

# Degradation of p12 Subunit by CRL4<sup>Cdt2</sup> E3 Ligase Inhibits Fork Progression after DNA Damage<sup>\*[5]</sup>

Received for publication, July 25, 2013, and in revised form, August 27, 2013  
Published, JBC Papers in Press, September 10, 2013, DOI 10.1074/jbc.C113.505586

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**Background:** The mechanism for inhibiting fork progression after DNA damage still remains.

**Results:** CRL4<sup>Cdt2</sup> promotes the degradation of the p12. Cells expressing a stable form of p12 exhibit UV-resistant DNA synthesis and decreased inhibition of fork progression.

**Conclusion:** p12 degradation by CRL4<sup>Cdt2</sup> is critical for inhibiting fork progression.

**Significance:** p12 degradation is one mechanism by which DNA damage in S-phase cells inhibits fork progression.

After acute DNA damage, the cell arrests S-phase progression by inhibiting origin initiation and fork progression to repair damaged DNA. The intra-S-phase checkpoint kinase Chk1 phosphorylates Cdc25A to target the latter for degradation by CRL1<sup>β-TrCP</sup> and so inhibit origin firing. The mechanism for inhibiting fork progression, however, has not been identified. Here, we show that degradation of p12, the fourth subunit of DNA polymerase δ, is critical for inhibiting fork progression. CRL4<sup>Cdt2</sup> is an E3 ligase that ubiquitinates and degrades p12 after UV treatment. Cells expressing a stable form of p12 exhibit UV-resistant DNA synthesis. DNA fiber assay and alkaline-sucrose gradient assay demonstrate that the impairment of fork progression after DNA damage requires p12 degradation. These results suggest that ubiquitination of p12 through CRL4<sup>Cdt2</sup> and subsequent degradation form one mechanism by which a cell responds to DNA damage to inhibit fork progression.

After acute DNA damage, the intra-S-phase checkpoint arrests S-phase progression to repair damaged DNA and rescue DNA replication so that cells can later enter mitosis without any DNA sequence changes (1, 2). DNA damage during S-phase delays or arrests DNA replication by inhibiting origin initiation and fork progression. Cdc25A, a phosphatase for activating cyclin-dependent kinase 2, is targeted for degradation by CRL1<sup>β-TrCP</sup> to inhibit origin firing (3–5). The mechanism for

inhibiting fork progression, however, has not been identified (6–8). p12, the smallest subunit of polymerase δ, is important for effective DNA replication and cell proliferation (9, 10). Furthermore, p12 is degraded immediately after DNA damage through polyubiquitination (11) by an unknown E3 ligase. Here, we show that p12 degradation is critical for inhibiting fork progression during the intra-S-phase checkpoint.

CRL4<sup>Cdt2</sup> is involved in the polyubiquitination of Cdt1 (12–15), p21 (16–18), and Set8 (19–22, 31) after DNA damage. All these substrates interact with PCNA<sup>2</sup> through a specialized PCNA-interacting motif (PIP degron) and are then recognized by CRL4<sup>Cdt2</sup> for polyubiquitination. Here we show that p12 has a similar PIP degron and that CRL4<sup>Cdt2</sup> ubiquitinates and degrades p12 after UV treatment. p12 degradation is critical for inhibiting fork progression.

## EXPERIMENTAL PROCEDURES

**Cell Lines and Reagents**—HCT116 and HCT116<sup>p53<sup>-/-</sup></sup> cells have been described (23). HeLa, 293T, and WI38 cells were purchased from ATCC. p12 cDNA was purchased from Invitrogen, and mutations were induced by PCR. Stable cell lines expressing p12 mutants were generated by MSCVpuro vector (Clontech). Cells stably expressing p12 proteins were selected in 1 μg/ml puromycin (Sigma). Antibodies directed against the indicated proteins were purchased from the following sources: PCNA (PC10) and Cul4 (C-19) (Santa Cruz Biotechnologies); phospho-Chk1, Chk2, phospho-Chk2, histone-H2AX, and phospho-histone-H2AX, β-TrCP (Cell Signaling Technology); p125 (BD Biosciences); Chk1 (Novus Biologicals); and DDB1 (Invitrogen). The antibody against p12 was kindly provided by Igor Stagljar (University of Toronto) (24). siRNA transfections into cell lines were performed using Oligofectamine (Invitrogen). The following siRNA oligonucleotides were purchased from Invitrogen: β-TrCP (1+2), 5'-ACAGGAUCAUCGGAU-UCCA-3' and 5'-UCAGUGCAGUUAAGUCGUA-3'; β-TrCP (1/2), 5'-GAUGUCCACAAAUCCACUUTT-3'. Other siRNA oligonucleotides were described before (21).

