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14. ABSTRACT We examined the effects of Cdt1 for gene amplification by using inducible system. We Obtained and validated reagents for inducible Cdt1-expression-system. Furthermore, we identified appropriated dose of MTX for testing gene amplification Cdt2 is a key molecule for inhibiting Cdt1 activity. The depletion of Cdt2 induces DNA re-replication in several cell lines. During experiment, we Identified a reduction of PCNA monoubiquitination in Cdt2 depleted MCF7 cells.Exogenous Cdt2 rescued the effect of si-Cdt2. Mutant of Cdt2 indicated that binding Cdt2 to Cul4/DDB1 was required for PCNA monoubiquitination. Furthermore, we identified that CRL4Cdt2 complex promotes PCNA-dependent translesion DNA synthesis.Additionally, we have identified p12 as a novel target of CRL4 complex. Cdt2 depletion canceled p12 degradation which regulated checkpoint pathway. Stable form of p12 promotes fork progression during intra-S-phase checkpoint.					
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Introduction:

Gene amplification is frequently observed in not only breast cancer but also in other malignant tumors. Overexpression and amplification of HER2/neu are reported in 25-30% of breast cancers. Patients with breast cancers that over-express HER2 have an aggressive form of the disease with poor prognosis. The number sites and extent of gene amplification also indicate a genetic instability of the cancer and predict a poor prognosis. Chromosomal replication origins are licensed by loading the Minichromosome maintenance 2–7 proteins (MCM2-7) complexes. Cdt1 is critical for loading MCM2-7, but it is inactivated by binding geminin and by ubiquitin mediated degradation after the onset of S phase so that origins are not licensed a second time in the cell cycle. Previous studies report that cells expressing excess Cdt1 re-replicated their DNA. Cdt1 is over-expressed in human breast cancer cells and over-expression of Cdt1 in mice predisposes them to tumorigenesis.

Cdt1 is degraded by ubiquitin-mediated proteolysis soon after the onset of S phase. There are two pathways for the degradation of Cdt1. In S and G2 phases, Cdt1 is phosphorylated by Cyclin E/A dependent CDK2 kinase, and the phosphorylated Cdt1 interacts with Skp2 to be targeted for degradation by SCF-mediated ubiquitination that depends on Skp1 and Cul1. Cul4A/DDB1 mediated ubiquitination is a second, CDK2-independent pathway of ubiquitinating Cdt1 during DNA replication. Indeed, Cdt1 has two signaling regions for degradation: a signal in residues 1-18 of Cdt1 for the Cul4A/DDB1 pathway and a cyclin-binding Cy-motif (around residue 62) and T29 signal for the Skp2-SCF pathway. Intriguingly, Cul4 is frequently amplified in breast cancers (Schindl, Gnant et al., 2007) suggesting that deregulation of Cdt1 stability may be common in breast cancers. However, there is no report yet on the levels of expression of Cdt1 in breast cancer.

The CRL4Cdt2 E3 ubiquitin ligase complex is a member of the cullin-RING family that was recently shown to promote the polyubiquitination and degradation of the replication licensing factor Cdt1 (Arias & Walter, 2006; Higa, Banks et al., 2006; Jin, Arias et al., 2006; Senga, Sivaprasad et al., 2006). The E3 ubiquitin ligase complex consists of Cul4A or Cul4B, DDB1 (damage-specific DNA binding protein 1), the RING-finger protein ROC1 and the DCAF substrate recognition factor, and the WD40 protein, Cdt2. Additional substrates for the CRL4Cdt2 ubiquitin ligase complex have been recently described including p21 (Kim, Starostina et al., 2008; Abbas, Sivaprasad et al., 2008; Nishitani, Shiomi et al., 2008), the *C. elegans* polymerase eta (Kim & Michael, 2008) and the *D. melanogaster* E2f1 transcription factor (Shibutani, de la Cruz et al., 2008). Notably, most identified CRL4Cdt2 substrates require their interaction with PCNA for their polyubiquitination. The exact role of PCNA is not clear but may lie in the recognition of substrate via Cdt2.

Body:**Validating Cdt1 expression by using Doxycycline in a dose-dependent manner**

Recently, a Doxycycline-inducible Cdt1-expression-system has reported by using Doxycycline (Liontos,

Koutsami et al., 2007). We obtained these cell lines and tested. As shown in Figure 1A, Cdt1 was nicely induced with 0.1 ug/ml of Doxycycline in U2OS colon cancer cell lines. Since this system can transfer to other cell lines, we will establish Cdt1 expressing in breast cancer cell lines.

Testing the toxicity of MTX in breast cancer cell lines

For Aim 3, I will test whether moderate over expression of Cdt1 promotes DNA amplification. Methotrexate (MTX) inhibits the DHFR gene, and a common method by which breast epithelial cells become resistant to MTX is through the amplification (and overexpression) of DHFR. To identify how much dose of MTX is required for inducing gene amplification, I tested the toxicity of MTX in breast cancer cell lines (Fig. 1B). A growth inhibition of cell was detected between 10-50 nM of MTX. Under these conditions, I will test whether Cdt1 over-expression promotes gene amplification.

Identifying a reduction of PCNA monoubiquitination in Cdt2 depleted MCF7 cells

Cdt2 is a key molecule for inhibiting Cdt1 activity. The depletion of Cdt2 induces DNA re-replication in several cell lines. Thus, we depleted Cdt2 in MCF7 breast cancer cell lines (Fig. 2). Notably, we identified that Cdt2 depleted cells showed lower level of PCNA monoubiquitination without UV treatment.

The monoubiquitination of PCNA is one of the best understood example of protein monoubiquitination at a conserved lysine residue (K164 in human PCNA) in response to DNA damage, increases its affinity for members of the Y-family of DNA bypass polymerases, and allows DNA synthesis across DNA lesions (Andersen, Xu et al., 2008). PCNA monoubiquitination in response to DNA damage is dependent on the activity of the ubiquitin conjugating E2 enzyme Rad6 and the ligase activity of the Rad18 E3 ubiquitin ligase (Kannouche, Wing et al., 2004; Hoege, Pfander et al., 2002) and cells deficient of either of these enzymes are extremely sensitive to a variety of DNA damaging agents (Andersen, Xu et al., 2008). Intriguingly, several reports demonstrated the presence of residual monoubiquitinated PCNA in Rad18-deficient cells, indicating that Rad18-independent ubiquitin ligase activity exists to promote PCNA monoubiquitination (Brun, Chiu et al., 2008; Huang, Nijman et al., 2006). Thus, we tested whether Cdt2 and CRL4 complex promote PCNA monoubiquitination.

The depletion of Cdt2 reduced basal level of PCNA mono-ubiquitination

Previously, it has been reported that the induction of PCNA monoubiquitination by depleting USP1, the enzyme responsible for deubiquitinating PCNA, is independent from Rad18 (Simpson, Ross et al., 2006). Thus, we hypothesized that knocking down Cdt2 may affect the basal level of PCNA monoubiquitination under USP1-depleted condition. As expected, treatment of U2OS cells with si-USP1 promoted PCNA monoubiquitination (Figure 3A). Furthermore, treatment of Rad18 siRNA reduced PCNA monoubiquitination in the absence of USP1. Surprisingly, knocking down of Cdt2 also reduced PCNA

monoubiquitination to levels observed with the depletion of Rad18 without affecting the basal level of Rad18. These results suggest that Cdt2 regulate the basal level of PCNA monoubiquitination.

Exogenous Cdt2 rescues the effect of si-Cdt2

To rule out the possibility that the reduction of PCNA monoubiquitination seen after si-Cdt2 was due to off-target activity, we tested whether exogenous siRNA-resistant Cdt2 can restore PCNA monoubiquitination in the absence of endogenous Cdt2. We developed U2OS cell which express either wild type Cdt2 or Cdt2 protein deficient in binding DDB1 (Cdt2-R246A) Since one Cdt2 siRNA is targeting ORF, both endogenous and exogenous Cdt2 were depleted. In contrast, another siRNA is designed for targeting 3'-UTR of Cdt2 coding gene and affects only endogenous. Indeed, si-Cdt2-2(targeting 3'-UTR) did not decrease PCNA monoubiquitination in cells expressing the exogenous wild type Cdt2 (Fig. 3B, lane 4). On the other hand, the mutant Cdt2-R246A could not restore PCNA monoubiquitination in cells transfected with siCdt2-2 (lane 8). These results demonstrate that Cdt2 knockdown specifically decreases PCNA monoubiquitination, and also that Cdt2 has to bind to DDB1 (and through it to the rest of the CRL4 complex) to monoubiquitinate PCNA.

CRL4Cdt2 complex promotes PCNA-dependent translesion DNA synthesis (TLS)

PCNA monoubiquitination plays a significant role in TLS by attracting the error-prone translesion polymerases to the site of DNA damage (Andersen, Xu et al., 2008). Because, CRL4Cdt2 promotes PCNA monoubiquitination under basal conditions, we tested whether such an activity impacts TLS in the absence of extrinsic DNA damage. We measured the TLS activity by measuring the mutation frequency in a supF gene (in a shuttle vector) subjected to UV-induced DNA damage before its introduction into the cells. Error-prone TLS activity will mutate the supF gene, which is scored by a blue-white colony screen after recovering the supF shuttle vector from the mammalian cells to transfect into bacteria. To increase the sensitivity of the assay, we performed these experiments after depleting 293T kidney epithelial cells of the high fidelity TLS enzyme, DNA polymerase eta (pol eta) (Huang, Nijman et al., 2006). Consistent with previous reports, cells depleted of USP1 showed a five-fold increase in mutation frequency compared to cells with USP1 (Figure 4A). Sequencing of the mutant supF genes, revealed a mutation spectrum consistent with error prone TLS (Supplemental Table 1). Significantly, the depletion of Cdt2 from these cells reduced the mutation frequency, indicating that the reduction in monoubiquitinated PCNA (Figure 1D) inhibits TLS activity in vivo. Similar results were obtained when 293T cells were depleted of the other components of the CRL4Cdt2, DDB1 and Cul4A/B, but not upon depletion of Cul1 or DDB2 (Figure 4B). These results demonstrate that the inhibition of TLS activity was a consequence of the specific inactivation of CRL4Cdt2 complex.

