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Direct Role for Proliferating Cell Nuclear Antigen in Substrate Recognition by the E3 Ubiquitin Ligase $CRL4^{Cdt2}$*[S]

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Background: $CRL4^{Cdt2}$ requires that a substrate bind to proliferating cell nuclear antigen (PCNA) on DNA prior to ligase recruitment, but the precise role of PCNA is unclear.
Results: A specific PCNA residue is required for destruction of $CRL4^{Cdt2}$ substrates.
Conclusion: $CRL4^{Cdt2}$ recognizes a composite surface composed of PCNA and substrate residues.
Significance: This is the first ubiquitin ligase whose substrate recognition requires creation of a bipartite substrate surface.

The E3 ubiquitin ligase Cullin-ring ligase 4-Cdt2 ($CRL4^{Cdt2}$) is emerging as an important cell cycle regulator that targets numerous proteins for destruction in S phase and after DNA damage, including Cdt1, p21, and Set8. $CRL4^{Cdt2}$ substrates contain a "PIP degron," which consists of a canonical proliferating cell nuclear antigen (PCNA) interaction motif (PIP box) and an adjacent basic amino acid. Substrates use their PIP box to form a binary complex with PCNA on chromatin and the basic residue to recruit $CRL4^{Cdt2}$ for substrate ubiquitylation. Using *Xenopus* egg extracts, we identify an acidic residue in PCNA that is essential to support destruction of all $CRL4^{Cdt2}$ substrates. This PCNA residue, which adjoins the basic amino acid of the bound PIP degron, is dispensable for substrate binding to PCNA but essential for $CRL4^{Cdt2}$ recruitment to chromatin. Our data show that the interaction of $CRL4^{Cdt2}$ with substrates requires molecular determinants not only in the substrate degron but also on PCNA. The results illustrate a potentially general mechanism by which E3 ligases can couple ubiquitylation to the formation of protein-protein interactions.

Eukaryotic cells contain hundreds of E3 ubiquitin ligases that each ubiquitylate one or more target proteins, modulating their activity or marking them for destruction by the 26 S proteasome (1). Several of the best-studied E3 ligases regulate cell cycle progression. For example, the Skp2-containing Cullin ring ligase 1 ($CRL1^{Skp2}$ also known as SCF^{Skp2})[2] is composed of a Cul1 scaffold, a Skp1 adaptor, the Skp2 substrate receptor, and Rbx1, which interacts with a ubiquitin-conjugating enzyme. Skp2 binds directly to substrates via a "phosphodegron," a short peptide motif on the substrate whose phosphorylation by cyclin-dependent kinases promotes its interaction with the leucine-rich repeat motif of Skp2 (2, 3). In late G_1 phase, when CDK activity rises, $CRL1^{Skp2}$ targets the CDK inhibitor p27 for destruction, promoting S phase entry. Another cell cycle-regulated ubiquitin ligase is the anaphase-promoting complex (APC^{Cdc20}). This multisubunit enzyme targets a number of factors for destruction, including Cyclin B and securin (4). In this case, the ligase itself is phosphorylated by mitotic CDKs, leading to substrate-ligase interactions.

Recently, an unusual ubiquitin ligase called $CRL4^{Cdt2}$ has been characterized, which promotes the ubiquitylation of several proteins in S phase and after DNA damage (5, 6). $CRL4^{Cdt2}$ is composed of a Cul4 scaffold, a Ddb1 adaptor, and Cdt2, the putative substrate receptor. In vertebrates, $CRL4^{Cdt2}$ targets the licensing factor Cdt1 (6–9), the CDK inhibitor p21 (Xic1 in frogs) (10–13), and the histone methyltransferase Set8 (14–18) for proteolysis in S phase. In all three cases, destruction appears to contribute to the block to re-replication. Set8 destruction also promotes transcription and prevents premature chromatin compaction (14, 15, 18). $CRL4^{Cdt2}$ targets the transcription factor E2F in flies (to shut off G_1 transcription in S phase and to regulate endocycles) (19, 20), the translesion DNA polymerase η in worms (perhaps to restrict access of this mutagenic polymerase to undamaged DNA) (21), and the ribonucleotide reductase inhibitor Spd1 in fission yeast (to activate nucleotide synthesis in S phase and after DNA damage) (22). Other substrates of $CRL4^{Cdt2}$ are likely to emerge.

The activity of $CRL4^{Cdt2}$ is coupled to DNA replication and damage via PCNA, a homotrimeric, ring-shaped molecule that encircles DNA. Given its topological embrace of DNA, PCNA tethers to DNA any proteins with which it interacts, including DNA polymerases, DNA ligases, chromatin-remodeling factors, and numerous other proteins involved in DNA replication and repair (23). Most proteins that bind PCNA do so via an eight-amino acid motif called a PIP box (see Fig. 1*A*, *green*

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[S] This article contains supplemental Figs. S1–S4 and Tables S1 and S2.
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[2] The abbreviations used are: CRL1, Cullin ring ligase 1; CRL4, Cullin ring ligase 4; MMS, methyl methane sulfonate; CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; IDCL, interdomain connector loop; HSS, high speed supernatant; LSS, low speed supernatant; TAP, tandem affinity purification.

amino acids). The aromatic and hydrophobic residues in the PIP box interact with a hydrophobic pocket underlying the interdomain connector loop (IDCL) (23–26). CRL4^{Cdt2} substrates contain a PIP box, through which they bind to chromatin-bound PCNA (PCNA^{chromatin}) at sites of DNA damage or at the replication fork. Most CRL4^{Cdt2} substrates also contain a TD motif at positions 5 and 6 of the PIP box (Fig. 1*A*, *blue* amino acids), which confers especially high affinity binding to PCNA (27, 28). However, a PIP box and TD motif are not sufficient for CRL4^{Cdt2} activity. All substrates also contain a basic residue four amino acids downstream of the PIP box (the "B+4" residue). When this residue is mutated to alanine in *Xenopus* Cdt1, the resulting protein binds normally to PCNA, but CRL4^{Cdt2} is not recruited to the Cdt1·PCNA complex (27). These data explain why most PIP box proteins, such as DNA polymerases, which lack the B+4 residue, are not destroyed. In summary, the above data indicate that, during CRL4^{Cdt2}-mediated proteolysis, a substrate docks onto PCNA^{chromatin}, leading to recruitment of CRL4^{Cdt2}, followed by ubiquitin transfer (see Fig. 1*B*). Notably, an alternative model has recently been proposed in which CRL4^{Cdt2} binds PCNA independently of a substrate, and in which the PCNA and Cdt2 binding regions within substrates can be separated (11). Thus, there is significant disagreement over the role of PCNA in promoting substrate recognition by CRL4^{Cdt2}.

In this report, we investigate the mechanism by which CRL4^{Cdt2} interacts with its substrates to trigger proteolytic degradation. First, we provide evidence against the recent proposal that CRL4^{Cdt2} and its substrates dock onto PCNA independently of one another (11), thereby affirming that CRL4^{Cdt2} is initially recruited to a PIP degron·PCNA complex. We then addressed whether the sole function of PCNA is to position the substrate's PIP degron for binding to CRL4^{Cdt2} (indirect role) or if a specific surface of PCNA is required, together with the PIP degron, to recruit CRL4^{Cdt2} (direct role). In support of the latter model, we identify an acidic residue (Asp-122) on the surface of PCNA that is essential for CRL4^{Cdt2} activity. Importantly, this residue is not necessary for PIP box binding to PCNA but is essential for CRL4^{Cdt2} recruitment to the PCNA·PIP degron complex on chromatin. Asp-122 is also essential for CRL4^{Cdt2} activity in fission yeast. Our findings support the idea that CRL4^{Cdt2} recognizes the PCNA·PIP degron interface by making direct contacts with residues in both polypeptides. This mechanism suggests new possibilities for the regulation of proteolysis.