**In Vitro Ubiquitination Assays**—The purification of CRL4<sup>Cdt2</sup> complex was described previously (21). Briefly, Cul4A-Myc, DDB1-Myc, FLAG-Roc1, and FLAG-Cdt2 were transiently overexpressed in 293T cells and purified by immunoprecipitation with anti-Myc antibody. Purified complexes were mixed with GST-tagged wild type or a T8D mutant of p12 in ubiquitination reaction buffer. The ubiquitination reactions were incubated at 37 °C for 1 h and terminated by adding Laemmli sample buffer. Proteins were resolved by SDS-PAGE and immunoblotted with anti-GST antibody (Sigma).

**Measurement of UV-resistant DNA Synthesis after UV Irradiation**—Experiments were performed as described previously (4). Briefly, 293T cells were treated with UVC light (254 nm), as measured by a UVX digital radiometer (UVP, Inc., San

\* This work was supported, in whole or in part, by National Institutes of Health Grants CA60499 and GM84465 (to A. D.) and CA140774 (to T. A.). This work was also supported by Department of Defense Breast Cancer Research Program and Uehara Memorial Foundation (to K. T.).

[5] This article contains supplemental Figs. 1–4.

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<sup>2</sup> The abbreviations used are: PCNA, proliferating cell nuclear antigen; TrCP, transducin repeat-containing protein; CldU, 5-chloro-2'-deoxyuridine; IdU, 5-iodo-2'-deoxyuridine; PIP, PCNA-interacting proteins; BLM, Bloom syndrome.

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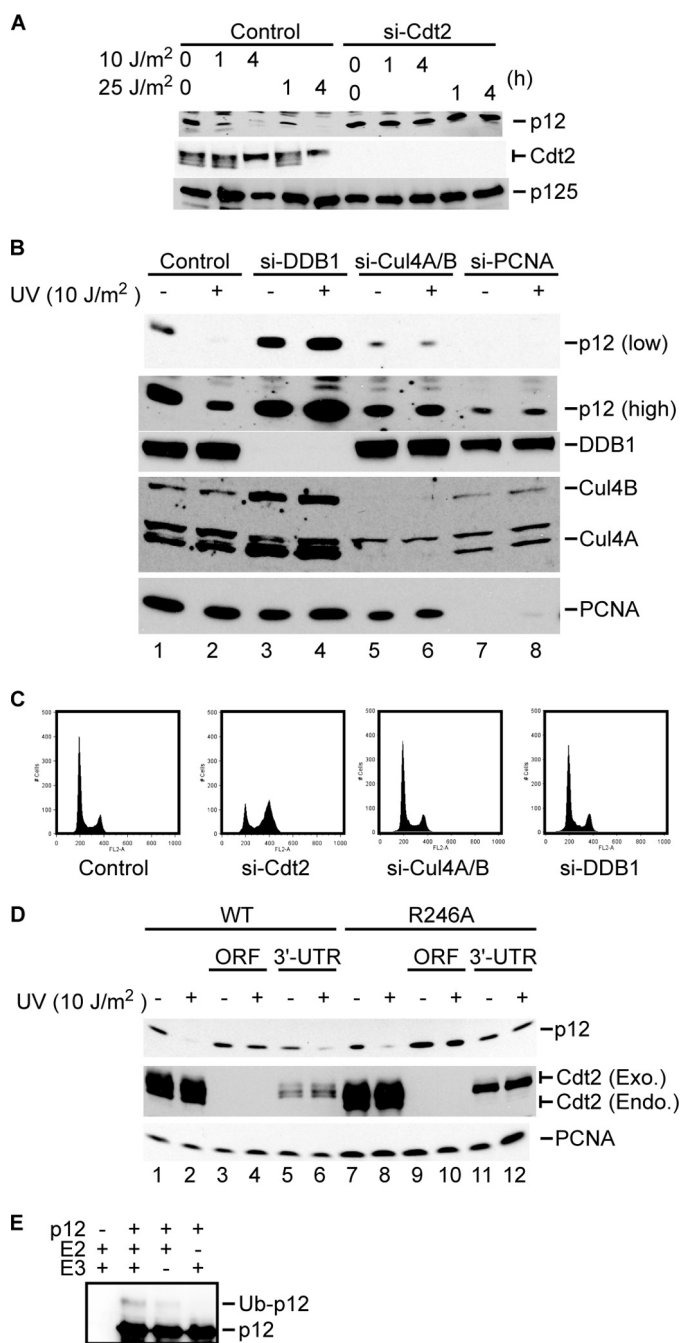
Gabriel, CA) and labeled with [<sup>3</sup>H]thymidine for 15 min before harvesting (1 μCi/ml; PerkinElmer Life Sciences). After removing medium, the cells were fixed by the addition of 5% trichloroacetic acid and washed three times with 5% trichloroacetic acid. DNA was eluted in 1% SDS and 10 mM NaOH, spotted into paper for drying, and transferred to a scintillation vial. After the addition of scintillation fluid, incorporation of [<sup>3</sup>H]thymidine was measured by scintillation counting. For normalization, cells were pre-labeled with [<sup>14</sup>C]thymidine for 24 h before the radiation, and DNA synthesis was measured as the <sup>3</sup>H counts normalized to the <sup>14</sup>C counts.

