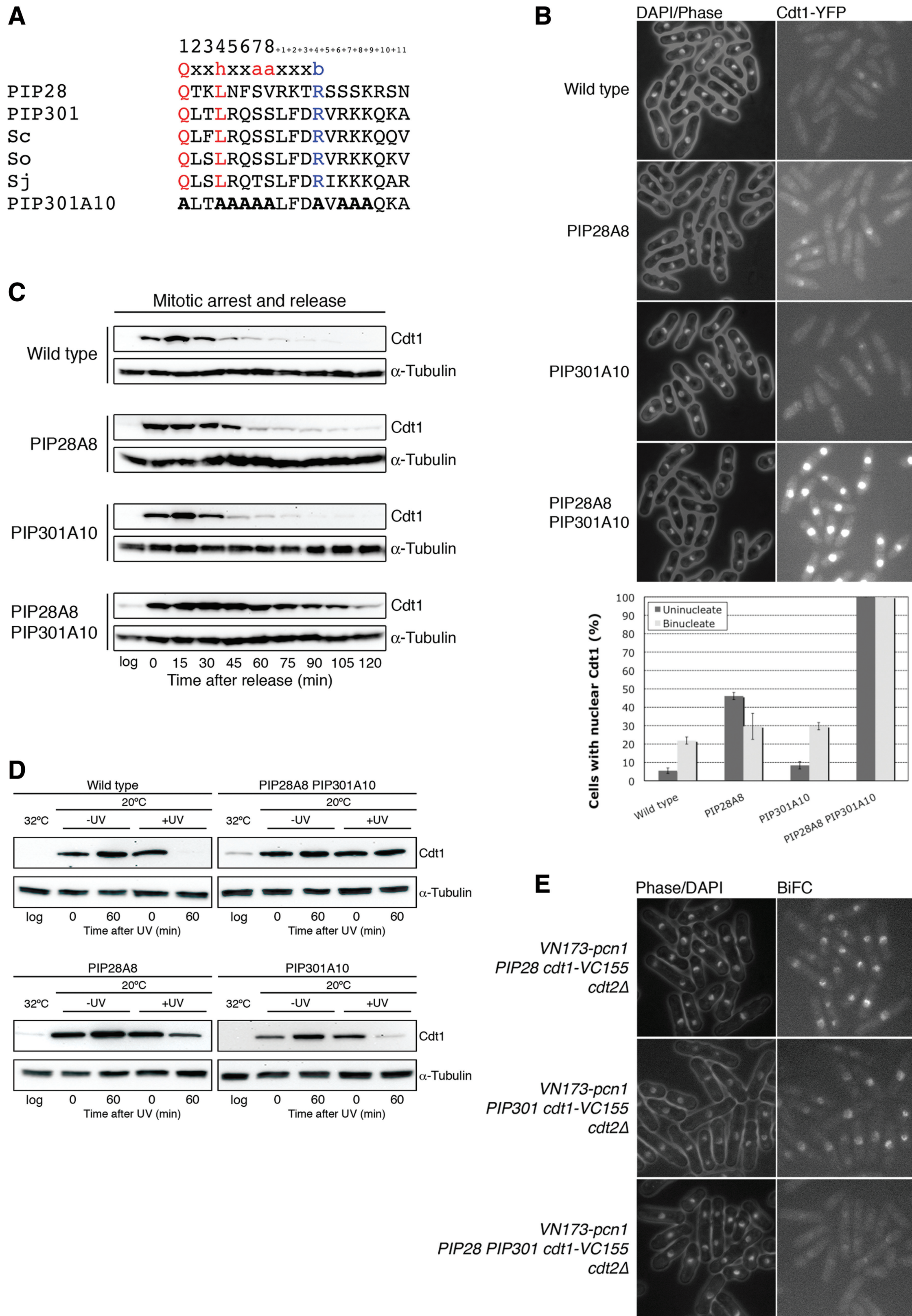

Figure 5. A second putative PIP box in Cdt1 is required for its degradation in S phase. (**A**) Alignment of *S. pombe* N-terminal (PIP28) and position 301 (PIP301) PIP boxes from *Schizosaccharomyces* species (abbreviations etc. as in Figure 3A); also shown is the $PIP301^{A10}$ mutation. (**B**) Cdt1–YFP imaged in exponentially growing cells in wild type (2337), $PIP28^{A8}$ mutant (2507), $PIP301^{A10}$ mutant (2513) and $PIP28^{A8}$ $PIP301^{A10}$ double-mutant cells (2500) (upper panel); lower panel shows the percentage of cells that show nuclear Cdt1-YFP. (**C**) Western blot analysis of Cdt1–YFP levels in the mutants shown in (B) after arresting in mitosis and releasing at the permissive temperature. (**D**) Cdt1-YFP levels following UV irradiation in wild type and PIP mutants [strains used as in (B)]; mitotically arrested cells were irradiated as described in Figure 1. (**E**) BiFC analysis of *cdt2Δ* cells expressing *VN173-pcn1* and *cdt1-VC155* containing single PIP28A8 (top panels, 2602), PIP301A10 (middle panels, 2603) or double PIP28A8, PIP301A10 mutations (lower panels, 2604). Both PCNA and Cdt1 constructs are expressed from native promoters.