Identifying a novel substrate, p12, which is polyubiquitinated by CRL4Cdt2

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 (Bartek, Lukas et al., 2004;Kaufmann, 2010). 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 CRL1beta-TRCP to inhibit origin firing (Mailand, Falck et al., 2000;Busino, Donzelli et al., 2003;Falck, Mailand et al., 2001). In the presence of DNA damage, ATM/ATR is activated and phosphorylates Chk1/Chk2. Activated Chk1/Chk2 phosphorylates Cdc25A that accelerates proteolysis of Cdc25A by CRL1beta-TRCP. The mechanism for inhibiting fork progression, however, has not been identified (Miao, Seiler et al., 2003;Chastain, Heffernan et al., 2006;Seiler, Conti et al., 2007).

DNA polymerase delta is an essential polymerase for replication machinery, consists of 4 subunits. p12, the smallest subunit of polymerase delta, is important for effective DNA replication and cell proliferation(Meng, Zhou et al., 2010;Huang, Akashi et al., 2010). In vitro situation, lacking of p12 in polymerase delta complex decreases its replication activity. Cells depleted p12 by using shRNA shows less proliferation and genomic instability. Furthermore, p12 is degraded immediately after DNA damage through polyubiquitination (Zhang, Zhou et al., 2007) by an unknown E3 ligase though its degradation is abrogated by checkpoint kinase inhibitor.

We identified that p12 degradation is critical for inhibiting fork progression during the intra-S-phase checkpoint. p12 is polyubiquitinated and degraded by CRL4Cdt2 in a PCNA-interaction-dependent manner.

CRL4Cdt2 E3 ligase complex promote p12 degradation

To test whether CRL4Cdt2 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. 5A). Depletion of Cdt2 increased the basal level of p12 and prevented the degradation of p12 following DNA damage. CRL4Cdt2 ligase complex is also composed of DDB1 and Cul4A or Cul4B. Consistent with our hypothesis, depletion of DDB1 or Cul4A and B also abrogated p12 degradation after UV treatment (Fig. 5B). Although PCNA depletion decreased the basal level of p12, degradation of the residual p12 in response to DNA damage was abrogated. Therefore p12 degradation post-UV-irradiation requires components of CRL4Cdt2 and PCNA, much like the other substrates of this E3, such as Cdt1, p21, and Set8.

N-terminus of p12 regulates its binding to PCNA and degradation

p12 binding to PCNA is reported to be essential for efficient activity of polymerase delta (Meng, Zhou et al., 2010;Huang, Akashi et al., 2010). Therefore, to address the physiological role of p12 degradation, we required a mutant of p12 that still interacts with PCNA but is not degraded post radiation. Substrates

of CRL4Cdt2, such as Cdt1, p21 and Set8, contain a threonine and a lysine in the context of the PIP box that are important for CRL4Cdt2 dependent degradation but not as important for PCNA binding, and the corresponding residues are present in p12 (Thr8 and Lys15) (Fig. 6A). However, mutation of Thr 8 (p12T8D) or Lys 15 (p12K15A) in p12 disrupted or decreased the interaction with PCNA, suggesting that both 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 (p12K4Q/S10Y) to generate a canonical PIP box sequence (Fig. 6A). p12K4Q/S10Y still maintained the binding to PCNA at the equivalent level as p12WT (Fig. 6B). In the K4Q/S10Y context, additional mutation of Lys 15 (p12K4Q/S10Y/K15A) 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 CRL4Cdt2 ligase like Cdt1 and p21, p12 Δ PIP was not degraded after UV treatment (Fig. 6C), suggesting that binding of p12 to PCNA is required for the degradation. p12K4Q/S10Y mutant was degraded similarly as the p12WT. On the other hand, this degradation was not seen in p12K4Q/S10Y/K15A, although this protein retained its ability to bind PCNA. Therefore, this mutant would allow us to test the role of DNA-damage dependent degradation of p12 without abolishing its binding to PCNA.

The degradation of p12 inhibits fork progression during S phase and is required for cell survival after DNA damage

Since p12 is required for effective DNA synthesis, and is degraded in response to DNA damage, we next measured RDS in cells expressing UV-degradation sensitive or insensitive mutant of p12. 293T cells stably expressing p12K4Q/S10Y or p12K4Q/S10Y/K15A mutants were irradiated with UV and labeled with 3H thymidine to label newly synthesized DNA (Fig. 7A). Cells expressing the stable p12K4Q/S10Y/K15A showed higher DNA synthesis after irradiation than cells expressing degradable p12K4Q/S10Y, suggesting that p12 degradation is required for the inhibition of DNA synthesis after DNA damage

The intra-S-phase checkpoint has two effects on DNA replication: decreasing the rate of fork progression, and inhibiting new origin initiation. To investigate whether the degradation of p12 post-radiation inhibits fork progression or initiation, we measured the size of newly synthesized DNA by separating 3H-labeled nascent single-stranded DNA using alkaline-sucrose gradient. Cells expressing either p12K4Q/S10Y or p12K4Q/S10Y/K15A were irradiated with UV followed by 30 minutes of 3H thymidine labeling. Newly labeled nascent DNA strands were fractionated by alkaline-sucrose gradient according to their size (Fig. 7B). Without UV treatment, the size distribution of newly synthesized DNA was similar between cells expressing p12K4Q/S10Y and p12K4Q/S10Y/K15A. After UV treatment, both short nascent DNA (from newly fired origins) and longer nascent DNA (from progression of

existing forks) were significantly reduced in p12K4Q/S10Y expressing cells due to the inhibition of origin firing and fork stalling, respectively. On the other hand, in p12K4Q/S10Y/K15A 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.

Key accomplishments:

- Obtained and validated reagents for an inducible Cdt1-expression-system
- Identified appropriate dose of MTX for testing gene amplification
- Identified that PCNA monoubiquitination induced by CRL4Cdt2 E3 ligase complex in MCF7 breast cancer cell lines.
- Identified that the depletion of Cdt2 reduces the steady-state level of PCNA mono-ubiquitination.
- Identified that exogenous Cdt2 rescues the effect of si-Cdt2.
- Identified that CRL4Cdt2 complex promotes PCNA-dependent translesion DNA synthesis
- Identified a novel substrate, p12, which is polyubiquitinated by CRL4Cdt2.
- Identified that the degradation of p12 inhibits fork progression during S phase and is required for cell survival after DNA damage

Conclusions:

Expression level of Cdt1 was induced by Doxycycline in a dose-dependent manner

By using Doxycycline, we confirmed inducible Cdt1 expression. Using the Doxycycline system, I will analyze DNA replication in breast cancer cells using FACS after inducing differing levels of Cdt1. From these results, I will estimate the highest and lowest dose of Doxycycline for testing whether moderate over expression of Cdt1 promotes DNA amplification.

CRL4Cdt2 E3 ubiquitin ligase monoubiquitinated PCNA to promoted translesion DNA synthesis.

Additionally, we have identified novel target of CRL4 complex. Cdt2 depletion reduced the basal level of monoubiquitinated PCNA and decreased the activity of translesion DNA synthesis. Since the monoubiquitination of PCNA is a key regulator for increasing the tolerance for DNA damage reagents, it is possible that depletion or inhibition of Cdt2 enhances the effect of anti-cancer drugs. Furthermore, TLS is required for cell survival but increases mutation frequency, it is tempting to speculate that the over-expression of Cul4A or Cdt2 observed in some human cancer may promote malignancy by enhancing PCNA-dependent and TLS-mediated mutagenesis.

Degradation of p12 by CRL4Cdt2 E3 ligase inhibits fork progression during intra-S-phase checkpoint

Additionally, we have identified p12 as a novel target of CRL4 complex. Cdt2 depletion canceled p12 degradation which regulated checkpoint pathway. Stable form of p12 promotes fork progression during intra-S-phase checkpoint. Since disruption of checkpoint pathway is a key event for tumorigenesis, our finding implicates that degradation of p12 can be a new target for anti-cancer drug.

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RESEARCH AND PROFESSIONAL EXPERIENCE

Concluding with your current position, list chronologically previous employment, experience and honors. Also list in chronological order the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application.

Research experience

From 01/04/2002 to 31/03/2006 : Osaka University (Suita, Japan) Graduate student Signal Transduction (Michiyuki Matsuda)

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Awards

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DoD Era of Hope Postdoctoral Fellowship, Jun 2008-Apr 2011

List of publications

Kurokawa K, Takaya A, **Terai K**, Fujioka A, Matsuda M. Visualizing the signal transduction pathways in living cells with GFP-based FRET probes. *Acta Histochemica et Cytochemica* 2004, 34, 347-355.

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Takegahara N, Takamatsu H, Toyofuku T, Tsujimura T, Okuno T, Yukawa K, Mizui M, Yamamoto M, Prasad DV, Suzuki K, Ishii M, **Terai K**, Moriya M, Nakatsuji Y, Sakoda S, Sato S, Akira S, Takeda K, Inui M,

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Abbas T, Sivaprasad U, **Terai K**, Amador V, Pagano M, Dutta A. PCNA-dependent regulation of p21 ubiquitylation and degradation via the CRL4Cdt2 ubiquitin ligase complex. *Genes Dev.* 2008 Sep 15;22(18):2496-506.

Matsunaga-Udagawa R, Fujita Y, Yoshiki S, **Terai K**, Kamioka Y, Kiyokawa E, Yugi K, Aoki K, Matsuda M. The scaffold protein Shoc2/SUR-8 accelerates the interaction of Ras and Raf. *J Biol Chem.* 2010 Mar 5;285(10):7818-26. Epub 2010 Jan 5.

