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A Novel Function of CRL4^{Cdt2}

*REGULATION OF THE SUBUNIT STRUCTURE OF DNA POLYMERASE δ IN RESPONSE TO DNA DAMAGE AND DURING THE S PHASE**

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Background: CRL4^{Cdt2} is an E3 ligase that is a central regulator of cell cycle progression.
Results: The p12 subunit of Pol δ is a novel substrate for CRL4^{Cdt2}.
Conclusion: CRL4^{Cdt2} regulates the degradation of the p12 subunit of Pol δ.
Significance: CRL4^{Cdt2} regulates Pol δ as a novel extension of its repertoire in cell cycle regulation and the response to DNA damage.

DNA polymerase δ (Pol δ4) is a heterotetrameric enzyme, whose p12 subunit is degraded in response to DNA damage, leaving behind a trimer (Pol δ3) with altered enzymatic characteristics that participate in gap filling during DNA repair. We demonstrate that CRL4^{Cdt2}, a key regulator of cell cycle progression that targets replication licensing factors, also targets the p12 subunit of Pol δ4 in response to DNA damage and on entry into S phase. Evidence for the involvement of CRL4^{Cdt2} included demonstration that p12 possesses a proliferating cell nuclear antigen-interacting protein-degron (PIP-degron) and that knockdown of the components of the CRL4^{Cdt2} complex inhibited the degradation of p12 in response to DNA damage. Analysis of p12 levels in synchronized cell populations showed that p12 is partially degraded in S phase and that this is affected by knockdowns of CUL4A or CUL4B. Laser scanning cytometry of overexpressed wild type p12 and a mutant resistant to degradation showed that the reduction in p12 levels during S phase was prevented by mutation of p12. Thus, CRL4^{Cdt2} also regulates the subunit composition of Pol δ during the cell cycle. These studies reveal a novel function of CRL4^{Cdt2}, *i.e.* the direct regulation of DNA polymerase δ, adding to its known functions in the regulation of the licensing of replication origins and expanding the scope of its overall control of DNA replication. The formation of Pol δ3 in S phase as a normal aspect of cell cycle progression leads to the novel implications that it is involved in DNA replication as well as DNA repair.

DNA polymerase δ (Pol δ)[2] plays a key role in eukaryotic DNA replication and is also involved in gap filling during DNA repair (1, 2). Human Pol δ is a heterotetramer (Pol δ4) composed of the p125 (catalytic), p50, p68, and p12 subunits (2–5). Evidence in yeast supports a model for a division of labor between Pol δ and Pol ϵ in which Pol δ is largely responsible for the synthesis of the lagging strand, and Pol ϵ is largely responsible for the synthesis of the leading strand (6–9). In human systems, evidence for a division of labor between Pol δ and Pol ϵ at the lagging and leading strands has not been fully established (10, 11). Current studies suggest that Pol δ and Pol ϵ may operate independently, be associated with replication of different types of chromatin, or function at different stages of S phase (12, 13). In addition to its roles in DNA replication, Pol δ also acts as the gap-filling enzyme in DNA repair processes. Evidence for this has been found in nucleotide excision repair (NER) (14, 15), heteroduplex extension during homologous recombination, double strand break repair (16, 17), and in translesion synthesis during the switch with translesion polymerases (18) in the DNA damage tolerance pathway (19, 20). There is also evidence that Pol δ participates in long patch base excision repair (21).

Human Pol δ4 is regulated in response to DNA damage (2, 22). The p12 subunit of Pol δ is degraded in response to UV radiation, alkylating agents, and replication stress under the control of ATR, resulting in the conversion of Pol δ4 to Pol δ3, the heterotrimer lacking p12 (22). Pol δ3 is the nearly exclusive form of Pol δ in UV-treated cells (22). Analysis of the subcellular recruitment of Pol δ subunits to sites of UV damage as determined by cyclobutane pyrimidine dimer co-localization demonstrated that the loss of p12, and the formation of Pol δ3, occurs at sites of DNA damage well before cyclobutane pyrimidine dimer repair is completed, *i.e.* the formation of Pol δ3 is temporally and spatially consistent with a role in DNA repair (23). Thus, Pol δ3 is the primary form of Pol δ activity present after DNA damage, so that it is the form of Pol δ likely involved in gap-filling reactions during DNA repair and also during

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[2] The abbreviations used are: Pol δ, DNA polymerase δ; ATR, ATM- and Rad3-related kinase; Cdt1, Cdc10-dependent transcript 1; Cdt2, Cdc10-dependent transcript 2; CHX, cycloheximide; CRL, cullin-ring ligase; CUL4, cullin 4; DDB1, damage specific DNA binding protein-1; p21, p21$^{CIP1/WAF1}$; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; SCF^{Skp2}, Skp1-CUL1-F-box protein-Skp2; LSC, laser scanning cytometry; PIP-degron, PCNA-interacting protein-degron; RFC, replication factor C.

translesion synthesis in S phase cells (23). Biochemical analyses of the properties of recombinant and native Pol δ3 and Pol δ4 have established that Pol δ3 has altered properties that represent a protective "gain of function" in regard to the maintenance of genomic stability when cells face genotoxic challenges. Pol δ3 is less prone to bypass base lesions, insert mismatched nucleotides, or extend mismatched primer ends (24). Pre-steady state kinetic analyses have shown that Pol δ3 has an increased rate constant ($k_{Pol\text{-}exo}$) for transfer of primer ends from the polymerase to the 3′- to 5′-exonuclease (exo) catalytic sites and a decrease in k_{Pol}, the rate constant for polymerization. These changes define Pol δ3 as having a greater proofreading ability than Pol δ4, providing a rationale for its formation in response to DNA damage (2, 24, 25).

The degradation of p12 requires an intact ubiquitination system (22). Therefore, the identity of the E3 ligase systems that are responsible for targeting p12 for degradation is central to understanding how the control of the conversion of Pol δ4 to Pol δ3 is integrated into DNA damage response signaling pathways. Recently, we identified RNF8 as an E3 ligase that participates in the targeting of p12 for degradation in response to UV damage (26). RNF8 plays a central role in the DNA damage response during the assembly of the complexes that orchestrate the activation of homologous recombination, checkpoints, and apoptotic signaling in response to ionizing radiation damage (27–29). In addition, RNF8 efficiently mono-ubiquitinates PCNA both *in vitro* and *in vivo* (30), a process central to the activation of translesion synthesis by Pol η and other translesion synthesis polymerases (19, 31).

In this study, we report the investigation of the role of CRL4^{Cdt2} in p12 degradation. CRL4^{Cdt2} is a member of the Cullin family of E3 ligases, the Cullin-Ring ubiquitin ligases (CRLs). The cullins serve as scaffolding proteins that permit modular assembly of protein factors involved in substrate recognition at their N termini and the E2 ubiquitin-conjugating enzymes at their C termini (32–34). The modular structure of the CRLs brings their substrates into a spatial context where ubiquitination by the E2-conjugating enzymes can take place. CRLs play important roles in the DNA damage response and the maintenance of genomic integrity during cell cycle progression (32–34). CRL4^{Cdt2} consists of cullin 4 (CUL4) whose substrate recognition system consists of the binding of an adaptor protein, DDB1, which in turn recruits a substrate recognition protein, Cdt2. The latter is only one of a number of DDB1- and CUL4-associated factors, also known as WD repeat-containing proteins, that can be assembled onto CRL4 (35).

CRL4^{Cdt2} has emerged as a "master coordinator of cell cycle progression and genome stability" (33) by virtue of its role as a key regulator of the licensing factors involved in the formation of the multiprotein pre-replicative complexes that are loaded at the replication origins during the G_1 phase of the cell cycle. Binding of origin recognition complex leads to recruitment of Cdt1 and Cdc6, which load the MCM proteins. On entry into S phase, replication is initiated when replication proteins, including the DNA polymerases, are assembled onto the pre-replicative complexes and activated by cyclin-dependent kinases and Dbf4-dependent kinase to initiate DNA replication (33). CRL4^{Cdt2} plays a central role in the management of replication licensing factor Cdt1 by targeting it for destruction on entry into S phase, thereby preventing re-licensing by Cdt1 and re-replication, events that lead to genomic instability. In human cells, Cdt1 is also regulated by geminin, which binds to and inactivates Cdt1. The degradation of Cdt1 (as well as geminin, Cdc6, and cyclins) is also regulated by SCF (Skp1/Cul1/F-box) and the anaphase-promoting complex/cyclosome complexes (36, 37). These mechanisms ensure that the genome is replicated only once per cell cycle. CRL4^{Cdt2} has two other mammalian substrates, Set8 (38–40) and p21 (p21$^{WAF1/CIP1}$) (41, 42), both of which are coordinately degraded with Cdt1 in S phase and contribute to the prevention of re-licensing. Set8 plays a central role in condensation of chromosomes in G_2/M, and its presence in S phase cells can lead to re-replication (40).