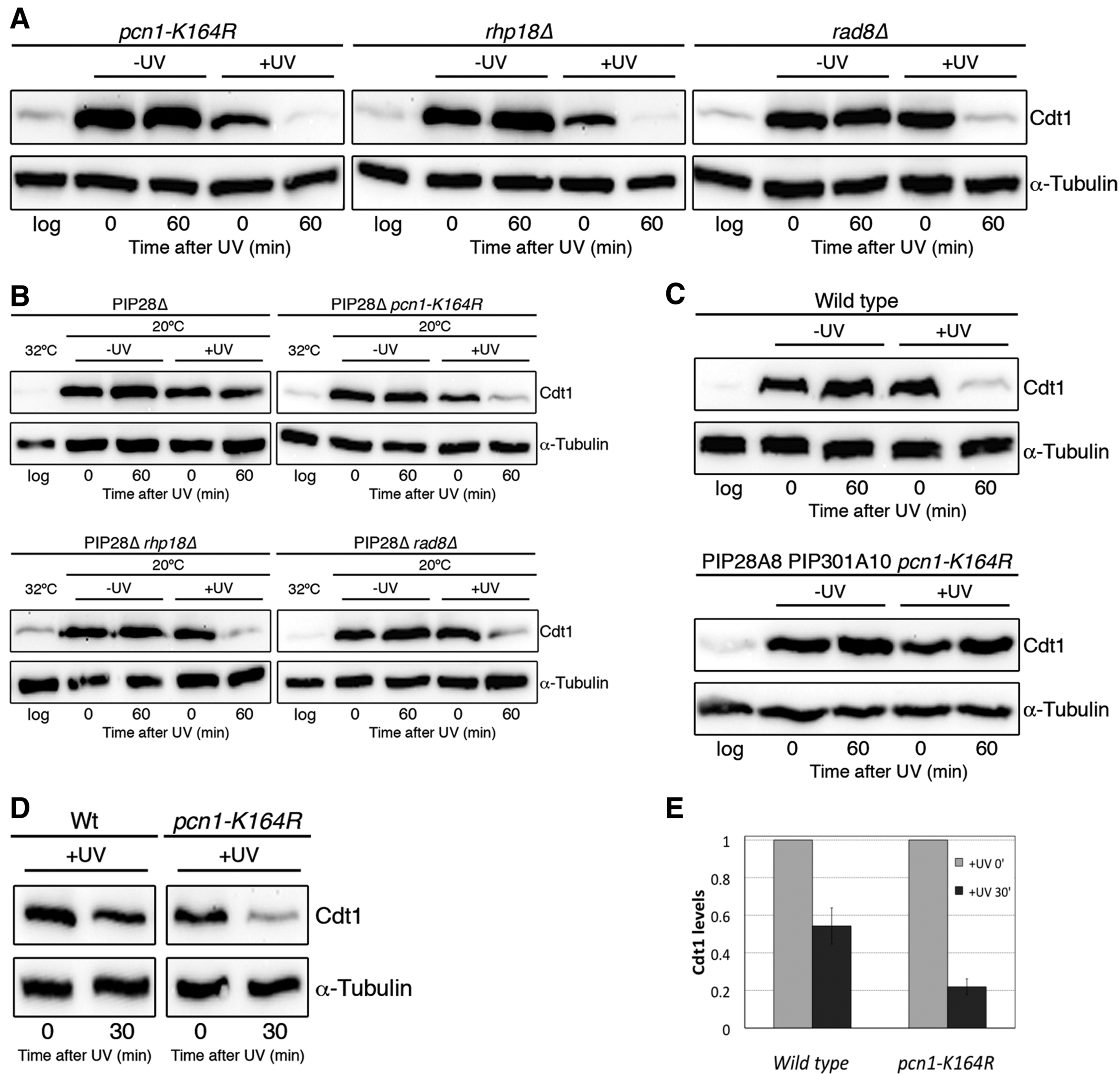

Figure 6. Ubiquitylation of PCNA impairs Cdt1 proteolysis. (**A**) Cdt1 levels following UV-induced DNA damage when PCNA mono- (*rhp18Δ*, strain 1945), poly (*rad8Δ*, 2139) or both mono- and poly-ubiquitylation (*pcn1-K164R*, strain 2138) were blocked. Mitotically arrested cells were UV irradiated as described in Figure 1. (**B**) As *A* but the N-terminal PIP box was deleted (strains 2520, 2508 and 2505, respectively); a $PIP28^{\Delta}$ single mutant is shown as a control (2238). (**C**) Western blot analysis of Cdt1 levels in wild type (2337) and $PIP28^{A8}PIP301^{A10}$ *pcn1-K164R* strains (2594) following irradiation as described in Figure 1. (**D**) Western blot analysis of Cdt1 levels in wild type (2069) and *pcn1-K164R* mutants (2138) following irradiation with a low dose of UV ($5\,J/m^2$). (**E**) Quantification of Cdt1 levels from experiment shown in (D); Graph shows the ratio of Cdt1 levels at $t = 30/t = 0$ after normalizing to the tubulin loading control (average of three independent experiments; error bars = SD).

mutations on Cdt1 proteolysis, a BiFC signal was observed when a single PIP degron was mutated (Figure 5E, top and middle panels) indicating interaction between Cdt1 and PCNA, but this was greatly reduced when both PIP degrons were mutated (Figure 5E, lower panels).

Ubiquitylation of PCNA can inhibit Cdt1 proteolysis after UV irradiation

In fission yeast, PCNA is modified after DNA damage by ubiquitylation of PCNA on lysine 164 (48). This modification also occurs in S phase, although to a lower extent. This modification is important as it changes the affinity of PCNA for different substrates; mono-ubiquitylation results in the association of translesion polymerases (TLS) with PCNA, while subsequent poly-ubiquitylation of PCNA at the same residue promotes error-free lesion bypass (49–51).