EXPERIMENTAL PROCEDURES

Xic1 in Vitro Transcription and Translation—pCS2+/Xic1 (11) was *in vitro* translated according to the manufacturer's protocol using TNT® SP6 Quick Coupled Transcription Translation kit from Promega (Madison, WI). In each reaction 125 ng of DNA was used per 10 μl of TNT® Quick Master Mix (Promega), and reactions were scaled according to the amount of Xic1 needed.

Egg Extract and Immunological Methods—High speed supernatant (HSS), low speed supernatant (LSS) (29–31), and chromatin spin-down assays (32) were performed as described. We used previously described antibodies against Cdt1 (32), Ddb1 (33), RPA (30), Cdt2 (7), GST (New England Biolabs), M2 and Rabbit FLAG (Sigma), *Xenopus* PCNA (34), and PCNA (Santa Cruz Biotechnology, sc-056).

Depletion of Xenopus PCNA—The polyclonal PCNA antibody used for PCNA depletion was generated as described previously (34). To deplete PCNA from HSS, three rounds of depletion were performed using 3 μl of PCNA antibody per 1 μl of rProtein A-Sepharose FastFlow resin (Amersham Biosciences). To deplete PCNA from LSS, two rounds of depletion were performed. The antibody and resin were pre-bound, and 0.2 volume of resin was used per microliter of egg extract.

Cloning and Protein Purification for Xenopus Egg Extract Experiments—Recombinant *Xenopus* Cdt1$^{1-243}$-3xNLS-GST-FLAG (27), human PCNA (27), and GST-FLAG-tagged human Set8 (15) were previously described. Mutations in human PCNA were generated using a QuikChange mutagenesis kit (Clontech) and the following primers and reverse compliments: D120A, GACTATGAAATGAAGTTGATGGCTTTAGATGTTGAACAACTTGG; D122A, GAAATGAAGTTGATGGATTTAGCTGTTGAACAACTTGGA; E124A, ATGGATTTAGATGTTGCACAACTTGGAATTCCAGAACAG; D122A/E124A, GATGGATTTAGCTGTTGCTCAACTTGGAATTCC; and D122K/E124R, GATGGATTTACGTGGTAAACAACTTGGAATTCC.

MMS DNA Preparation and Bead Spin-down Assay—Methylated DNA was generated as described previously (35). A biotinylated 1-kb PCR product was generated, treated with methyl methane sulfonate (MMS), and coupled to M-280 streptavidin Dynabeads (Invitrogen) as previously described (27). To spin down MMS-DNA beads, we used a modification of our standard chromatin spin-down protocol (33), in which the egg lysis buffer (10 mM HEPES (pH 7.7), 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$) wash step was supplemented with 0.6% Triton X-100.

Fission Yeast General Methods—Strains used in this study are listed in supplemental Table S1. Standard genetic methods and flow cytometry were used as described previously (36, 37).

PCNA Mutagenesis in Fission Yeast—To construct the *pcn1-D122A* mutant strain 2640, two partially complementary fragments for the mutation (NsiI-fragment and BamHI-fragment) were amplified using primers containing the mutation and an NsiI or BamHI restriction site; subsequently, the fragments were annealed and amplified. After DNA purification, the fragment was cloned into NsiI- and BamHI-digested pSMH and integrated at the *pcn1*$^{+}$ locus after linearizing with XhoI (in the strain 2069). Hygromycin-resistant transformants were selected, and the correct integration of the plasmid was checked with the primers 619 and 979. The plasmid and all the strains constructed were checked by sequencing (primer 978). Primers used are shown in supplemental Table S2.

Cell Cycle Synchronization and UV Irradiation Experiments—Cells were arrested in G_1 phase in Edinburgh minimal medium (65) lacking NH_4Cl for 16 h at 25 °C (38), then released into the cell cycle in rich yeast extract and supplements medium (65) at 32 °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. For UV exposure, cells were resuspended in water and irradiated with

100 J/m^2 of 254 nm UV light in a 6-mm deep stirred suspension at 20 °C.

Protein Analysis of Fission Yeast Samples—Protein extracts were made by TCA extraction and analyzed by Western blotting as described previously (39). Tandam affinity purification (TAP)-tagged Cdt1 was detected with peroxidase·anti-peroxidase-soluble complex (P1291, Sigma), and α-tubulin was used as loading control and detected with antibody T5168 (Sigma).

RESULTS

To study the mechanism of CRL4^{Cdt2}-mediated proteolysis, we used two approaches that involve different types of *Xenopus* egg extracts (summarized in supplemental Fig. S1). First, DNA that had been treated with MMS to induce methylation damage was added to an HSS of egg cytoplasm. Nucleotide excision repair of the methylation damage involves a PCNA-dependent gap-filling step that promotes destruction of endogenous Cdt1 as well as other substrates by CRL4^{Cdt2} (7, 15). Second, sperm chromatin was added to an LSS of egg cytoplasm. Upon nuclear-envelope assembly around the sperm, chromosomal DNA replication initiates, leading to PCNA-dependent, CRL4^{Cdt2}-mediated proteolysis (7, 33). Most experiments were performed using DNA damage-induced destruction in HSS, but key conclusions were confirmed using the S phase pathway in LSS.

The B+4 Residue Can Be Partially Compensated for in Xic1 by Residues Upstream of the PIP Box—The B+4 residue is conserved in all CRL4^{Cdt2} substrates (Fig. 1*A*), and it is known to be essential for Cdt1 destruction (27, 28). We wanted to know whether B+4 is also important for destruction of other substrates. We therefore examined Xic1 and Set8. When added to *Xenopus* egg extracts, Xic1, a *Xenopus* CDK inhibitor, is destroyed in a manner that requires its PIP box, chromatin-loaded PCNA, and Cdt2 (11, 40–42). However, in contrast to Cdt1^{B+4A}, which was completely stable (27), Xic1^{B+4A} was still destroyed, albeit more slowly than Xic1^{WT} (Fig. 2*A*, *bottom*, *light blue trace*). This residual destruction of Xic1^{B+4A} was PCNA-dependent (data not shown). We next tested the histone H4 lysine 20 methyltransferase Set8, which is also destroyed during S phase and after DNA damage in human cells or when added to *Xenopus* egg extracts (14–18). Like Xic1^{B+4A}, Set8$^{B+3/4A}$ was destroyed slowly (Fig. 2*B*, *lanes 7–9*; note that in Set8$^{B+3/4A}$, the B+3 residue was also mutated to alanine in case of charge redundancy), and the residual destruction was still PCNA-dependent (see below). Similar to Cdt1^{B+4A} (27), Set8$^{B+3/4A}$ bound normally to PCNA^{chromatin}, and its slow destruction was due to reduced recruitment of CRL4^{Cdt2} to chromatin, resulting in reduced ubiquitylation of Set8$^{B+3/4A}$ (Fig. 2*C*). In summary, as seen in Cdt1, the B+4 residue in Xic1 and Set8 is important for efficient destruction due to a role in CRL4^{Cdt2} recruitment, although it is less crucial for destruction of the latter substrates.

