Direct Role for Proliferating Cell Nuclear Antigen in Substrate Recognition by the E3 Ubiquitin Ligase CRL4\(^{\text{Cdt2}}\)^*[5]

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Background: CRL4\(^{\text{Cdt2}}\) 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\(^{\text{Cdt2}}\) substrates.

Conclusion: CRL4\(^{\text{Cdt2}}\) 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\(^{\text{Cdt2}}\)) 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\(^{\text{Cdt2}}\) 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\(^{\text{Cdt2}}\) for substrate ubiquitylation. Using Xenopus egg extracts, we identify an acidic residue in PCNA that is essential to support destruction of all CRL4\(^{\text{Cdt2}}\) 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\(^{\text{Cdt2}}\) recruitment to chromatin. Our data show that the interaction of CRL4\(^{\text{Cdt2}}\) 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\(^{\text{Skp2}}\) also known as SCF\(^{\text{Skp2}}\))^2 is composed of a Cul1 scaffold, a Skp1 adapter, 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\(^{\text{Skp2}}\) targets the CDK inhibitor p27 for destruction, promoting S phase entry. Another cell cycle-regulated ubiquitin ligase is the anaphase-promoting complex (APC\(^{\text{Cdc20}}\)). 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\(^{\text{Cdt2}}\) has been characterized, which promotes the ubiquitylation of several proteins in S phase and after DNA damage (5, 6). CRL4\(^{\text{Cdt2}}\) is composed of a Cul4 scaffold, a Ddb1 adaptor, and Cdt2, the putative substrate receptor. In vertebrates, CRL4\(^{\text{Cdt2}}\) 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\(^{\text{Cdt2}}\) 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\(^{\text{Cdt2}}\) are likely to emerge.

The activity of CRL4\(^{\text{Cdt2}}\) 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. 1A, green
a 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<sup>Cdt2</sup> substrates contain a PIP box, through which they bind to chromatin-bound PCNA (PCNA<sub>chromatin</sub>) at sites of DNA damage or at the replication fork. Most CRL4<sup>Cdt2</sup> substrates also contain a TD motif at positions 5 and 6 of the PIP box (Fig. 1A, 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<sup>Cdt2</sup> 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 <i>Xenopus</i> Cdt1, the resulting protein binds normally to PCNA, but CRL4<sup>Cdt2</sup> 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<sup>Cdt2</sup>-mediated proteolysis, a substrate docks onto PCNA<sub>chromatin</sub>, leading to recruitment of CRL4<sup>Cdt2</sup>, followed by ubiquitin transfer (see Fig. 1B). Notably, an alternative model has recently been proposed 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<sup>Cdt2</sup>.

In this report, we investigate the mechanism by which CRL4<sup>Cdt2</sup> interacts with its substrates to trigger proteolytic degradation. First, we provide evidence against the recent proposal that CRL4<sup>Cdt2</sup> and its substrates dock onto PCNA independently of one another (11), thereby affirming that CRL4<sup>Cdt2</sup> 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<sup>Cdt2</sup> (indirect role) or if a specific surface of PCNA is required, together with the PIP degron, to recruit CRL4<sup>Cdt2</sup> (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<sup>Cdt2</sup> activity. Importantly, this residue is not necessary for PIP box binding to PCNA but is essential for CRL4<sup>Cdt2</sup> recruitment to the PCNA-PIP degron complex on chromatin. Asp-122 is also essential for CRL4<sup>Cdt2</sup> activity in fission yeast. Our findings support the idea that CRL4<sup>Cdt2</sup> 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 <i>in vitro</i> translated according to the manufacturer’s protocol using TNT<sup>®</sup> SP6 Quick Coupled Transcription Translation kit from Promega (Madison, WI). In each reaction 125 ng of DNA was used per 10 μl of TNT<sup>®</sup> 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), Db1 (33), RPA (30), Cdt2 (7), GST (New England Biolabs), M2 and Rabbit FLAG (Sigma), <i>Xenopus</i> 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<sub>1–243</sub>-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, GACTATGAATAAGTTGAGGCTTGGATGTTGAAACAATTGG; D122A, GAAATGAAGTGATGGGTTTAGCTGCTGTGGGAACACTTGG; D122A, ATGGATTTAGATGTTGCAACACTTTGGAATCAGAG; D122A/El24A, GATGGATTAGTGGCTGCTCAACTTTGGAGATCC; and D122K/El24AR, GATGGATTACGG-TAAAACACTTTGGAGATCC.