In addition to the S phase degradation of its substrates, CRL4^{Cdt2} also participates in the UV-induced degradation of Cdt1, p21 (41, 43), and Set8 (40). This UV-induced degradation occurs in all cell cycle phases. The degradation of Cdt1 is thought to contribute to the cell cycle checkpoints that occur on DNA damage. p21, in addition to inhibition of the cyclin-dependent kinases, binds to PCNA and may also inhibit enzymes that require PCNA, including Pol δ (2, 44). p21 also affects the mono-ubiquitination of PCNA that is essential for triggering translesion synthesis at replication forks stalled at UV damage sites (41, 43). Recent studies have revealed the cellular and molecular mechanisms whereby Cdt1 is degraded on entry into S phase by CRL4^{Cdt2}. Studies in *Xenopus* egg and human systems have shown that the recognition of CRL4^{Cdt2} substrates by Cdt2 is unusual, because it recognizes substrates bound to PCNA and DNA through a specialized PIP-box (referred to as the PIP-degron), with a conserved Thr-Asp sequence within the PIP-box and a basic residue at the +4 position (32, 33). The utilization of a common PIP-degron provides an elegant molecular mechanism for integrating key proteins important for the cell cycle progression and DNA damage response under the control of CRL4^{Cdt2}.

In this study, we present evidence that the p12 subunit of Pol δ possesses a PIP-degron and that CRL4^{Cdt2} contributes to the UV- and DNA damage-induced degradation of p12. In addition, we show that p12 is also degraded in S phase under the control of CRL4^{Cdt2}. The latter findings have significant implications for the potential role of Pol δ3 in DNA replication in addition to its role in DNA repair, and it illuminates an important new function of CRL4^{Cdt2} in its dominion over the overall process of DNA replication by direct regulation of Pol δ.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments with UV and Alkylating Agents—HeLa and HEK293T cells were maintained in DMEM; A549 and H1299 cells were maintained in RPMI 1640 medium (22, 26). Cells were treated with UVC as described previously (22). Briefly, the medium was decanted, and the cells were washed once with PBS. After UV exposure, fresh medium was added. The degradation of p12 in response to DNA damage in the chosen cell lines has previously been documented (22).

Cell Synchronization—H1299 cells were arrested in G_2/M by addition of 40 ng/ml of nocodazole to the media for 12 h (45, 46). Mitotic cells were shaken off, centrifuged down, and

washed once with PBS. Cells were released by transfer to fresh medium and analyzed at different time points. Cells entered S phase at about 12–14 h after release from nocodazole.

CUL4A, CUL4B, and Cdt2 Knockdown Cell Lines—Human CUL4A, CUL4B shRNA, and control shRNA were as described previously (47, 48). Human Cdt2-shRNA (mature sense sequence, GGTTATCAGTGCAGTGGT) was obtained from Open Biosystems. The shRNAs were packaged into lentivirus by co-transfection with the packaging plasmids Δ8.9 and VSVG into HEK293T cells. Forty eight hours after transfection, the supernatant was filtered through a sterile 0.45-μm filter and used for infection of target cells (H1299 and A549). Following infection, knockdown cells were selected by addition of 2–3 μg/ml puromycin, and the cultures were grown for 1 week and further maintained in 0.5 μg/ml puromycin for experimental use.

Ectopic Expression of Wild Type and Mutant His-p12—His-p12 (4) was subcloned into the PSMPUW-IRES-Puro lentivirus expression vector (Cell Biolabs, Inc.). The p12$^{TD/AA}$ (^{8}TD^{9} to ^{8}AA^{9}) and p12$^{KRR/AAA}$ (^{15}KRR^{17} to ^{15}AAA^{17}) mutants were generated by PCR mutagenesis of the His-p12-PSMPUW-IRES-Puro plasmid; confirmation of the mutagenesis was performed by DNA sequencing. The His-p12, p12$^{TD/AA}$, and p12$^{KRR/AAA}$ sequences were packaged into lentivirus by co-transfection of the plasmids with the packaging plasmids Δ8.9 and VSVG into HEK293T cells. Forty eight hours after transfection, the culture supernatants were filtered through sterile 0.45-μm filters and used for infection of A549 cells. Selection for cells expressing His-p12 was performed as described above for shRNA expression.

Western Blotting—Western blotting for p12 and other proteins was performed as described previously (22). Antibodies against individual Pol δ subunits were as described previously (23). Antibodies against the following proteins and their sources were: PCNA (PC10) and cyclin A (Santa Cruz Biotechnology Inc); Cdt1, p21, Chk1-p, γ-H2AX, and cyclin B1 (Cell Signaling Technology); Cdt2 and CUL4A (Bethyl Laboratories, Inc); CUL4B (Proteintech); anti-His (Sigma).

Laser Scanning Cytometry—A549 cells in which His-p12 WT or p12$^{KRR/AAA}$ were stably expressed were grown on glass-chambered slides, fixed, and stained as described previously (23). His-p12 and its mutant were detected using anti-p12 antibody as the primary antibody, which was detected with secondary antibody labeled with the green wavelength emitting fluorochrome AlexaFluor488. Cellular DNA was detected by the blue fluorescence of DAPI staining. Laser scanning cytometry (LSC) analysis was performed on an iCys Research Imaging Cytometer (CompuCyte, Westwood, MA). The primary contours were set using DAPI fluorescence to define the nucleus. At least 3,000 cells were measured per sample. Methods used for gating analysis were essentially as described previously (23, 49).

RESULTS

p12 Has a PIP-box That Conforms in Sequence to the PIP-degrons Recognized by CRL4^{Cdt2}—The p12 subunit of Pol δ is a small protein (107 amino acid residues) that interacts with both the p125 and p50 subunits and has a functional PCNA-binding motif (PIP-box) (4). The PIP-box motif consists of eight residues (50), with a glutamine at position 1, an aliphatic residue ("h") at position 4, and two aromatic residues, phenylalanine or tyrosine at positions 7 and 8 (Fig. 1*A*). The PIP-box of the p68 subunit of Pol δ is shown as an example (Fig. 1*A*). The fact that p12 is degraded in response to UV and other forms of DNA damage, like the CRL4^{Cdt2} substrates Cdt1, p21, and Set8, raises the obvious question as to whether p12 is also regulated by CRL4^{Cdt2}. CRL4^{Cdt2} recognizes an extended PIP-degron that is present in Cdt1, p21, and Set8. The PIP-degron is essentially a PIP-box harboring a "TD" motif within the PIP-box sequence and a basic residue at the +4 position C-terminal to the PIP-box (32, 51, 52).

Sequence alignment of the PIP-degrons of the three currently known human CRL4^{Cdt2} substrates (p21, Cdt1, and Set8) with the N-terminal region of p12 harboring its PIP-box reveals the presence of a well defined and recognizable PIP-degron (Fig. 1*A*), *i.e.* it possesses the TD motif within the PIP-box, as well as a basic residue at the B+4 position. The alignment also shows the B+4 residue is generally one of a cluster of basic residues, all of which may have interactions with PCNA (52, 53). Both p12 and Set8 have similarities in the N-terminal part of the PIP-box where they lack the conserved glutamine (Fig. 1*A*).

Mutational Analysis of the Putative PIP-Degron of p12 Shows That It Is Functional in Vivo—To establish that p12 indeed contains a PIP-degron, we examined the effects of UV on p12 and its mutants that were stably overexpressed in A549 cells (see "Experimental Procedures"). The mutants were p12$^{TD/AA}$, in which the conserved ^{8}TD^{9} residues of the degron were mutated to ^{8}AA^{9}, and the p12$^{KRR/AAA}$ (^{15}KRR^{17} to ^{15}AAA^{17}) mutant in which all three of the basic residues (+4, +5,and +6) C-terminal to the PIP-box were mutated to alanines (Fig. 1*A*). Similar types of mutants have been used to establish the importance of the TD sequence and B+4 basic residue in defining the degron determinants in Cdt1 and p21 (32, 51, 52). Mutation of all three basic residues was performed as it has been observed that all three are required for recruitment of CRL4^{Cdt2} (52, 53).

