Molecular Cell
Article

Docking of a Specialized PIP Box onto Chromatin-Bound PCNA Creates a Degron for the Ubiquitin Ligase CRL4^{Cdt2}

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DOI 10.1016/j.molcel.2009.05.012

SUMMARY

Substrates of the E3 ubiquitin ligase CRL4^{Cdt2}, including Cdt1 and p21, contain a PCNA-binding motif called a PIP box. Upon binding of the PIP box to PCNA on chromatin, CRL4^{Cdt2} is recruited and the substrate is ubiquitylated. Importantly, a PIP box cannot be sufficient for destruction, as most PIP box proteins are stable. Using *Xenopus* egg extracts, we identify two sequence elements in CRL4^{Cdt2} substrates that promote their proteolysis: a specialized PIP box that confers exceptionally efficient PCNA binding and a basic amino acid 4 residues downstream of the PIP box, which recruits CRL4^{Cdt2} to the substrate-PCNA complex. We also identify two mechanisms that couple CRL4^{Cdt2}-dependent proteolysis to the chromatin-bound form of PCNA, ensuring that this proteolysis pathway is active only in S phase or after DNA damage. Thus, CRL4^{Cdt2} recognizes an unusual degron, which is assembled specifically on chromatin via the binding of a specialized PIP box to PCNA.

INTRODUCTION

Cells use ubiquitin-mediated proteolysis to regulate many processes, including cell-cycle progression, transcription, signaling, DNA replication, and DNA repair. During ubiquitin-mediated proteolysis, an E3 ubiquitin ligase attaches a polyubiquitin chain to the substrate, which targets it for destruction by the 26S proteasome (Nakayama and Nakayama, 2006; Petroski and Deshaies, 2005). Many ubiquitin ligases recognize substrates via a short amino acid motif called a "degron" (Nakayama and Nakayama, 2006; Petroski and Deshaies, 2005). Because the binding of the ligase to its cognate degron is frequently a key point of regulation, ligase-degron interactions have been the subject of intense investigation.

Several E3 ubiquitin ligases control cell-cycle progression by binding to specific degron motifs. One of these, the anaphase-promoting complex (APC), is active from mitosis until the end of the G1 phase. The APC binds the activating subunits Cdc20 or Cdh1 (Peters, 2006). These activators function as "substrate receptors" by binding directly to a degron motif within the substrate. APC^{Cdc20} is positively regulated via cyclin-dependent kinase 1 (CDK1)-dependent phosphorylation of Cdc20, and it recognizes proteins with a "D box" degron, whose consensus is R-X-X-L (Glotzer et al., 1991). In contrast to APC^{Cdc20}, the activity of APC^{Cdh1} is negatively regulated by CDK, such that it only functions from anaphase until late G_1, when CDK activity is low. APC^{Cdh1} recognizes proteins that contain either a D box or a "KEN box," which comprises the motif K-E-N (Pfleger and Kirschner, 2000).

Another example of a cell-cycle-regulated E3 ubiquitin ligase is SCF, now known as Cullin-ring ligase 1 (CRL1). The CRL1$^{F\ box}$ complex is composed of Cul1, a scaffolding protein; Skp1, an adaptor protein; Rbx1, a ring protein that interacts with the E2; and one of numerous substrate receptors called F box proteins that bind Skp1 (Nakayama and Nakayama, 2006; Petroski and Deshaies, 2005). Recognition by CRL1$^{F\ box}$ often requires substrate phosphorylation at serine or threonine residues, which creates a "phosphodegron" that interacts with the F box protein. For example, proteins destroyed by CRL1$^{\beta\text{-}TRCP}$ typically employ the degron D-pS-G-X-X-pS (Ang and Wade Harper, 2005; Nakayama and Nakayama, 2006), whereas the degron for CRL1^{Fbw7} is L-X-pT-P-P-X-pS (Ang and Wade Harper, 2005).

Recently, CRL4^{DCAF} has emerged as another E3 ubiquitin ligase that is an essential regulator of cell-cycle progression and genome stability. In this E3 ligase, the Cul4 scaffold links to the Ddb1 adaptor, which, in turn, binds to different putative substrate receptors called DCAFs (Jin et al., 2006; Lee and Zhou, 2007; O'Connell and Harper, 2007). Within this family, CRL4^{Cdt2} targets the replication licensing factor Cdt1 for destruction in S phase of the cell cycle in all metazoans (Arias and Walter, 2006; Jin et al., 2006; Zhong et al., 2003). Cdt1 is required to recruit the MCM2-7 complex to origins in the G1 phase of the cell cycle ("licensing"), and CRL4^{Cdt2}-mediated destruction of Cdt1 prevents de novo licensing in S phase, thereby limiting DNA replication to one round per cell cycle (Jin et al., 2006; Kim and Kipreos, 2007; Lovejoy et al., 2006; Sansam et al., 2006; Zhong et al., 2003). CRL4^{Cdt2} likely also targets the *Xenopus* CDK inhibitor Xic1 (Chuang and Yew, 2001, 2005; Chuang et al., 2005; You et al., 2002), and it was recently shown to target p21 (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008), CKI-1 (Kim et al., 2008), E2F (Shibutani et al., 2008), and DNA polymerase η (Kim and Michael, 2008) for destruction. Interestingly, Cdt1 and Xic1 destruction in S phase is coupled directly to DNA replication (Arias and Walter, 2005; Chuang and Yew, 2001; May et al., 2005; Nishitani et al., 2006). Cdt1

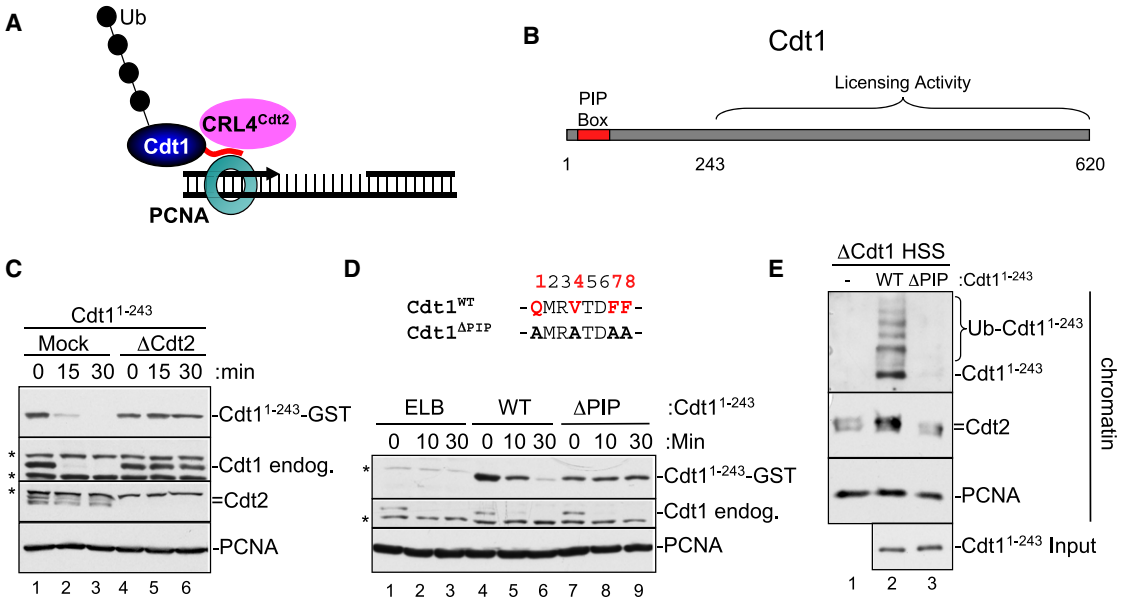

Figure 1. Cdt1$^{1–243}$ Is Destroyed in a CRL4^{Cdt2}- and PCNA-Dependent Manner and Interacts with Chromatin-Bound PCNA

(A) Model of PCNA-Cdt1-CRL4^{Cdt2} complex on DNA.

(B) Schematic of PCNA binding and licensing regions of *Xenopus* Cdt1.

(C) Cdt2-dependent destruction of endogenous Cdt1 and recombinant Cdt1$^{1–243}$ in HSS. Mock-depleted or Cdt2-depleted HSS was supplemented with 50 nM recombinant Cdt1$^{1–243}$-GST-Flag (Cdt1$^{1–243}$) and 5 ng/μl MMS plasmid. At different times, samples were blotted for the indicated proteins. Asterisks indicate background bands.

(D) Cdt1$^{1–243}$ destruction is PIP box dependent. HSS was supplemented with 1 kb MMS DNA and Cdt1$^{1–243}$ or Cdt1$^{1–243/ΔPIP}$. At different times, samples were blotted for the indicated proteins. The sequence of the ΔPIP mutant is shown.