**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 eugarynogenic buffer (10 mM HEPES (pH 7.7), 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>) 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<sup>Δ</sup>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 <i>pcn1</i><sup>Δ</sup> locus after linearizing with XhoI (in the strain 2609). 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 G1 phase in Edinburgh minimal medium (65) lacking NH<sub>4</sub>Cl for 16 h at 25 °C (38), then released into the cell cycle in rich yeast extract and supplements medium (65) at 32 °C. <i>nda3–311</i> 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
Substrate Recognition by CRL4\textsuperscript{Cdt2}

100 J/m\textsuperscript{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). Tandem 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\textsuperscript{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\textsuperscript{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\textsuperscript{Cdt2}-mediated proteolysis around the sperm, chromosomal DNA chromatin was added to an LSS of egg cytoplasm. Upon nucleolar-envelope assembly around the sperm, chromosomal DNA replication initiates, leading to PCNA-dependent, CRL4\textsuperscript{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\textsuperscript{Cdt2} substrates (Fig. 1A), 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\textsuperscript{B + 4A}, which was completely stable (27), Xic1\textsuperscript{B + 4A} was still destroyed, albeit more slowly than Xic1\textsuperscript{WT} (Fig. 2A, bottom, light blue trace). This residual destruction of Xic1\textsuperscript{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\textsuperscript{B + 4A}, Set8\textsuperscript{B + 3/4A} was destroyed slowly (Fig. 2B, lanes 7–9; note that in Set8\textsuperscript{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\textsuperscript{B + 4A} (27), Set8\textsuperscript{B + 3/4A} bound normally to PCNA\textsuperscript{chromatin}, and its slow destruction was due to reduced recruitment of CRL4\textsuperscript{Cdt2} to chromatin, resulting in reduced ubiquitylation of Set8\textsuperscript{B + 3/4A} (Fig. 2C). 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\textsuperscript{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\textsuperscript{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. 1A, pink). We speculated that these 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\textsuperscript{B\textsuperscript{RRKR}/AAAA} (Fig. 2A, top). Mutation of these residues alone had little or no effect on Xic1 destruction (Fig. 2A, yellow trace). However, when combined with the B + 4A mutant, these mutations rendered the protein completely stable, similar to Xic1\textsuperscript{ΔPIP} (Fig. 2A, green trace). Together, these data show that, in a CRL4\textsuperscript{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\textsuperscript{Cdt2}-mediated destruction, but they are only essential in the absence of the B + 4A mutant. The upstream basic residues likely promote substrate destruction in a manner that is distinct from the downstream basic residues, including B + 4.

**Figure 1.** PCNA-dependent recognition of PIP degrons by CRL4\textsuperscript{Cdt2}.

A, sequence alignment of CRL4\textsuperscript{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, model of substrate recognition on PCNA by CRL4\textsuperscript{Cdt2} on the immobilized 1-kb DNA template.

The PCNA and CRL4\textsuperscript{Cdt2} Binding Motifs of Substrates Cannot Be Separated—Recently, Yew and colleagues proposed a new model for substrate recognition by CRL4\textsuperscript{Cdt2} in which the substrate and CRL4\textsuperscript{Cdt2} initially bind independently to PCNA before coming together in a complex (Fig. 3A). 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...
to recruit some CRL4Cdt2 and thus promote destruction via PCNA subunits (Fig. 3). The PIP box might bind cooperatively via interactions with two mutated. Thus, the mutated Xic1 PIP box and the added p21 site (Ile-174) in the endogenous Xic1 PIP box was not mutated, likely still allowing binding to Cdt2. Second, only the CRL4Cdt2-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 p21PIP-Xic1I174A (Fig. 3). This construct was destroyed normally in egg extracts (11) even though it was thought to contain well recognized a composite surface created by the two proteins. Unlike p21PIP-Xic1I174A, p21PIP-Xic1ΔPP was destroyed much less efficiently than Xic1WT (Fig. 3, green line). From these data we conclude that ~40% of p21PIP-Xic1I174A 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, p21PIP/B+4A-Xic1ΔPP (Fig. 3C), was destroyed at background levels (Fig. 3D, purple line), indicating that the p21 PIP box retained Cdt2 binding capacity. Thus, the original p21PIP-Xic1I174A 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 CRL4Cdt2-mediated Destruction—We next wanted to further characterize what role PCNA plays in the recognition of the PIP degron by CRL4Cdt2. Specifically, we wished to distinguish between direct and indirect roles for PCNA. In the “indirect” model, CRL4Cdt2 only contacts residues in the substrate’s PIP degron, and the sole function of PCNA is to position the degron binding capacity. Therefore, the original p21PIP-Xic1I174A 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.