Given the different effects of the B+4A mutation on different CRL4^{Cdt2} substrates, we re-examined their sequences. Notably, the PIP box of Cdt1 is located at the extreme amino terminus, whereas the PIP boxes of p21, Xic1, and Set8 are internal or C-terminal to these proteins. Additionally, the latter substrates contain one or more basic amino acids immediately upstream of the PIP box (Fig. 1*A*, *pink*) (27). We speculated that these

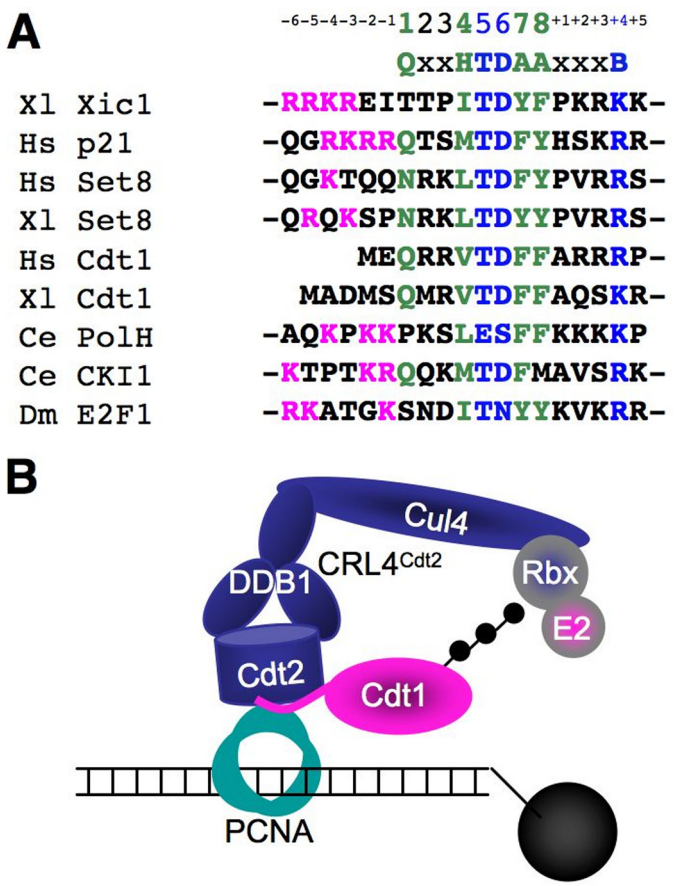

FIGURE 1. **PCNA-dependent recognition of PIP degrons by CRL4^{Cdt2}.** *A*, sequence alignment of CRL4^{Cdt2} substrate PIP degrons. Canonical PIP box residues are shown in *green*. *H* stands for a hydrophobic residue (Ile, Leu, Val, or Met) and *A* stands for an aromatic residue (Phe or Tyr). The PIP degron residues are shown in *blue*, *B* stands for any basic residue (Lys or Arg). The upstream basic residues are shown in *pink*. *B*, model of substrate recognition on PCNA by CRL4^{Cdt2} on the immobilized 1-kb DNA template.

basic residues might contribute to destruction in the absence of the B+4 residue. To test this hypothesis, we mutated the basic residues N-terminal of the PIP box to alanines in Xic1, yielding Xic1$^{RRKR/AAAA}$ (Fig. 2*A*, *top*). Mutation of these residues alone had little or no effect on Xic1 destruction (Fig. 2*A*, *yellow trace*). However, when combined with the B+4 mutant, these mutations rendered the protein completely stable, similar to Xic1$^{\Delta PIP}$ (Fig. 2*A*, *green trace*). Together, these data show that, in a CRL4^{Cdt2} substrate where the PIP degron is not located at the extreme N terminus, additional basic residues located just upstream of the PIP box can contribute to CRL4^{Cdt2}-mediated destruction, but they are only essential in the absence of the B+4 mutant. The upstream basic residues likely promote substrate destruction in a manner that is distinct from the downstream basic residues, including B+4.

The PCNA and CRL4^{Cdt2} Binding Motifs of Substrates Cannot Be Separated—Recently, Yew and colleagues proposed a new model for substrate recognition by CRL4^{Cdt2} in which the substrate and CRL4^{Cdt2} initially bind independently to PCNA before coming together in a complex (Fig. 3*A*). This model was based on two considerations. First, they showed that the C terminus of Cdt2 can bind to PCNA independently of substrate (11). However, our previous data established that Cdt2 does not

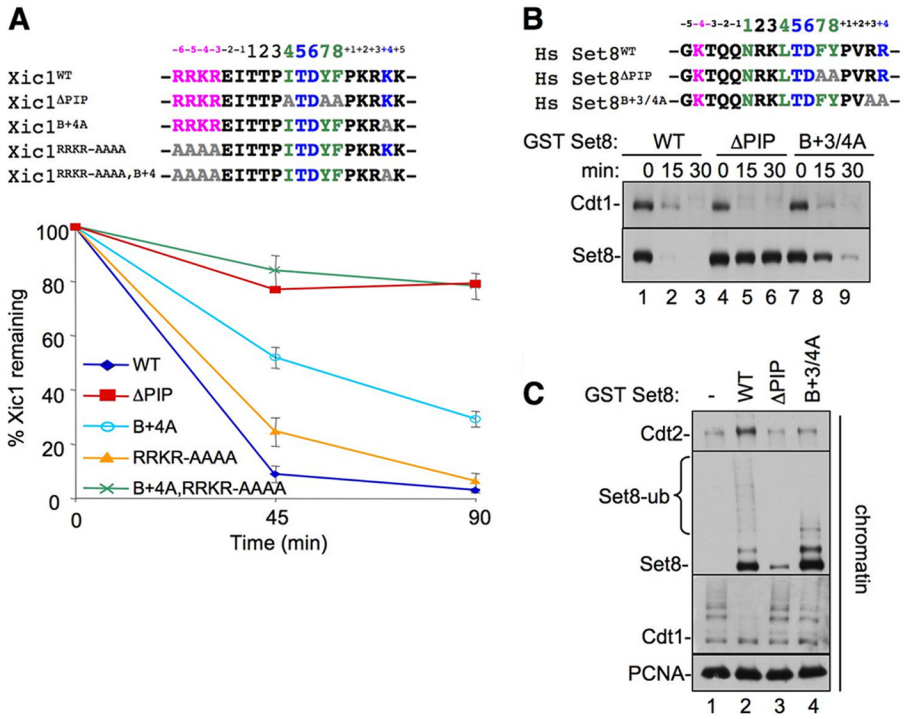

FIGURE 2. **The B+4 residue can be partially compensated for by residues upstream of the PIP box in Xic1 and Set8.** In *A*: *Top*, sequence comparison of Xic1 PIP degron with upstream basic residues and the various mutants examined. *Bottom*, *graph* showing the percentage of *in vitro* translated ^{35}S-labeled Xic1 remaining after it was added to HSS in the presence of 5 ng/μl MMS-damaged plasmid. Reactions were stopped at the indicated time points, and the amount of Xic1 remaining was quantified by autoradiography. Results from three independent experiments were averaged and graphed. *Bars* represent the standard error of the mean. In *B*: *Top*, sequence alignment of Set8 PIP degron with upstream residues and the various mutants examined. *Bottom*, HSS was supplemented with 5 ng/μl MMS plasmid and 50 nM human Set8^{WT}, Set8$^{\Delta PIP}$, or Set8$^{B+3/4A}$. At the indicated times, samples were blotted for endogenous Cdt1 or Set8. *C*, HSS was supplemented with immobilized 1-kb MMS DNA and 2 mg/ml methyl ubiquitin. Buffer and 50 nM Set8^{WT}, Set8$^{\Delta PIP}$, or Set8$^{B+3/4A}$ was also added, and after 10 min, chromatin was recovered from the extract and washed, and the indicated proteins were visualized by Western blotting.

bind to PCNA on chromatin in the absence of substrate (7, 27), demonstrating that a Cdt2-PCNA interaction is insufficient to support chromatin recruitment of the ligase. Second, Yew and colleagues proposed that, within the primary amino acid sequence of Xic1, PIP box and Cdt2 binding motifs can be widely separated and still promote destruction (11). Their conclusion, which would further indicate that CRL4^{Cdt2} does not bind PCNA in the context of substrate, was based on the following experiment. The p21 PIP degron, in which a proposed Cdt2-binding region had been deleted, was fused onto the N terminus of Xic1, in which the endogenous PIP box had been compromised through mutation of Ile-174 to alanine, yielding p21^{PIP}-Xic1^{I174A} (Fig. 3, *B* and *C*; originally named NPIP2-Xic1^{I174A} in Ref. 11). This construct was destroyed normally in egg extracts (11) even though it was thought to contain well separated PCNA and Cdt2 interaction motifs, as illustrated in Fig. 3*B*. We repeated this experiment and obtained the same result (Fig. 3*D*, *yellow trace*). However, this experiment has two caveats. First, the B+4 residue of the added p21 PIP degron was not mutated, likely still allowing binding to Cdt2. Second, only one residue (Ile-174) in the endogenous Xic1 PIP box was mutated. Thus, the mutated Xic1 PIP box and the added p21 PIP box might bind cooperatively via interactions with two PCNA subunits (Fig. 3*E*). In this case, each PIP box might be able to recruit some CRL4^{Cdt2} and thus promote destruction via the mechanism we previously proposed (Fig. 3*E*) (27).