**Alkaline Sucrose Gradient Centrifugation**—Cells were irradiated with UV, labeled with [<sup>3</sup>H]thymidine (5 μCi/ml), and lysed in lysis buffer (0.5 M NaOH, 0.02 M EDTA, pH 12.5, 0.1% Nonidet P-40). Lysates were layered on top of a linear sucrose gradient (5–15% sucrose in 0.1 M NaOH, 0.9 M NaCl, 0.01 M EDTA, pH 12.5) and subsequently centrifuged at 26,000 rpm for 2 h at 20 °C. After collecting each fraction, DNAs were precipitated on glass microfiber filters, and radioactivity measured by scintillation counting.

**DNA Fiber Assay**—Cells were incubated with 50 μM CldU for 20 min. After irradiation of UV, cells were immediately incubated with 200 μM IdU for 30 min. Labeled cells were lysed (0.5% SDS, 50 mM EDTA, 200 mM Tris-HCl, pH 7.4) on glass slides and spread for DNA isolation. DNA fiber was fixed with methanol-acetic acid (3:1) and denatured with 1.5 M HCl for 30 min. For immunofluorescence, denatured DNA was stained for CldU with rat anti-BrdU monoclonal antibody (AbD Serotec, 1:200) and for IdU with mouse anti-BrdU antibody (BD Biosciences, 1:200) for 4 h at room temperature followed by an Alexa Fluor 594-conjugated anti-rat IgG (Molecular Probes) (red) and an Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) (green) at 1:200 dilution for 1 h at room temperature. Lengths of green tracks were determined by ImageJ software.

## RESULTS AND DISCUSSION

Because p12 has a PIP box similar to the PIP degron identified in other CRL4<sup>Cdt2</sup> substrates, we tested whether CRL4<sup>Cdt2</sup> is responsible for p12 degradation. We depleted Cdt2 by using siRNA and measured p12 levels after UV treatment. As expected, UV treatment reduced the level of p12 in HeLa cells (Fig. 1A). Depletion of Cdt2 increased the basal level of p12 and prevented the degradation of p12 following DNA damage. CRL4<sup>Cdt2</sup> ligase complex is also composed of DDB1 and Cul4A or Cul4B. Consistent with our hypothesis, depletion of DDB1 or Cul4A and Cul4B also abrogated p12 degradation after UV treatment (Fig. 1B). The decrease of p12 after knockdown of PCNA suggests that PCNA is required for p12 stabilization in normal cells. Although PCNA depletion decreased the basal level of p12, degradation of the residual p12 in response to DNA damage was abrogated, suggesting that the PCNA is also important for the degradation of p12 after DNA damage. Therefore, p12 degradation after UV irradiation requires components of CRL4<sup>Cdt2</sup> and PCNA, much like the other substrates of this E3, such as Cdt1, p21, and Set8. si-Cdt2 caused accumulation of the cells in G<sub>2</sub> as reported (15), but the depletion of Cul4A/B or DDB1 did not alter cell cycle profile (Fig. 1C), suggesting that the stabilization of p12 after depletion of CRL4