The effects of UV on the degradation of the p12$^{TD/AA}$ (Fig. 1*B*) and p12$^{KRR/AAA}$ mutants expressed in A549 cells show that both mutants were significantly stabilized compared with wild type p12 in response to UV doses from 20 to 40 J/m^2 (Fig. 1*C*). The effects of mutation of the internal TD sequence and the basic cluster C-terminal to the PIP-box are similar to findings in previous studies of the mammalian PIP-degron of p21 (52). Thus, these results confirm that p12 contains a functional PIP-degron for CRL4^{Cdt2}. Similar results were obtained using FLAG-tagged p12 constructs (data not shown). Moreover, the basic residues at +3 and +4 are required for p21 degradation (52). In p12, the basic residues are at the +4 to +6 position, with a valine at +3. However, it is noted that the cluster of basic residues most likely interacts with a well defined acidic patch on PCNA, based on structural information of the only PIP-degron-PCNA structure that has been solved (54), *i.e.* p21 (see below).

The crystal structure of a C-terminal p21 peptide harboring the PIP-degron in complex with PCNA shows an extensive interaction of the 17 residues (^{144}QTSMTDFYHSK*RRLIFS*^{160}) of p21 with PCNA (Protein Data Bank code 1AXC), especially

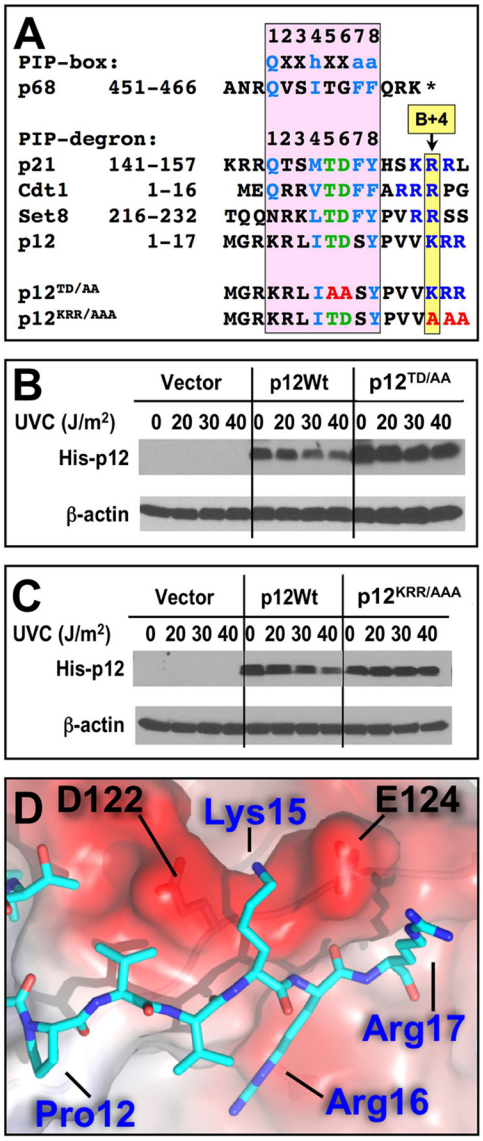

FIGURE 1. **p12 possesses an operational PIP-degron at its N terminus**. *A*, alignment of the PIP-box of p12 with the PIP-degron sequences of the three known human CRL4^{Cdt2} substrates (p21, Cdt1, and Set8). The *1st line* shows the canonical PIP-box (*shaded box in pink*), with the four conserved residues shown in *aqua* (*Q* = glutamine, *h* = aliphatic residue, and *aa* = two aromatic residues, commonly Phe or Tyr), with the PIP-box for the p68 subunit of human Pol δ below as an example. Next are shown the PIP-degrons of p21, Cdt1, and Set8, aligned with the corresponding region of p12. The TD motif within the PIP-box is indicated in *green*, and the presence of a basic residue at the +4 position is shown in *blue* (*yellow box*). Basic residues adjacent to the +4 base are also shown in *blue*. The two p12 mutants (p12$^{TD/AA}$ and p12$^{KRR/AAA}$) in which the determinants for the PIP-degron were mutated are shown in *red*. *B*, A549 cells in which His-p12 WT and the p12$^{TD/AA}$ mutant were stably expressed were treated with increasing doses of UV and immunoblotted with anti-His antibody. β-Actin was used as a loading control. *C*, similar as for *B*, except that cells expressing the His-p12$^{KRR/AAA}$ mutant were used. *D*, model of the basic region of the p12 PIP-degron interacting with the acidic surface of the interdomain connecting loop of PCNA. The model is based on the structure of PCNA (Protein Data Bank code 1AXC) in complex with a peptide from p21 (54) through mutating residues based on the sequence alignment in *A* with Coot (80) and rendered with PyMOL. The side-chain orientations were optimized to avoid collisions. Residues of p12 are in *cyan* as *sticks*, whereas PCNA is shown as an electrostatic surface with acidic regions in *red*. Basic residues in p12 are shown in *blue lettering* and acidic residues in PCNA in *black lettering*.

the residues C-terminal to the PIP-box. The side chains of the conserved N-terminal hydrophobic residues within the PIP-box degron (underlined) fit into a hydrophobic cleft on PCNA, whereas the C-terminal residues have an extended interaction with the interdomain connecting loop of PCNA in the form of an anti-parallel β-sheet (italicized residues). This structure has been used to visualize the interaction of other PIP-degrons with PCNA. A similar view of the basic region of p12 interacting with PCNA is modeled in Fig. 1*D*, which illustrates the acidic patch on PCNA that provides interaction for the basic residues in the PIP-degrons. In p12, the residue at the +4 position is Lys-15, which could be modeled between Asp-122 and Glu-124 of PCNA to display ionic interactions. Asp-122 and Glu-124 have been shown to be required for CRL4^{Cdt2} recruitment (53), indicating that the basic residue at +4 as well as Asp-122 and Glu-124 are required for CRL4^{Cdt2} interaction.

Effects of CUL4A and CUL4B Knockdowns on the UV-induced Degradation of p12—To confirm that CRL4^{Cdt2} plays a role *in vivo* in regulating p12 degradation in response to DNA damage, we examined the effects of UV on p12 levels in cells in which CUL4 was knocked down by the use of lentiviral shRNA (see under "Experimental Procedures"). As there are two paralogs of CUL4 in human cells, CUL4A and CUL4B (34, 55), cells with individual lentiviral shRNA knockdowns of CUL4A or CUL4B were used (these shRNAs had previously been demonstrated to be effective for knocking down CUL4A and CUL4B (47, 56)). The effectiveness of the knockdowns of CUL4A and CUL4B in the A549 cells is shown in Fig. 2*A* (*1st two rows*). We examined the levels of the p12, p125, and p68 subunits of Pol δ and of PCNA. p12 was partially stabilized in knockdowns of either CUL4A or CUL4B, although levels of the p125 and p68 subunits of Pol δ and PCNA were unaffected (Fig. 2*A*). The observations that p125 and p68 levels are not affected are consistent with our previous studies and also with the fact that the loss of p12 leads to the generation of Pol δ3 *in vivo* (22).

The UV dose response of p12 degradation was then examined in more detail for the CUL4A knockdown cells (Fig. 2*B*). p12 was heavily degraded at the lowest dose of UV (5 J/m^2) tested in the control cells (control shRNA, Fig. 2*B*, *left column*). Levels of p21 and Cdt1 exhibited comparable dose responses. In the CUL4A knockdowns, comparable degradation required a 4–5-fold higher UV dose of 20–25 J/m^2 for p12. Similar stabilization of p21 and Cdt1 was observed (Fig. 2*B*, *right column*) The efficacy of the knockdown was demonstrated by Western blots for CUL4A (Fig. 2*B*). Chk1-Ser(P)-345 was Western blotted to confirm activation of ATR by UV.