To test whether the destruction of p21^{PIP}-Xic1^{I174A} involved residual binding of the Xic1 PIP box to PCNA, we mutated all three Xic1 PIP box residues, creating p21^{PIP}-Xic1^{I174A,Y177A,F178A} (Fig. 3*C*; abbreviated as p21^{PIP}-Xic1$^{\Delta PIP}$). Unlike p21^{PIP}-Xic1^{I174A}, p21^{PIP}-Xic1$^{\Delta PIP}$ was destroyed much less efficiently than Xic1^{WT} (Fig. 3*D*, *green line*). From these data we conclude that ~40% of p21^{PIP}-Xic1^{I174A} destruction can be attributed to residual binding of the Xic1 PIP box to PCNA. When the B+4 residue of the added p21 PIP box was also mutated to alanine, the resulting protein, p21$^{PIP/B+4A}$-Xic1$^{\Delta PIP}$ (Fig. 3*C*), was destroyed at background levels (Fig. 3*D*, *purple line*), indicating that the p21 PIP box retained Cdt2 binding capacity. Thus, the original p21^{PIP}-Xic1^{I174A} construct did not contain adequately separated PCNA and Cdt2-binding functions. When these domains are effectively separated, the resulting fusion protein is not destroyed. Therefore, there is no evidence that these domains can function separately.

Identification of a PCNA Residue That Is Essential for CRL4^{Cdt2}-mediated Destruction—We next wanted to further characterize what role PCNA plays in the recognition of the PIP degron by CRL4^{Cdt2}. Specifically, we wished to distinguish between direct and indirect roles for PCNA. In the "indirect" model, CRL4^{Cdt2} only contacts residues in the substrate's PIP degron, and the sole function of PCNA is to position the degron for recognition by CRL4^{Cdt2}. In the "direct" model, CRL4^{Cdt2} recognizes a composite surface created by the two proteins. The second model predicts that substrate recognition by CRL4^{Cdt2} requires residues in PCNA that do not influence PIP degron binding. To look for such residues, we examined the co-crystal structure of PCNA with the PIP degron of p21

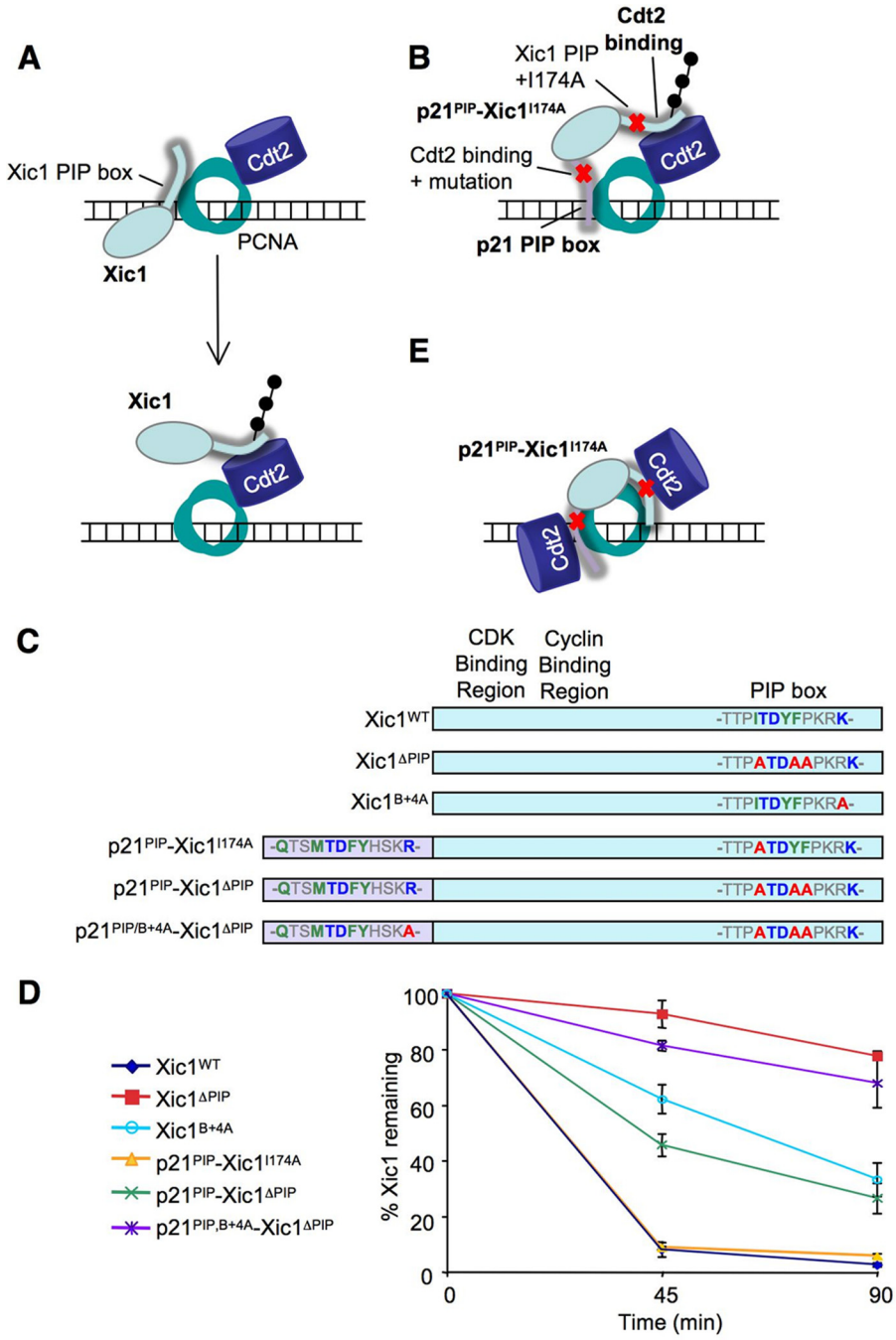

FIGURE 3. **The Cdt2 and PCNA binding motifs of Xic1 cannot be separated.** *A*, model of CRL4^{Cdt2}-mediated destruction of Xic1, in which Xic1 and Cdt2 both dock onto PCNA separately (*top*) and then come together for ubiquitin transfer (*bottom*). Adapted from Ref. 11. *B*, model for destruction of p21^{PIP}-Xic1^{I174A} using separable Cdt2 and PCNA binding motifs. Yew and colleagues fused residues 135–164 of p21 containing the PIP box to the N terminus of Xic1 to create a p21 PIP box-Xic1 fusion protein (11). In this construct one canonical PIP box residue in Xic1 (Ile-174) was mutated to an alanine to inhibit PCNA binding, and six residues (156–161) just past the B+4 residue of p21 (residues +5 to +10) were deleted to inhibit the Cdt2-binding region proposed by Yew and colleges. *C*, *scheme* of Xic1 and p21^{PIP}-Xic1 mutations used in our experiments. *D*, *graph* showing the percentage of ^{35}S-labeled Xic1 mutants remaining when incubated with HSS and 5 ng/μl MMS-damaged plasmid at the indicated times. Results from three independent experiments were averaged and graphed. *Bars* represent the standard error of the mean. *E*, our model for CRL4^{Cdt2}-mediated destruction of p21^{PIP}-Xic1^{I174A} binding to two PCNA monomers.

(Fig. 4*A*) (24). Interestingly, the structure shows that two conserved acidic residues, Asp-122 and Glu-124 on the IDCL of PCNA, cradle the B+4 residue of p21 (Fig. 4, *A* and *B*, *purple*). Because the B+4 residue is not required for binding to PCNA (27), we postulated that Asp-122 and/or Glu-124 contact CRL4^{Cdt2} and help recruit it to the PCNA·PIP degron complex.