**FIGURE 1. CRL4<sup>Cdt2</sup> depletion abrogates the degradation of p12 by UV irradiation.** A and B, HeLa cells were transfected with the indicated siRNA and analyzed by immunoblotting with anti-p12, anti-Cdt2, anti-p125, anti-DDB1, anti-Cul4A/B, or si-DDB1. C, HeLa cells were transfected with si-Cdt2, si-Cul4A/B, or si-DDB1. After 48 h, cell cycle was analyzed by propidium iodide staining followed by flow cytometry. D, HeLa cells stably expressing HA-tagged Cdt2 or Cdt2-R246A were transfected with si-Cdt2 targeting the ORF (ORF) or si-Cdt2 targeting the 3'-UTR (3'-UTR). Cells were irradiated with UV and lysed 1 h after irradiation. Protein lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-PCNA, anti-Cdt2, or anti-p12 antibody. *Exo.*, exogenous; *Endo.*, endogenous. E, immunopurified CRL4<sup>Cdt2</sup> complexes (E3) were mixed with p12<sup>WT</sup>, E1, and UbcH5 (E2). Ubiquitinated p12 (Ub-p12) was detected by Western blotting after separating the reaction products by SDS-PAGE.

components or PCNA is not an indirect effect of cell cycle change.

To rule out the possibility that the stabilization of p12 by si-Cdt2 was due to an off-target effect of the siRNA, we tested



whether siRNA-resistant exogenous Cdt2 can restore p12 degradation in the absence of endogenous Cdt2 (Fig. 1D). Cdt2 is the substrate recognition adaptor of CRL4<sup>Cdt2</sup> and itself interacts with the DDB1 subunit of the E3 in a manner that is dependent on Arg-246 of Cdt2 (12). HeLa cells stably expressing either wild type HA-Cdt2 or HA-Cdt2-R246A, which is deficient in binding to DDB1, were transfected with two different siRNAs, one against the open reading frame (ORF) and the other against the 3'-untranslated region (3'-UTR) of Cdt2. Because both wild type Cdt2 and Cdt2-R246A are expressed from cDNAs without the Cdt2 3'-UTR, si-Cdt2 targeting the ORF depleted both endogenous and exogenous Cdt2, whereas si-Cdt2 targeting the 3'-UTR of Cdt2 depleted only the endogenous Cdt2 (Fig. 1D). Depletion of both endogenous and exogenous Cdt2 by the ORF si-Cdt2 stabilized p12, whereas depletion of endogenous Cdt2 by 3'-UTR si-Cdt2 and sparing of exogenous wild type Cdt2 allowed p12 degradation after DNA damage (lanes 5 and 6). On the other hand, the mutant Cdt2-R246A could not restore p12 degradation in cells transfected with si-Cdt2 targeting the 3'-UTR (lanes 11 and 12). These results demonstrate that knockdown of Cdt2 specifically abrogates p12 degradation and that Cdt2 has to bind to DDB1 for p12 degradation. On the other hand, depletion of  $\beta$ -TrCP, the E3 ligase that targets Cdc25A, did not affect p12 degradation after UV treatment (supplemental Fig. 1).

To address whether CRL4<sup>Cdt2</sup> ubiquitinates p12 directly, we tested its ability to ubiquitinate p12 *in vitro*. GST-tagged p12 was expressed in 293T cells and purified as substrate (with associated PCNA). CRL4<sup>Cdt2</sup> E3 ligase complex was obtained as reported previously (18, 21). The ubiquitination reaction was performed in the presence of E1, UbcH5c as E2, and ATP. After incubation of p12-GST and CRL4<sup>Cdt2</sup>, only the monoubiquitinated form of p12 appeared (Fig. 1E). One possibility is that the *in vitro* reaction is inefficient at polyubiquitinating p12, although CRL4<sup>Cdt2</sup> polyubiquitinates p12 *in vivo*, possibly because we are using the wrong E2 in the reaction as compared with what may be used *in vivo*. Alternatively, the monoubiquitin added by CRL4<sup>Cdt2</sup> may be extended *in vivo* by an E4 type E3 ligase different from CRL4<sup>Cdt2</sup>. Finally, it has recently been shown that small proteins, less than 150 amino acids, can be degraded by monoubiquitination (25) so that it is possible that p12 is also degraded *in vivo* directly after monoubiquitination by CRL4<sup>Cdt2</sup>.

p12 contains an atypical PIP box (where Gln and Phe are replaced by Lys and Ser, Fig. 2A). However, GST-tagged p12<sup>WT</sup> expressed in 293T cells co-precipitated endogenous PCNA when pulled down on glutathione-Sepharose beads (Fig. 2B and supplemental Fig. 2A). Mutation of the PIP box in p12 (p12 <sup>$\Delta$ PIP</sup>) totally abrogated its binding to PCNA (lane 10), confirming that this is the interaction site.