We performed similar experiments with CUL4B knockdown cells. The degradation of p12 and p21 in CUL4B knockdown cells both exhibited a significant reduction in sensitivity to UV at comparable dose ranges as for CUL4A (Fig. 2*C*). Thus, these experiments show that both CUL4A and CUL4B contribute to p12 degradation in A549 cells.

The decreased dose response for UV-induced degradation of p12 in cells in which CUL4A or CUL4B were knocked down suggests a decreased turnover of p12 by CRL4^{Cdt2}. To confirm this, we examined the time course of p12 degradation after a fixed UV dose in cycloheximide-treated control and CUL4A knockdown cells (Fig. 2*D*) (control experiments with no UV treatment are shown in Fig. 2*E*). Degradation of p12 was significantly slowed down in the CUL4A knockdown cells, as was the degradation of p21 and Cdt1 (Fig. 2*D*). The blots for p12 were

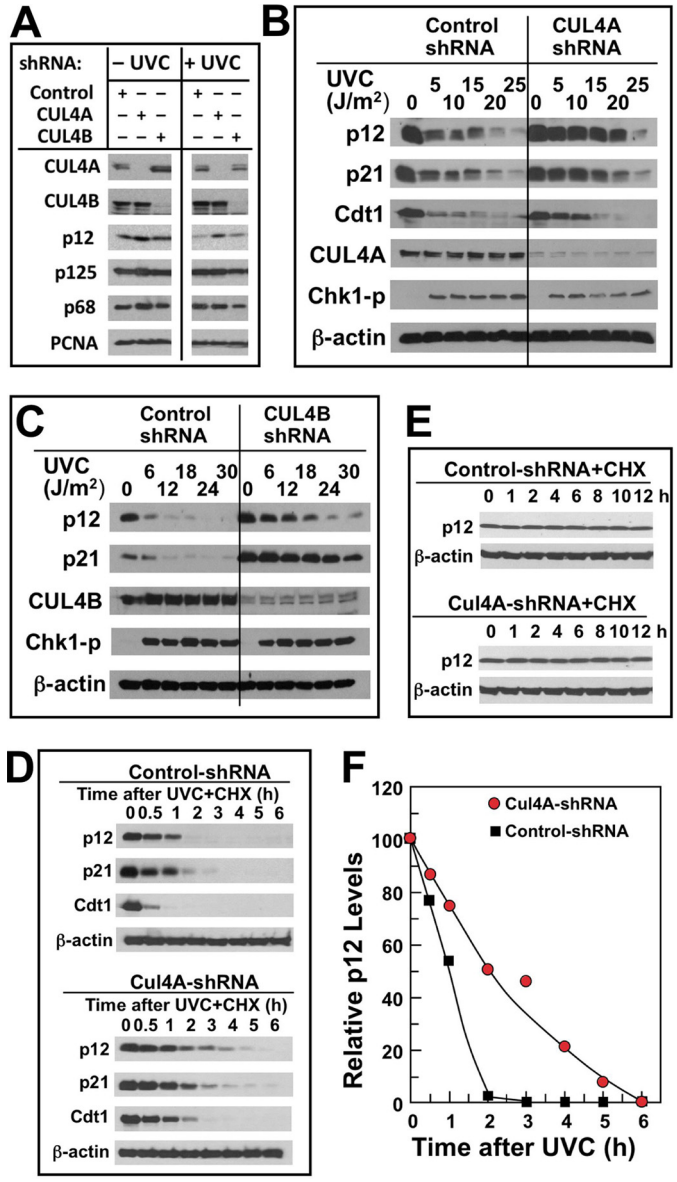

FIGURE 2. **Knockdown of CUL4 reduces the sensitivity of p12 to UVC degradation and decreases the rate of degradation of p12.** *A,* stably transfected A549 cells in which either CUL4A or CUL4B were knocked down by shRNA (see "Experimental Procedures") were treated with UVC (6.6 J/m^2) and analyzed 4 h later. The cell lysates were Western blotted for CUL4A, CUL4B, p12, as well for the p125, and p68 subunits of Pol δ; PCNA was used here as a loading control. *B,* CUL4A knockdown (*right panel*) and control A549 cells (*left panel*) were treated with increasing doses of UVC as indicated and analyzed 4 h later. The lysates were Western-blotted for p12, p21, Cdt1, Chk1-Ser(P)-345, and β-actin. *C,* CUL4B knockdown (*right panel*) and control A549 cells (*left panel*) were treated with increasing doses of UVC as indicated and analyzed 4 h later. The lysates were Western-blotted for p12, p21, Chk1-Ser(P)-345, CUL4B, and β-actin. *D,* control cells and CUL4A knockdown cells were treated with UVC (12 J/m^2) in the presence of cycloheximide (10 μg/ml) and analyzed at varying times after UV exposure. The cell lysates were Western-blotted for p12, p21, Cdt1, and β-actin. *E,* control and CUL4A knockdown cells were treated with cycloheximide (10 μg/ml) without UVC treatment. p12 levels were examined by Western blotting at the indicated times after addition of cycloheximide. *F,* blots for p12 for the data in *D* were scanned by densitometry, and the relative levels of p12 (normalized to β-actin as a loading control) were plotted against time. Data for the control cells are shown as *solid squares,* and those for the CUL4A knockdown cells as *red circles*.

analyzed by densitometry, and relative levels of p12 were plotted against time to illustrate the time course for degradation (Fig. 2*F*). At 2 h after UV exposure, ~50% of the p12 remained in the CUL4A knockdown cells, whereas p12 was almost absent in the control cells (Fig. 2*F*).

Because p12 is degraded in response to other genotoxic agents in addition to UV, we examined the effects of alkylating agents on p12 degradation in CUL4A knockdown cells. p12 degradation by the alkylating agents, *N*-methyl-*N*′-nitroso-*N*-nitrosoguanidine and methyl methylsulfonate, was significantly reduced in CUL4A knockdown cells by comparison with cells treated with control shRNAs (data not shown). The ability of alkylating agents to induce CRL4^{Cdt2} degradation of its substrates has been previously documented in *Xenopus* (53) and mammalian cells (57).

Effects of DDB1 and Cdt2 Depletion on the UV-induced Degradation of p12—Next, we examined the effects of depletion of two other components of the substrate recognition modules of CRL4^{Cdt2}, DDB1 and Cdt2. For the ablation of DDB1, we used DDB1$^{flox/flox}$ mouse epithelial fibroblasts in which the floxed DDB1 was acutely deleted by infection of adenoviral-Cre adenovirus (58). In uninfected cells, low UVC doses of 2.2 and 4.4 J/m^2 were sufficient to lead to degradation of p12, which was significantly blocked in the Ade-Cre-infected DDB1$^{flox/flox}$ mouse epithelial fibroblasts (Fig. 3*A*).

For depletion of Cdt2, we used shRNA knockdown cells (see under "Experimental Procedures"). Control and Cdt2 knockdown cells were exposed to increasing doses of UVC and immunoblotted for p12, p21, and Cdt2 (Fig. 3*B*). p12 levels in the Cdt2 knockdown cells were elevated in the untreated cells and significantly (but not completely) stabilized in UV-treated cells. Similar effects were observed for the behavior of p21 (Fig. 3*B*). The relative levels of p12 were determined by densitometric analysis and were plotted against UV dose (Fig. 3*C*). The plots show that the knockdown of Cdt2 significantly reduces the sensitivity of p12 to UV. In the control cells p12 levels were reduced by 50% at 15 J/m^2, although a dose of 40 J/m^2 was required for the same response in the Cdt2 knockdown cells. Collectively, these results indicate a critical role of CRL4^{Cdt2} in p12 degradation in response to UVC damage.

p12 possesses a PIP-degron sequence that is recognized by CRL4^{Cdt2} *in vivo*, and depletion of individual CUL4A, CUL4B, DDB1, or Cdt2 components of CRL4^{Cdt2} all attenuate UV-induced degradation of p12. Our results indicate that both CUL4A and CUL4B act redundantly in the degradation of p12. This redundancy obviously cannot be generalized for other cell types, because it would be dependent on their relative expression levels, and we cannot discount the possibility that compensatory alterations take place. Examples of where one or the other CUL4 genes exhibit predominant roles are in the regulation of DDB2 and p21 in mouse epithelial cells by CUL4A, and in mouse models where CUL4A is essential for spermatogenesis and male fertility (59). Deficiency of CUL4B is associated with syndromic X-linked mental retardation in humans (60), and mice with targeted disruption of CUL4B show that it has an essential role in extra-embryonic tissue development during embryogenesis (48).