We first tested whether Asp-122 and Glu-124, as well as another nearby acidic residue, Asp-120, are required for destruction of CRL4^{Cdt2} substrates. Asp-120, Asp-122, and Glu-124, or Asp-122 and Glu-124, were mutated to alanines, and the resulting proteins (PCNA^{D120A}, PCNA^{D122A}, PCNA^{E124A}, and PCNA$^{DE/AA}$) were purified (supplemental Fig. S2*A*). PCNA-depleted HSS was supplemented with the differ-

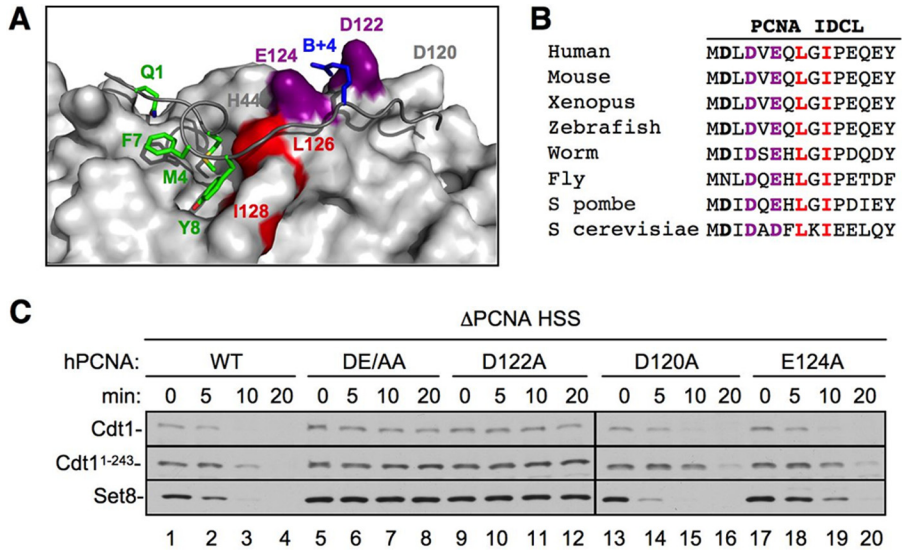

FIGURE 4. **Identification of a PCNA residue that is essential for CRL4^{Cdt2}-mediated destruction.** *A*, an image of the PCNA-p21 co-crystal structure (24) was generated using PDB accession number 1AXC and PyMOL (available on-line). PIP box residues of p21 are shown in *green*, the B+4 of p21 is shown in *blue*, Leu-126 and Ile-128 (of the PCNA-79 mutant) are shown in *red*, and PCNA acidic residues Asp-122 and Glu-124 are shown in *purple*. *B*, sequence alignment of the interdomain connector loop of PCNA (amino acids 119–133) from different species. Leu-126 and Ile-128 are shown in *red*; Asp-122 and Glu-124 are shown in *purple*. *C*, PCNA-depleted HSS was supplemented with recombinant human PCNA^{WT}, PCNA$^{DE/AA}$ (PCNA$^{D122A/E124A}$), PCNA^{D122A}, PCNA^{D120A}, or PCNA^{E124A}. Recombinant Set8^{WT}, Cdt1$^{1-243}$, and 5 ng/μl MMS plasmid were also added to the extract. Reactions were stopped at the indicated times and blotted for Cdt1 (both endogenous and Cdt1$^{1-243}$) or Set8. Samples were run on two separate gels that were processed under the same conditions. Additionally, PCNA^{WT} samples were run on both gels, exposures were matched, and similar exposures were used.

ent recombinant PCNA proteins, damaged DNA, as well as Set8 and Cdt1$^{1-243}$, an N-terminal fragment of Cdt1 that is destroyed by the same mechanism as Cdt1^{WT} (27). Although PCNA^{D120A} behaved essentially like PCNA^{WT}, PCNA^{E124A} displayed a noticeable, but minor defect, especially in Set8 destruction (Fig. 4*C*, *lanes 17–20*). Strikingly, PCNA^{D122A} was completely inactive for destruction of Cdt1, Cdt1$^{1-243}$, and Set8 (Fig. 4*C*, *lanes 9–12*). PCNA^{D122A} was also unable to support efficient Xic1 destruction (supplemental Fig. S2*C*). As expected, PCNA$^{DE/AA}$ was unable to support CRL4^{Cdt2} activity in HSS extracts (Fig. 4*C* and supplemental Fig. S2*B*). In addition, PCNA$^{DE/AA}$ did not support replication-dependent Cdt1 destruction in the context of sperm chromatin replication carried out in LSS extracts (supplemental Fig. S3*A*).

We examined the role of other PCNA residues near the IDCL. Alanine substitution of His-44, which contacts the 5 and 6 positions of bound PIP boxes, caused a slight defect in destruction of Cdt1, due to deficient substrate binding to PCNA, whereas S42A and S230A, which reside on either side of the IDCL, had no effect.[3]

It was important to rule out the possibility that the D122A and E124A mutations in PCNA inhibited CRL4^{Cdt2} activity due to indirect effects on DNA replication. As shown in supplemental Fig. S2*D*, PCNA$^{DE/AA}$ supported normal levels of M13 DNA replication in HSS, a model for leading strand synthesis in *Xenopus* egg extracts, implying that this mutant is also loaded normally on chromatin. PCNA$^{DE/AA}$ was also fully competent for sperm chromatin replication in LSS (supplemental Fig. S3*B*). Together, our results show that the D122A and E124A residues of PCNA play a specific role in potentiating CRL4^{Cdt2} function.

[3] B. Morris, C. G. Havens, and J. C. Walter, data not shown.

Asp-122 Is Required for CRL4^{Cdt2} Docking, but Not Substrate Binding to PCNA—To determine why the Asp-122 residue of PCNA is required for CRL4^{Cdt2} function, we first examined whether it mediates binding of substrates to PCNA. We took advantage of the fact that Cdt1$^{1-243}$ only binds to chromatin via PCNA in a PIP box-dependent manner (27). MMS-treated DNA coupled to magnetic beads was added to PCNA-depleted HSS supplemented with Cdt1$^{1-243}$ and either buffer, PCNA^{WT}, PCNA$^{DE/AA}$, or PCNA$^{DE/KR}$, a charge-reversal mutant that also failed to support CRL4^{Cdt2} function and behaved identically to PCNA$^{DE/AA}$ (supplemental Figs. S2*B* and S3*B*). We also included PCNA$^{LI/AA}$, a PCNA mutant previously studied in *Saccharomyces cerevisiae* (25), which carries mutations in residues Leu-126 and Ile-128 in the IDCL of PCNA and does not bind well to PIP box proteins (24–26, 43). Each of the recombinant PCNAs bound to chromatin at similar levels (Fig. 5*A*, PCNA), suggesting that none of the mutations affected replication factor C-mediated loading of PCNA onto DNA. Indeed, it was previously reported that even PCNA$^{LI/AA}$ is efficiently loaded onto DNA by replication factor C (25). Importantly, equal amounts of Cdt1$^{1-243}$ bound to DNA in the presence of PCNA^{WT}, PCNA$^{DE/AA}$, and PCNA$^{DE/KR}$ (Fig. 5*A*, *lanes 4*, *6*, and *7*). In contrast, PCNA$^{IL/AA}$ did not support Cdt1 loading above background levels, as expected, based on previous studies using DNA polymerases (Fig. 5*A*, compare *lanes 2* and *5*) (25, 26). In conclusion, the defect in CRL4^{Cdt2} function observed with mutations in Asp-122 and Glu-124 was not due to a failure to recruit the substrate to chromatin-bound PCNA.