Terai K, Abbas T, Jazaeri AA, Dutta A. CRL4(Cdt2) E3 ubiquitin ligase monoubiquitinates PCNA to promote translesion DNA synthesis. *Mol Cell.* 2010 Jan 15;37(1):143-9.

Fig. 1A

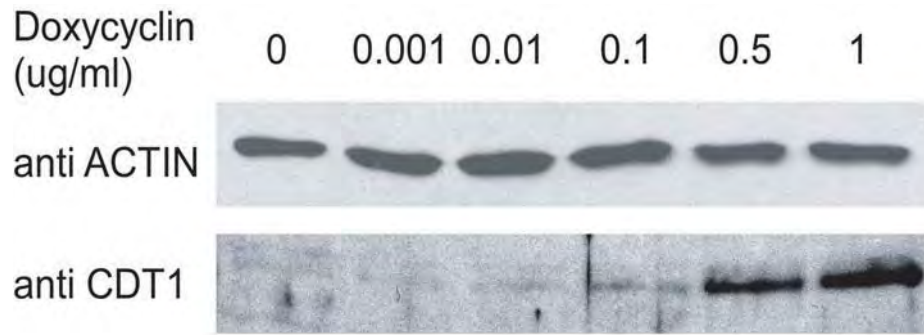
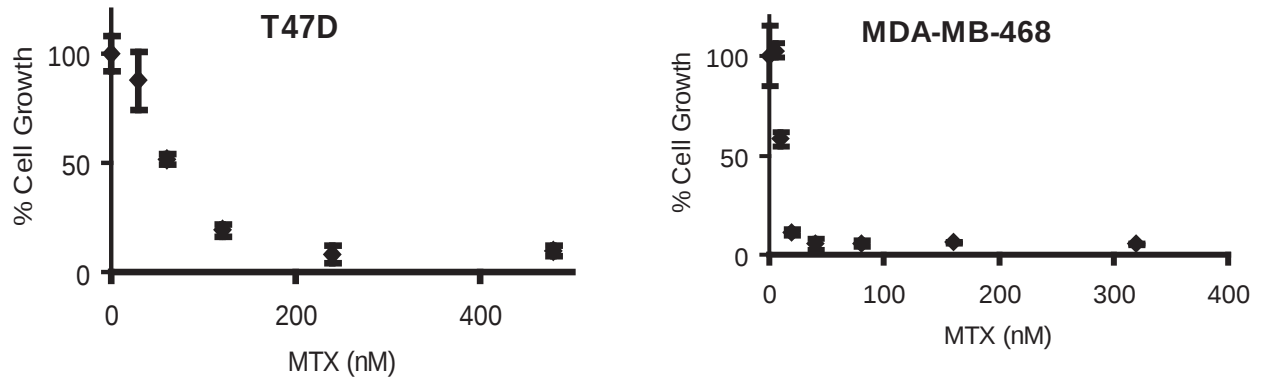
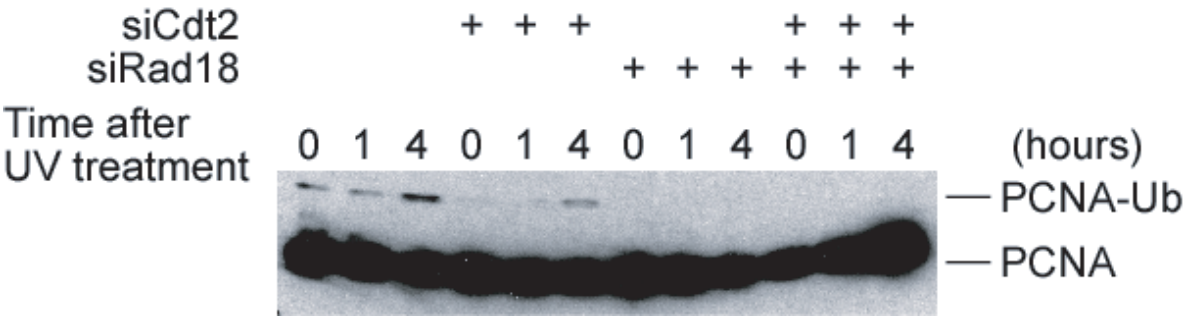


Fig. 1B



(A) U2OS-TetON-hCdt1 cells were treated with a different dose of doxycycline. After two days, cells were lysed and analyzed with Cdt1 antibody. (B) T47D and MDA-MB-468 cell lines were treated with indicated dose of MTX. Five days after treatment, numbers of cells were counted.

Fig. 2



MCF7 cells were transfected siRNA of Cdt2, Rad18, or Cdt2 and Rad18. After two days, cells were irradiated with UV at 50 J/m² and lysised indicated time points. Cell lysates were probed with PCNA antibody.

Fig. 3A

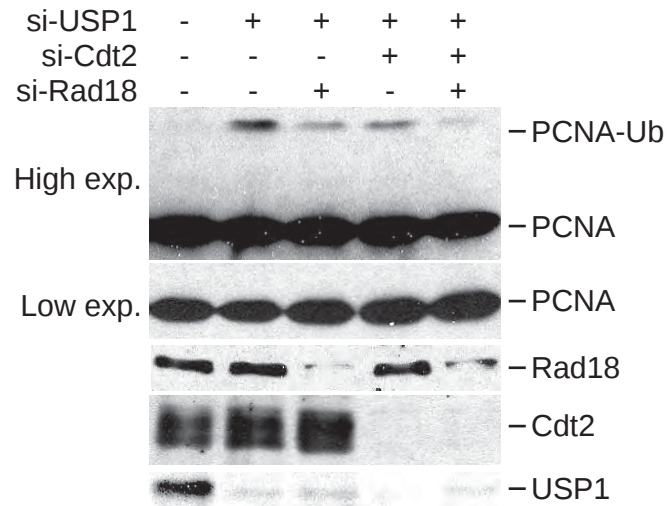
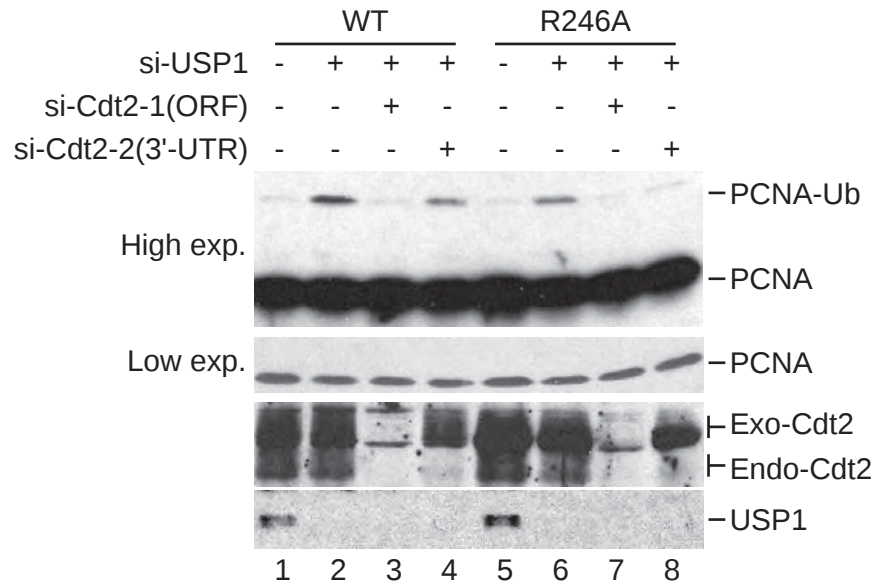


Fig. 3B



(A) U2OS cells were transfected with the indicated siRNA. After 2 days, cells were analyzed by immunoblotting with anti-PCNA, anti-Rad18, anti-Cdt2, or anti-USP1 antibody. (B) U2OS cells stably expressing either wild type and flag-tagged Cdt2 or Cdt2 mutant protein (Cdt2-R246A) were transfected with si-Cdt2-1 (targeting the ORF) or with si-Cdt2-2 (targeting the 3'-UTR). Where indicated cells were also transfected with si-USP1. Two days after transfections, cells were harvested and the cell lysates analyzed by immunoblotting with anti-PCNA, anti-Cdt2, or anti-USP1 antibody. The anti-Cdt2 blot shows endogenous and exogenous Cdt2.