It is noted that p12 degradation in response to UV was not completely abolished in our experiments. This may be attributed to the fact that both CUL4A and CUL4B contribute to UV-induced p12 degradation. In addition, CRL4^{Cdt2} is not the

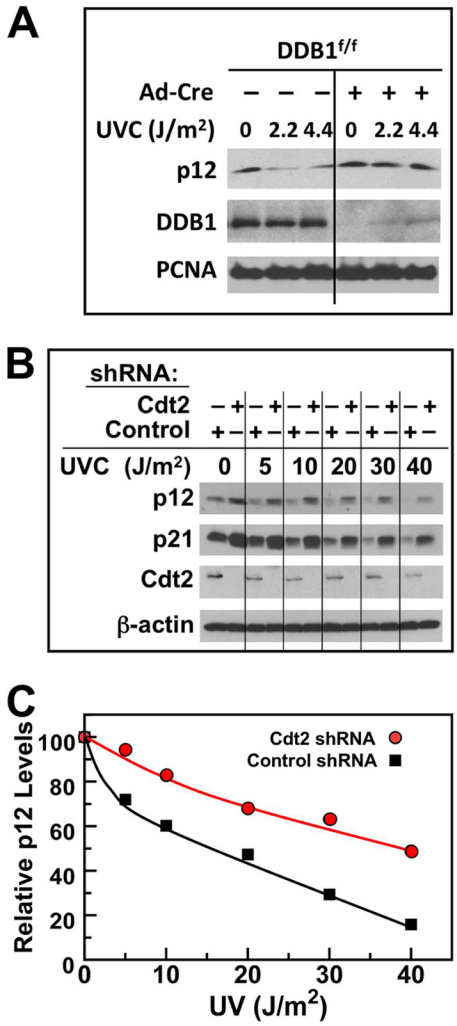

FIGURE 3. **Depletion of DDB1 and Cdt2 stabilize p12 to UVC-induced degradation.** *A,* effects of knockdown of DDB1 were examined in mouse epithelial cells (DDB1$^{f/f}$) in which the DDB1 gene was conditionally deleted by use of Cre/lox methodology (58). Inactivation of the DDB1 gene was performed by infection with an adenoviral vector expressing Cre recombinase or an empty vector (58). Control cells (infected with empty vector, shown as *Ad-Cre* −) and cells infected with adenovirus expressing Cre recombinase (*Ad-Cre* +) were treated with the indicated doses of UVC and immunoblotted for p12, DDB1, and PCNA after 4 h. *B,* effects of Cdt2 knockdown on p12 expression were performed in A549 cells in which Cdt2 was stably knocked down by shRNA (see "Experimental Procedures"). Cells were treated with the indicated dosages of UVC and immunoblotted for p12, p21, Cdt2, and β-actin after 4 h. *C,* blots for p12 in control (*solid squares*) and Cdt2 knockdown cells (*red solid circles*) were quantitated by densitometric analysis, normalized to β-actin, and the relative p12 levels plotted against UV dose.

sole ubiquitin ligase involved in the regulation of p12 in response to DNA damage, as RNF8 is also involved in DNA damage-induced targeting of p12 for degradation (26). As we have demonstrated previously with RNF8, only partial stabilization of p12 was observed, which is expected if there is more than one targeting pathway that is activated by DNA damage.

p12 Exhibits Cell Cycle-associated Degradation during S Phase That Is Mediated by CRL4^{Cdt2}—One of the critical functions of CRL4^{Cdt2} is the targeting of Cdt1 for destruction to prevent re-replication during S phase (32, 33). The question is raised as to whether and to what extent cell cycle degradation of p12 takes place given that we have established that p12 possesses a PIP-degron. We therefore examined p12 levels in synchronized cells to determine whether p12 levels were decreased during S phase. For this purpose we used nocodazole, which arrests cells in G_2/M by disrupting microtubular structures (26). It is noteworthy that use of other methods, *i.e.* thymidine blockade, hydroxyurea, or aphidicolin were precluded because they cause replication stress that results in p12 degradation (22). H1299 cells (control and CUL4A-shRNA knockdown cells) were arrested in G_2/M by nocodazole, and were subsequently released to allow cell cycle progression over a 24-h period (see "Experimental Procedures"). Cell extracts were immunoblotted for p12 and p21. Cyclin A and cyclin B1 were used as markers for cell cycle progression, and β-actin was used as a loading control. Representative blots for one of four replicate experiments is shown in Fig. 4*A*. Densitometric analysis of the p12 blots was used to quantify the relative p12 levels, which were plotted in Fig. 4*B* as the mean ± S.D. of four replicate experiments. In control cells, p12 levels exhibited a trough-shaped curve that starts to fall at 8 h during cell cycle progression, reaching a low (~35% of the G_2/M arrested cells) between 12 and 16 h, and then it rises again. The appearance of the trough is consistent with the period when the cells are in S phase as gauged by the onset of the cyclin A increase at 12 h. In the CUL4A-shRNA knockdown cells, the fall in p12 levels in S phase was significantly diminished, so that the lowest levels were ~70% of the starting levels. In contrast, the levels of the p125, p68, and p50 subunits of Pol δ did not change with cell cycle progression (Fig. 4*A*). This is consistent with the fact that only the p12 subunit is affected by DNA damage (22). The changes in p12 levels can be used to estimate the relative amounts of Pol δ3 and Pol δ4. In the control cells, a fall of p12 to 35% of the levels present in G_2/M and G_1 suggests that Pol δ3 represents ~65% of the total Pol δ present in S phase cells, given that the other Pol δ subunits do not exhibit changes during the cell cycle.

In these same experiments, we examined the levels of p21, which also exhibited an S phase decline (Fig. 4*C*). The p21 levels in the control cells fell to ~20% of the starting levels during S phase; in the CUL4A knockdown cells, the decline was blunted and fell to levels of ~40% of the starting levels. In addition, the p21 levels started decreasing earlier than p12, beginning in G_1. However, it is known that p21 levels are also regulated by SCF^{Skp2}/CRL1^{Skp2} during G_1 and S (61) and anaphase-promoting complex/cyclosome and its activator Cdc20 during G_2/M (62).

The levels of cyclin A and cyclin B1 for the experiment of Fig. 4*A* were quantitated and plotted against time (data not shown). These revealed that there were no significant changes between the time courses in control and CUL4A shRNA knockdown cells, suggesting that there were minimal perturbations to the cell cycle.

We compared the effects of CUL4A and CUL4B knockdowns on changes in p12 levels in a separate set of experiments (Fig. 5*A*). The blots for p12 were scanned, and the relative p12 levels were determined. The levels of p12 in the control and CUL4A knockdown cells were ~40 and ~80% of the starting levels, respectively (Fig. 5*B*), consistent with the data of Fig. 4*B*. Data for the CUL4B knockdown experiment are plotted in Fig. 5*C*. A similar S phase decline in p12 levels was observed, with the