To test whether the destruction of p21PIP-Xic1I174A involved residual binding of the Xic1 PIP box to PCNA, we mutated all three Xic1 PIP box residues, creating p21PIP-Xic1WT, p21PIP-Xic1ΔPP, or p21PIP-Xic1ΔPP, or p21PIP-B+3AA. At the indicated times, samples were blotted for endogenous Cdt1 or Set8. C, HSS was supplemented with 5 ng/µM MMS plasmid and 50 ng human Set8WT, Set8ΔPP, or Set8B+3AA. At the indicated times, samples were blotted for endogenous Cdt1 or Set8.

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 labeling Xic1 remaining after it was added to HSS in the presence of 5 ng/µM 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/µM MMS plasmid and 50 ng human Set8WT, Set8ΔPP, or Set8B+3AA. 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 Set8WT, Set8ΔPP, or Set8B+3AA was also added, and after 10 min, chromatin was recovered from the extract and washed, and the indicated proteins were visualized by Western blotting.
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. 4A). Because the B+4 residue is not required for binding to PCNA (27), we postulated that Asp-122 and/or Glu-124 contact CRL4<sup>cdt2</sup> 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<sup>cdt2</sup> substrates. Asp-120, Asp-122, and Glu-124, or Asp-122 and Glu-124, were mutated to alanines, and the resulting proteins (PCNA<sup>△120A</sup>, PCNA<sup>△122A</sup>, PCNA<sup>△124A</sup>, and PCNA<sup>△DE/A</sup>) were purified (supplemental Fig. S2A). PCNA-depleted HSS was supplemented with the differ-
ent recombinant PCNA proteins, damaged DNA, as well as Set8 and Cdt11–243, an N-terminal fragment of Cdt1 that is destroyed by the same mechanism as Cdt1WT (27). Although PCNADE/AA behaved essentially like PCNAWT, PCNAE124A displayed a noticeable, but minor defect, especially in Set8 destruction (Fig. 4C, lanes 17–20). Strikingly, PCNADE/AA was completely inactive for destruction of Cdt1, Cdt11–243, and Set8 (Fig. 4C, lanes 9–12). PCNADE/AA was also unable to support efficient Xic1 destruction (supplemental Fig. S2C). As expected, PCNADE/AA was unable to support CRL4Cdt2 activity in HSS extracts (Fig. 4C and supplemental Fig. S2B). In addition, PCNADE/AA did not support replication-dependent Cdt1 destruction in the context of sperm chromatin replication carried out in LSS extracts (supplemental Fig. S3A).

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.  

It was important to rule out the possibility that the D122A and E124A mutations in PCNA inhibited CRL4Cdt2 activity due to indirect effects on DNA replication. As shown in supplemental Fig. S2D, PCNADE/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. PCNADE/AA was also fully competent for sperm chromatin replication in LSS (supplemental Fig. S3B). Together, our results show that the D122A and E124A residues of PCNA play a specific role in potentiating CRL4Cdt2 function.