Fig. 4A

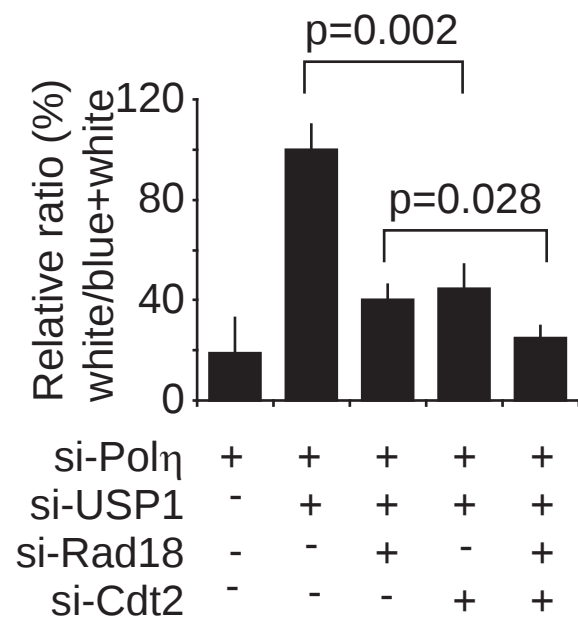
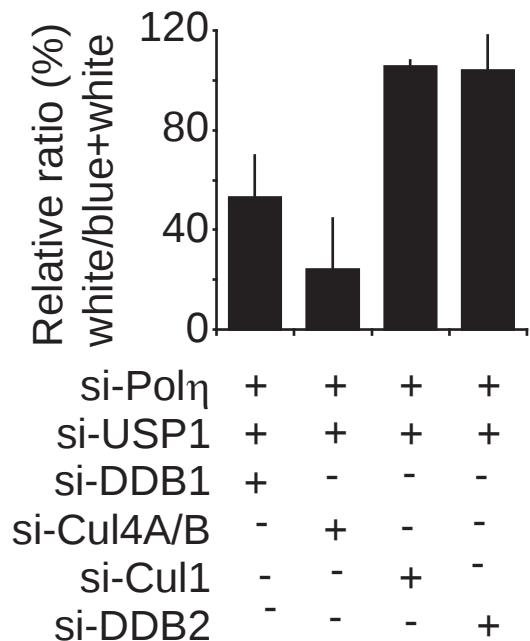


Fig. 4B



(A and B) 293T cells were transfected with the indicated siRNA. Twenty-four hours after incubation, UV-treated reporter plasmid was introduced into the 293T cells. Forty-eight hours after this, plasmids were purified from cells and analyzed for mutation frequency expressed as ratio of white to total colonies. The ratio seen after si-USP1+ si-pol eta is shown as 100%. Mean and S.D. of 3 experiments

	Control					si-Cdt2					
10 J/m ²	0	1	4			0	1	4			
25 J/m ²	0			1	4	0			1	4	(h)
											– p12
											– Cdt2
											– p125

	Control		si-DDB1		si-Cul4A/B		si-PCNA		
UV (10 J/m ²)	-	+	-	+	-	+	-	+	
									-p12 (low)
									-p12 (high)
									-DDB1
									-Cul4B
									-Cul4A
									-PCNA
	1	2	3	4	5	6	7	8	

(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-Cul4/B, anti-betaTrcp antibody, or anti-PCNA antibody.

Fig. 6A

	PCNA binding	Degradation
p12 _m grKrlI <u>TD</u> S <u>Y</u> pvv K r-	++	+
Cdt1 me <u>Q</u> rrV <u>TD</u> F <u>F</u> arr R p-		
p21-rr <u>Q</u> ts <u>M</u> <u>TD</u> F <u>Y</u> hsk R r-		
T8D _m grKrlI <u>DD</u> S <u>Y</u> pvv K r-	-	n. d.
K15A _m grKrlI <u>TD</u> S <u>Y</u> pvv A r-	+	n. d.
ΔPIP _m grArlA <u>TD</u> A <u>A</u> pvv K r-	-	-
K4Q/S10Y _m gr <u>Q</u> rlI <u>TD</u> <u>Y</u> <u>Y</u> pvv K r-	++	+
K4Q/S10Y/K15A _m gr <u>Q</u> rlI <u>TD</u> <u>Y</u> <u>Y</u> pvv A r-	++	-

Fig. 6B

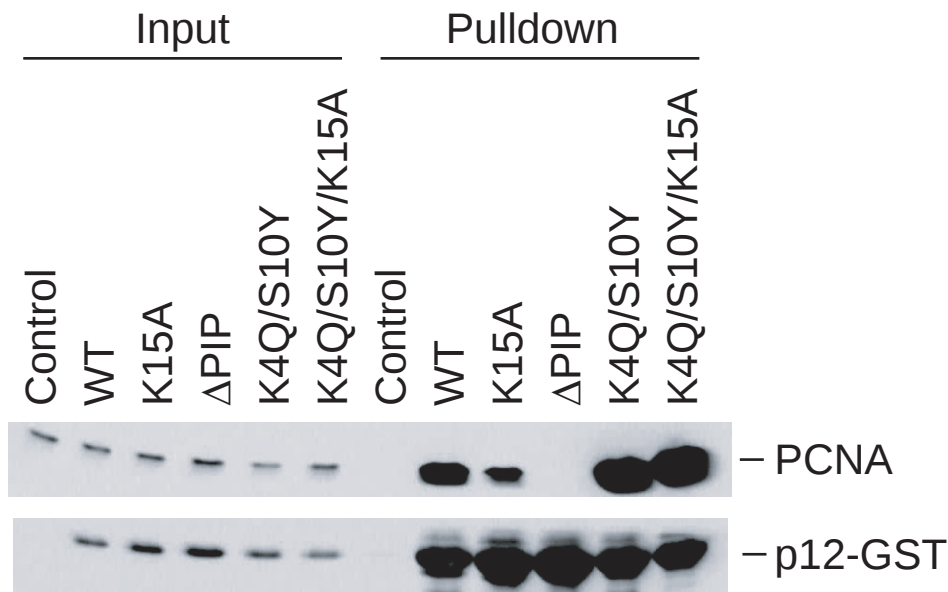
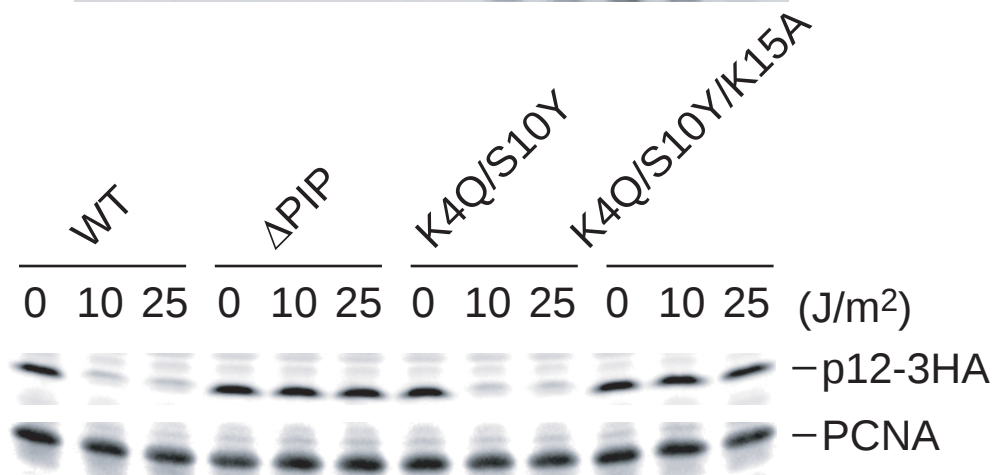


Fig. 6C



(A) Alignment of PIP boxes in p12, Cdt1, and p21. Underlined residues constitute a canonical PIP box. Bold letters are highly conserved residues that predict polyubiquitination by CRL4Cdt2. 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, lysed and pulled down by glutathione beads. The cell lysates (Input) and eluates (Pull-down) were analyzed by immunoblotting with anti-PCNA and anti-GST antibody. Two exposures of the PCNA blot are shown in B. (C) 293T cells stably expressing HA-tagged p12 were irradiated with UV at indicated doses. One hour later, cells were lysed and analyzed by immunoblotting with anti-HA or anti-PCNA antibody.

Fig. 7A

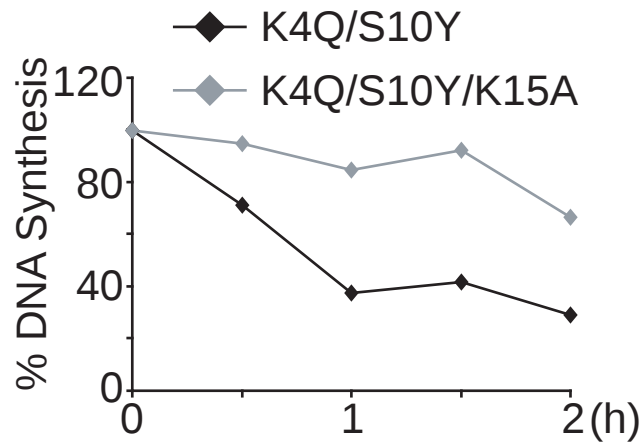
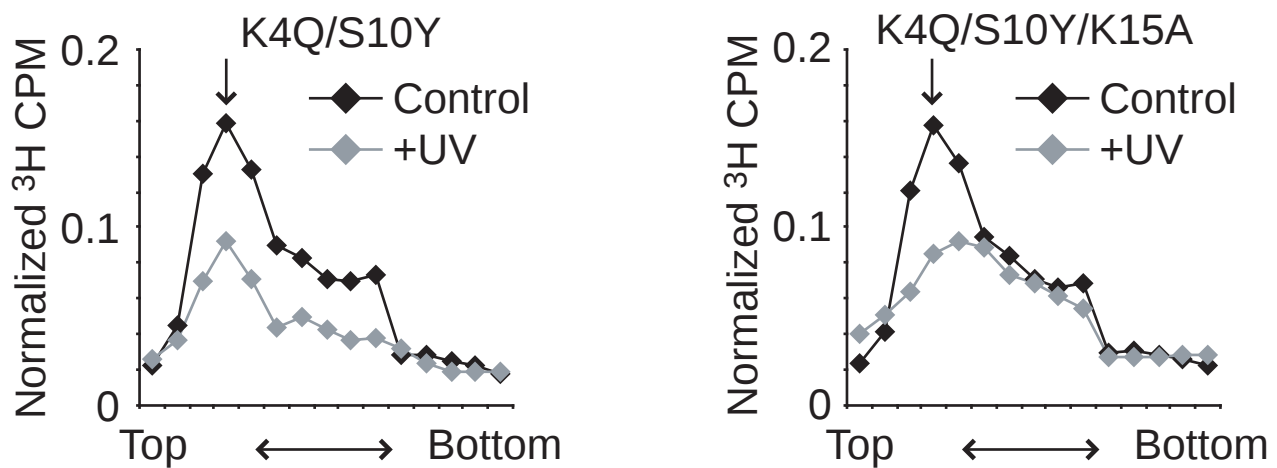


Fig. 7B



(A) 293T cells expressing p12K4Q/S10Y or p12K4Q/S10Y/K15A were irradiated with UV (15 J/m²). 15 minutes before harvesting at indicated times, cells were labeled with ^3H thymidine and incorporated radioactivity measured. (B) 293T cells expressing indicated p12 mutant were irradiated with UV (15 J/m²) and harvested one hour after irradiation. 15 minutes before harvesting, cells were labeled with ^3H thymidine and incorporated radioactivity measured. The size distribution of nascent DNAs was determined by alkaline-sucrose gradient. Arrow indicates a peak of lambda DNA (48 Kbp).