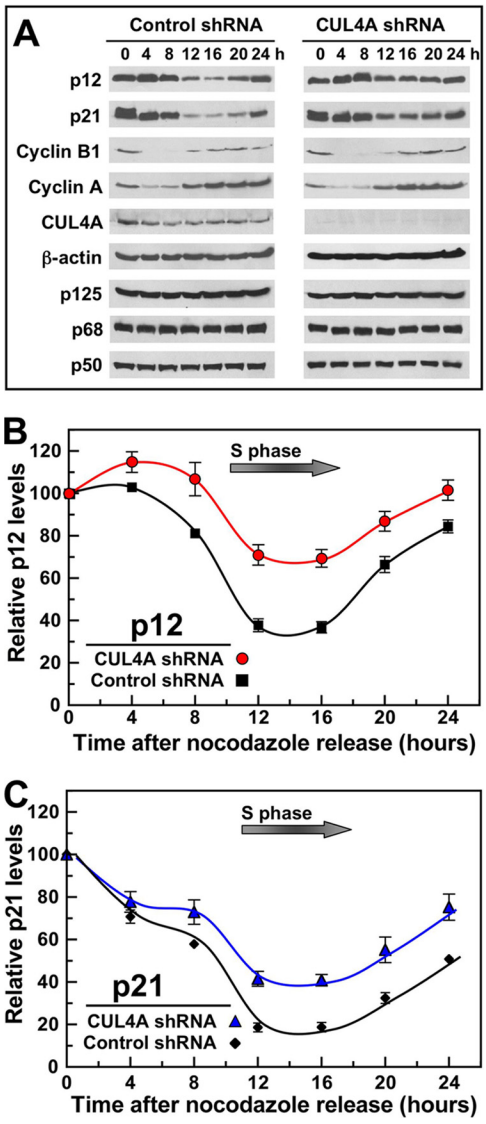

FIGURE 4. **Analysis of p12 levels during cell progression in control and CUL4A knockdown cells.** Control and CUL4A knockdown H1299 cells were synchronized by nocodazole treatment (40 ng/ml, 12 h) and allowed to progress through the cell cycle by transfer to fresh medium (see "Experimental Procedures"). Cells were harvested at 4-h intervals for 24 h and immunoblotted for p12, p21, cyclin B1, cyclin A, CUL4A, β-actin, and the Pol δ subunits p125, p68, and p50. Quadruplicate experiments were performed. *A,* representative blots for one of the four replicate experiments are shown. *B* and *C,* levels of p12 (*B*) and p21 (*C*) were determined and plotted against time after release from nocodazole. The blots for p12 and p21 from each replicate experiment were analyzed by densitometry and normalized to zero time after correction against β-actin levels. Mean values ± S.D. for p12 in the control are shown as *solid squares* and in the CUL4A knockdown cells as *red circles* in *B*. Mean values ± S.D. for p21 in the control are shown as *solid diamonds* and in the CUL4A knockdown cells as *blue triangles* in *C*. The approximate duration of S phase is shown by the *gray arrows*.

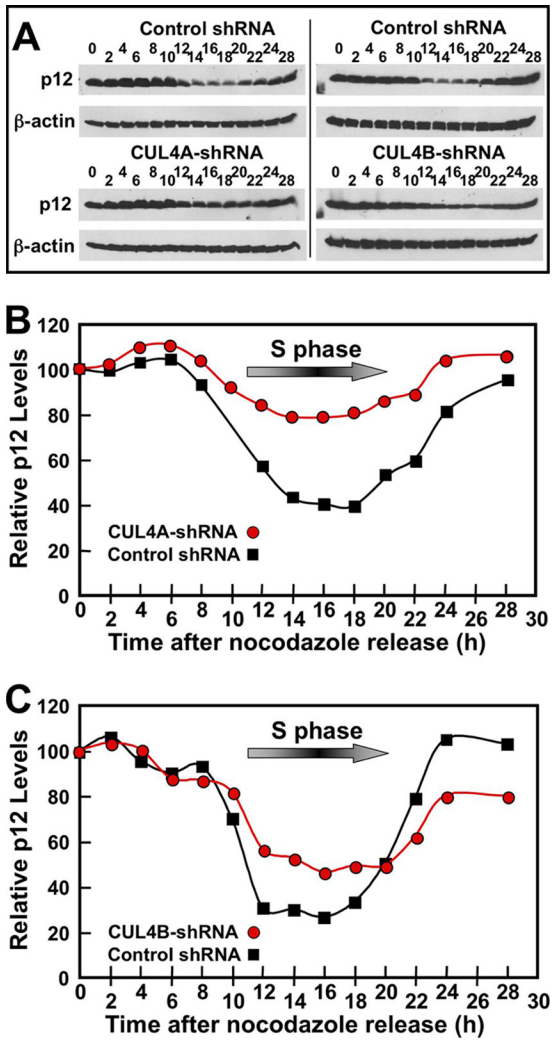

FIGURE 5. **p12 levels during cell progression in control is affected by both CUL4A and CUL4B knockdown.** CUL4A and CUL4B knockdown H1299 cells were synchronized by nocodazole in parallel experiments and analyzed over a period of 28 h at 2-h intervals. *A,* cells were immunoblotted for p12 and β-actin for CUL4A shRNA knockdown cells (*left lanes*) and CUL4B shRNA knockdown cells (*right lanes*). *B,* immunoblots for p12 for the CUL4A knockdown cells were analyzed by densitometry and normalized to the starting time point values after correction against β-actin levels. Data for the control cells are shown as *solid squares* and those for the CUL4A shRNA knockdown cells are shown as *red circles*. *C,* immunoblots for p12 for the CUL4B knockdown cells were analyzed by densitometry and normalized to the starting time point values after correction against β-actin levels. Data for the control cells are shown as *solid squares* and those for the CUL4B shRNA knockdown cells are shown as *red circles*. The approximate duration of S phase is shown by the *gray arrows*.

lowest S phase values of ~30 and 50% for control and CUL4B knockdown cells, respectively. The data for CUL4B show that it is also involved in the regulation of p12 during the S phase. Thus, there is redundancy in the roles of CUL4A and CUL4B in the regulation of p12 during the cell cycle.

These studies are the first to examine p12 levels during the cell cycle progression in synchronized cells, and they provide direct evidence that p12 levels fall during S phase in unperturbed cells. In other experiments (data not shown), we had found that the p125, p50, and p68 subunits of Pol δ do not exhibit cell cycle changes in S phase, so that the degradation of p12 is consistent with generation of Pol δ3 during the cell cycle. The second finding is that the reduction in p12 levels in S phase during the cell cycle progression is mediated by CUL4A and CUL4B. Taken together with the identification of a PIP-degron in p12, these results firmly place p12 degradation in S phase under the control of CRL4^{Cdt2}. Moreover, these data indicate that Pol δ3 is formed during normal cell cycle progression in the S phase under the control of CRL4^{Cdt2}.

Mutation of the p12-degron Abolishes the S Phase Degradation of p12 during Cell Cycle Progression—To confirm that the CRL4^{Cdt2}-mediated decline in p12 levels in the S phase during

cell cycle progression is due to a direct effect on p12 degradation, we analyzed the cell cycle distribution of p12 and the p12$^{KRR/AAA}$ mutant in cells where these were overexpressed as the His-tagged proteins (see "Experimental Procedures"). This analysis was performed by laser scanning cytometry (LSC). The expression of p12 in wild type and p12$^{KRR/AAA}$ mutant was assessed by immunofluorescence staining of the His-p12 (green fluorescence). The DNA-specific fluorochrome DAPI (blue fluorescence) was used to set the primary contour to the nucleus, allowing information to be obtained for p12 immunofluorescence in individual nuclei. The integral value of intensity of p12 immunofluorescence represents the expression of this subunit in the nucleus. It is evident from the bivariate distribution analysis (scatter plots) representing p12 expression *versus* cellular DNA content in the p12 wild type cells that the expression of p12 drops dramatically in S phase (Fig. 6*A*). These data show that the overexpressed p12 behaves in a similar manner to endogenous p12 as we had previously observed in studies of the expression of all four subunits of Pol δ by LSC (23).

The scatter plot representing expression of the p12$^{KRR/AAA}$ mutant shows a radical difference compared with WT p12 cells as there is no evidence of any decrease in its expression during S phase (Fig. 6*B*). Indeed, the expression of p12$^{KRR/AAA}$ exhibits a remarkable resemblance to the behavior of the p125, p50, and p68 subunits that we previously assessed by LSC, *viz.* a steady increase in the mean value of expression from G_1 through S to G_2/M, which is consistent with the increase in DNA content and cell size during the cell cycle progression (23).

The scatter plot analysis provides information on the expression levels of p12 in the cell population at various stages of cell cycle progression that allows further quantitative analysis. One useful method is to use the gating analysis to identify cells in G_1, S, and G_2/M (as shown in Fig. 6), estimate the mean p12 expression for cells in each phase, and divide it per mean DNA content of cells in that phase. This approach effectively relates p12 expression with the DNA content, a method we previously used for examination of cell cycle expression of all four (p125, p50, p68, and p12) subunits of Pol δ (23). It is noted that these mean integral values are derived from individual measurements of >3000 cells. This analysis for p12 and the p12$^{KRR/AAA}$ mutant is shown in Table 1, where the p12/DNA ratios have been normalized to the value for G_1. For p12 wild type, the ratio drops to 0.58 during S phase and rises again to 0.9 in G_2/M. The ratios for p12$^{KRR/AAA}$ remain fairly constant at unity, a behavior consistent with a complete immunity from S phase destruction. In our previous studies, we had noted also that the p125, p50, and p68 ratios remained close to unity throughout the cell cycle, although the ratio for p12 dropped to 0.37 (23). The LSC data (Table 1) provide evidence that suggests that CRL4^{Cdt2} provides the primary regulation of p12 levels during the observed decline in S phase. These findings are significant because of the likely redundancy of CUL4A and CUL4B, so that only partial effects of their knockdowns could be observed in our knockdown experiments (Figs. 4 and 5).