We investigated whether this modification is relevant to activation of Cdt1 proteolysis. The major pathway whereby lysine 164 is modified in fission yeast involves Rhp6-Rhp18, which carries out mono-ubiquitylation and Mms2-Ubc13-Rad8, which performs poly-ubiquitylation. $CRL4^{Cdt2}$ has also been reported to mono-ubiquitylate this residue in mammalian cells (52). We first determined whether this modification was necessary for activation of Cdt1 proteolysis after DNA damage by arresting *rhp18Δ* or *rad8Δ* cells in mitosis and irradiating the cells. No stabilization of Cdt1 was seen and a similar result was seen with a *pcn1-K164R* strain, where ubiquitylation of PCNA is blocked by mutation of the accepting lysine (Figure 6A).

This indicates that PCNA ubiquitylation does not promote Cdt1 proteolysis after DNA damage.

We next checked whether the modification could have a negative role relevant to the phenotype of the PIP28 mutants, which are defective in Cdt1 proteolysis after DNA damage. As described previously, Cdt1 levels remain high after UV-mediated DNA damage in a Cdt1 mutant where only PIP28 is mutated ($PIP28^{\Delta}$), but if PCNA ubiquitylation is blocked as well with a *pcn1-K164R* mutation Cdt1 levels fall dramatically (Figure 6B). To examine whether this effect requires mono- or poly-ubiquitylation of PCNA, we analysed Cdt1 proteolysis in $PIP28^{\Delta}$ mutants where only mono-ubiquitylation occurs (*rad8Δ*), or where all ubiquitylation is blocked (*rhp18Δ*). In both strains, Cdt1 levels were reduced after UV-mediated DNA damage similar to the level seen with the *pcn1-K164R* mutant (Figure 6B), indicating that poly-ubiquitylation of PCNA is necessary for inhibition of $PIP28^{\Delta}$–PCNA proteolysis. One interpretation of these results is that PCNA ubiquitylation inhibits interaction between PIP301 and PCNA, rather than inhibiting proteolysis through some other mechanism. If this were the case, no stimulation of proteolysis by the K164R mutation would be expected when both PIP boxes are mutated. Consistent with this expectation, Cdt1 levels are stable after DNA damage in a *pcn1-K164R* $PIP28^{A8}$ $PIP301^{A10}$ strain (Figure 6C), as seen in a wild-type PCNA genetic background.