CRL4^{Cdt2} E3 Ubiquitin Ligase Monoubiquitinates PCNA to Promote Translesion DNA Synthesis

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SUMMARY

Monoubiquitination of proliferating cell nuclear antigen (PCNA) is a critical posttranslational modification essential for DNA repair by translesion DNA synthesis (TLS). The Rad18 E3 ubiquitin ligase cooperates with the E2 Rad6 to monoubiquitinate PCNA in response to DNA damage. How PCNA is monoubiquitinated in unperturbed cells and whether this plays a role in the repair of DNA associated with replication is not known. We show that the CRL4^{Cdt2} E3 ubiquitin ligase complex promotes PCNA monoubiquitination in proliferating cells in the absence of external DNA damage independent of Rad18. PCNA monoubiquitination via CRL4^{Cdt2} is constitutively antagonized by the action of the ubiquitin-specific protease 1 (USP1). In vitro, CRL4^{Cdt2} monoubiquitinates PCNA at Lys164, the same residue that is monoubiquitinated by Rad18. Significantly, CRL4^{Cdt2} is required for TLS in nondamaged cells via a mechanism that is dependent on PCNA monoubiquitination. We propose that CRL4^{Cdt2} regulates PCNA-dependent TLS associated with stresses accompanying DNA replication.

INTRODUCTION

Protein ubiquitination is an essential posttranslational modification involved in a variety of physiological processes including cell-cycle control, DNA replication, and repair. Whereas the covalent attachment of polyubiquitin chains to proteins frequently results in the targeted proteolysis of the ubiquitinated protein, the attachment of a single ubiquitin moiety often results in the modification of its activity by regulating its interactions with other proteins, cellular localization, or enzymatic activity. The monoubiquitination of PCNA at a conserved lysine residue (Lys164 in human PCNA) in response to DNA damage increases its affinity for members of the Y family of DNA bypass polymerases and allows DNA synthesis across DNA lesions (Andersen et al., 2008). DNA damage-dependent PCNA monoubiquitination requires the activities of the Rad6 E2 ubiquitin-conjugating enzyme and the Rad18 E3 ubiquitin ligase (Hoegge et al., 2002; Kannouche et al., 2004), and cells deficient of either of these enzymes are extremely sensitive to a variety of DNA-damaging

agents (Andersen et al., 2008). Recently, RNF8 has been suggested to function as an E3 ligase for PCNA monoubiquitination in cells irradiated with UV (Zhang et al., 2008). Huang et al., however, showed that UV-induced PCNA monoubiquitination is not affected in HeLa cells depleted of RNF8 or in MEFs deficient of RNF8 (Huang et al., 2009). The basis for these conflicting results are currently unclear, but the available evidence suggest that, at least under some conditions, other ubiquitin ligases are capable of promoting PCNA monoubiquitination. Indeed, several reports demonstrated the presence of residual monoubiquitinated PCNA in Rad18-deficient cells (Brun et al., 2008; Huang et al., 2006; Simpson et al., 2006).

The CRL4^{Cdt2} E3 ubiquitin ligase complex is a member of the cullin-RING family that promotes the polyubiquitination and degradation of the replication licensing factor Cdt1 (Arias and Walter, 2006; Higa et al., 2006; Jin et al., 2006; Senga et al., 2006). CRL4^{Cdt2} consists of Cul4A or Cul4B, damage-specific DNA-binding protein 1 (DDB1), the RING-finger protein ROC1 and the DDB1 and Cul4-associated factor (DCAF), and substrate recognition factor/WD40 protein Cdt2. Additional substrates for CRL4^{Cdt2} have been recently described, including the CDK inhibitor p21 (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008), the *C. elegans* polymerase eta (Kim and Michael, 2008), and the *D. melanogaster* E2f1 transcription factor (Shibutani et al., 2008). Notably, most identified CRL4^{Cdt2} substrates require their interaction with PCNA for their polyubiquitination.

Because of the physical association of CRL4^{Cdt2} with PCNA in the context of substrate ubiquitination, we examined whether CRL4^{Cdt2} monoubiquitinates PCNA. We report here that in normally cycling mammalian cells, CRL4^{Cdt2} monoubiquitinates PCNA and that this activity is constitutively antagonized by the deubiquitinating enzyme USP1 (Huang et al., 2006). In response to DNA damage, however, Rad6/Rad18 is most critical for PCNA monoubiquitination, with Cdt2 depletion affecting this monoubiquitination in some cell lines but not all. We finally show that PCNA monoubiquitination via CRL4^{Cdt2} synergizes with Rad6/18 in promoting TLS in undamaged cells.

RESULTS

Cdt2 Associates with CRL4 to Promote PCNA Monoubiquitination in Normally Cycling Cells

We and others have recently shown that Cdt1 and p21 ubiquitination via the CRL4^{Cdt2} requires association with PCNA

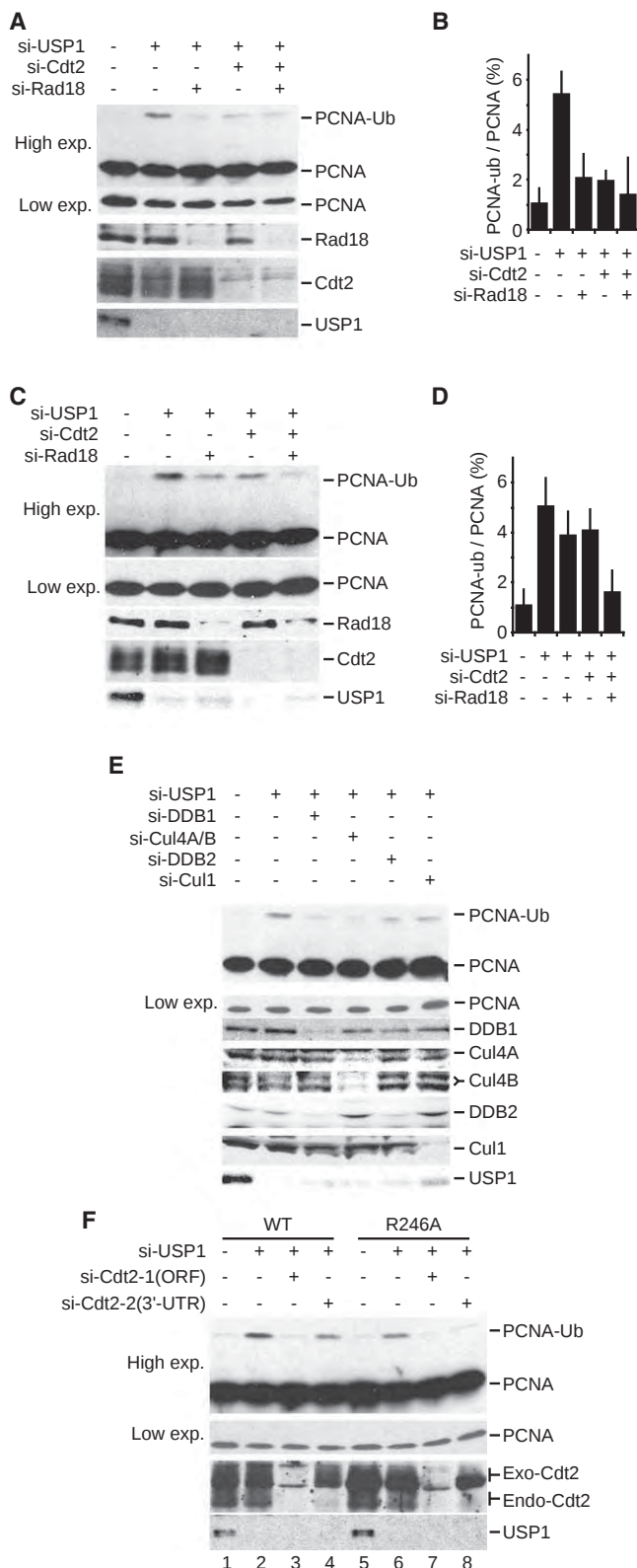


Figure 1. CRL4^{Cdt2} Depletion Reduces the Steady-State Level of PCNA Monoubiquitination

(A–D) HeLa cells (A and B) or U2OS cells (C and D) were transfected with the indicated siRNA and analyzed by immunoblotting with anti-PCNA, anti-

(Abbas et al., 2008; Arias and Walter, 2006; Kim et al., 2008; Nishitani et al., 2008; Senga et al., 2006), raising the possibility that CRL4^{Cdt2} may regulate PCNA ubiquitination. It has been reported that USP1 depletion from Rad18-deficient cells induces PCNA monoubiquitination, indicating that other E3 ubiquitin ligases carry out this function independent of Rad18 (Simpson et al., 2006). Thus, we tested whether CRL4^{Cdt2} inactivation may affect basal PCNA monoubiquitination under USP1-depleted condition. As expected, transfection of HeLa cells with si-USP1 enhanced PCNA monoubiquitination, which was reduced by the codepletion of Rad18 (Figures 1A and 1B). Surprisingly, depletion of Cdt2 also reduced PCNA monoubiquitination to levels observed with Rad18 depletion without affecting the steady-state level of Rad18 (Figures 1A and 1B). However, we did not detect an additive effect when both Cdt2 and Rad18 are depleted in this cell line, most likely because each of the depletions repressed PCNA monoubiquitination to near baseline levels (see below). Similar results were observed in U2OS cells, although the best decrease in PCNA monoubiquitination in this cell line was seen when Rad18 and Cdt2 were codepleted (Figures 1C and 1D). Thus in these cells Rad18 and Cdt2 appear to have equal and independent roles in basal monoubiquitination of PCNA.