(E) Cdt1-depleted HSS was supplemented with immobilized 1 kb MMS DNA and 2 mg/ml methyl ubiquitin. We added 50 nM Cdt1$^{1–243}$ or Cdt1$^{1–243/ΔPIP}$ as indicated, and after 10 min, chromatin was recovered from the extract, washed, and blotted for the indicated proteins.

and p21 are also destroyed after DNA damage in a manner that depends on PCNA and CRL4^{Cdt2} (Abbas et al., 2008; Arias and Walter, 2006; Higa et al., 2003; Hu et al., 2004; Jin et al., 2006; Kim et al., 2008; Lovejoy et al., 2006; Nishitani et al., 2008; Ralph et al., 2006; Sansam et al., 2006). CRL4^{Cdt2}- and PCNA-dependent proteolysis of two substrates (Cdt1 and Spd1) after DNA damage have also been detected in *S. pombe*, but not in *S. cerevisiae* (Liu et al., 2005; Ralph et al., 2006). Currently, there is no evidence that CDK, ATR, ATM, or any other protein kinase is required for Cdt1 destruction in S phase or after DNA damage, and all of the data suggest that the mechanism of destruction in these two contexts is identical.

Many proteins involved in DNA metabolism interact with PCNA, including DNA polymerases, ligases, endonucleases, chromatin remodeling factors, and CDK inhibitors (Moldovan et al., 2007). PCNA is a homotrimeric ring that encircles DNA and thereby tethers interacting proteins to the DNA. Most PCNA-binding proteins contain a PCNA interaction protein motif, or PIP box, through which they interact with PCNA. PIP boxes are 8 amino acids long and are thought to contain 4 essential residues (Q-x-x-Ψ-x-x-ϑ-ϑ, in which Ψ is any moderately hydrophobic amino acid L, V, I, or M and ϑ is an aromatic residue, Y or F). The PIP box interacts with the interdomain connector loop (IDCL) and a hydrophobic pocket of PCNA (Moldovan et al., 2007). Importantly, all known substrates of CRL4^{Cdt2} contain a PIP box, and in each case, mutation of the PIP box abolishes destruction by CRL4^{Cdt2}. An unresolved puzzle in the field is why the vast majority of PIP box proteins are not targeted for destruction by CRL4^{Cdt2}.

Another important question is how PCNA promotes CRL4^{Cdt2}-dependent proteolysis. Our results in *Xenopus* egg extracts showed that Cdt1 ubiquitylation occurs exclusively on chromatin in a PIP box-, PCNA-, and replication- or damage-dependent manner, suggesting that chromatin-bound PCNA (PCNA^{Chromatin}) serves as a platform for Cdt1 ubiquitylation (Arias and Walter, 2005, 2006). Consistent with this idea, CRL4^{Cdt2} binds to chromatin during replication and in a fashion that depends on Cdt1's PIP box (Arias and Walter, 2006; Jin et al., 2006). These data suggest that Cdt1 docks onto PCNA^{Chromatin} and that the PCNA-Cdt1 complex recruits CRL4^{Cdt2}, after which ubiquitin transfer occurs (Figure 1A). This model explains why Cdt1 is destroyed only in S phase and after DNA damage because PCNA is bound to chromatin under both circumstances. However, the model does not address why unbound PCNA (PCNA^{Free}) is not competent to support Cdt1 destruction, a critical feature that prevents aberrant Cdt1 destruction in G1. One idea is that Cdt1 does not bind efficiently to PCNA^{Free}. In apparent support of this explanation, we failed to detect an interaction between Cdt1 and PCNA^{Free} in extracts lacking DNA, although we were unable to verify binding to PCNA^{Chromatin} under the same conditions (unpublished results cited in Arias and Walter, 2006; Jin et al., 2006). Another possibility is that only the complex of PCNA^{Chromatin} and Cdt1 is competent to interact with CRL4^{Cdt2}.

To define the sequence elements that comprise the CRL4^{Cdt2} degron and to understand why Cdt1 destruction depends on PCNA^{Chromatin}, we studied the DNA-damage-induced proteolysis of Cdt1 in *Xenopus* egg extracts. Here, we demonstrate that Cdt1 binds much more efficiently to PCNA^{Chromatin} than to PCNA^{Free}. In addition, an artificial CRL4^{Cdt2} substrate that binds to PCNA^{Free} and PCNA^{Chromatin} alike only recruits CRL4^{Cdt2} in the context of PCNA^{Chromatin}. These results suggest that Cdt1 destruction is limited to S phase and damaged cells because Cdt1 only binds to PCNA^{Chromatin} and because DNA is a necessary cofactor for CRL4^{Cdt2} recruitment. We also show that the degron of Cdt1 contains a TD motif at positions 5 and 6 of the PIP box, which confers high-affinity PCNA^{Chromatin} binding, and a positively charged amino acid 4 residues downstream of the PIP box that recruits CRL4^{Cdt2} to the Cdt1-PCNA^{Chromatin} complex. When transferred to Fen1, a PIP box protein that is normally not destroyed, these sequence elements induce CRL4^{Cdt2}-dependent proteolysis. Together, the data support the idea that a select number of proteins in eukaryotic cells contain a specialized PIP box that assembles on PCNA^{Chromatin} to form the degron for CRL4^{Cdt2}.

RESULTS

Selective Binding of Cdt1 to PCNA^{Chromatin}

We wanted to directly test whether Cdt1 has a higher affinity for PCNA^{Chromatin} than PCNA^{Free} (Arias and Walter, 2006; Jin et al., 2006). Our goal was complicated by the fact that Cdt1 can associate with chromatin in two modes, via PCNA^{Chromatin} (Arias and Walter, 2006) and via ORC (Ferenbach et al., 2005; Maiorano et al., 2000), which obscures binding to PCNA^{Chromatin} (Figure S1 available online). To circumvent this problem, we exploited the fact that Cdt1's PIP box resides at the N terminus of the protein (Arias and Walter, 2006; Nishitani et al., 2006; Senga et al., 2006), whereas the ORC-binding domain is located in residues 243–620 (Figure 1B; Ferenbach et al., 2005). Therefore, we prepared *Xenopus* Cdt1$^{1–243}$ with a GST and Flag tag on the C terminus. Like endogenous Cdt1 (Jin et al., 2006), this protein was destroyed when added to a high-speed supernatant of egg cytoplasm (HSS) containing plasmid that had been damaged with Methyl methanesulfonate (MMS) (Figure 1C, top, lanes 1–3). As expected, Cdt1$^{1–243}$ was stable in Cdt2-depleted extract (Figure 1C, lanes 4–6). In addition, a protein in which the four consensus PIP box residues were mutated (Cdt1$^{1–243/\Delta PIP}$) was stable (Figure 1D, top, compare lanes 4–6 and 7–9), as was Cdt1$^{1–243}$ after addition of a competitor PIP box peptide derived from p21 (Figure S2, lanes 4–6). Therefore, Cdt1$^{1–243}$ is destroyed in a PIP box- and CRL4^{Cdt2}-dependent manner, similar to endogenous Cdt1.

To examine the binding of Cdt1$^{1–243}$ to chromatin, we coupled MMS-treated 1 kb linear DNA to magnetic beads and added these to Cdt1-depleted HSS. The DNA fragment caused efficient destruction of the endogenous Cdt1, as well as recombinant Cdt1$^{1–243}$ (Figure 1D, lanes 4–6). In all experiments in which chromatin was isolated, methyl-ubiquitin was added to help visualize Cdt1 ubiquitylation on chromatin (Arias and Walter, 2005). Cdt1$^{1–243}$, but not Cdt1$^{1–243/\Delta PIP}$, was recovered on the DNA beads (Figure 1E, top, compare lanes 2 and 3), and recovery was inhibited after PCNA depletion (Figure S3). Together, these results show that this assay detects a direct interaction between the Cdt1$^{1–243}$ PIP box and PCNA^{Chromatin}. Importantly, Cdt1$^{1–243}$, but not Cdt1$^{1–243/\Delta PIP}$, was ubiquitylated on chromatin and induced loading of Cdt2 (Figure 1E, compare lanes 2 and 3). The Cdt2 protein that bound to chromatin in the absence of added Cdt1 (Figure 1E, lane 1) does not reflect a nonspecific background given that it disappears upon inhibition of PCNA loading by preincubation with a competitor PIP box peptide (Figure S1, compare lanes 5 and 7). Therefore, this basal Cdt2 is likely recruited to DNA via unknown, endogenous CRL4^{Cdt2} substrates other than Cdt1. Because we have eliminated the binding of Cdt1 to ORC, these results clearly show that Cdt1 interacts directly with PCNA^{Chromatin} during CRL4^{Cdt2}-mediated ubiquitylation.

We next asked whether Cdt1 interacts more efficiently with PCNA^{Chromatin} than with PCNA^{Free}. In the presence or absence of damaged DNA, Cdt1$^{1–243}$ or Cdt1$^{1–243/\Delta PIP}$ were added to HSS and then immunoprecipitated (IP-ed) from the extract using the C-terminal Flag tag. Equal amounts of Cdt1$^{1–243}$ were recovered from the extract under all conditions (Figure 2A, lanes 2, 3, 5, and 6). As expected, Cdt1$^{1–243}$ was only ubiquitylated in the presence of MMS DNA and a PIP box (Figure 2A, top, compare lane 2 with lanes 3 and 5). Strikingly, Cdt1$^{1–243}$ coimmunoprecipitated (coIP-ed) with PCNA only in the presence of damaged DNA (Figure 2A, PCNA panel, compare lanes 2 and 5) and a PIP box (Figure 2A, PCNA panel, compare lanes 2 and 3). Considering that the large majority of PCNA is soluble (Figure 2A, compare lanes 7–9 and 11–13), these results demonstrate that Cdt1 binds much more efficiently to PCNA^{Chromatin} than to PCNA^{Free}. Importantly, CRL4^{Cdt2} only coIP-ed with Cdt1$^{1–243}$ in the presence of PCNA^{Chromatin} (Figure 2A, Cdt2 and Ddb1 panels, compare lanes 2 and 5), whereas Cdt1$^{1–243/\Delta PIP}$ did not interact with Cdt2 (lanes 3 and 6). Furthermore, it was possible to extract DNA from the anti-Flag IP only when Cdt1$^{1–243}$ contained a PIP box (Figure 2B, lane 2). In contrast to Cdt1, Fen1, a PIP box protein that is not destroyed by CRL4^{Cdt2} (Figure S4), was able to bind efficiently to PCNA^{Free} (Figure 2C, lane 5), suggesting that not all PIP box proteins exhibit selective binding for PCNA^{Chromatin}. Together, these results demonstrate that Cdt1 interacts efficiently only with PCNA^{Chromatin}, providing one explanation for why Cdt1 is destroyed only in S phase and after DNA damage.