Asp-122 Is Required for CRL4Cdt2 Docking, but Not Substrate Binding to PCNA—To determine why the Asp-122 residue of PCNA is required for CRL4Cdt2 function, we first examined whether it mediates binding of substrates to PCNA. We took advantage of the fact that Cdt11–243 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 Cdt11–243 and either buffer, PCNAWT, PCNADE/AA, or PCNADE/KR, a charge-reversal mutant that also failed to support CRL4Cdt2 function and behaved identically to PCNADE/AA (supplemental Figs. S2B and S3B). We also included PCNALI/AA, a PCNA mutant previously studied in Saccharomyces cerevisiae (25), which carries mutations in residues Leu-126 and Ile-128 (of the PCNA-79 mutant) are shown in purple, Saccharomyces cerevisiae (25), which carries mutations in residues Leu-126 and Ile-128 (of the PCNA-79 mutant) are shown in purple, SucCharomyces cerevisiae (25), which carries mutations in residues Leu-126 and Ile-128 (of the PCNA-79 mutant) are shown in purple, S. cerevisiae MDIGUGFIDKQYE, W. morris, C. G. Havens, and J. C. Walter, data not shown.
were inactive for Cdt2 recruitment and Cdt11–243 ubiquitylation (Fig. 5B). PCNA \textsuperscript{DE/AA} was unaffected, whereas PCNA \textsuperscript{DE/KR} behaved like PCNA \textsuperscript{DE/AA} with respect to Cdt1 binding, CRL4\textsuperscript{Cdt2} recruitment, and ubiquitylation (Fig. 5C, lanes 3 and 4). Consistent with its intermediate effects on Cdt1 destruction, PCNA \textsuperscript{AE124A} supported intermediate levels of Cdt2 recruitment (Fig. 5C, lane 5). Similar results were observed for Set8 (Fig. 5D). PCNA \textsuperscript{DE/AA} and PCNA \textsuperscript{DE/KR} also did not support Cdt2 recruitment or Cdt1 ubiquitylation in the context of sperm chromatin replication (Fig. 5E). Together, these data show that Asp-122 is essential and Glu-124 is important for substrate-dependent CRL4\textsuperscript{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\textsuperscript{Cdt2}-mediated Destruction Independently of the B+4 Residue—Based on the above results, we postulated that CRL4\textsuperscript{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\textsuperscript{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\textsuperscript{Cdt2} sub-
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 Set8B+3/4A in PCNA-depleted extracts supplemented with PCNA WT or PCNA DE/KR. As shown in Fig. 5F, the intermediate level of Set8B+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 CRL4Cdt2-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 CRL4Cdt2-mediated Destruction in Fission Yeast—In Schizosaccharomyces pombe, CRL4Cdt2 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 CRL4Cdt2 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. 6A, compare lanes 5 and 10). In addition, analysis of Cdt1 levels in cells released from a G1 arrest showed that the S phase destruction of Cdt1 is inhibited in the mutant (Fig. 6B, 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. 6C, 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 CRL4Cdt2 activity during replication and after DNA damage.

DISCUSSION

In this study we explore the molecular mechanism by which CRL4Cdt2 recognizes its substrates in the context of PCNA. We provide evidence that recognition by CRL4Cdt2 requires amino acids not only in the substrate’s PIP degron, but also in PCNA.
Substrate Recognition by CRL4<sub>Cdt2</sub>

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

The simplest interpretation is that CRL4<sup>Cdt2</sup> 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<sup>Cdt2</sup> 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<sup>Cdt2</sup> substrates is the creation of a composite surface composed of PCNA and the substrate. Conversely, CRL1<sub>Tir1</sub>-auxin (SCF<sub>Tir1</sub>-auxin) (45) and CRL1<sub>Skp2</sub>-Cks1 (SCF<sub>Skp2</sub>-Cks1) (46) require ligase cofactor interactions to recognize their substrates. In the case of CRL1<sub>Tir1</sub>-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<sub>Skp2</sub> 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<sub>Skp2</sub>-Cks1-mediated proteolysis is promoted by a phosphorylation event on threonine 187 of the substrate p27, which mediates the p27-CRL1<sub>Skp2</sub>-Cks1 interaction (3, 46, 49).