Cdt2 is the substrate recognition subunit of the CRL4^{Cdt2} complex that includes Cul4A or Cul4B and DDB1. Depletion of DDB1, or Cul4A and Cul4B, also inhibited PCNA monoubiquitination in the absence of USP1 (Figure 1E), demonstrating that the effect of Cdt2 on PCNA monoubiquitination is mediated via its assembly in CRL4 ubiquitin ligase complexes. The requirement of CRL4^{Cdt2} in promoting PCNA monoubiquitination was specific, since there was significant PCNA monoubiquitination in cells depleted of Cul1, a Cul4-related E3 ligase, or DDB2, another DCAF that assembles with the CRL4 ligase (Figure 1E). Furthermore, we tested whether exogenous siRNA-resistant Cdt2 can restore PCNA monoubiquitination in the absence of endogenous Cdt2. We developed U2OS cells that express either wild-type Cdt2 or Cdt2 protein deficient in binding DDB1 (Cdt2-R246A) (Jin et al., 2006). Both versions of the protein are expressed from a cDNA without the 3'UTR. si-Cdt2-1, targeting the ORF of Cdt2 and so targeting both endogenous and exogenous Cdt2, reduced PCNA monoubiquitination in the absence of USP1 (Figure 1F). si-Cdt2-2 was designed to target the 3'UTR of Cdt2 and so exclusively targets the endogenous gene while sparing exogenous Cdt2. Indeed, si-Cdt2-2 did not decrease PCNA monoubiquitination in cells overexpressing wild-type Cdt2 (Figure 1F, lane 4). On the other hand, Cdt2-R246A could

Rad18, anti-Cdt2, or anti-USP1 antibody. A low exposure of the nonubiquitinated PCNA is also shown. The ratio of monoubiquitinated PCNA to total PCNA is blotted in (B) and (D), respectively. Mean $\pm$ SD of three experiments. The quantitative analysis of PCNA monoubiquitination is shown in Figure S1. (E) HeLa cells transfected with the indicated si-RNA and the level of PCNA monoubiquitination were analyzed as in (A) and (C).

(F) U2OS cells stably expressing flag-tagged wild-type Cdt2 or Cdt2-R246A were transfected with si-Cdt2-1 (targeting the ORF) or with si-Cdt2-2 (targeting the 3'UTR). Where indicated, cells were also transfected with si-USP1. Protein lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-PCNA, anti-Cdt2, or anti-USP1 antibody.

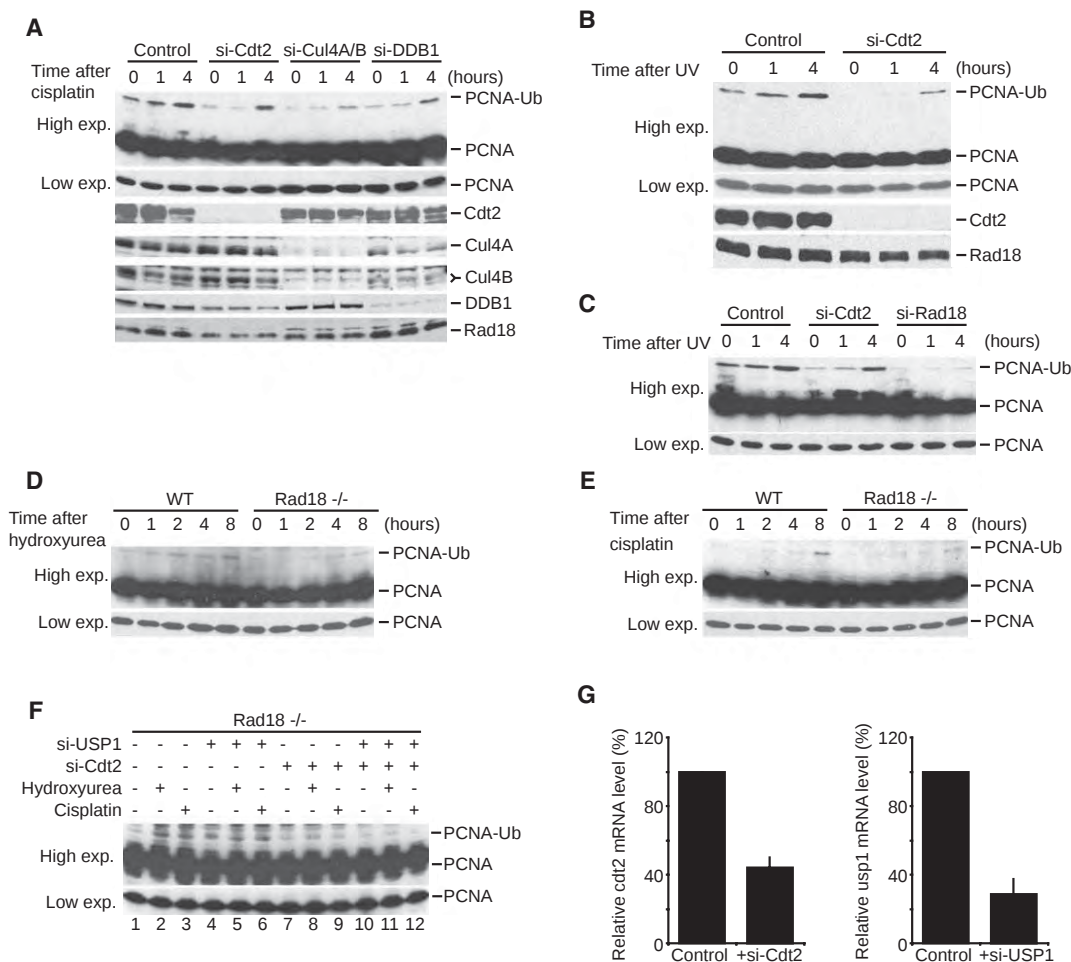


Figure 2. CRL4^{Cdt2} Contributes to DNA Damage-Induced PCNA Monoubiquitination

(A) ES2 cells were transfected with the indicated siRNA. After 48 hr, cells were treated with 500 μ M cisplatin for the indicated time. Cell lysates were analyzed by immunoblotting with anti-PCNA, anti-Cdt2, anti-Cul4, anti-DDB1, and anti-Rad18 antibodies.

(B and C) 293T cells (B) and MCF7 cells (C) were transfected with si-Cdt2. After 48 hr, cells were irradiated with UV (50 J/m²) and harvested at the indicated time postirradiation. Cell lysates were analyzed as above.

(D and E) DT40 cells or DT40 cells deficient of Rad18 (*Rad18*^{-/-}) were treated with 50 μ M cisplatin or 1 mM hydroxyurea for the indicated time and analyzed as above.

(F) *DT40*^{Rad18^{-/-}} cells were transfected with the indicated siRNA. Three days after transfection, cells were either left untreated or treated with 50 μ M cisplatin or 1 mM hydroxyurea for 8 hr before harvesting. Lysates were immunoblotted with anti-PCNA antibody.

(G) Total RNA was prepared from *DT40*^{Rad18^{-/-}} cells with or without siRNA treatment. Using β -actin as a control, *cdt2* and *usp1* mRNA were analyzed by quantitative real-time PCR and expressed relative to the control si-GL2 sample. Mean $\pm$ SD of three experiments.

not restore PCNA monoubiquitination in cells transfected with si-Cdt2-2 (Figure 1F, lane 8). Collectively, these results demonstrate that CRL4^{Cdt2} specifically promotes basal PCNA monoubiquitination and that this activity is counteracted by USP1.

CRL4^{Cdt2} Contributes to DNA Damage-Induced PCNA Monoubiquitination

ES2 ovarian cancer cells, 293T kidney cancer cells, and MCF7 breast cancer cells showed higher levels of basal monoubiquitinated PCNA that could be detected without USP1 depletion (0 hr lanes in Figures 2A–2C). Interestingly, in these cell lines, Cdt2 depletion reduced both basal and damage-dependent PCNA monoubiquitination. Furthermore, depletion of Cul4 and DDB1

in ES2 cells also inhibited cisplatin-induced PCNA monoubiquitination (Figure 2A) without affecting Rad18 expression levels. The depletion of Cul4A or DDB1 did not alter cell-cycle profile, suggesting that the reduction of PCNA monoubiquitination is independent from cell-cycle effects (see Figure S2 available online). Although the kinetics of PCNA monoubiquitination after DNA damage was delayed, all these cells eventually monoubiquitinated PCNA in the absence of Cdt2, consistent with the well-accepted role of Rad18 in this process. Thus, in several human cell lines with a high level of basal PCNA monoubiquitination, CRL4^{Cdt2} seems to be important for both basal monoubiquitination and the kinetics of the DNA damage-induced monoubiquitination of PCNA.

CRL4^{Cdt2} Promotes PCNA Monoubiquitination Independent of Rad18

It is conceivable that CRL4^{Cdt2} may affect PCNA indirectly via the modulation of Rad18 activity. To rule out this possibility, we tested the effect of CRL4^{Cdt2} on PCNA monoubiquitination in DT40 chicken cells that do not express Rad18 (*DT40^{Rad18-/-}*). Wild-type and Rad18-deficient DT40 cells were treated with the DNA-damaging agents cisplatin or hydroxyurea. Consistent with previous reports, *DT40^{Rad18-/-}* cells exhibited PCNA monoubiquitination that was significantly less than that observed in Rad18-proficient cells (Figures 2D and 2E). Importantly, Cdt2 depletion in *DT40^{Rad18-/-}* cells decreased DNA damage-induced PCNA monoubiquitination (Figure 2F, lanes 8 and 9 compared with lanes 2 and 3) as well as basal monoubiquitination seen after depleting cells of USP1 (lane 10 compared with lane 4). Thus, CRL4^{Cdt2} promotes PCNA monoubiquitination independent of Rad18 and can substitute for its activity in response to DNA damage in Rad18-deficient cells.