We next examined CRL4^{Cdt2} recruitment to chromatin in the presence of the PCNA mutants. MMS-treated 1-kb DNA conjugated to magnetic beads was added to PCNA-depleted extract supplemented with recombinant PCNAs and Cdt1$^{1-243}$. Unlike PCNA^{WT}, PCNA$^{DE/KR}$ and PCNA$^{DE/AA}$

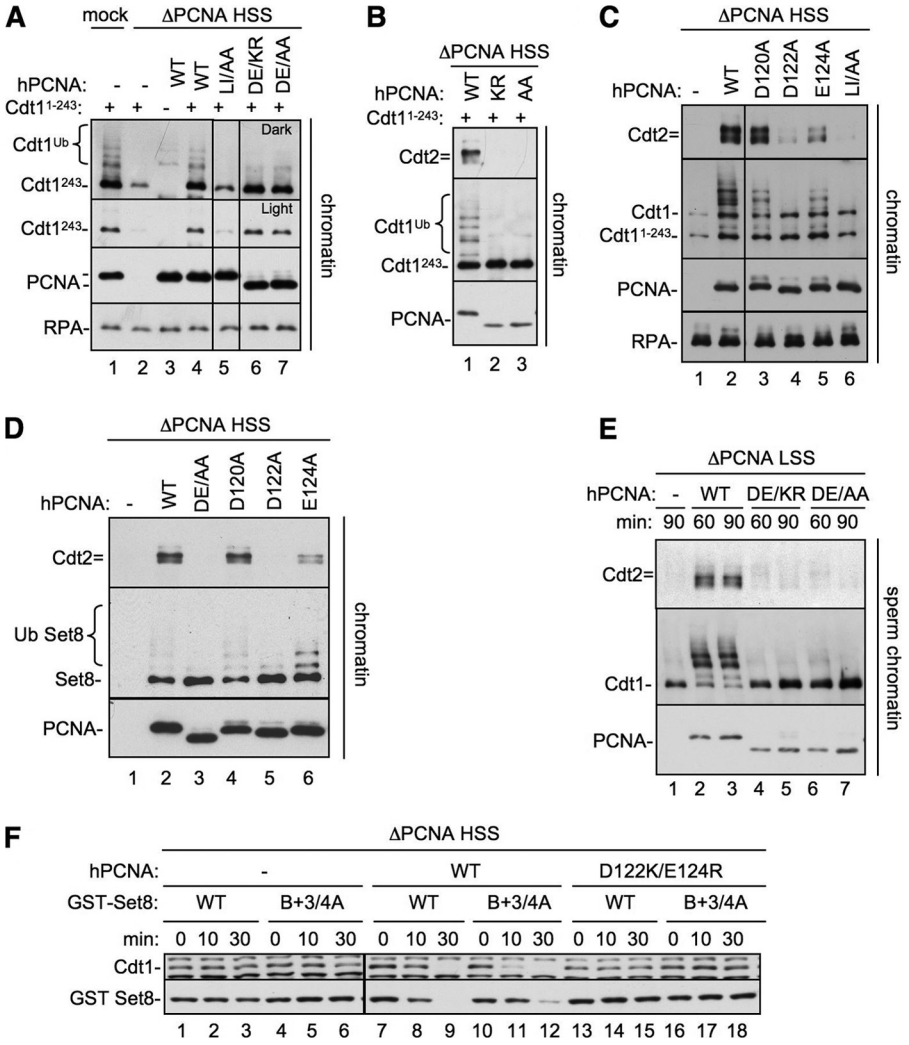

FIGURE 5. **PCNA Asp-122 is required for CRL4^{Cdt2} recruitment, but not substrate binding.** *A*, Cdt1$^{1-243}$ binding to PCNA mutants. PCNA-depleted HSS was supplemented with 1-kb MMS DNA, methyl ubiquitin, Cdt1$^{1-243}$, and either buffer, PCNA^{WT}, PCNA$^{LI/AA}$ (PCNA$^{L126A/I128A}$), PCNA$^{DE/AA}$ (PCNA$^{D122A/E124A}$), or PCNA$^{DE/KR}$ (PCNA$^{D122K/E124R}$). After 10 min, chromatin was recovered from extract, washed with 200 mM KCl-ELB, and blotted for the indicated proteins. All samples were run on the same gel, but some irrelevant lanes were removed. Note that PCNA$^{DE/AA}$ and PCNA$^{DE/KR}$ migrate faster than PCNA^{WT} likely due to the change in charges. *B*, Cdt2 recruitment to chromatin by PCNA mutants. PCNA-depleted HSS was supplemented with 1 kb of immobilized MMS DNA, methyl ubiquitin, Cdt1$^{1-243}$, and either buffer, PCNA^{WT}, PCNA$^{DE/AA}$ (PCNA$^{D122A/E124A}$), or PCNA$^{DE/KR}$ (PCNA$^{D122K/E124R}$). After 10 min chromatin was recovered from extract, washed, and blotted for the indicated proteins. *C*, PCNA-depleted HSS was supplemented with 1 kb of MMS DNA, methyl ubiquitin, Cdt1$^{1-243}$, and either buffer, PCNA^{WT}, PCNA^{D120A}, PCNA^{D122A}, PCNA^{E124A}, or PCNA$^{LI/AA}$. After 10 min chromatin was recovered from extract, washed, and blotted for the indicated proteins. All samples were run on the same gel, but some irrelevant lanes were removed. *D*, PCNA-depleted HSS was supplemented with 1 kb of MMS DNA, methyl ubiquitin, Set8^{WT}, and either buffer, PCNA^{WT}, PCNA$^{DE/AA}$, PCNA$^{DE/KR}$, PCNA^{D120A}, PCNA^{D122A}, PCNA^{E124A}, or PCNA$^{LI/AA}$. After 10 min chromatin was recovered from extract, washed, and blotted for the indicated proteins. *E*, PCNA-depleted LSS was supplemented with sperm chromatin, methyl ubiquitin and buffer, PCNA^{WT}, PCNA$^{DE/KR}$, or PCNA$^{DE/AA}$. After 60 or 90 min, chromatin was recovered from extract, washed, and blotted for the indicated proteins. *F*, PCNA-depleted HSS was supplemented with MMS plasmid, Set8^{WT}, or Set8$^{B+3/4A}$ and either buffer, PCNA^{WT}, or PCNA$^{DE/KR}$. The reactions were stopped at the indicated times and blotted for Cdt1 or Set8. Samples were run on two separate gels that were processed under the same conditions. Additionally, one sample set was run on both gels, exposures were matched, and similar exposures were used.

were inactive for Cdt2 recruitment and Cdt1$^{1-243}$ ubiquitylation (Fig. 5*B*). PCNA^{D120A} was unaffected, whereas PCNA^{D122A} behaved like PCNA$^{DE/AA}$ with respect to Cdt1 binding, CRL4^{Cdt2} recruitment, and ubiquitylation (Fig. 5*C*, *lanes 3* and *4*). Consistent with its intermediate effects on Cdt1 destruction, PCNA^{E124A} supported intermediate levels of Cdt2 recruitment (Fig. 5*C*, *lane 5*). Similar results were observed for Set8 (Fig. 5*D*). PCNA$^{DE/AA}$ and PCNA$^{DE/KR}$ also did not support Cdt2 recruitment or Cdt1 ubiquitylation in the context of sperm chromatin replication (Fig. 5*E*). Together, these data show that Asp-122 is essential and Glu-124 is important for substrate-dependent CRL4^{Cdt2} recruitment to chromatin, whereas these residues have no role in mediating the binding of PIP box proteins to PCNA.