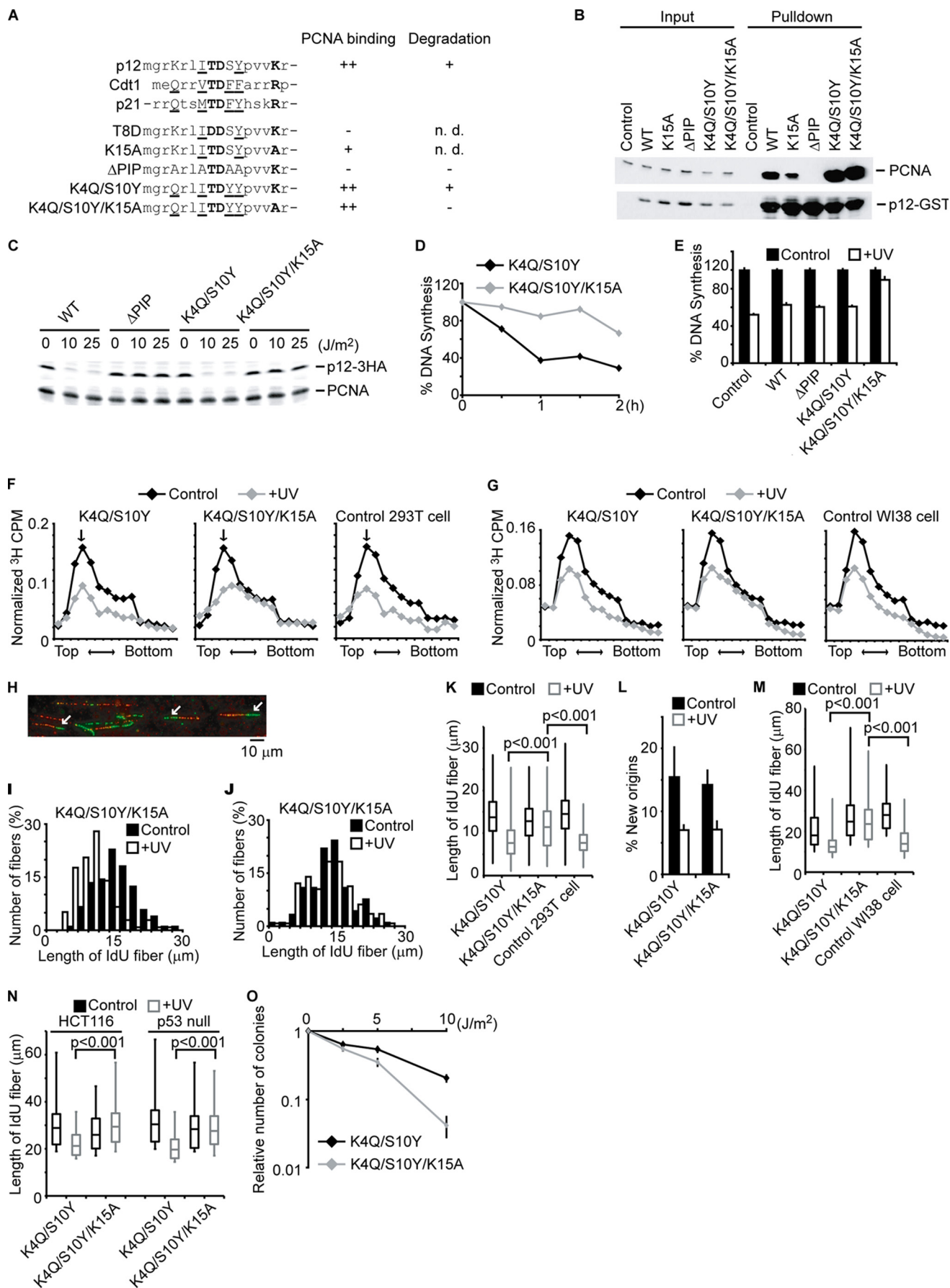
p12 binding to PCNA is reported to be essential for efficient activity of polymerase  $\delta$  (9, 10). Therefore, to address the physiological role of p12 degradation, we required a mutant of p12 that still interacts with PCNA but is not degraded after radiation. Substrates of CRL4<sup>Cdt2</sup>, such as Cdt1, p21, and Set8, contain a threonine and a lysine in the context of the PIP box that are important for CRL4<sup>Cdt2</sup>-dependent degradation (15, 26) but not as important for PCNA binding, and the corresponding