CRL4^{Cdt2} Promotes PCNA Monoubiquitination In Vitro

To address whether the CRL4^{Cdt2} can directly monoubiquitinate PCNA, we tested whether PCNA can serve as a direct substrate for CRL4^{Cdt2} in vitro. In the presence of E1, E2, and ATP, PCNA was efficiently monoubiquitinated by the CRL4^{Cdt2} complex immunopurified from 293T cells (Figures 3A and 3B). Furthermore, CRL4^{Cdt2} monoubiquitinated PCNA specifically at the conserved lys164, as a mutant PCNA in which this residue was mutated to arginine (PCNA-K164R) was not monoubiquitinated (Figure 3B, lane 4). Thus, CRL4^{Cdt2} monoubiquitinates PCNA at the same site that is monoubiquitinated via Rad18 (Hoegge et al., 2002). Importantly, depletion of 293T cells of Rad18 did not prevent CRL4^{Cdt2}-dependent PCNA monoubiquitination (Figure 3C, lane 9), ruling out that the in vitro enzymatic activity of CRL4^{Cdt2} is indirectly mediated by Rad18.

CRL4^{Cdt2} Promotes PCNA-Dependent Translesion DNA Synthesis

PCNA monoubiquitination plays a significant role in TLS by recruiting the error-prone translesion polymerases to sites of DNA damage (Andersen et al., 2008). Because CRL4^{Cdt2} promotes PCNA monoubiquitination under basal conditions, we tested whether such an activity impacts TLS in the absence of extrinsic DNA damage. We measured TLS activity by measuring the mutation frequency in a supF gene (in a shuttle vector) (Parris and Seidman, 1992). Error-prone TLS activity in 293T cells will be dependent on basal level of PCNA monoubiquitination and will mutate the damaged supF gene. The mutation rate in replicated plasmids is scored by a blue-white colony screen after recovering the supF shuttle vector from the mammalian cells and transfection into bacteria. To increase the sensitivity of the assay, we performed these experiments first after depleting 293T cells of the high-fidelity TLS enzyme, DNA polymerase ϵ . We measured mutation frequency in cells depleted of USP1 and polymerase ϵ using non-UV-treated supF plasmid. However, no white colony was observed among more than 3000 colonies. Thus, we used plasmids subjected to UV-induced DNA damage before its introduction into undamaged cells. Consistent with previous reports, cells depleted of

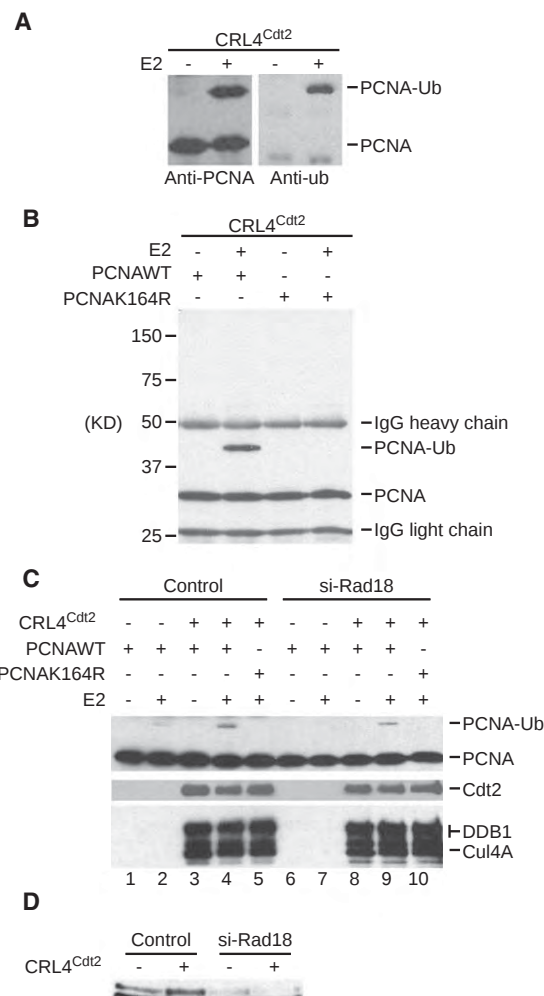


Figure 3. CRL4^{Cdt2} Complex Promotes PCNA Monoubiquitination at Lys164 In Vitro

(A and B) Immunopurified CRL4^{Cdt2} complexes were mixed with recombinant wild-type or K164R mutant PCNA, E1, and UbcH5. Ubiquitinated PCNA was then detected by western blotting after separating the reaction products by SDS-PAGE.

(C) Same as in (A), except that the CRL4^{Cdt2} immunocomplexes were either purified from 293T cells or from 293T cells that were predepleted of Rad18 by siRNA. Immunoblots for Cdt2 (anti-Cdt2), Cul4A, and DDB1 (anti-myc) are also shown.

(D) Western blot of Rad18 to show effectiveness of si-Rad18 in (C).

USP1 showed a 5-fold increase in mutation frequency compared to cells with USP1 (Figure 4A). Sequencing of the mutant supF genes revealed a mutation spectrum consistent with error-prone TLS (Table S1). Interestingly, Cdt2 depletion significantly reduced the mutation frequency, indicating that the reduction in basal PCNA monoubiquitination seen after Cdt2 depletion in 293T cells (Figure 2B, the 0 hr time points) inhibits TLS activity in vivo. Similar results were obtained when 293T cells were depleted of DDB1 or Cul4A/B, but not upon depletion of Cul1 or DDB2 (Figure 4B). These results demonstrate that the inhibition of TLS activity was a consequence of the specific inactivation of CRL4^{Cdt2} complex. As shown in Figures 1C and 1D,

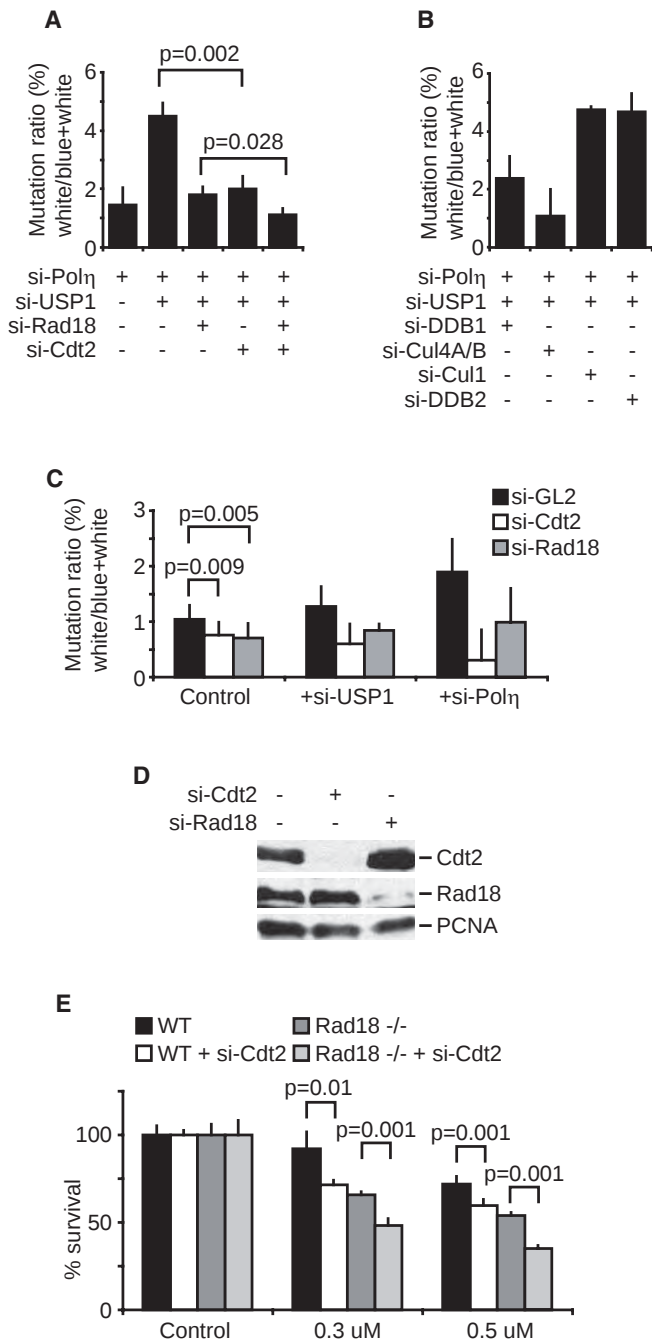


Figure 4. CRL4^{Cdt2} E3 Ubiquitin Ligase Complex Promotes Translesion DNA Synthesis in Unperturbed Proliferating Cells

(A–C) 293T cells were transfected with the indicated siRNA for 24 hr. UV-irradiated reporter plasmid was transfected into 293T cells. Replicated plasmids were recovered, DpnI digested, and used to transform bacteria. Mutation frequency (see the Experimental Procedures) is expressed as the ratio of white to total (blue and white) colonies. Mean $\pm$ SD of three experiments (A and B) or ten experiments (C).

(D) Western blot of Cdt2 and Rad18 to show effectiveness of si-Cdt2 and si-Rad18 in 293T cells.

(E) DT40 cells were transfected with si-Cdt2 for 24 hr and treated with cisplatin for 72 hr. Viable cells were counted and expressed relative to the control si-GL2 sample. Mean $\pm$ SD of three experiments.

codepletion of Cdt2 and Rad18 decreased basal PCNA monoubiquitination in a synergistic manner in some cells. Indeed, in 293T cells, codepletion of Cdt2 and Rad18 reduced the mutagenic frequency further than the individual depletions to almost background levels (Figure 4A). Thus, CRL4^{Cdt2} and Rad18 independently promote TLS in normal cycling 293T cells.