A PIP Box Peptide Is Sufficient to Recruit CRL4^{Cdt2} to Chromatin

Previous results showed that a short peptide derived from Cdt1 promotes DNA-damage-induced destruction (Nishitani et al., 2006; Senga et al., 2006). We wanted to determine whether a PIP box peptide is sufficient to recruit CRL4^{Cdt2} to PCNA^{Chromatin}, as predicted by these results. We prepared a synthetic peptide containing the Cdt1 PIP box, but it was highly insoluble (data not shown). Therefore, we used a 20 amino acid peptide containing the PIP box of p21 (Figure 3A Mattock et al., 2001), as this protein was recently identified as a target of CRL4^{Cdt2} (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008). When added to Cdt1-depleted HSS, the p21 peptide, but not a mutant peptide lacking core PIP box residues, was able to recruit Cdt2 and Ddb1 onto immobilized MMS DNA above basal levels (Figure 3B, lanes 1 and 2).

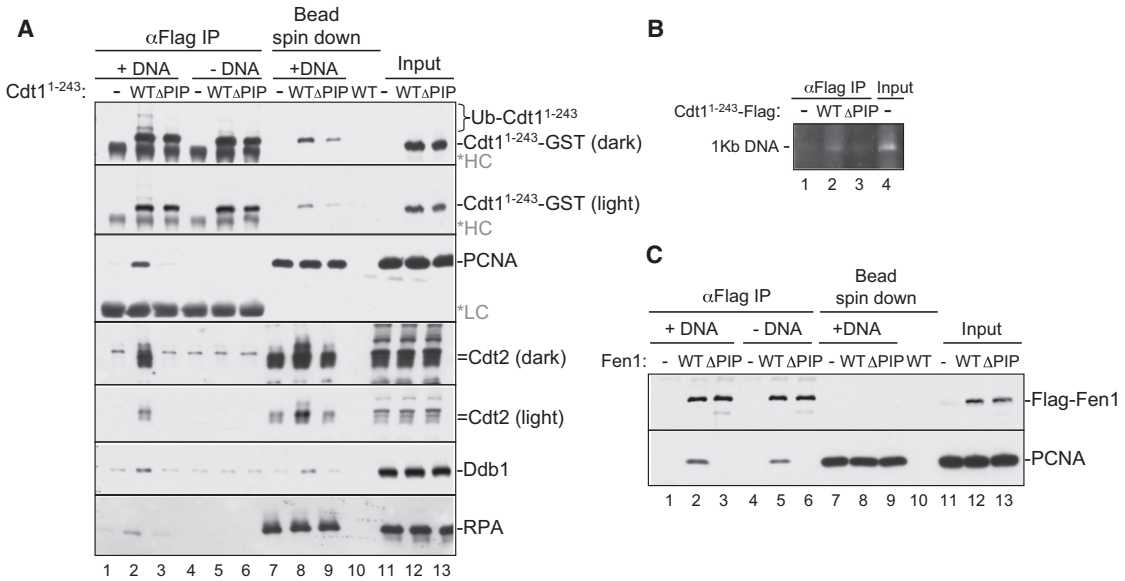

Figure 2. Specific Assembly of the PCNA-Cdt1-CRL4^{Cdt2} Complex on Chromatin

(A) Aliquots of HSS supplemented with methyl ubiquitin (10 μl) were mixed with 350 ng of 1 kb MMS DNA (lanes 1–3), no DNA (lanes 4–6), or 350 ng immobilized 1 kb MMS DNA template (lanes 7–9), as well as 250 nM Cdt1$^{1-243}$-Flag or Cdt1$^{1-243/\Delta PIP}$-Flag for 10 min. For lanes 1–6, Cdt1$^{1-243}$ was precipitated with flag antibody, and the IPs were analyzed. For lanes 7–10, the beads were recovered and associated proteins analyzed. In lanes 11–13, input extract was analyzed. The material recovered from the equivalent of 2 μl of HSS was loaded in lanes 1–10, whereas 1 μl of HSS was loaded in lanes 11–13. HC, heavy chain; LC, light chain.

(B) For reactions described in lanes 1–3 of (A), DNA was extracted from the Flag IP; 50% was analyzed on an agarose gel and then stained with SYBER gold alongside the total input DNA, which was also extracted but not exposed to extract.

(C) Aliquots of HSS supplemented with methyl ubiquitin (10 μl) were mixed with 350 ng of 1 kb MMS DNA template (lanes 1–3), or 350 ng immobilized 1 kb MMS DNA template (lanes 7–9), as well as 250 nM Flag-Fen1 or Flag-Fen1$^{\Delta PIP}$, for 10 min. For lanes 1–6, Flag-Fen1 was precipitated with flag antibody, and the IPs were analyzed. For lanes 7–10, the DNA was recovered and associated proteins analyzed. In lanes 11–13, input extract was analyzed. The material recovered from the equivalent of 2 μl of HSS was loaded in lanes 1–10, whereas 0.5 μl of HSS was loaded in lanes 11–13.

We wanted to determine whether a PIP box is also sufficient to recruit CRL4^{Cdt2} to PCNA^{Chromatin} in the context of chromosomal DNA replication. To this end, we employed a low-speed supernatant of egg cytoplasm (LSS), which assembles added sperm chromatin into nuclei that undergo chromosomal DNA replication and support Cdt1 destruction (Arias and Walter, 2006). Endogenous Cdt1 was depleted from LSS and replaced with Cdt1$^{\Delta PIP}$ to allow DNA replication but prevent Cdt1-mediated CRL4^{Cdt2} loading. Addition of p21 peptide, but not mutant peptide, to this extract induced efficient CRL4^{Cdt2} recruitment (Figure 3C, compare lanes 2 and 3). Together with previous data (Chuang et al., 2005; Nishitani et al., 2006; Senga et al., 2006; You et al., 2002), our results demonstrate that a short PIP box containing peptide is sufficient to recruit the CRL4^{Cdt2} ubiquitin ligase to PCNA^{Chromatin}.

Sequence Alignment Identifies a Potential "PIP Degron" Motif

A critical question is why some PIP box proteins, such as Cdt1, Xic1, p21, and E2F, are destroyed by CRL4^{Cdt2}, whereas the vast majority of PIP box proteins are stable. Given that a short PIP box peptide is sufficient to recruit CRL4^{Cdt2}, we reasoned that the PIP boxes of CRL4^{Cdt2} substrates might contain sequences that distinguish them from the PIP boxes of stable PCNA-binding proteins. Therefore, we aligned the PIP boxes of Cdt1 from multiple species with that of Xic1 (Figure 4A; other CRL4^{Cdt2} targets had not yet been identified) and compared this set to the PIP boxes from numerous proteins, which are presumably stable (Figure 4B). For example, under conditions that induce Cdt1 destruction, endogenous Fen1 is completely stable (Figure S4). When comparing PIP boxes of different proteins, we refer to the position of an amino acid relative to the PIP box, rather than its absolute position within the amino acid sequence of the protein (Figure 4A). The alignment revealed that Cdt1 and Xic1 contain two unique features. First, with the exception of *Drosophila* Cdt1, all of the proteins in this group contain a TD motif at positions 5 and 6 of the PIP box (Figure 4A, in blue). In addition, all of the proteins contain a positively charged residue 4 amino acids downstream of the PIP box (Figure 4A; "K/R+4"). Importantly, we found no examples of canonical PIP boxes that contain both of these features (Figure 4B). Together, these observations suggested that CRL4^{Cdt2} might recognize a degron with the following consensus: Q-x-x-Ψ-T-D-ϑ-ϑ-x-x-x-B (Ψ = I, L, M, or V; ϑ = Y or F; B = K or R). In support of this idea, the recently identified CRL4^{Cdt2} substrates (human p21, worm CKI, fly E2F, and worm DNA pol η) also conform closely to this consensus (Figure 4A).