PCNA Residues Asp-122 and Glu-124 Are Required for CRL4^{Cdt2}-mediated Destruction Independently of the B+4 Residue—Based on the above results, we postulated that CRL4^{Cdt2} binds directly to residues in the PIP degron (B+4), as well as in PCNA (Asp-122 and Glu-124). However, one alternative explanation was that Asp-122 and Glu-124 do not directly contact CRL4^{Cdt2} (or an unknown ligase cofactor) but rather function to position B+4 for interaction with the ligase. To address this possibility, we examined whether mutations in Asp-122 and Glu-124 still affect destruction of a CRL4^{Cdt2} sub-

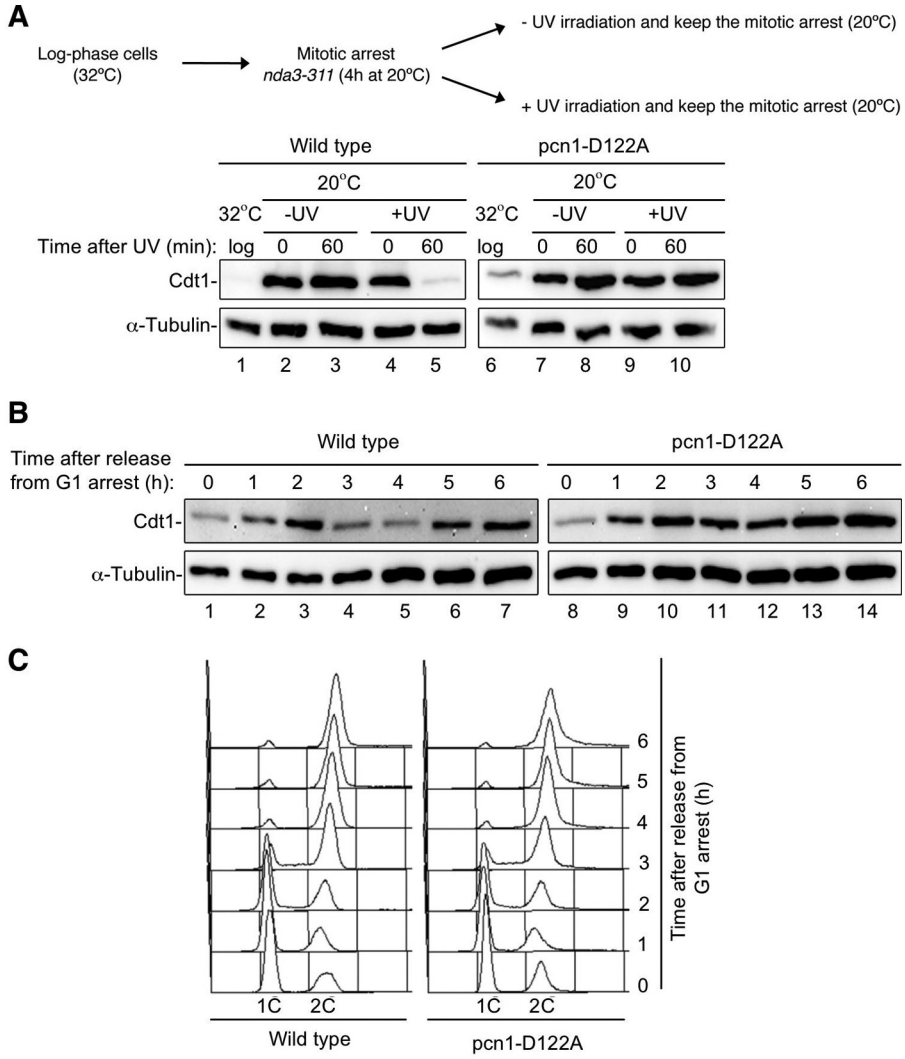

FIGURE 6. **The Asp-122 residue of PCNA is required for CRL4^{Cdt2}-mediated destruction in fission yeast.** *A*, Western blot analysis of Cdt1-TAP levels in cell extracts from a wild-type strain (2069) and a *pcn1-D122A* mutant (2640) prepared after UV irradiation (100 J/m^2) or mock irradiated. Both strains 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 to maintain the mitotic arrest and prevent entry into S phase. *B*, Cdt1-TAP levels in wild-type (1540) and *pcn1-D122A* (2660) in G_1-arrested cells, subsequently released into the cell cycle. *C*, flow cytometric analysis of DNA contents of cells shown in *B*.

strate lacking the B+4 residue. This experiment was not possible in the context of Cdt1, where mutation of B+4 alone completely eliminates destruction (27). Therefore, we examined the destruction of Set8$^{B+3/4A}$ in PCNA-depleted extracts supplemented with PCNA^{WT} or PCNA$^{DE/KR}$. As shown in Fig. 5*F*, the intermediate level of Set8$^{B+3/4A}$ destruction was completely abolished in the presence of PCNA$^{DE/KR}$ (compare *lanes 10–12* with *16–18*). Thus, PCNA residues 122 and 124 are critical for CRL4^{Cdt2}-mediated destruction even in the absence of the B+4 residue, indicating that residues on PCNA are directly involved in ligase recruitment.

PCNA Residue Asp-122 Is Required for CRL4^{Cdt2}-mediated Destruction in Fission Yeast—In *Schizosaccharomyces pombe*, CRL4^{Cdt2} targets Cdt1 for destruction in a PCNA-dependent manner during S phase and after DNA damage (39, 44). To determine whether the function of aspartic acid 122 in PCNA is conserved and whether it affects endogenous CRL4^{Cdt2} substrates in an *in vivo* model, we made the D122A mutation in the *S. pombe* PCNA gene, *pcn1*. Upon UV irradiation, cells expressing wild-type PCNA destroyed Cdt1, while cells expressing PCNA^{D122A} did not (Fig. 6*A*, compare *lanes 5* and *10*). In addition, analysis of Cdt1 levels in cells released from a G_1 arrest showed that the S phase destruction of Cdt1 is inhibited in the mutant (Fig. 6*B*, compare *lanes 4* and *5* to *lanes 11* and *12*). Stabilization of TAP-tagged Cdt1 by PCNA^{D122A} appeared to cause a minor delay in either S phase entry or progression through S phase (Fig. 6*C*, compare 3-h time points), but this could not explain the effect on destruction. Similar results for Cdt1 stabilization were seen in cells released from a mitotic block (supplemental Fig. S4). Therefore, as in *Xenopus* egg extracts, Asp-122 in *S. pombe* PCNA is required to support CRL4^{Cdt2} activity during replication and after DNA damage.

DISCUSSION

In this study we explore the molecular mechanism by which CRL4^{Cdt2} recognizes its substrates in the context of PCNA. We provide evidence that recognition by CRL4^{Cdt2} requires amino acids not only in the substrate's PIP degron, but also in PCNA

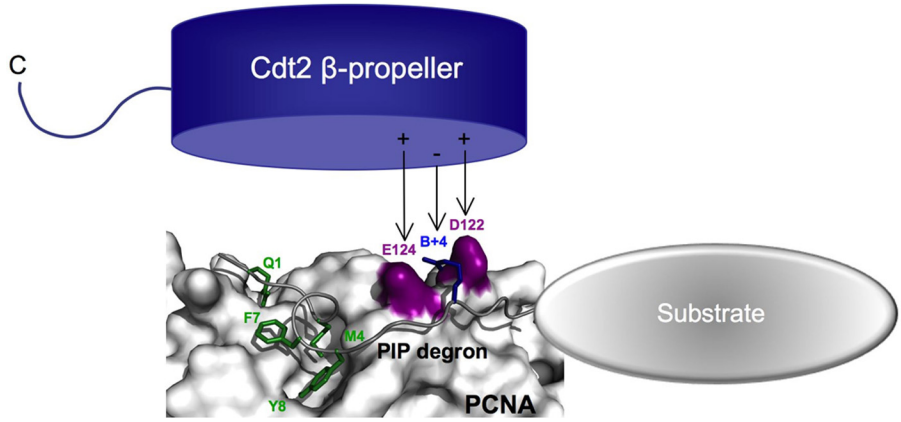

FIGURE 7. **Putative model of CRL4^{Cdt2} recruitment to the PCNA·substrate complex.** Substrate-dependent recruitment of CRL4^{Cdt2} to PCNA^{DNA} requires residues in the PIP degron (B+4), as well as PCNA Asp-122. PCNA Glu-124 also contributes to CRL4^{Cdt2} recruitment but is not essential. We speculate charged residues in Cdt2 make direct contacts with B+4, Asp-122, and Glu-124.

itself. The simplest interpretation is that CRL4^{Cdt2} makes direct contacts with both polypeptides during substrate recognition. This mechanism appears to be conserved from humans to fission yeast.