This result led us to analyse whether the negative effect of PCNA ubiquitylation is specific to the $PIP28^{\Delta}$ mutant or also affects wild-type Cdt1. Previous irradiation experiments were performed with a UV dose of $100\,J/m^2$ which results in >90% reduction in Cdt1 levels and would make it difficult to detect any increased proteolysis. Thus, we reduced the UV dose to $5\,J/m^2$, which Callegari and Kelly (53) calculated as equivalent to the steady-state lesion burden of continuous sunlight exposure. At this dose, Cdt1 levels were reduced to around 50%, a level similar to the levels obtained at a high dose of UV in the PIP28Δ mutant (Figure 6D, compare with PIP28Δ in Figure 6B). If PCNA ubiquitylation was blocked however, using the *pcn1-K164R* mutation, the degradation of Cdt1 was significantly increased (Figure 6D and E). These results suggest that when DNA is damaged by low (physiological) UV exposure, PCNA ubiquitylation can impair Cdt1 proteolysis.

DISCUSSION

In Metazoa, Cdt1 is tightly regulated in the cell cycle by a number of mechanisms to ensure that replication origins fire only a single time in S phase. This study shows that in fission yeast, a single mechanism is involved which requires chromatin-bound PCNA to trigger Cdt1 proteolysis in S phase and after UV irradiation. We identify two sequences in fission yeast Cdt1 showing a partial match to the PIP consensus, starting at amino acid positions 28 and 301, which are involved in the cell cycle and DNA-damage-dependent regulation of the protein. These matches both lack the conserved aromatic amino acids in the canonical PIP consensus. Mutation of both PIP motifs is required to stabilize the protein in S phase, although the N-terminal PIP box appears to play a dominant role in Cdt1 proteolysis after DNA damage and in S phase. Finally, we demonstrate that, at a physiological UV dose, PCNA poly-ubiquitylation impairs Cdt1 proteolysis and that this effect is also observed at a higher UV dose when Cdt1 degradation is compromised by mutation of one PIP degron.

The involvement of PCNA in Cdt1 degradation has been documented in human, *Xenopus* and fission yeast cells (14,16,25,54). Interestingly, studies in *Xenopus* have demonstrated that Cdt1 binds more efficiently to chromatin-bound PCNA than to free PCNA (21) although by BiFC we are able to detect an interaction at all the stages of the cell cycle. This result is however consistent with size-exclusion chromatography of mammalian cell extracts, which indicates that Cdt1 and PCNA form a complex off chromatin (54), although we cannot exclude the possibility that once the two Venus–YFP fragments interact they remain associated when PCNA is displaced from DNA.

The interaction between chromatin-bound PCNA and Cdt1 is proposed to recruit $CRL4^{Cdt2}$ and lead to Cdt1 ubiquitylation on chromatin (21,24,25,55), thus providing a mechanism to link replication fork assembly with degradation of a factor needed for licensing. Similarly, in this study, we demonstrate that compromising the DNA association of PCNA by inactivating RFC leads to stabilization of Cdt1 levels in S phase and after UV irradiation. Why only chromatin-bound PCNA promotes Cdt1 ubiquitylation by $CRL4^{Cdt2}$ is unknown but it has been suggested that the binding of PCNA to DNA may result in a conformational change or a modification that enhances its ability to interact with Cdt1 (21).

Fission yeast Cdt1 is unusual in having two PIP boxes that contribute to the regulation of the protein and mutation of both boxes appears to be necessary to prevent Cdt1–PCNA interaction as detected by BiFC. The concept of two regulatory motifs has been documented in detail in human Cdt1, where the protein contains both a PIP box and a cyclin binding (Cy) motif (14), although these are subject to regulation by distinct ubiquitin ligases. The Cy motif mediates the interaction between Cdt1 and cyclin-A-dependent kinases, which phosphorylate Cdt1 and target it for SCF^{Skp2}-dependent ubiquitylation in S and G2 phases (14,18,19,56). The PIP box in the extreme N-terminus is required for the interaction with PCNA and targets Cdt1 for $CRL4^{Cdt2}$-dependent ubiquitylation in S phase and after DNA damage (14,16). Redundancy between the SCF^{Skp2} and $CRL4^{Cdt2}$ pathway in Cdt1 regulation does not appear to be conserved in fission yeast, *Caenorhabditis elegans*, *Drosophila* or *Xenopus*, leading to suggestion that the SCF^{Skp2} regulatory pathway evolved more recently than the $CRL4^{Cdt2}$ pathway (4,15,25,57–59). Why fission yeast Cdt1 has two motifs functioning redundantly in its proteolysis is unclear. Since fission yeast does not have other regulatory mechanisms for Cdt1 based on SCF ubiquitylation or geminin, provision of two PIP degrons

may be important for robust downregulation of the protein in S phase.