Cdt1 T5 and K+4 Residues Are Essential for Cdt1 Destruction

The above sequence alignment predicts that T5, D6, and B+4 are required for PCNA-dependent protein destruction. Indeed, Dutta

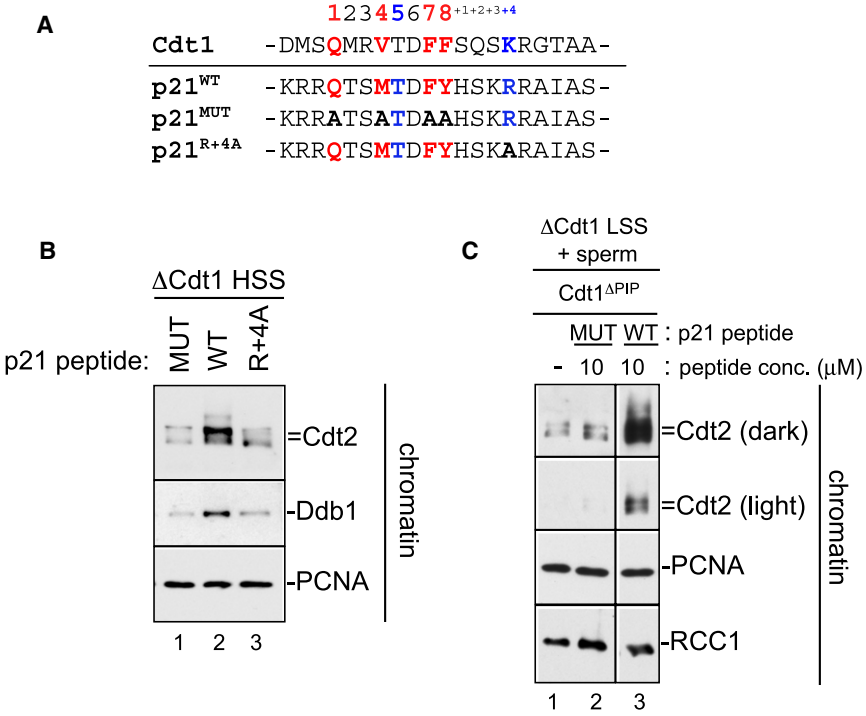

Figure 3. The p21 PIP Box Peptide Is Sufficient to Recruit CRL4^{Cdt2} to Chromatin

(A) Sequence of human p21 PIP box peptides used in (B) and (C) aligned with *Xenopus* Cdt1.

(B) Cdt1-depleted HSS was mixed with methyl ubiquitin and immobilized 1 kb MMS DNA. Three minutes after the addition of DNA, 10 μM p21$^{\Delta PIP}$ peptide (MUT), p21 peptide (WT), or p21^{R+4A} peptide (R+4A) were added to the reaction. Chromatin was recovered, and samples were stopped with sample buffer after 10 min and blotted for the indicated proteins.

(C) Cdt1-depleted LSS was supplemented with Cdt1$^{\Delta PIP}$, methyl ubiquitin, sperm chromatin, and buffer, p21$^{\Delta PIP}$ peptide (Mut) or p21 peptide (WT). After 45 min, chromatin was isolated, washed, and blotted for the indicated proteins. All samples were run on the same gel, but some irrelevant lanes were removed between lanes 2 and 3.

and colleagues previously showed that mutation of T5 to alanine in human Cdt1 reduced damage-dependent destruction (Senga et al., 2006). Consistent with these data, mutation of T5 to alanine in *Xenopus* Cdt1$^{1–243}$ (Cdt1$^{1–243/T5A}$) abolished destruction of the protein in response to DNA damage (Figure 5A, compare lanes 1–3 and 7–9). Similar results were obtained when the T5A mutation was made in the context of full-length Cdt1 and assayed for DNA-damage- or replication-dependent destruction (Figures S5A and S5B). Dutta and colleagues also reported that mutation of D6 to alanine had no effect on Cdt1 stability (Senga et al., 2006). We found that, although Cdt1$^{1–243/D6A}$ was still degraded, the rate was reduced compared to the WT protein (Figure 5B, compare lanes 1–4 to 9–12).

More recently, simultaneous mutation of positions +3, +4, and +5 in human p21 (KRR to AAA) was shown to prevent destruction (Nishitani et al., 2008), suggesting that at least one positively charged residue beyond the core PIP box is important for PCNA-dependent destruction. Consistent with the sequence alignment shown above, we found that mutation of only the lysine at position +4 (Cdt1$^{1–243/K+4A}$) resulted in a completely stable protein (Figure 5A). In contrast, Cdt1$^{1–243/R+5A}$ was still degraded, albeit at a reduced rate (Figure 5C, lanes 5–8). Together, these data argue that the T5 and K+4 residues are essential for Cdt1 destruction. In addition, D6 and R+5, though not essential, enhance the rate of destruction.

The TD Motif Confers Highly Efficient PCNA^{Chromatin} Binding, whereas the K+4 Residue Is Required for CRL4^{Cdt2} Recruitment

We next wished to determine what contribution the TD and K+4 residues make to Cdt1 PCNA^{Chromatin} binding and CRL4^{Cdt2} recruitment. The cocrystal structure of soluble PCNA with p21's PIP box reveals that the core PIP box resides (Figure 4C, red) interact directly with the hydrophobic pocket of PCNA (Gulbis et al., 1996). Interestingly, T5, D6, and B+4, the residues that are specific to CRL4^{Cdt2} substrates, all protrude from the surface of PCNA into solution (Figure 4C, blue). Although this structure does not contain DNA, it suggests that T5, D6, and B+4 might be dispensable for PCNA binding while being specifically required to recruit CRL4^{Cdt2} to the PIP box-PCNA^{Chromatin} complex. Consistent with this prediction, Cdt1$^{1–243/K+4A}$ bound as efficiently to PCNA^{Chromatin} as Cdt1$^{1–243}$ did (Figure 6A, compare lanes 1–3 and 7–9). However, unlike Cdt1$^{1–243}$, Cdt1$^{1–243/K+4A}$ was not ubiquitylated (Figure 6A), consistent with it being completely stable (Figure 5A). Notably, we found no concentration at which Cdt1$^{1–243/K+4A}$ was able to recruit Cdt2 above the basal level seen in the absence of added Cdt1 (Figure 6B, compare lanes 5–8 with lane 9). Similarly, when the basic residue at +4 in the p21 peptide was changed to an alanine, it could no longer recruit Cdt2 to chromatin (Figure 3B, lane 3). In contrast, Cdt1$^{1–243/R+5A}$ bound PCNA^{Chromatin} and recruited Cdt2, but not quite as efficiently as Cdt1$^{1–243}$ (Figure S6, compare lanes 1 and 2 to 4 and 5), consistent with Cdt1$^{1–243/R+5A}$ being destroyed at a slightly reduced rate (Figure 5C). We conclude that K+4 is not essential for efficient PCNA^{Chromatin} binding but, rather, is specifically required for the recruitment of CRL4^{Cdt2} to the Cdt1-PCNA^{Chromatin} complex.

Strikingly, Cdt1$^{1-243/T5A}$ did not bind detectably to PCNA^{Chromatin} (Figure 6A, lanes 10–12), which explains why it failed to be destroyed (Figure 5A) or to recruit Cdt2 (Figure 6C). Like Cdt1$^{1–243/T5A}$, full-length Cdt1^{T5A} also failed to bind efficiently to PCNA on chromatin or to recruit Cdt2 (data not shown). We considered the possibility that an alanine is not well tolerated at this position of Cdt1's PIP box. Indeed, alanine is significantly underrepresented at position 5 of the PIP box in *Xenopus* proteins. In contrast, a considerable number of these proteins contain an aspartic acid at this position. Therefore, we prepared Cdt1$^{1–243/T5D}$ and found that it was destroyed, albeit slowly (Figure 5C, lanes

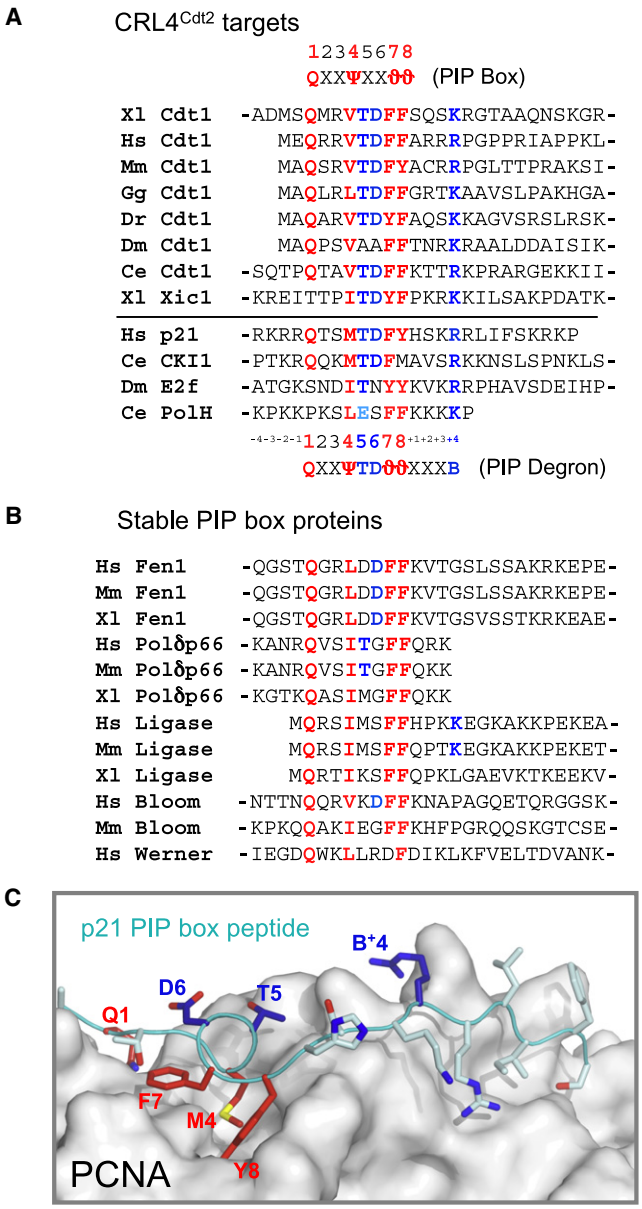

Figure 4. Sequence Alignment of PIP Box Proteins

(A) Alignment of PIP boxes from proteins that are known to be targeted for destruction by CRL4^{Cdt2}. Canonical PIP box residues are shown in red, and putative "degron-specific" residues are shown in blue. The PIP box consensus and the putative PIP degron consensus are shown (Ψ = I/L/M/V; ϑ = Y/F; B = K/R). The absence of a dash indicates the N or C terminus.