Mechanism of Substrate Recognition by CRL4<sup>Cdt2</sup>—Our data suggest the following model for the assembly of the ternary PCNA<sup>DNA</sup>-PIP degron-CRL4<sup>Cdt2</sup> complex. First, substrates bind PCNA<sup>DNA</sup> via their PIP degron, an event that does not require CRL4<sup>Cdt2</sup> (27) and therefore almost certainly precedes binding of the ligase. Next, CRL4<sup>Cdt2</sup> docks onto the PCNA-PIP degron complex, likely using the WD40-repeat-containing β-propeller of Cdt2. A model for the structure of CRL4<sup>Ddb2</sup>, 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<sup>Cdt2</sup>. 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<sup>Cdt2</sup>. 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<sup>Cdt2</sup>. 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<sup>Cdt2</sup> has not been reconstituted with purified components, we cannot rule out the possibility that the binding of CRL4<sup>Cdt2</sup> 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<sup>Cdt2</sup> 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<sup>Cdt2</sup> 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<sup>Cdt2</sup> bind independently to two different subunits of PCNA and only later come together for Xic1 ubiquitylation (Fig. 3A) (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. 3B). 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<sup>Cdt2</sup> recruitment and activity, the data strongly favor a model in which Xic1 and CRL4<sup>Cdt2</sup> bind independently to two different subunits of PCNA and only later come together for Xic1 ubiquitylation (Fig. 3A) (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. 3B). However, we show here that the motifs were in fact not well separated (Fig. 3, C and D).

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<sup>Cdt2</sup> to the PCNA-PIP degron complex. Importantly, the
residual destruction of Set8B+3/4A (Fig. 5F) and Xic1B+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 CRL4Cdtd2. 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 CRL4Cdtd2 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 CRL4Cdtd2 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 CRL4Cdtd2-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 CRL4Cdtd2 (60). UBC8 cooperates with CRL4Cdtd2 to target p21 and Set8, whereas UBE2G1 and UBE2G2 collaborate with CRL4Cdtd2 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 CRL4Cdtd2 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 CRL4Cdtd2 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 CRL4Cdtd2. 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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REFERENCES
1. Deshaies, R. J., and Joazeiro, C. A. (2009) RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. 78, 399–434
2. Nguyen, H., Gitig, D. M., and Koff, A. (1999) Cell-free degradation of p27kip1, a G1 cyclin-dependent kinase inhibitor, is dependent on CDK2 activity and the proteasome. Mol. Cell. Biol. 19, 1190–1201
3. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. 13, 1181–1189
4. Yu, H. (2007) Cdc20. A WD40 activator for a cell cycle degradation machine. Mol. Cell 27, 3–16
5. Abbas, T., and Dutta, A. (2011) CRL4Cdtd2. Master coordinator of cell cycle progression and genome stability. Cell Cycle 10, 241–249
6. Havens, C. G., and Walter, J. C. (2011) Mechanism of CRL4Cdtd2. A PCNA-dependent E3 ubiquitin ligase. Genes Dev. 25, 1568–1582
7. Jin, J., Arias, E. E., Chen, J., Harper, J. W., and Walter, J. C. (2006) A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. Mol. Cell 23, 709–721
8. Higa, L. A., Banks, D., Wu, M., Kobayashi, R., Sun, H., and Zhang, H. (2006) L2DTL/CDT2 interacts with the Cul4/DDB1 complex and PCNA and regulates CDT1 proteolysis in response to DNA damage. Cell Cycle 5, 1675–1680
9. Sansam, C. L., Shepard, J. L., Lai, K., Ianari, A., Danielian, P. S., Amst¨am, A., Hopkins, N., and Lees, J. A. (2006) DTL/CDT2 is essential for both CDT1 regulation and the early G2/M checkpoint. Genes Dev. 20, 3117–3129
10. Abbas, T., Sivaprasad, U., Terai, K., Amador, V., Pagano, M., and Dutta, A. (2008) PCNA-dependent regulation of p21 ubiquitylation and degradation via the CRL4Cdtd2 ubiquitin ligase complex. Genes Dev. 22, 2496–2506
11. Kim, D. H., Budhavarapu, V. N., Herrera, C. R., Nam, H. W., Kim, Y. S., and Yew, P. R. (2010) The CRL4Cdtd2 ubiquitin ligase mediates the proteolysis of cyclin-dependent kinase inhibitor Xic1 through a direct association with PCNA. Mol. Cell. Biol. 30, 4120–4133
12. Kim, Y., Starostina, N. G., and Kipreos, E. T. (2008) The CRL4Cdtd2 ubiquitin ligase targets the degradation of p21Cip1 to control replication licensing. Genes Dev. 22, 2507–2519
13. Nishitani, H., Shiomi, Y., Iida, H., Michishita, M., Takami, T., and Tsumoto, T. (2008) CDK inhibitor p21 is degraded by a proliferating cell nuclear antigen-coupled Cul4-DDB1-Cdt2 pathway during S phase and after UV irradiation. J. Biol. Chem. 283, 29045–29052
14. Abbas, T., Shibata, E., Park, J., Jha, S., Karnani, N., and Dutta, A. (2010) CRL4Cdtd2 regulates cell proliferation and histone gene expression by targeting PR-Set7/Set8 for degradation. Mol Cell 40, 9–21
15. Centore, R. C., Havens, C. G., Manning, A. L., Li, J. M., Flynn, R. L., Tse, A., Jin, J., Dyson, N. J., Walter, J. C., and Zou, L. (2010) CRL4Cdtd2-mediated destruction of the histone methyltransferase Set8 prevents premature chromatin compaction in S phase. Mol. Cell. 40, 22–33
16. Oda, H., Hubner, M. R., Beck, D. B., Vermeulen, M., Hurwitz, J., Spector, D. L., and Reinberg, D. (2010) Regulation of the histone H4 monomethylase PR-Set7 by CRL4Cdtd2-mediated PCNA-dependent degradation during DNA damage. Mol. Cell 39, 364–376
17. Tardat, M., Brustel, J., Kirsh, O., Lefebvre, C., Callanan, M., Sardet, C., and Julien, E. (2010) The histone H4 Lys 20 methyltransferase PR-Set7 regu- lates replication origins in mammalian cells. Nat. Cell Biol. 12, 1086–1093
18. Irgensens, S., Eskildsen, M., Fugger, K., Hansen, L., Larsen, M. S., Kousholt, A. N., Syljuåsen, R. G., Trelle, M. B., Jensen, O. N., Helin, K., and Sørensen, C. S. (2011) SET8 is degraded via PCNA-coupled CRL4(CDT2) ubiquitylation in S phase and after UV irradiation. J. Cell Biol. 192, 43–54
19. Shibutani, S. T., de la Cruz, A. F., Tran, V., Turbyfill, W. J., 3rd, Reis, T., Edgar, B. A., and Duronio, R. J. (2008) Intrinsic negative cell cycle regula-
Substrate Recognition by CRL4Cdt2