The activity of most ubiquitin ligases is regulated either by the assembly of ligase subunits, or post-translational modification of the ligase or substrate. To our knowledge, CRL4^{Cdt2} is the first example of a ubiquitin ligase whose activity is regulated by the creation of a bipartite surface when a substrate interacts with another polypeptide. Specifically, the event that triggers destruction of CRL4^{Cdt2} substrates is the creation of a composite surface composed of PCNA and the substrate. Conversely, CRL1^{Tir}-auxin (SCF^{Tir1}-auxin) (45) and CRL1^{Skp2}-Cks1 (SCF^{Skp2}-Cks1) (46) require ligase cofactor interactions to recognize their substrates. In the case of CRL1^{Tir1}-auxin, binding of auxin to the substrate receptor Tir1 creates a surface on the ligase that mediates binding to and ubiquitylation of its substrates (45). Although ubiquitylation of the CRL1^{Skp2} substrate p27 requires prior formation of a complex between the substrate receptor Skp2 and its cofactor Cks1, this interaction is not the initiating event to trigger p27 destruction (46, 47). Rather, CRL1^{Skp2}-Cks1-mediated proteolysis is promoted by a phosphorylation event on threonine 187 of the substrate p27, which mediates the p27-CRL1^{Skp2}-Cks1 interaction (3, 46, 49).

Mechanism of Substrate Recognition by CRL4^{Cdt2}—Our data suggest the following model for the assembly of the ternary PCNA^{DNA}·PIP degron·CRL4^{Cdt2} complex. First, substrates bind PCNA^{DNA} via their PIP degron, an event that does not require CRL4^{Cdt2} (27) and therefore almost certainly precedes binding of the ligase. Next, CRL4^{Cdt2} docks onto the PCNA·PIP degron complex, likely using the WD40-repeat-containing β-propeller of Cdt2. A model for the structure of CRL4^{Ddb2}, which ubiquitylates xeroderma pigmentosum, complementation group C (XPC), in the context of nucleotide excision repair (50–52), provides a framework for the possible structure of CRL4^{Cdt2}. Thus, like the β-propeller protein Ddb2, Cdt2 likely contacts the adaptor protein Ddb1 via a helix located near the bottom surface of its propeller (50, 53). Accordingly, the top surface of the propeller of Cdt2 would interact with the PCNA·PIP degron complex. We have shown that the B+4 residue within the PIP degron and at least one residue on PCNA that cradles B+4 are essential for stably recruiting CRL4^{Cdt2}. It is presently unclear whether residues in the PIP degron other than B+4 or residues in PCNA other than Asp-122 and Glu-124 make contact with CRL4^{Cdt2}. Our data suggest that Cdt2 contains a surface with an appropriate arrangement of positive and negative charges that binds the PCNA·PIP degron complex (Fig. 7). Importantly, because substrate recognition by CRL4^{Cdt2} has not been reconstituted with purified components, we cannot rule out the possibility that the binding of CRL4^{Cdt2} to the PCNA·PIP degron complex is indirect. However, given the direct binding of several other β-propeller WD40 proteins to substrate (50, 54–58), this appears unlikely.

An important question is whether PCNA functions primarily as a match-maker that promotes interactions between CRL4^{Cdt2} and its substrates (as illustrated in Fig. 7), or whether it also regulates ubiquitin transfer allosterically, by inducing conformational changes in the substrate or ligase. Our identification of residues on PCNA that are specifically required to recruit CRL4^{Cdt2} to the PCNA·substrate complex provides strong evidence for the former view, although it leaves open the possibility that PCNA could play additional roles in ubiquitin transfer.

Recently, Yew and colleagues proposed a two-step recognition model in which Xic1 and CRL4^{Cdt2} bind independently to two different subunits of PCNA and only later come together for Xic1 ubiquitylation (Fig. 3*A*) (11). This conclusion was based in part on an experiment in which a hybrid Xic1 substrate was constructed that was thought to have well separated PCNA and Cdt2 recognition motifs (Fig. 3*B*). However, we show here that the motifs were in fact not well separated (Fig. 3, *C* and *D*). Together with the observation that short peptides derived from Cdt1 (59) and p21 (27) are sufficient to support CRL4^{Cdt2} recruitment and activity, the data strongly favor a model in which the PCNA- and CRL4^{Cdt2}-binding activities in the substrate are closely linked.

Contribution of Positively Charged Residues Upstream of the PIP Box—In Cdt1, mutation of the B+4 residue completely abolished destruction (27, 28), whereas in Xic1 and Set8, destruction was slowed but not eliminated (this report). In all cases, the mutation dramatically reduced the recruitment of CRL4^{Cdt2} to the PCNA·PIP degron complex. Importantly, the

residual destruction of Set8$^{B+3/4A}$ (Fig. 5*F*) and Xic1^{B+4A} (data not shown) still required Asp-122, arguing that this residue does not merely function to position the B+4 residue for recognition by CRL4^{Cdt2}. Notably, the PIP degron of Cdt1 is located at the extreme N terminus of the protein, whereas in Set8, Xic1, and p21, this is not the case. The latter class of substrates also contains a cluster of basic residues immediately upstream of the PIP box. Mutation of these residues alone didn't interfere with destruction of Xic1. However, when they were mutated in combination with B+4, Xic1 was no longer degraded. The upstream basic residues are likely to contribute to the free energy of ternary complex formation, but this contribution is only readily detectable when the PIP degron is otherwise compromised through mutation of B+4. The discovery that Set8 and Xic1 lacking the B+4 residue can be inefficiently destroyed at reduced rates suggests that CRL4^{Cdt2} could also modify proteins that lack the B+4. However, the absence of this residue would have to be compensated for by other features to enhance CRL4^{Cdt2} recruitment, as illustrated by the basic residues upstream of the Xic1 PIP box. In addition, we know that the +5 downstream basic residue near the B+4 enhances PCNA binding, which contributes to efficient CRL4^{Cdt2}-mediated destruction (13, 24, 27, 28). In fact, if the dimensions of the Cdt2 β-propeller are similar to those of Ddb2, separate areas of the "top" surface of Cdt2 could simultaneously contact the B+4 and upstream basic residues in the p21 PIP degron·PCNA complex.

Recently two distinct E2 ubiquitin-conjugating enzymes were identified that mediate ubiquitylation of substrates with CRL4^{Cdt2} (60). UBCH8 cooperates with CRL4^{Cdt2} to target p21 and Set8, whereas UBE2G1 and UBE2G2 collaborate with CRL4^{Cdt2} to ubiquitylate Cdt1. During polyubiquitin chain formation, E2s can recognize the surface of ubiquitin or the substrate near the lysine to be ubiquitylated (48, 61–64). Therefore, given the different PIP box locations and additional contributions of upstream residues of CRL4^{Cdt2} substrates, it is tempting to speculate that these different recognition determinants could contribute to the use of distinct E2s for Cdt1 *versus* p21 and Set8.

New Perspectives on the Degron—Our work raises interesting questions about the nature of degrons and the regulation of proteolysis. Because substrate recognition by CRL4^{Cdt2} requires residues in the PIP degron and in PCNA, PCNA could be considered part of the degron. However, to conform to the field's implicit understanding of the term, we propose that "degron" be reserved for recognition elements within the protein that gets destroyed. Eukaryotic cells contain hundreds of distinct ubiquitin ligases, most of which are completely uncharacterized, and we speculate that some of these might be regulated similarly to CRL4^{Cdt2}. Thus, we propose that, among the thousands of transient protein-protein interactions that form during cellular growth and metabolism, some create a composite recognition surface that attracts a specific E3 ubiquitin ligase. Degron recognition could also involve the binding of protein substrates to other macromolecules such as nucleic acids, sugars, or lipids. The utility of this strategy is that it couples proteolysis to the final outcome of signaling events, which is usually the assembly of macromolecular complexes.

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