residues are present in p12 (Thr-8 and Lys-15) (Fig. 2A). However, mutation of Thr-8 (p12<sup>T8D</sup>) or Lys-15 (p12<sup>K15A</sup>) in p12 disrupted or decreased the interaction with PCNA (supplemental Fig. 2A), suggesting that both of these residues are important for efficient binding to PCNA in the context of the atypical PIP box of p12. We therefore substituted the Lys-4 and Ser-10 of p12 (p12<sup>K4Q/S10Y</sup>) to generate a canonical PIP box sequence (Fig. 2A). p12<sup>K4Q/S10Y</sup> still binds to PCNA at the equivalent level as p12<sup>WT</sup> (Fig. 2B). In the K4Q/S10Y context, additional mutation of Lys-15 (p12<sup>K4Q/S10Y/K15A</sup>) did not affect the affinity of p12 for PCNA. To test the stability of these mutants in response to UV, we stably expressed HA-tagged versions of these mutants in 293T cells by retroviral transduction and checked the protein levels before and after UV treatment. As with the PIP mutants of other substrates of CRL4<sup>Cdt2</sup> ligase such as Cdt1 and p21, p12 <sup>$\Delta$ PIP</sup> was not degraded after UV treatment (Fig. 2C), suggesting that binding of p12 to PCNA is required for the degradation. p12<sup>K4Q/S10Y</sup> mutant was degraded similarly as the p12<sup>WT</sup>. On the other hand, this degradation was not seen in p12<sup>K4Q/S10Y/K15A</sup>, although this protein retained its ability to bind PCNA (Fig. 2B). Therefore, this mutant would allow us to test the role of DNA damage-dependent degradation of p12 without abolishing its binding to PCNA (see below).

We next confirmed that cell cycle progression and checkpoint kinase activation were normal in cells expressing stable p12 (supplemental Fig. 2, B and C). There was no difference in cell cycle profile or phosphorylation of Chk1, Chk2, and histone H2AX after UV irradiation between cells expressing the two forms of p12. These results imply that comparison between p12<sup>K4Q/S10Y</sup> and p12<sup>K4Q/S10Y/K15A</sup> will be useful for investigating whether p12 degradation is important for the response of the cell when it is damaged in S-phase.

Because p12 is required for effective DNA synthesis and is degraded in response to DNA damage, we next tested whether p12 degradation contributes to the intra-S-phase replication arrest following DNA damage. Increasing doses of UV decreased DNA synthesis in 293T (supplemental Fig. 3A). We measured UV-resistant DNA synthesis in cells expressing UV-labile or UV-stable mutant of p12. 293T cells stably expressing p12<sup>K4Q/S10Y</sup> or p12<sup>K4Q/S10Y/K15A</sup> mutants were irradiated with UV and labeled with [<sup>3</sup>H]thymidine to label newly synthesized DNA (Fig. 2D). Cells expressing the stable p12<sup>K4Q/S10Y/K15A</sup> showed higher UV-resistant DNA synthesis after irradiation than cells expressing degradable p12<sup>K4Q/S10Y</sup> (Fig. 2, D and E), suggesting that p12 degradation is required for inhibition of DNA synthesis after DNA damage. Cells treated with ionizing radiation also showed similar results as cells treated with UV irradiation (supplemental Fig. 3B). Notably, p12 <sup>$\Delta$ PIP</sup>, which is as stable as p12<sup>K4Q/S10Y/K15A</sup>, did not increase UV-resistant DNA synthesis (Fig. 2E). These results demonstrated that both the stabilization of p12 and the binding of the stable p12 to PCNA are required to rescue DNA synthesis when a cell responds to DNA damage. Similar results were obtained with WI38, a normal human fibroblast cell line (supplemental Fig. 3, D–F), and in p53 mutant H1299 lung cancer cells (supplemental Fig. 3G), suggesting that stable p12 restores DNA synthesis in damaged cells independent of cell transformation or of p53 status.