(B) Alignment of PIP boxes from proteins that are not likely targets of CRL4^{Cdt2}.

(C) An image of the PCNA-p21 peptide cocrystal structure (Gulbis et al., 1996) was generated using PDB accession number 1AXC and PyMOL (www.pymol.org).

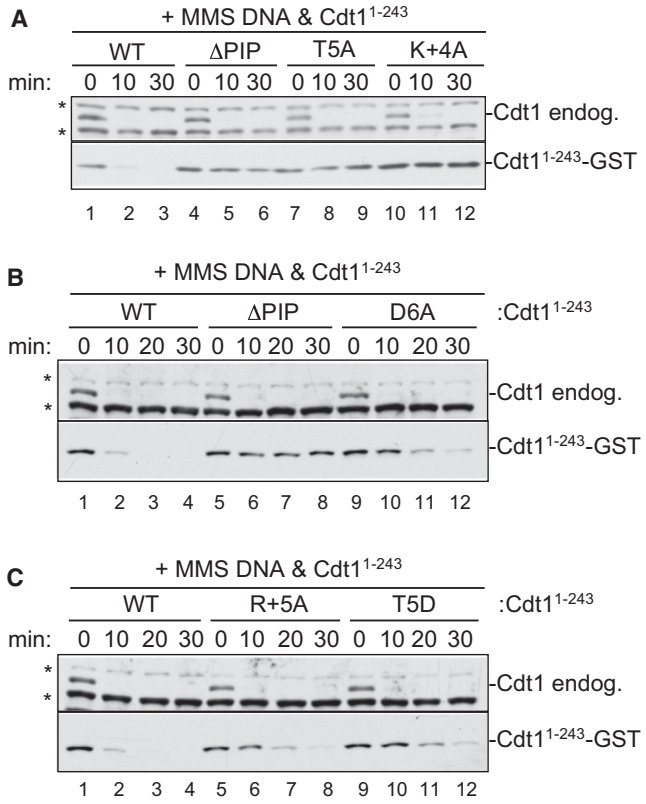

Figure 5. Mutational Analysis of the Cdt1 PIP Box

(A) HSS was mixed with MMS plasmid and 50 nM recombinant Cdt1$^{1–243}$, Cdt1$^{1–243/ΔPIP}$, Cdt1$^{1–243/T5A}$, or Cdt1$^{1–243/K+4A}$. Reactions were stopped at different times and blotted for Cdt1 or Cdt1$^{1–243}$-GST (anti-GST antibody).

(B) HSS was mixed with MMS plasmid and 50 nM recombinant Cdt1$^{1–243}$, Cdt1$^{1–243/ΔPIP}$, or Cdt1$^{1–243/D6A}$. Reactions were stopped at different times and blotted as in (A).

(C) HSS was mixed with MMS plasmid and 50 nM recombinant Cdt1$^{1–243}$, Cdt1$^{1–243/ΔPIP}$, Cdt1$^{1–243/R+5A}$, or Cdt1$^{1–243/T5D}$. Reactions were stopped at the indicated times and blotted as in (A).

9–12), and it still bound poorly to PCNA^{Chromatin} (Figure 6C, lanes 8 and 9). When we titrated Cdt1$^{1–243/T5D}$ into the extract, we found that, at high levels, it was able to recruit Cdt2 efficiently (Figure 6C, lanes 8 and 9). Based on these results, we conclude that T5 does not form a critical interaction with CRL4^{Cdt2} but, instead, mediates tight binding of Cdt1 to PCNA^{Chromatin} (see below). Similar to Cdt1$^{1–243/T5D}$ and Cdt1$^{1–243/R+5A}$, Cdt1$^{1–243/D6A}$ did not bind PCNA^{Chromatin} as well as Cdt1$^{1–243}$ did (Figure 6D, compare lanes 2 and 3), but it was able to recruit Cdt2 when added at higher concentrations (data not shown).

In summary, in order to be destroyed, Cdt1 requires a canonical PIP box and a basic residue at position +4 to recruit CRL4^{Cdt2}. In addition, T5, D6, and a basic residue at position +5 each enhance the binding of Cdt1 to chromatin-bound PCNA, with T5 providing the most important and essential contribution.

Binding of Cdt1 to PCNA^{Chromatin} Does Not Require Cdt2

We have shown that the K+4A mutant of Cdt1 binds to PCNA^{Chromatin} but does not interact with CRL4^{Cdt2} (Figures 6A and 6B), indicating that the ligase is not required for efficient binding of Cdt1 to PCNA. In agreement with this conclusion, Cdt1$^{1–243}$ bound to PCNA^{Chromatin} with similar efficiency in control-depleted and Cdt2-depleted extracts (Figure S7, compare

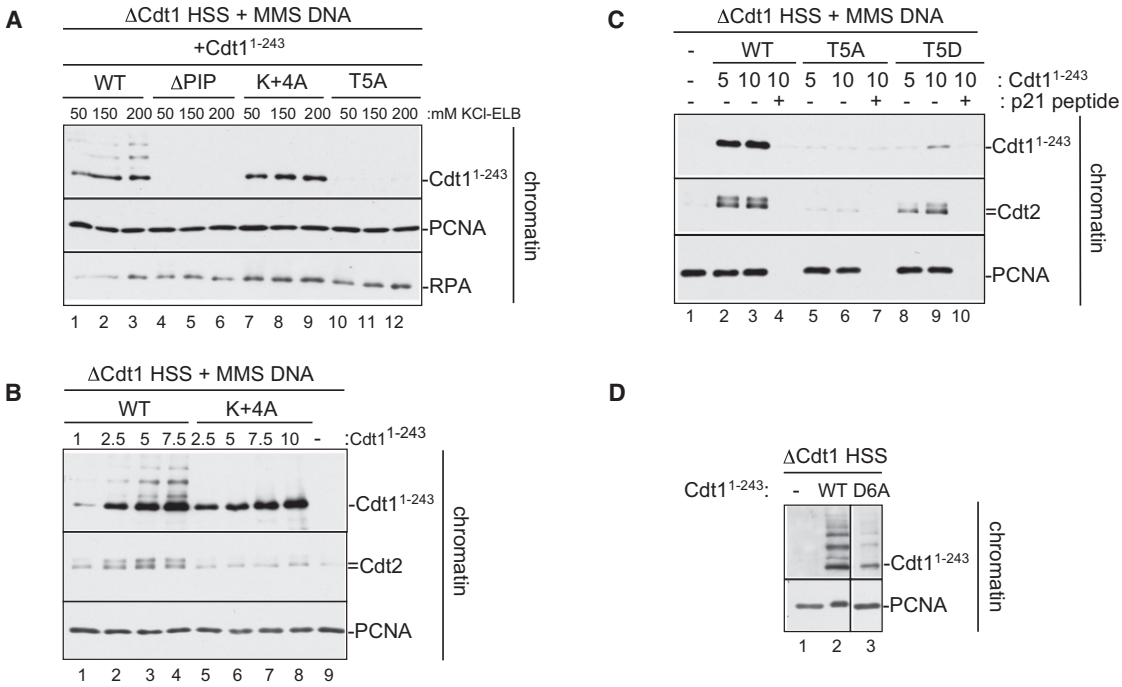

Figure 6. PCNA^{Chromatin} Binding and Cdt2 Recruitment of Cdt1$^{1–243}$ Mutants

(A) Cdt1-depleted HSS was supplemented with immobilized 1 kb MMS DNA, methyl ubiquitin, and 50 nM Cdt1$^{1–243}$, Cdt1$^{1–243/ΔPIP}$, Cdt1$^{1–243/K+4A}$, or Cdt1$^{1–243/T5A}$. After 10 min, the beads were recovered, washed with buffer containing different concentrations of salt, and blotted for the indicated proteins.

(B) Cdt1-depleted HSS was mixed with immobilized 1 kb MMS DNA, methyl ubiquitin, and 50 nM (1×) to 500 nM (10×) Cdt1$^{1–243}$, or Cdt1$^{1–243/K+4A}$. After 10 min, the beads were recovered and blotted for the indicated proteins.

(C) Cdt1-depleted HSS was mixed with immobilized 1 kb MMS DNA, methyl ubiquitin, and 250 (5×) or 500 nM (10×) Cdt1$^{1–243}$, Cdt1$^{1–243/T5A}$, or Cdt1$^{1–243/T5D}$. Chromatin-bound proteins were analyzed as in (B).