We next performed the experiment without USP1 depletion and/or polymerase eta depletion (Figure 4C). Under control conditions (when USP1 or polymerase eta was intact), Cdt2 depletion significantly decreased the mutation frequency much like si-Rad18, demonstrating that effect of CRL4^{Cdt2} on TLS reported above was not an artifact of USP1 or polymerase eta depletion. Similar results were obtained when USP1 or polymerase eta was individually depleted (Figure 4C). Together, these findings demonstrate that CRL4^{Cdt2} cooperates with Rad18 to promote PCNA-dependent TLS activity in unperturbed proliferating cells.

It has been shown that TLS activity and PCNA monoubiquitination are required for cell survival after DNA damage (Hoege et al., 2002; Kannouche et al., 2004; Simpson et al., 2006). We therefore tested the sensitivity of the Cdt2-depleted DT40 cells to cisplatin treatment. Cdt2-depleted cells were significantly more sensitive than the control DT40 cells (Figure 4E). The sensitization was also seen in DT40^{Rad18^{-/-}}, as expected from the Rad18-independent role of Cdt2 in monoubiquitinating PCNA. Collectively, these results demonstrate that CRL4^{Cdt2} monoubiquitinates PCNA to promote TLS and is required for cell survival after DNA damage.

DISCUSSION

CRL4^{Cdt2} and Rad18 Promote PCNA Monoubiquitination Synergistically in the Absence of Extrinsic DNA Damage

It is well established that Rad18 is essential for PCNA monoubiquitination in response to DNA damage (Shiomi et al., 2007; Watanabe et al., 2004; Yamashita et al., 2002). Our results support this conclusion. Several reports, however, demonstrated that in the absence of Rad18, PCNA continues to be monoubiquitinated (albeit to a lower extent) in a variety of cell lines (Brun et al., 2008; Huang et al., 2006; Simpson et al., 2006). We demonstrated here that CRL4^{Cdt2} monoubiquitinates PCNA independent of Rad18 in non-DNA-damaged cells. In vitro, CRL4^{Cdt2} was sufficient to promote PCNA monoubiquitination specifically at Lys164, the same residue that is ubiquitinated via Rad18. Although CRL4^{Cdt2} inactivation in mammalian cells did not prevent the induction of PCNA monoubiquitination after DNA damage, it prevented the monoubiquitination in normally cycling cells and delayed the kinetics of damage-induced monoubiquitination in several cells. Intriguingly, the depletion of Rad18 also decreased basal monoubiquitination of PCNA in some cell lines, and this is further decreased by CRL4^{Cdt2} inactivation in other cells. Thus, Rad18 and CRL4^{Cdt2} seem to function independently to promote basal PCNA monoubiquitination in normal cycling cells. It is important to note that the term “basal” used in this study refers to cells that are not subjected to extrinsic DNA damage, but of course, the basal monoubiquitination could result from intrinsic DNA damage caused by and/or sensed by replication forks.

CRL4^{Cdt2} Monoubiquitinates PCNA in DNA-Damaged Cells Independent of Rad18

In several human cell lines, the requirement of CRL4^{Cdt2} for PCNA monoubiquitination was observed also after treating the cells with UV or cisplatin, as it delayed, but did not prevent, the induction of PCNA monoubiquitination (Figure 2). Furthermore, Cdt2 is required not only for monoubiquitinating PCNA in undamaged cells but also in response to DNA damage in the Rad18-deficient DT40 cells. Thus it seems likely that although Rad18 is essential for PCNA monoubiquitination in all human cell lines tested, CRL4^{Cdt2} plays a supportive role to ensure optimal PCNA monoubiquitination after DNA damage.

Whereas CRL4^{Cdt2} appears to be required for monoubiquitinating PCNA in 293T, ES2, and MCF7 cells following DNA damage, it is dispensable in HeLa cells following UV irradiation (Figure S3). Additionally, although basal PCNA monoubiquitination in most of the cells we examined is dependent on Cdt2, there are rare exceptions, like HCT116 colon cancer cells, where Cdt2 does not appear to be required for PCNA monoubiquitination in undamaged cells (data not shown). We hypothesize that differences in levels (or activation) of PCNA, Rad6/Rad18, or CRL4^{Cdt2} in different cell lines account for the variable requirement of CRL4^{Cdt2} in basal or damage-induced monoubiquitination of PCNA in different cell lines.

PCNA Monoubiquitination via the CRL4^{Cdt2} E3 Ubiquitin Ligase Complex; A Surveillance Mechanism to Monitor and Respond to DNA Lesions Associated with Replication Stress

Because CRL4^{Cdt2}-dependent monoubiquitination of PCNA is constitutively counteracted by USP1, our data support the hypothesis that in normally cycling cells, PCNA monoubiquitination is regulated by the balanced activity of CRL4^{Cdt2} and USP1. We propose that stress associated with DNA replication may interfere with the activity of USP1, thus allowing CRL4^{Cdt2} to promote PCNA monoubiquitination necessary for bypassing DNA lesions. This hypothesis is supported by the observation that CRL4^{Cdt2} inactivation specifically impairs TLS activity in undamaged cells (Figure 4). Recently, it has been shown that polymerase η is required for surveillance of the genome and S phase progression in normally cycling cells (Rey et al., 2009), demonstrating the functional significance of regulating basal PCNA monoubiquitination in normally proliferating cells in the absence of external DNA damage and highlighting the significant role of CRL4^{Cdt2} in promoting such an activity. Since TLS is required for cell survival but increases mutation frequency, it is tempting to speculate that the overexpression of Cul4A or Cdt2 observed in some human cancer (Pan et al., 2006; Schindl et al., 2007) may promote malignancy by enhancing PCNA-dependent and TLS-mediated mutagenesis.

CRL4^{Cdt2} Induces Mono- and Polyubiquitination

Although CRL4^{Cdt2} complex promotes the polyubiquitination and degradation of several substrates, polyubiquitination of PCNA by CRL4^{Cdt2} was not detected in vitro. Monoubiquitination versus polyubiquitination by the same E3 ligase has been observed in other contexts. For example, the CRL4^{DDB2} E3 ubiquitin ligase not only monoubiquitinates histone H3 and H4 (Wang

et al., 2006) and promotes the monoubiquitination of Histone H2A at UV-induced DNA damage sites (Kapetanaki et al., 2006; Guerrero-Santoro et al., 2008), it also promotes the polyubiquitination of XPC protein (Sugasawa et al., 2005). Since monoubiquitinated PCNA has a high-affinity interaction with Y family polymerases through an ubiquitin-binding domain and a PIP box, it is possible that the bound polymerase sterically hinders the continued interaction of PCNA with CRL4^{Cdt2}. In such a model, it is also possible that PCNA monoubiquitination could inhibit CRL4^{Cdt2}-dependent polyubiquitination of other substrates by displacing the CRL4^{Cdt2} from the substrate-bound PCNA ring.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents

HeLa, 293T, and U2OS cell lines were obtained from ATCC. Wild-type DT40 and DT40^{Rad18-/-} cells were kindly provided by Shunichi Takeda (Kyoto University). The antibodies were purchased as indicated: against PCNA (PC10), Cul1 (C-5), Cul4 (C-19) (Santa Cruz Biotechnologies); against Rad18, USP1, DDB2 (Rockland Immunochemicals); and against DDB1 (Invitrogen).

Gene Silencing by RNAi

siRNA transfections into human cell lines were performed using oligofectamine (Invitrogen). For introducing siRNA into DT40 cells, Amaxa Nucleofector Kit T was used. The following siRNA oligonucleotides were purchased from Invitrogen: polymerase η , 5'-GUGGAGCAGCGGCAAAUUCdTdT-3' human USP1, 5'-AGCUACAAGUGAUACAUUAdTdT-3'; chicken USP1, 5'-AGUGAAAGUUACAGAGGAAdTdT-3'; and chicken Cdt2, 5'-CCAAAGAGCUUGUGAU GAAdTdT-3'.

Other siRNA oligonucleotides were described before (Abbas et al., 2008). mRNA of β -actin, Cdt2, and USP1 in DT40 cells were measured by using the following primers: USP1, 5'-GCTTTGTGCATCTCCAACACAGG-3' and 5'-CACCTATTACACACTTTGC-3'; Cdt2, 5'-CCTTACAGATTGAGCCTGAAGC-3' and 5'-GCTAGTGATCTTGTCTACC-3'; and β -actin, 5'-CATTGCTGACAGG ATGCAAGG-3' and 5'-TGCTTGCTGATCCACATCTGCTGG-3'.

In Vitro Ubiquitination Assays

The purification of CRL4^{Cdt2} complex was previously described (Abbas et al., 2008). 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 recombinant wild-type or a K164A mutant of PCNA in ubiquitination reaction buffer. The ubiquitination reactions were incubated at 30°C for 1 hr and terminated by adding Laemmli sample buffer. Proteins were resolved by SDS-PAGE and immunoblotted with anti-PCNA or anti-ubiquitin antibody (Sigma).

Mutation Frequency Assays

293T cells were transfected with siRNAs as described above. Twenty-four hours later, pSP189 plasmids (Parris and Seidman, 1992) were irradiated with UV (1000 J/m²) and transfected into the cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours after, cells were harvested for plasmid purification using DNA miniprep kit (QIAGEN). Purified plasmids were DpnI digested and introduced by electroporation into the MBM7070 bacterial strain. The mutation frequency in the SupF coding region was analyzed by counting the ratio between blue (wild-type) and white (mutant) colonies. Mutations in the SupF coding region were confirmed by sequencing.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at doi:10.1016/j.molcel.2009.12.018.

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