39. Ralph, E., Boye, E., and Kearsey, S. E. (2006) DNA damage induces Cdt1 proteolysis in fission yeast through a pathway dependent on Cdt2 and Ddb1. EMBO Rep. 7, 1134–1139

40. You, Z., Harvey, K., Kong, L., and Newport, J. (2002) Xic1 degradation in Xenopus egg extracts is coupled to initiation of DNA replication. Genes Dev. 16, 1182–1194

41. Chuang, L. C., and Yew, P. R. (2005) Proliferating cell nuclear antigen recruits cyclin-dependent kinase inhibitor Xic1 to DNA and couples its proteolysis to DNA polymerase switching. J. Biol. Chem. 280, 35299–35309

42. Chuang, L. C., Zhu, X. N., Herrera, C. R., Tseng, H. M., Pfleger, C. M., Block, K., and Yew, P. R. (2005) The C-terminal domain of the Xenopus cyclin-dependent kinase inhibitor, p27Xic1, is both necessary and sufficient for phosphorilation-independent proteolysis. J. Biol. Chem. 280, 35290–35298

43. Gomes, V. X., and Burgers, P. M. (2000) Two modes of FEN1 binding to PCNA regulated by DNA. EMBO J. 19, 3811–3821

44. Guarino, E., Shepherd, M. E., Salguero, I., Hua, H., Deegan, R. S., and Karsey, S. E. (2011) Cdt1 proteolysis is promoted by dual PIP degrons and is modulated by PCNA ubiquitylation. Nucleic Acids Res. 39, 5978–5990

45. Tan, X., Calderon-Villalobos, L. I., Shar, M., Zheng, C., Robinson, C. V., Estelle, M., and Zheng, N. (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446, 640–645

46. Hao, B., Zheng, N., Schulman, B. A., Wu, G., Miller, J. J., Pagano, M., and Pavletich, N. P. (2005) Structural basis of the Csk1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase. Mol. Cell. 20, 9–19