(D) Cdt1-depleted HSS was mixed with immobilized 1 kb MMS DNA, methyl ubiquitin, and 50 nM Cdt1$^{1–243}$ or Cdt1$^{1–243/D6A}$. Chromatin-bound proteins were analyzed as in (B). All samples were run on the same gel, but some irrelevant lanes were removed between lanes 2 and 3.

lanes 1 and 3). These results establish a hierarchical assembly pathway, in which Cdt1 first docks onto chromatin-bound PCNA, followed by recruitment of CRL4^{Cdt2}.

PCNA- and CRL4^{Cdt2}-Dependent Destruction of a Canonical PIP Box Protein

To determine whether the degron motifs that we defined in Cdt1 are sufficient to induce CRL4^{Cdt2}-dependent proteolysis, we examined Fen1, a PIP box protein that is normally not destroyed (Figure S4). To this end, the aspartic acid in position 5 of Fen1's PIP box (Figure 7A) was changed to a threonine ("Fen1^{T}"), the glycine in the +4 position was changed to a lysine residue ("Fen1^{K}"), or both residues were altered ("Fen1^{TK}"). Like Fen1, Fen1^{K} was not destroyed in response to damaged DNA (data not shown). Fen1^{T} was also not destroyed (Figure 7B, compare lanes 4–6 and 7–9). However, Fen1^{T} (but not Fen1^{K}) bound more efficiently than Fen1 to PCNA^{Chromatin} (Figure 7E, compare lanes 1 and 3, and data not shown), consistent with the results from Cdt1, which indicate that PIP boxes containing T5 bind exceptionally well to PCNA^{Chromatin}.

Unlike Fen1, Fen1^{TK} was destroyed in the presence of DNA damage (Figure 7B, lanes 10–12) and in a manner that depended on Cdt2 (Figure 7C, compare lanes 1–3 and 4–6). In addition, Fen1^{TK} destruction was PCNA dependent because addition of a competing PIP box peptide (but not a mutated peptide) also stabilized the protein (Figure 7D, compare lanes 1–3 and 4–6). Finally, Fen1^{TK} was ubiquitylated, and it recruited Cdt2 to the chromatin above basal levels (Figure 7E, compare lanes 1 and 4). Therefore, the specific sequence elements that we identified in Cdt1 as being important for PCNA binding and CRL4^{Cdt2} recruitment are transferable to another substrate. We propose to call these sequence elements a "PIP degron" (see Discussion).

CRL4^{Cdt2} Only Binds to the Cdt1-PCNA^{Chromatin} Complex

Having defined the CRL4^{Cdt2} degron, we revisited the question of why Cdt1 destruction is critically dependent on PCNA^{Chromatin}. We were curious whether a PIP degron protein that *is* able to bind PCNA^{Free} would also recruit CRL4^{Cdt2}. Unlike Cdt1, Fen1 binds efficiently to PCNA^{Free} (Figure 2). Therefore, we examined the binding of Fen1^{TK}, which contains a PIP degron, to Cdt2 in the presence of PCNA^{Free} or PCNA^{Chromatin}. Like Fen1 (Figure 2C), Fen1^{TK} bound efficiently to PCNA^{Free}, demonstrating that a PIP degron does not necessarily impose selective binding to PCNA^{Chromatin} (Figure 7F, compare lanes 3 and 6). In addition, Fen1^{T} and Fen1^{TK} both bound PCNA^{Free} and PCNA^{Chromatin} more efficiently than Fen1 did (Figure 7E and data not shown), demonstrating that the T5 position of the PIP box enhances binding to PCNA^{Free} and PCNA^{Chromatin} alike. Most importantly,

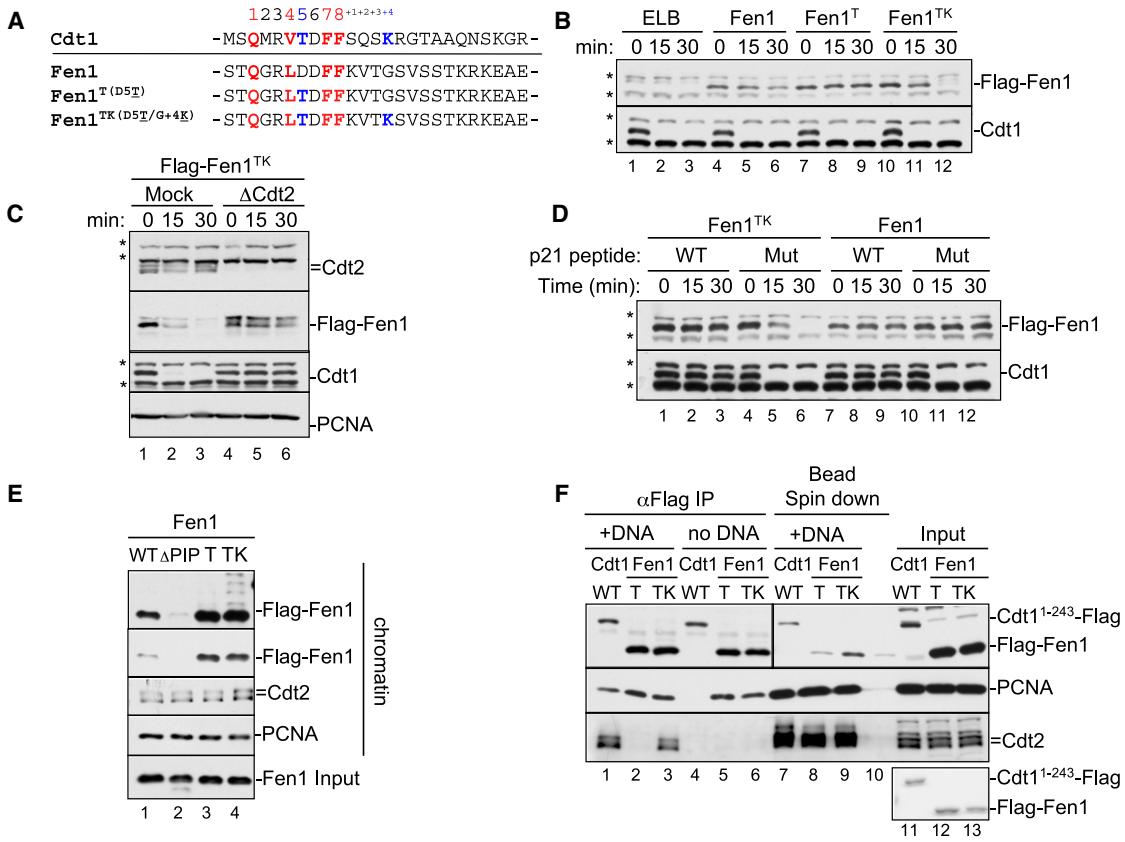

Figure 7. The PIP Degron Is Portable

(A) Sequence comparison of *Xenopus* Cdt1's PIP box with that of *Xenopus* Fen1 and various mutants of Fen1.

(B) HSS was mixed with MMS plasmid and 50 nM recombinant Fen1, Fen1^{T}, or Fen1^{TK}. Reactions were stopped at the indicated times and blotted for Cdt1 and the Flag peptide to visualize Flag-Fen1.

(C) Mock-depleted or Cdt2-depleted HSS was supplemented with 5 ng/μl MMS plasmid and 50 nM Fen1 or Fen1^{TK}. Samples were blotted for the indicated proteins.

(D) HSS was incubated with 50 μM mutant or WT p21 PIP peptide and then supplemented with 50 nM recombinant Fen1^{TK} or Fen1 and 5 ng/μl MMS plasmid. At different times, samples were blotted for the indicated proteins.

(E) HSS was mixed with immobilized 1 kb MMS DNA, methyl ubiquitin, and 200 nM Fen1, Fen1$^{\Delta PIP}$, Fen1^{T}, or Fen1^{TK}. After 10 min, the beads were recovered and blotted for the indicated proteins.

(F) Aliquots of HSS supplemented with methyl ubiquitin (10 μl) were mixed with 350 ng of 1 kb MMS DNA (lanes 1–3) or 350 ng immobilized 1 kb MMS DNA (lanes 7–9), as well as 250 nM Cdt1$^{1–243}$-Flag, Flag-Fen1^{T}, or Flag-Fen1^{TK} for 10 min. Lane 10 contained all three proteins, HSS, and magnetic beads, but no DNA. For lanes 1–6, Flag-tagged proteins were precipitated with flag antibody, and the IPs were analyzed. For lanes 7–10, the beads were recovered and associated proteins analyzed. In lanes 11–13, input extract was analyzed. The material recovered from the equivalent of 2 μl of HSS was loaded in lanes 1–10, whereas 0.5 μl of HSS was loaded in lanes 11–13. All samples were from the same experiment, but for the anti-Flag panel, a darker exposure of lanes 7–13 is shown. (Bottom) Coomassie gel showing that equal amounts of recombinant proteins were used. Note that Cdt1$^{1–243}$-Flag contains a single C-terminal Flag tag, whereas Flag-Fen1 contains two N-terminal Flag tags.

although Fen1^{TK} bound stably to PCNA^{Free}, it did not recruit Cdt2 in this context (Figure 7F, compare lanes 3 and 6). Therefore, binding of a PIP degron to PCNA^{Free} is not sufficient to promote assembly of the Cdt1-PCNA-CRL4^{Cdt2} complex.