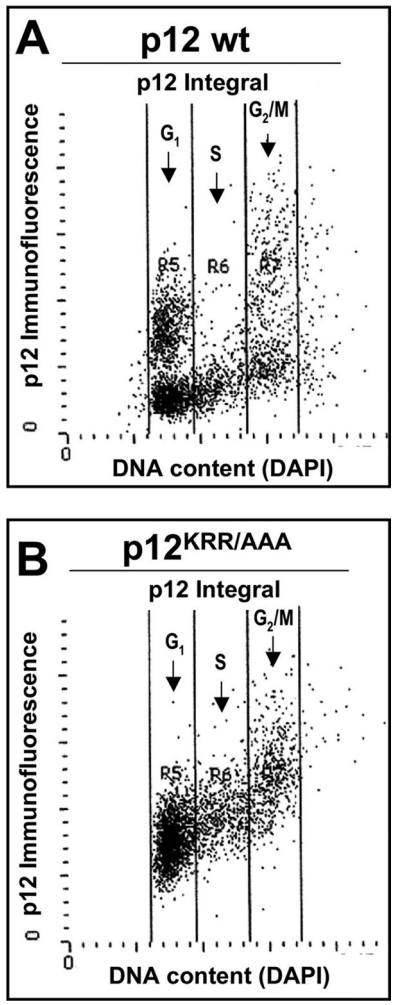

FIGURE 6. **Laser scanning cytometry analysis of the cell cycle distribution of overexpressed His-p12 and the His-p12$^{KRR/AAA}$ mutant.** A549 cells in which His-p12 and the p12$^{KRR/AAA}$ mutant were stably expressed were grown on glass-chambered slides and stained for p12 with anti-p12 antibody and AlexaFluor4-labeled secondary antibody (green fluorescence). DAPI was used to detect DNA (blue fluorescence). Cells were analyzed by laser scanning cytometry (see "Experimental Procedures"). *A,* scatter plot of the mean integral for p12 WT immunofluorescence against DNA (blue fluorescence). *B,* scatter plot of the mean integral for p12$^{KRR/AAA}$ immunofluorescence against DNA (blue fluorescence).

TABLE 1
Levels of p12 WT and p12$^{KRR/AAA}$ in different cell cycle phases

Cell cycle phase	Mean integral values ($\times 10^{-6}$)[a]		p12/DNA (normalized)[b]
	Blue (DAPI)	Green (p12)	
p12 WT			
G_1	8.0	2.9	1.00
S	11.6	2.5	0.58
$G_{2/M}$	15.9	5.2	0.90
p12KRR/AAA			
G_1	11.3	4.6	1.00
S	16.3	5.7	0.85
$G_{2/M}$	22.0	7.5	0.83

[a] Data were determined from LSC data (Fig. 6).
[b] Mean green integral was divided by mean integral values (DAPI) and normalized to the values for G_1 set to 1.00 (23).

DISCUSSION

The p12 subunit of the heterotetrameric form of Pol δ is rapidly degraded in response to UV damage, alkylating agents, and replication stress under the control of the ATR/Chk1 signaling kinases (2, 22). The significance of the degradation of p12 is that the process converts Pol δ4 to Pol δ3, the trimer lacking p12, which displays properties that are consistent with its hav-

ing a role in DNA repair processes (2). In this study we provide evidence that the CRL4^{Cdt2} ubiquitin ligase participates in the DNA damage-induced targeting of p12 for degradation. Our evidence includes the identification of a PIP-degron in p12 that is common to Cdt1, Set8, and p21, the other three known vertebrate substrates of CRL4^{Cdt2}, and the blunting of the degradation of p12 by depletion of CUL4A, CUL4B, DDB1, or Cdt2. CRL4^{Cdt2} is well established to target Cdt1, Set8, and p21 for degradation in response to DNA damage (32, 33). Thus, our studies now expand the role of CRL4^{Cdt2} to the control of p12 degradation.

The degradation of the licensing factors Cdt1 and Set8 in response to DNA damage by CRL4^{Cdt2} provides an integrated control that regulates entry into S phase (32, 33). p21 may have diverse activities besides inhibition of cyclin/cyclin-dependent kinases, because of its very high affinity for PCNA compared with most PCNA-binding proteins that possess a simpler PIP-box (63), and thus may have the inherent ability to inhibit the recruitment of other PCNA-binding proteins, which may include DNA polymerases such as Pol η (43, 64) and Pol δ (44, 65–67) as well as RFC (67).

The inclusion of p12 as a target of CRL4^{Cdt2} further expands our understanding of how signaling of the degradation of p12 is integrated into the network of DNA damage response pathways. We have recently identified RNF8 as an E3 ubiquitin ligase that participates in DNA damage-induced degradation of p12 (26). RNF8 is recruited to sites of DNA damage in all phases of the cell cycle, together with Pol δ. Moreover, RNF8 is able to mono-ubiquitinate PCNA, indicating that it may promote translesion synthesis at replication forks stalled by UV damage in S phase (30). Interestingly, CRL4^{Cdt2} has also been implicated as having a role in regulating translesion synthesis, because it mono-ubiquitinates PCNA (68). In addition, Pol η is a target of CRL4^{Cdt2} in *Caenorhabditis elegans* (69). Thus, it is now evident that there are multiple E3 ligase pathways for the targeting of p12 degradation. Such redundancy also is present in the targeting of Cdt1, p21, and Set8 by the SCF^{Skp2} ubiquitin ligase (33).

In this study, we provide strong evidence that p12 levels are reduced during the S phase under the control of CRL4^{Cdt2} during normal cell cycle progression (Figs. 4 and 5). The LSC analysis of the cell cycle distribution of WT p12 and the p12$^{KRR/AAA}$ mutant (Fig. 6 and Table 1) suggests that CRL4^{Cdt2} is the primary regulator of p12 during cell cycle progression. The data also show that a complete depletion of p12 during the S phase does not take place. Because the depletion of p12 is coupled with the conversion of Pol δ4 to Pol δ3, this means that during S phase we can predict that both forms of Pol δ are present, a situation that is different from that in UV damage, where p12 depletion is nearly complete. These are important new findings that greatly impact our understanding of the cellular functions of Pol δ3, which previously has been focused on its role in DNA repair processes (2). The generation of Pol δ3 under the control of CRL4^{Cdt2} argues that it serves some significant function in S phase, specifically in DNA replication. In this regard, our findings raise important new questions regarding the roles of Pol δ3 and Pol δ4 in DNA replication. Pol δ3 has altered properties that could be crucial in the context of DNA replication, and in fact it has an intrinsically greater fidelity than Pol δ4 (2, 25). Several hypotheses can be advanced for potential roles of both Pol δ3 and Pol δ4 in higher eukaryotic DNA replication. The first is that Pol δ3 may be more adapted for Okazaki fragment processing, although Pol δ4 is more adapted to participate in leading strand synthesis; in this regard, the increased stalling that is exhibited by Pol δ3 suggests that it has more limited strand displacement abilities (24), one of the properties advanced for the adaptation of yeast Pol δ to Okazaki fragment processing (1, 70). As noted above (see Introduction), there is a strong precedent in the yeast system that there may be a division of labor between the replicative DNA polymerases in the synthesis of the lagging and leading strands at the replication fork (6, 71). We have recently examined the abilities of Pol δ3 and Pol δ4 to perform Okazaki fragment processing in a reconstituted human system, and we observed significant differences that argue for a role of Pol δ3 in Okazaki fragment processing.[3]

Our discovery that p12 is a substrate for CRL4^{Cdt2} does not allow a simple insertion of p12 into current models for the recruitment of CRL4^{Cdt2} to its PCNA-bound substrates, because p12 is a subunit of Pol δ and not a free protein. The model that we propose for recruitment envisages Pol δ4 as the substrate as shown in Fig. 7, and it is consistent with the wealth of intensive studies of components of the DNA replication and repair systems (1). The first step in the recruitment of PCNA to chromatin, in both the initiation of DNA replication (Fig. 7*A*) or the gap-filling step in DNA repair (Fig. 7*B*), is the creation of a primer with a free 3′ end ("DNA/primer"). In the early stages of initiation of DNA synthesis, this occurs by synthesis of the RNA/DNA primer by Pol α/primase and by the excision step during NER. One of the key observations in studies of the *Xenopus* egg system is that the degradation of Cdt1 (and PIP-degron substrates) is replication- and DNA damage-dependent and requires the presence of PCNA loaded onto DNA (PCNA^{chromatin}) (32, 51). Thus, we would characterize the bound PCNA as PCNA$^{DNA/primer}$ in the context of both DNA replication and DNA repair.