47. Xu, S., Abbasian, M., Patel, P., Jensen-Pergakes, K., Lombardo, C. R., Cathers, B. E., Xie, W., Mercurio, F., Pagano, M., Giegel, D., and Cox, S. (2007) Substrate recognition and ubiquitination of SCF(Skp2/Csk1 ubiquitin-protein isopeptide ligase. J. Biol. Chem. 282, 15462–15470

48. Wickliffe, K. E., Lorenz, S., Weimer, D. E., Kuriyan, J., and Rape, M. (2011) The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. Cell 144, 769–781

49. Sitry, D., Seeliger, M. A., Ko, T. K., Ghanotakis, D., Breward, S. E., Itzhaki, L. S., Pagano, M., and Hershko, A. (2002) Three different binding sites of Csk1 are required for p27-ubiquitin ligation. J. Biol. Chem. 277, 42233–42240

50. Scrima, A., Fischer, E. S., Lingaraju, G. M., Böhm, K., Cavadini, S., and Tomà, N. H. (2008) Structural basis of UV DNA-damage recognition by the DDB1-DDB2 complex. Cell 135, 1213–1223

51. Angers, S., Li, T., Xi, Y., MacCoss, M. J., Moon, R. T., and Zheng, N. (2006) Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. Nature 443, 590–593

52. Scrima, A., Birkenmeier, J., Han, J., Yang, M., and Pavletich, N. P. (2004) Structure of a Fbw7-Skp1-cyclin E complex. Multisite-phosphorylation motif binding and lysine specificity of the SCF(β-TrCP1) ubiquitin ligase. Mol. Cell 11, 1445–1456

53. Hao, B., Oehlmann, S., Sowa, M. E., Harper, J. W., and Pavletich, N. P. (2007) Structure of a Fbw7-Skp1-cyclin E complex. Multisite-phosphorylated substrate recognition by SCF ubiquitin ligases. Mol. Cell 26, 131–143

54. Couture, J. F., Collazo, E., and Trievel, R. C. (2006) Molecular recognition of histone H3 by the WD40 protein WDR5. Nat. Struct. Mol. Biol. 13, 698–703

55. Schuetz, A., Allali-Hassani, A., Martin, F., Loppnau, P., Vedadi, M., Bochkarev, A., Plotnikov, A. N., Arrowsmith, C. H., and Min, J. (2006) Structural basis for molecular recognition and presentation of histone H3 by WDR5. EMBO J. 25, 4245–4252

56. Han, Z., Guo, L., Wang, H., Shen, Y., Deng, X. W., and Chai, J. (2006) Structural basis for the specific recognition of methylated histone H3 lysine 4 by the WD-40 protein WDR5. Mol. Cell 22, 137–144

57. Senga, T., Sivaprasad, U., Zhu, W., Park, J. H., Arias, E. E., Walter, J. C., and Dutta, A. (2006) PCNA is a cofactor for Cdt1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination. J. Biol. Chem. 281, 6246–6252

58. Shibata, E., Abbas, T., Huang, X., Wohlschlegel, J. A., and Dutta, A. (2011) Selective ubiquitilation of p21 and Cdt1 by UBC88 and UBE2G ubiquitin-conjugating enzymes via the CRL4Cdt2 ubiquitin ligase complex. Mol. Cell. Biol. 31, 3136–3145
61. Ye, Y., and Rape, M. (2009) Building ubiquitin chains. E2 enzymes at work. Nat. Rev. Mol. Cell Biol. 10, 755–764
62. Jin, L., Williamson, A., Banerjee, S., Philipp, I., and Rape, M. (2008) Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell 133, 653–665
63. Rodrigo-Brenni, M. C., Foster, S. A., and Morgan, D. O. (2010) Catalysis of lysine 48-specific ubiquitin chain assembly by residues in E2 and ubiquitin. Mol. Cell 39, 548–559
64. Saha, A., Lewis, S., Kleiger, G., Kuhlman, B., and Deshaies, R. J. (2011) Essential role for ubiquitin-ubiquitin-conjugating enzyme interaction in ubiquitin discharge from Cdc34 to substrate. Mol. Cell 42, 75–83
65. Moreno, S., Klar, A., and Nurse, P. (1991) Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. 194, 795–823