DISCUSSION

In this paper, we identify the *cis*-acting sequences within Cdt1 that target it for destruction via CRL4^{Cdt2}. Two elements are important to promote efficient Cdt1 destruction: a PIP box that binds PCNA with high affinity and a basic residue at the +4 position relative to the PIP box that recruits CRL4^{Cdt2}. In addition, we identify two mechanisms that ensure that CRL4^{Cdt2} targets are destroyed only in the presence of chromatin-bound PCNA (PCNA^{Chromatin}), limiting the activity of this pathway to S phase and damaged cells. Thus, our data show that CRL4^{Cdt2} recognizes a highly unusual degron that is formed on chromatin via the interaction between a specialized PIP box and PCNA.

Coupling of CRL4^{Cdt2}-Dependent Proteolysis to PCNA^{Chromatin}

We identify two mechanisms that explain our previous observation that Cdt1 destruction is strictly dependent on PCNA^{Chromatin} (Arias and Walter, 2006; Jin et al., 2006). First, we show that Cdt1

binds efficiently to PCNA^{Chromatin}, whereas there is no detectable interaction with PCNA^{Free} (Figure 2). Consistent with this observation, Cdt1 only coIP-ed with CRL4^{Cdt2} in the presence of PCNA^{Chromatin}. The highly selective binding of Cdt1 to PCNA^{Chromatin} is not a general property of PIP box proteins, as illustrated by Fen1, which also binds to PCNA^{Free}. Selective PCNA^{Chromatin}-binding is not an obligatory property of the PIP degron because Fen1^{TK}, which was engineered to contain the PIP degron, still binds efficiently to PCNA^{Free} (Figure 7). The mechanistic basis of Cdt1's selectivity for PCNA^{Chromatin} is presently unclear. It could involve a conformational change in PCNA^{Chromatin}, chromatin-specific modification of PCNA, or cooperative binding of Cdt1 to PCNA and DNA. In support of the latter model, mouse Cdt1 is reported to bind DNA (Yanagi et al., 2002), and the destruction of *Drosophila* E2F by CRL4^{Cdt2} was recently reported to require the DP protein (Shibutani et al., 2008), which binds E2F and recruits it to DNA.

We discovered a second mechanism that couples CRL4^{Cdt2}-dependent ubiquitylation to PCNA^{Chromatin} when we examined Fen1^{TK}. This mutant of Fen1, which contains a PIP degron, binds efficiently to PCNA^{Free} but is only able to recruit CRL4^{Cdt2} in the context of PCNA^{Chromatin} (Figure 7F). Thus, even if a PIP degron protein should inadvertently bind to PCNA^{Free}, it will not be destroyed. We speculate that the conformations of PCNA^{Free} and PCNA^{Chromatin} differ and that Cdt2 is able to interact with the PIP degron only when it is displayed on PCNA^{Chromatin}. However, another explanation is that other unknown DNA-binding proteins cooperate with PCNA^{Chromatin} to recruit CRL4^{Cdt2}, although such factors would have to be shared between the replication and repair machineries. The two mechanisms that we have described, which couple CRL4^{Cdt2}-dependent proteolysis to PCNA^{Chromatin}, together ensure that this pathway is only active in S phase and after DNA damage. It remains to be seen whether bona fide CRL4^{Cdt2} substrates other than Cdt1 employ both mechanisms.

Interestingly, it has been reported that, in the absence of DNA, Cdt1 and p21 co-IP with PCNA^{Free} (Abbas et al., 2008; Arias and Walter, 2006; Hu and Xiong, 2006; Kim et al., 2008; Nishitani et al., 2006, 2008). However, the experiments were performed with purified proteins or in lysates of cells overexpressing these proteins, calling into question whether such interactions really occur in vivo. Even if they do, our results show that a PIP degron-PCNA^{Free} complex does not support CRL4^{Cdt2} recruitment. In addition, Cdt1 and p21 were found to bind to CRL4^{Cdt2} in the absence of PCNA or a PIP box (Abbas et al., 2008; Higa et al., 2006; Hu et al., 2004; Kim et al., 2008; Nishitani et al., 2008). These observations are difficult to reconcile with the fact that these substrates are only destroyed in S phase and after DNA damage unless PCNA^{Chromatin} is not only required to join CRL4^{Cdt2} with its substrates, but also to facilitate ubiquitin transfer.

Anatomy of the PIP Degron

In this study, we identify two functional elements that are necessary and sufficient to create a degron for CRL4^{Cdt2}. The first is a basic residue 4 amino acids downstream of the PIP box. Every known CRL4^{Cdt2} substrate contains a K or R residue at the +4 position (Figure 4A). This residue is dispensable for substrate binding to PCNA^{Chromatin} but is essential to recruit CRL4^{Cdt2} to the substrate-PCNA^{Chromatin} complex. In support of this conclusion, we show that Cdt1$^{1–243/K+4A}$ is completely stable and binds PCNA^{Chromatin} normally but fails to recruit Cdt2 to chromatin. Moreover, Fen1 (Figure 7) and p21 peptide (Figure 3) only recruit CRL4^{Cdt2} if a positively charged amino acid is present at the +4 position. Although numerous CRL4^{Cdt2} targets contain multiple positive charges downstream of the PIP box, only the +4 position is essential. This is apparent from the fact that some targets have only a single positive charge, which is always at the +4 position. In addition, several proteins that were engineered to contain only the positive charge at the +4 position are destroyed (Cdt1$^{1–243/R+5A}$ and Fen1^{TK}). Although Cdt1$^{1–243/R+5A}$ is destroyed slowly, this is not due to defective CRL4^{Cdt2} recruitment but, rather, due to poor binding to PCNA^{Chromatin} (see below). Thus, the basic residue at +4 is an indispensable determinant of the degron that enables CRL4^{Cdt2} recruitment.

The second element required for efficient destruction by CRL4^{Cdt2} is a PIP box. Importantly, for the PIP box to promote destruction, it appears that it must have a very high affinity for PCNA^{Chromatin}. For most CRL4^{Cdt2} substrates, high-affinity PCNA^{Chromatin} binding is conferred by a TD motif, which is located at positions 5 and 6 of the PIP box (Figure 4A). Thus, we found that mutation of T5 to A dramatically decreases the binding of Cdt1 to PCNA^{Chromatin}, thereby preventing destruction, and that insertion of a T5 in Fen1 strongly enhances PCNA^{Chromatin} binding and enables proteolysis when a basic residue is also present at +4. These results are further supported by data from Xic1, a likely CRL4^{Cdt2} target, in which a T5A mutation blocked proteolysis and greatly attenuated PCNA binding (Chuang et al., 2005). Interestingly, Cdt1$^{1–243/T5D}$ was still destroyed, albeit with reduced kinetics. Although this raised the possibility that T5 might be phosphorylated, numerous lines of evidence in diverse systems argue against this idea (see Supplemental Discussion). Instead, it seems that D5 simply allows better binding to PCNA^{Chromatin} than A5 does (Figure 6C). Importantly, we also found that mutation of D6 to A in Cdt1 significantly reduced PCNA^{Chromatin} binding and slowed but did not eliminate Cdt1 destruction. Together, the data argue that a TD motif confers high-affinity binding to PCNA^{Chromatin} and that, in most CRL4^{Cdt2} substrates, this motif is essential for destruction.

Notably, three proteins that are destroyed by CRL4^{Cdt2} (worm DNA pol η, fly Cdt1, and fly E2F) lack the TD motif (Figure 4A). We speculate that these proteins are destroyed efficiently despite the absence of a TD motif because they achieve high-affinity PCNA^{Chromatin} binding by other means. In the case of fly Cdt1, it is conceivable that an AA motif at positions 5 and 6 is just as adept at binding PCNA^{Chromatin} as TD is. However, we consider it more likely that other sequence elements compensate for the absence of TD motifs in these proteins. Specifically, it is conspicuous that all three proteins contain many positively charged amino acids downstream of the PIP box (at positions +1 to +5) and, in the case of pol η, immediately upstream of the PIP box (at positions −1 to −4). Interestingly, the cocrystal structure of p21 and PCNA shows that the analogous basic residues in p21 form salt bridges with acidic residues in PCNA, and PIP residues −1 through −4 form poorly ordered ionic interactions with the C terminus of PCNA (Gulbis et al., 1996). In support of these

interactions being relevant for proteolysis, we found that Cdt1$^{1–243/R+5A}$ exhibits reduced PCNA^{Chromatin} binding and destruction kinetics. Importantly, this was not due to defective CRL4^{Cdt2} recruitment because at equivalent levels of chromatin binding, Cdt1$^{1–243/R+5A}$ and Cdt1$^{1–243}$ recruited the same amount of ligase (Figure S6). Further, in p21, mutation of the $^{-4}$RKRR^{-1} motif inhibited destruction (Nishitani et al., 2008). Together, these data suggest that positively charged residues might be able to compensate for the absence of a TD motif and/or an imperfect core PIP box in some CRL4^{Cdt2} substrates.