Once this primer is formed, PCNA is loaded onto the primer terminus. In the context of Pol δ and its role in DNA replication as well as DNA repair, the loading of PCNA requires a clamp loader, RFC. Structural studies of RFC have shown that it loads PCNA in a spatially oriented manner with regard to the direction of elongation of the primer (72–74). During initiation of DNA replication, loading of PCNA onto the primer synthesized by Pol α/primase is an essential step that allows loading of Pol δ or Pol ε. Similarly, within the context of DNA repair, PCNA loading takes place on a primer terminus generated by the excision step of NER. The loading of PCNA by RFC is illustrated with the loaded PCNA designated as PCNA$^{DNA/primer}$ to take into account the previous considerations (Fig. 7*C*, *1*). Next, Pol δ4 is recruited to the primer terminus (Fig. 7*C*, *2*). Current views on the recruitment of Pol δ subsequent to RFC loading of PCNA on the primer terminus favors a hand-off process,

[3] Lin, S. H. S., Wang, X., Zhang, S., Zhang, Z., Lee, E. Y. C., and Lee, M. Y. W. T. (2013) Dynamics of enzymatic interactions during short flap human Okazaki fragment processing by two forms of human DNA polymerase δ. *DNA Repair* http://dx.doi.org/10.1016/j.dnarep.2013.08.008.

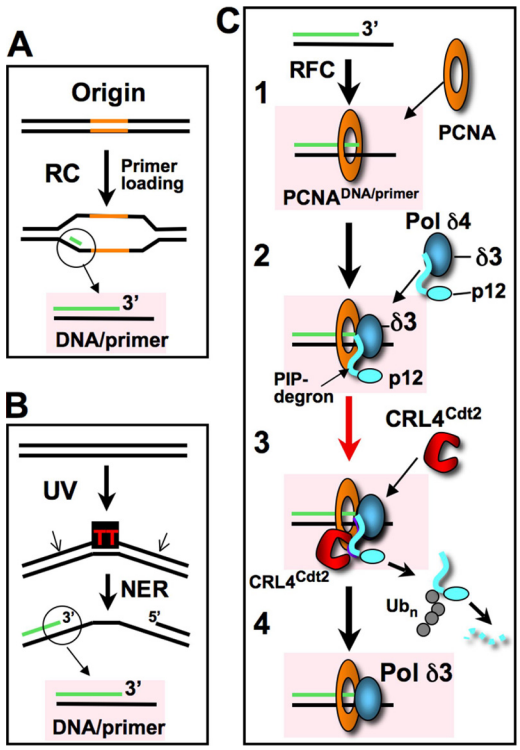

FIGURE 7. **Model for the degradation of p12 by CRL4^{Cdt2} during DNA replication and DNA repair.** *A,* this diagram shows a representation of the replication origin (*orange*) where a primer is synthesized on at the replication complex (*RC*). This generates a 3′ end primer (shown as DNA/primer) that serves for the point of elongation by the replication complex. *B,* this diagram shows the formation of a UV lesion, a TT dimer, on the double-stranded DNA as a consequence of UV damage. During NER, the lesion and a section of surrounding DNA is excised (*arrows*), leaving in place a 3′-end primer (DNA/primer, *green*) and a single-stranded DNA gap. *C,* common steps of recruitment of PCNA and Pol δ4 to initiate extension of the primer end are shown first. *1,* PCNA is loaded onto the primer terminus by RFC. *2,* Pol δ4 is recruited to PCNA. Here, the three subunits of Pol δ that include Pol δ3 is shown as the *blue ovoid*, and p12 is shown in *aqua*, with its N-terminal containing the PIP-degron as a *solid line*. *3,* CRL4^{Cdt2} (shown as the *red C-shape*) is then recruited to Pol δ4 bound to PNCA$^{DNA/primer}$. *4,* p12 is polyubiquitinated and detaches to be degraded by proteasomes, leaving Pol δ3 bound to PCNA and the DNA primer terminus.

because dissociation of RFC would allow PCNA to slide away from the primer terminus. The interaction of Pol δ with the primer terminus greatly enhances its affinity for PCNA, and human Pol δ binds to its primer/template in the presence of PCNA with a K_D of ~35 nM (25). Thus, we propose that Pol δ4 is the substrate for CRL4^{Cdt2}, rather than free p12. (There is no current evidence that p12 is freely dissociable from Pol δ4, and it is noted that the association of p12 with Pol δ4 occurs via interaction with both the p125 and p50 subunits. Moreover, in addition to the interaction of p12 as a high affinity binding partner for PCNA, Pol δ has multiple binding interactions with PCNA, via the p125 and p68 subunits (2).) The recruitment of CRL4^{Cdt2} to Pol δ4/PCNA$^{DNA/primer}$ (Fig. 7*C, 3*) then leads to the ubiquitination of p12, following which it is directed toward proteasomal degradation (Fig. 7*C, 4*), leaving Pol δ3 *in situ* on the DNA where it is poised to engage in elongation of the primer.

Although our model is similar to that proposed for free PIP-degron-containing ligands, it presents some new insights, in that we can define the nature of the DNA (a template/primer) that is the site of location of the p12 bound to PCNA and DNA. Studies of PCNA loading onto singly primed M13 DNA templates have shown that a single PCNA molecule is loaded and that RFC is retained after Pol δ is recruited and DNA synthesis is initiated; multiple loading of PCNA is only observed after extended DNA synthesis (75). In our present state of information, it would appear that CRL4^{Cdt2} bound to its PIP-degron-containing ligand is free to slide away from the primer terminus. Whether an as yet unknown mechanism allows the PCNA-degron substrates to remain associated with the primer terminus is also a question of interest. Another issue is the trivalent nature of PCNA, so that in principle three PIP-degrons could be bound on PCNA. Multivalent interactions of PCNA have been demonstrated by surface plasmon resonance measurements where 2.3 molecules of p21 were found per PCNA trimer (65–67). How the hierarchy for the temporal sequence of the degradation of the various CRL4^{Cdt2} substrates is established and whether this is a stochastic process is unknown.

Another question with our model is whether CRL4^{Cdt2} has access to the PIP-degron of p12 when Pol δ4 is bound to PCNA$^{DNA/primer}$. The PIP-degron of p12 is located at its N terminus and is able to interact with PCNA, although it is associated with the Pol δ4 enzyme (4). In addition, there may be conformational flexibility of Pol δ bound to PCNA, a property that has been proposed in models for Okazaki fragment processing where PCNA simultaneously hosts DNA polymerase, Fen1, and DNA ligase I (76) based on its trivalent nature. Flexible conformations for DNA polymerases residing on sliding clamps are also invoked in models for switching of DNA polymerases bound to sliding clamps (77–79). Thus, the model we propose (Fig. 7) is not incongruent with current models for CRL4^{Cdt2} recruitment to PCNA^{chromatin} (32, 33), with the added hypothesis that Pol δ/PCNA$^{DNA/primer}$ might function as a platform for recruitment of PIP-degron-containing ligands and CRL4^{Cdt2}.

In summary, we have identified a novel function of CRL4^{Cdt2} as a regulator of p12 degradation in the DNA damage response and during the S phase of cell cycle progression. The discovery that p12 is degraded during the S phase under the control of CRL4^{Cdt2} is highly significant and expands the cellular role of CRL4^{Cdt2} to encompass regulation of DNA replication at the level of Pol δ. Moreover our findings provide the first evidence that Pol δ3 is formed during the S phase and may therefore be a participant in DNA replication. These studies point to important avenues for future studies that may further our understanding of the significance and molecular and cellular roles of Pol δ3.

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