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DNA damage has two immediate effects on DNA replication: decreasing the rate of fork progression and inhibiting new origin initiation. To investigate whether the degradation of p12 after radiation inhibits fork progression or initiation, we measured the size of newly synthesized DNA by separating  $^3\text{H}$ -labeled nascent single-stranded DNA using alkaline-sucrose gradient. 293T (Fig. 2F) or WI38 (Fig. 2G) cells expressing either p12<sup>K4Q/S10Y</sup> or p12<sup>K4Q/S10Y/K15A</sup> were irradiated with UV followed by 30 min of [ $^3\text{H}$ ]thymidine labeling. Newly labeled nascent DNA strands were fractionated by alkaline-sucrose gradient according to their size. Without UV treatment, the size distribution of newly synthesized DNA was similar between cells expressing p12<sup>K4Q/S10Y</sup> and p12<sup>K4Q/S10Y/K15A</sup>. The shorter nascent DNA near the top of the gradient arises from newly fired origins both in cells already in S-phase and in cells newly entering S-phase from G<sub>1</sub>. The longer nascent DNA nearer the bottom of the gradient is a measure of progression of already existing forks. UV irradiation of p12<sup>K4Q/S10Y</sup>-expressing cells decreases short strands due to inhibition of origin firing (including inhibition of S-phase entry) and decreases long strands due to inhibition of fork progression. On the other hand, in p12<sup>K4Q/S10Y/K15A</sup>-expressing cells irradiated with UV, the longer nascent DNA produced by fork progression was selectively spared. This result suggests that p12 degradation following radiation is responsible for the decrease in fork progression following UV damage. Similar results were obtained in 293T cells and WI38 cells (Fig. 2, F and G).

Although much is known about how DNA damage blocks firing of origins, not much is known about how fork progression is slowed by DNA damage. Because the nascent strand sizing experiments suggested the exciting possibility that p12 degradation contributes to this slowing, we decided to test this hypothesis by an independent assay. Therefore, the fork progression rate was measured by DNA fiber assay in 293T cells expressing CRL4<sup>Cdt2</sup>-insensitive p12 mutant protein. Cells were first labeled with CldU for 20 min, irradiated with UV, and then labeled with IdU for another 20 min. Genomic DNA was extracted from the cells, and single DNA molecules were stretched and denatured for immunodetection of CldU (*red*) and IdU (*green*) (Fig. 2H). Progression of existing forks was identified by a green IdU track extending a red CldU track. The rate of fork progression was measured by the lengths of these

green IdU tracks (Fig. 2, I–K). The reduction of fork rate progression in response to UV treatment was smaller in cells expressing the stable p12<sup>K4Q/S10Y/K15A</sup> as compared with cells expressing the degradable p12<sup>K4Q/S10Y</sup>. Interestingly, new origin firing was still suppressed in cells expressing p12 (Fig. 2L), demonstrating the clear separation of the firing suppression and elongation suppression by DNA damage. WI38 fibroblast, HCT116 wild type, and HCT116 p53 null cells showed similar results (Fig. 2, M and N), supporting our model that p12 degradation by CRL4<sup>Cdt2</sup> is essential for suppressing fork progression in response to DNA damage, and this is independent of cell transformation or p53 status.

It has been shown that disturbing the response of the cell to DNA damage, specifically allowing continued DNA replication immediately after DNA damage, sensitizes cells to DNA damage (1, 2). We therefore tested the sensitivity of the stable p12-expressing cells to UV irradiation by colony formation assay. Cells expressing stable p12<sup>K4Q/S10Y/K15A</sup> were significantly more sensitive than the cells expressing p12<sup>K4Q/S10Y</sup> (Fig. 2O). Collectively, these results demonstrate that p12 degradation is required for cell survival after DNA damage.

It is already known that inhibition of fork progression is dependent on Chk1 activity (7, 8, 27) and that p12 degradation is inhibited by caffeine (7), suggesting that p12 degradation is one of the pathways by which the checkpoint inhibits fork progression. However, it is entirely possible that the checkpoint kinases activate additional pathways by which to inhibit fork progression that are independent of p12. An interesting possibility is that the checkpoint pathways inactivate or degrade other components of the replication fork to inhibit fork progression.