Although the $^{-4}$RKRR^{-1} motif appears to play a role in p21 destruction (assuming that mutation of these residues to alanines did not grossly disorder the protein's structure), a cluster of positive charges upstream of the PIP box is clearly not a universal feature of the PIP degron. Thus, although numerous CRL4^{Cdt2} substrates contain positively charged amino acids in this area (or they are located at the extreme N terminus, which provides a positive charge), several substrates (frog and worm Cdt1, Fen1^{TK}) lack positive charges (Figure 4A). Therefore, although positively charged residues upstream of the PIP box might enhance PCNA^{Chromatin} binding, they are not essential elements of the PIP degron.

In summary, our data suggest that a protein will be a target of CRL4^{Cdt2} as long as it binds to PCNA^{Chromatin} with sufficient affinity, contains a basic residue at +4, and has an accessible degron. We speculate that high-affinity PCNA^{Chromatin} binding is essential for proteolysis because it allows processive ubiquitylation of Cdt1 by CRL4^{Cdt2} and thereby mediates synthesis of polyubiquitin chains. Although the basic residue at the +4 position is the only residue that is essential for recruitment of CRL4^{Cdt2} to the PCNA^{Chromatin}-PIP box complex, the binding must involve other interactions. CRL4^{Cdt2} might recognize other general features of the PIP box (such as the hydrophobic residues at 4, 7, and 8 positions) and/or specific residues that lie on the surface of PCNA^{Chromatin}.

PCNA-Binding Affinity and Canonical PIP Box Proteins

A large number of stable proteins that function in chromosome metabolism bind to PCNA^{Chromatin} via a PIP box (Moldovan et al., 2007), but it appears that most of these have nonoptimal PCNA-binding affinity. This conclusion is based on the fact that the vast majority of PIP box proteins lack a TD motif and the finding that, in all cases in which the TD motif has been mutated, binding to PCNA^{Chromatin} or PCNA^{Free} is diminished (Cdt1 in this paper; Chuang et al., 2005; Nakanishi et al., 1995; Warbrick et al., 1995). Moreover, when we introduced a TD motif into Fen1's PIP box, its binding to PCNA^{Chromatin} increased substantially (Figure 7E). Therefore, we conclude that the TD motif can enhance PCNA^{Chromatin} binding in the context of many and perhaps all PIP boxes, and the question arises as to why more PCNA-interacting proteins do not contain this motif. Given the number of proteins that need to access PCNA^{Chromatin} in the course of chromosomal DNA replication (polymerases, ligases, nucleases, and chromatin assembly factors) and the requirement for frequent exchange between these factors, we speculate that replication would be adversely affected if the affinity of these proteins for PCNA^{Chromatin} was very high. In contrast, high-affinity PCNA^{Chromatin} binding by CRL4^{Cdt2} substrates is not deleterious because they are rapidly destroyed upon binding to PCNA^{Chromatin}.

A New Paradigm for Temporally Regulated Proteolysis?

Most E3 ubiquitin ligases recognize short degron motifs, and regulation of proteolysis usually involves posttranslational modifications of the substrate or the ligase, most notably by phosphorylation. The degron for CRL4^{Cdt2} departs from this paradigm in two ways. First, there is currently no evidence that posttranslational modifications regulate the binding of CRL4^{Cdt2} to its substrates. Rather, binding requires the assembly of a cell-cycle-regulated structure, PCNA^{Chromatin}, and this ensures that substrates are only destroyed in S phase and after DNA damage. Second, the degron is formed via the interaction of two proteins. Because the only unique determinant for ligase recruitment in the substrate is a single basic residue, it is almost certainly true that other essential determinants for CRL4^{Cdt2} recognition are provided by residues on PCNA. Thus, CRL4^{Cdt2} is an example of a ubiquitin ligase that appears to recognize a specific surface created by two different proteins. We will be surprised if this principle is not exploited repeatedly by cells to couple proteolysis to the formation of other transient protein-protein interactions.

EXPERIMENTAL PROCEDURES

Egg Extract and Immunological Methods

HSS, LSS (Walter and Newport, 2000; Walter et al., 1998), and chromatin spin-downs (Arias and Walter, 2005) were performed as described. We used previously described antibodies against Orc2 (Walter et al., 1998), Cdt1 (Arias and Walter, 2005), RCC1 (Dasso et al., 1992), Ddb1 (Arias and Walter, 2006), RPA (Walter and Newport, 2000), Cdt2 (Jin et al., 2006), Fen1 (Cell Signaling Technology), GST (New England Bio Labs), M2 and Rabbit Flag (Sigma), and PCNA (Santa Cruz sc-056). Whenever a western blot was performed with anti-Flag or anti-GST antibody, the protein being detected is indicated with its tag to the right of the panel.

For Cdt1$^{1-243}$-Flag and Flag-Fen1 IPs, HSS extract was first precleared with anti-Flag agarose beads (Sigma). Then, recombinant proteins and DNA were added, and 10 min later, Flag-tagged proteins were IP-ed using anti-Flag agarose resin. The resin was washed three times in 2.5 mM $MgCl_2$, 150 mM KCl, 250 mM sucrose, 10 mM HEPES (pH 7.7), and 0.6% Triton and then resuspended in sample buffer for SDS-PAGE. DNA from the Flag IP was extracted, run on an agarose gel, and stained with SYBER gold (Molecular Probes). The p21 WT and ΔPIP peptides were previously published (Arias and Walter, 2006), and the p21^{R155A} peptide (CKRRQTSMTDFYHSKARAIAS) was synthesized by the Tufts University Core Facility (Boston, MA).

Plasmid Construction and Protein Purification

Recombinant full-length *Xenopus* Cdt1 was purified as described (Arias and Walter, 2005). Recombinant *Xenopus* Cdt1$^{1–243}$-3 × NLS-GST-Flag was cloned into pDONR201 and produced in SF9 cells using the BaculoDirect Baculovirus Expression System (Invitrogen). All Cdt1 mutants were cloned using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). See Supplemental Data for primers and cloning information. For purification of Cdt1$^{1–243}$, insect cell pellets were lysed in lysis buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100, and 10 mM Imidazole), sonicated, and spun for 30 min at 30,000 × g. The supernatant was loaded onto Ni-NTA beads (QIAGEN) and washed in lysis buffer with 30 mM Imidazole, and Cdt1$^{1–243}$ was eluted in lysis buffer with 250 mM Imidazole. The eluted Cdt1$^{1–243}$ was incubated with GST-sepharose (Amersham), which was washed to remove Imidazole and Triton (50 mM Tris [pH 7.5], 300 mM NaCl, 50 mM KCl, and 5 mM $MgCl_2$). Cdt1$^{1–243}$ was eluted with 10 mM Tris (pH 8.0), 10 mM reduced glutathione, 250 mM NaCl, and 10% glycerol.

See Supplemental Data for details on *Xenopus* Fen1 cloning. For purification of Fen1, pET28b-2 × Flag-Fen1-GST was transformed into BL-21, grown at 37°C to an $OD_{600\ nm}$ of 0.6, and induced with 1 mM IPTG overnight at 19°C. Pellets were frozen at −80°C and then purified in buffer A plus protease inhibitors (500 mM NaCl, 20 mM HEPES [pH 8], 10% glycerol, 2 mM β-mercaptoethanol, and 0.1% NP-40) (Hohl et al., 2007), except that the lysate was loaded onto Ni-NTA resin (QIAGEN) in buffer A containing 7.5 mM Imidazole. The resin was washed with buffer A containing 20 mM Imidazole and Fen1 eluted in 250 mM Imidazole. A second purification step using Glutathione Sepharose 4 Fast Flow was used to remove the Imidazole, transfer Fen1 into freezing buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 10% glycerol, 1 mM EDTA, and 1 mM DTT), and cleave the GST tag from the C terminus using PreScission Protease (GE Healthcare).

MMS DNA Preparation and Bead Spin-Down Assay

Methylated DNA was generated as described (Stokes and Michael, 2003). A 2 × biotinylated double-stranded 1 kb linear DNA was generated by PCR (see Supplemental Data for primer sequences). The DNA product was then purified using a PCR purification kit (QIAGEN), MMS treated, and then coupled to M-280 Streptavidin Dynabeads (Invitrogen). Biotinylated MMS treated PCR product (100 ng) was used per 10 μg of Dynabeads. DNA and beads were bound for 2 hr at 25°C in 10 mM Tris 8.0, 100 mM NaCl, and 1 mM EDTA, incubated overnight at 4°C in the presence of excess streptavidin to block any free biotin ends, and finally washed three times. Sixty to seventy percent of DNA bound to beads. To spin down MMS-DNA beads, we used a modification of our standard chromatin spin-down protocol (Arias and Walter, 2006), in which the ELB wash step was supplemented with 0.6% Trition X-100.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Discussion, Supplemental Experimental Procedures, and seven figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00345-1.

ACKNOWLEDGMENTS

We are grateful to Yoshihiro Matsumoto for the gift of *Xenopus* Fen1 cDNA; Yi Zhang and Jarrod Marto for the mass spectrometry analysis of Cdt1 phosphorylation sites; Puck Knipscheer for generating Figure 4C and for helpful discussions; and Anna Kochaniak for the gift of the *Xenopus* PCNA antibody. We thank Emily Arias, Xiaolu Ang, David Long, and Randall King for critical reading of the manuscript. This research was funded by NIH-F32-GM082014 to C.G.H. and NIH-R01-GM080676 to J.C.W.

Received: February 6, 2009
Revised: April 14, 2009
Accepted: May 6, 2009
Published: July 9, 2009

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