In fission yeast it appears that the N-terminal PIP28 plays a more dominant role in the regulation of Cdt1 degradation after UV irradiation and to some extent during S phase. Mutation of key residues in the PIP28 prevented the efficient degradation of Cdt1 after DNA damage, a phenotype not seen to the same extent in the $PIP301^{A10}$ mutant. The relative contribution that the two PIP boxes make to Cdt1 regulation may result from the sequence contexts of the two PIP box regions or their relative accessibility. Analysis of human PCNA bound to the CDK inhibitor p21, flap endonuclease (FEN1) and p66 subunit of pol δ demonstrate that variation in the sequence of the PIP box and its flanking regions determines PCNA-binding affinity (60,61).

Work on the *Xenopus* Cdt1 degron demonstrates the importance of the TD motif at positions 5 and 6 of the PIP box which is important for efficient binding of Cdt1 to PCNA, and the lysine at the +4 position which is important for $CRL4^{Cdt2}$ recruitment (21). Fission yeast Cdt1 does not contain TD residues at position 5 and 6 of either PIP box. This motif is widely, but not completely, conserved in Cdt1 homologues from a range of organisms (21). For example, *Drosophila* Cdt1 does not contain the TD motif but the protein has recently been documented to be regulated via a PIP-box-dependent pathway (59). In fission yeast Cdt1, mutation of the arginine corresponding to the position of the +4 lysine in the *Xenopus* degron had no effect on Cdt1 regulation but mutation of five positively charged residues downstream of the PIP box stabilized the protein after DNA damage. Our work confirms the importance of the downstream sequence, but shows that a positively charged region instead of a single residue is required for efficient Cdt1 proteolysis and thus there may be significant variation in the structure of $CRL4^{Cdt2}$ degrons. This view is also supported by analysis of the Xic1 degron, which has a more extended positively charged region in the vicinity of the PIP motif (62).

Previous work has demonstrated the importance of PCNA ubiquitylation after DNA damage in promoting the association of translesion polymerases and affecting the choice of repair pathway (32). We show here a novel finding in that poly-ubiquitylation downregulates Cdt1 proteolysis. The mechanism for this inhibition has yet to be clarified but could result from poly-ubiquitylation (or a protein binding to the poly-ubiquitin chain) directly affecting the ability of Cdt1 to bind to PCNA. Since blocking PCNA ubiquitylation has a dramatic result when the N-terminal PIP28 box is inactivated, ubiquitylation may inhibit interaction of the C-terminal PIP301 box with PCNA. Since PCNA poly-ubiquitylation occurs to a lower extent in S phase than after DNA damage (48), the effect of this modification would be to lower the activity of the $CRL4^{Cdt2}$ pathway after DNA damage but not in S phase. This could have the effect of preserving Cdt1 after physiological levels of DNA damage in M/G1, allowing it to carry out its essential role in DNA replication. It will be interesting to determine if other targets of the $CRL4^{Cdt2}$ pathway are also subject to this regulatory mechanism, which might differentially affect the ability of PCNA to interact with PIP-box containing proteins at replication forks and at DNA damage sites.

One uncertainty in this discussion is the fact that the biological significance of Cdt1 degradation after DNA damage is unclear. It may constitute a G1 checkpoint to delay S-phase entry (22) or help prevent origin re-licensing that could inadvertently occur following CDK suppression in response to DNA damage checkpoint activation (20). An alternative view is that Cdt1 might inhibit DNA repair more directly. Given that various repair enzymes interact with PCNA via PIP boxes (32) it is possible that some Cdt1 proteolysis is necessary to improve the accessibility of PCNA to these enzymes, or Cdt1 might directly inhibit repair by virtue of its ability to recruit Mcm2-7 (55). Generation of a stable form of Cdt1 described here will enable us to address the biological relevance of Cdt1 proteolysis after DNA damage. It will be interesting to examine the effect of a non-degradable form of Cdt1 on the timing of origin licensing and replication as well the efficiency of DNA repair and to see if these effects are conserved more generally in eukaryotes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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