Recently, RNF8 was reported as an E3 ubiquitin ligase for p12 (28). Under our conditions, however, siRNA-mediated knockdown of RNF8 did not stabilize p12 after UV irradiation (supplemental Fig. 4). We suspect that differences in cell lines, cellular growth conditions, extent of RNF8 knockdown, or radiation conditions account for this difference.

How does p12 degradation lead to inhibition of fork progression? In an *in vitro* system, the lack of p12 in the polymerase  $\delta$  complex decreases polymerase activity (10, 29) so that fork progression may be retarded simply from failure of the elongating polymerase in the absence of p12. Activity of BLM, a RecQ

**FIGURE 2. The degradation of p12 inhibits fork progression during S-phase and is required for cell survival after DNA damage.** A, alignment of PIP degrons in p12, Cdt1, and p21. *Underlined residues* constitute a canonical PIP box. *Bold letters* are highly conserved residues that predict PIP degrons, promoting ubiquitination by CRL4<sup>Cdt2</sup>. A summary of the PCNA binding and post-UV degradation of the mutants is shown. B, 293T cells were transfected with the plasmids coding GST-tagged p12 (WT or mutant), lysed, and pulled down by glutathione beads. The cell lysates (*Input*) and eluates (*Pulldown*) were analyzed by immunoblotting with anti-PCNA and anti-GST antibody. *n.d.*, not determined. C, 293T cells stably expressing HA-tagged p12 (WT or mutant) were irradiated with UV at the indicated doses. One hour later, cells were lysed and analyzed by immunoblotting with anti-HA or anti-PCNA antibody. D, 293T cells expressing p12<sup>K4Q/S10Y</sup> or p12<sup>K4Q/S10Y/K15A</sup> were irradiated with UV (15 J/m<sup>2</sup>). 15 min before harvesting at the indicated times, cells were labeled with [ $^3\text{H}$ ]thymidine, and incorporated radioactivity was measured. E, 293T cells expressing the indicated p12 mutant were irradiated with UV (15 J/m<sup>2</sup>) and harvested 1 h after irradiation. 15 min before harvesting, cells were labeled with [ $^3\text{H}$ ]thymidine, and incorporated radioactivity was measured. *Error bars* indicate mean  $\pm$  S.D. of three experiments. F, similar experiments were performed as in C. The size distribution of nascent DNAs was determined by alkaline-sucrose gradient. The *arrow* indicates a peak of  $\lambda$  DNA (48 Kb). G, similar experiments were performed as in F by using WI38 cells. H, a representative image of DNA fiber assay is shown. The *arrows* indicate ongoing forks where a *green IdU track* extends a *red CldU track*. I–K, 293T cells expressing mutant p12 were incubated with CldU for 20 min. After irradiation of UV (15 J/m<sup>2</sup>), cells were immediately incubated with IdU for 30 min. DNA was spread on a glass slide and detected by immunofluorescence. More than 100 fibers of ongoing replication fork progression (*green IdU tracks* that extend *red CldU tracks*) were measured. Histograms of green track lengths are shown in I and J. K, 293T cells without (*Control*) or with stable expression of p12 mutants were subjected to similar experiments as I and J. Distributions of green track lengths are shown as box plot. The *p* values were calculated using the Mann-Whitney *U* test. L, new origin firing rates were measured by counting green-only tracks divided by the total of red + green or red-only tracks. The *bars* and *lines* represent the mean  $\pm$  S.E. of three independent experiments. M and N, WI38 cells, HCT116, and HCT116<sup>p53-/-</sup> cells were tested as in K. Distributions of green track lengths are shown as box plot. The *p* values were calculated using the Mann-Whitney *U* test. O, 293T cells expressing mutant p12 were irradiated with the indicated dose of UV. The number of colonies was counted 10 days after irradiation. The number of colonies relative to nonirradiated cells is shown. *Error bars* indicate mean  $\pm$  S.D. of three experiments.



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family DNA helicase, is required for efficient replication fork restart after DNA damage (30), and depletion of p12 also affects BLM activity (24). Thus, another possibility is that degradation of p12 inhibits BLM activity at damaged forks and retards fork progression in the presence of DNA damage. The results reported in this study provide one of the long sought mechanisms by which fork progression is slowed by DNA damage to maintain genomic health in DNA-damaged cells.

*Acknowledgments*—We thank members of the Dutta laboratory for helpful comments.

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