

Rad18 guides pol η to replication stalling sites through physical interaction and PCNA monoubiquitination

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The DNA replication machinery stalls at damaged sites on templates, but normally restarts by switching to a specialized DNA polymerase(s) that carries out translesion DNA synthesis (TLS). In human cells, DNA polymerase η (pol η) accumulates at stalling sites as nuclear foci, and is involved in ultraviolet (UV)-induced TLS. Here we show that pol η does not form nuclear foci in *RAD18*^{-/-} cells after UV irradiation. Both Rad18 and Rad6 are required for pol η focus formation. In wild-type cells, UV irradiation induces relocalization of Rad18 in the nucleus, thereby stimulating colocalization with proliferating cell nuclear antigen (PCNA), and Rad18/Rad6-dependent PCNA monoubiquitination. Purified Rad18 and Rad6B monoubiquitinate PCNA *in vitro*. Rad18 associates with pol η constitutively through domains on their C-terminal regions, and this complex accumulates at the foci after UV irradiation. Furthermore, pol η interacts preferentially with monoubiquitinated PCNA, but pol δ does not. These results suggest that Rad18 is crucial for recruitment of pol η to the damaged site through protein–protein interaction and PCNA monoubiquitination.

The EMBO Journal (2004) 23, 3886–3896. doi:10.1038/sj.emboj.7600383; Published online 9 September 2004

Subject Categories: genome stability & dynamics

Keywords: PCNA; polymerase η ; Rad6; Rad18; ubiquitination

Introduction

Exposure of cells to ultraviolet (UV) light causes several types of DNA damage. Among these, cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts are major DNA lesions. In normal vertebrate cells, 6-4 photoproducts are efficiently repaired by nucleotide-excision repair, but nearly 50% of CPDs remain unrepaired even at 24 h after UV irradiation

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Received: 3 May 2004; accepted: 4 August 2004; published online: 9 September 2004

(Mitchell and Nairn, 1989). In such a situation, the DNA replication machinery often encounters the lesion during the S-phase of the cell cycle, and stalls at the replication fork, resulting in a gap opposite the site of damage in the newly synthesized DNA strand. Cell death may be imminent unless the gap is filled. This gap-filling process is operationally defined as postreplication repair (PRR), which is characterized by restarting of DNA replication without removal of the lesion on a template strand. PRR is observed in diverse species from *Escherichia coli* to humans. It is hypothesized that PRR is mediated by either translesion DNA synthesis (TLS) or recombination to resolve the stalled replication fork (Broomfield *et al*, 2001).

In the budding yeast *Saccharomyces cerevisiae*, genes belonging to the *RAD6* epistasis group are involved in the PRR pathway, where Rad18 (a putative ubiquitin ligase) and Rad6 (a ubiquitin-conjugating enzyme, E2) play a pivotal role (Bailly *et al*, 1994, 1997a). *rad6* and *rad18* mutants are highly susceptible to various DNA-damaging agents including UV and methylmethanesulfonate (MMS) (Hynes and Kunz, 1981). *rad6* and *rad18* mutants, however, show reduced mutation frequency following treatments with UV and MMS, possibly because error-prone TLS does not work without Rad18/Rad6. Because Rad18 protein binds to single-stranded DNA and forms a tight complex with Rad6 protein (Bailly *et al*, 1994, 1997b), it is proposed that Rad18 recruits Rad6 protein at replication stalling sites through binding to gap regions, and that the Rad18 complex ubiquitinates some target molecules on the stalled replication forks. Recently, proliferating cell nuclear antigen (PCNA) was shown to be monoubiquitinated in a Rad18/Rad6-dependent manner, which is necessary for tolerance to DNA damage (Hoege *et al*, 2002; Stelter and Ulrich, 2003). Interaction with PCNA is essential for the function of Rad30 (Haracska *et al*, 2001a), a yeast homolog of polymerase η , which is a member of *RAD6* epistasis group (McDonald *et al*, 1997). These results suggest that PCNA might be a major target of Rad18/Rad6 in the PRR process.

In vertebrate cells, thus far only a single homolog of *RAD18* has been identified (Tateishi *et al*, 2000). Human and mouse Rad18 interacts with two forms of the Rad6 homolog, Rad6A and Rad6B, both *in vitro* and *in vivo* (Tateishi *et al*, 2000, 2003; Xin *et al*, 2000). *RAD18* knockout mouse embryonic stem (ES) cells and chicken DT40 cells manifest sensitivity to various DNA-damaging agents and enhanced genomic instability as determined by increased sister-chromatid exchange (SCE) and frequency of stable transformation (Yamashita *et al*, 2002; Tateishi *et al*, 2003).

Vertebrate polymerase η (pol η), a homolog of the *RAD30* gene product of the yeast, is a member of a recently discovered Y-family of novel DNA polymerases including pol ι and pol κ (Burgers *et al*, 2001; Ohmori *et al*, 2001). They are shown to be involved in TLS *in vitro*, and structurally related

to each other, but unrelated to the replicative polymerases (pol δ and pol ϵ). Pol η has a highly distributive, rather than processive, mode of DNA synthesis on undamaged templates and a relatively low stringency (Johnson *et al*, 2000; Matsuda *et al*, 2000). However, pol η can insert correct nucleotides opposite CPDs in TLS (Johnson *et al*, 1999; McCulloch *et al*, 2004). The gene encoding pol η is mutated in a cancer-prone hereditary disorder, xeroderma pigmentosum variant (XPV) (Masutani *et al*, 1999). It is possible that without normal pol η CPD becomes highly mutagenic probably due to TLS by some other error-prone polymerase(s), resulting in skin cancers of sun-exposed areas.

When the replicative machinery encounters unrepaired CPD lesions, it is expected that the replicative polymerase is switched to pol η to carry out TLS in normal cells. In UV-irradiated human cells, pol η forms discrete nuclear foci in a UV-dose- and time-dependent manner (Kannouche *et al*, 2001). The sites of these foci are colocalized with PCNA, suggesting that these are sites of stalled replication. Because a pol η deletion mutant, which has a polymerase activity but does not show focus formation following UV irradiation, cannot complement the sensitivity of XPV cells to UV irradiation, foci formation is an essential step of pol η function. However, molecular mechanisms of how pol η forms nuclear foci in UV-irradiated cells are largely unknown.

In the study reported here, in order to understand the role of Rad18 in tolerance to UV-induced DNA damage, we used *RAD18*^{-/-} mouse fibroblasts from *RAD18* knockout mice to show that Rad18 functions as an essential coordinator of the formation of pol η foci through PCNA monoubiquitination and physical interaction with pol η .

Results

Requirement of Rad18 for PCNA monoubiquitination

To investigate the role of Rad18 in UV-induced TLS, we established cell lines from *RAD18* knockout mice (Tateishi *et al*, 2003). These cells did not express detectable levels of Rad18 protein, but showed normal levels of Rad6A/B (Figure 1A) and normal growth rates (Figure 1B). In wild-type (WT) cells, a band of PCNA corresponding to 44 kDa increased in a UV dose- and time-dependent manner, while in *RAD18*^{-/-} cells, the band remained at the control level up to 8 h after UV irradiation even at 40 J/m² (Figure 1C and D). Similar modification of PCNA in MMS-treated HeLa cells was reported (Hoegge *et al*, 2002). We concluded that this band represented a monoubiquitinated form of PCNA for two reasons. (i) Unmodified PCNA was detected at 36 kDa in SDS-PAGE (Figure 1C-E) and, when lysates of UV-irradiated cells expressing transfected HA-tagged ubiquitin were immunoprecipitated, bands of 45 and 44 kDa were detected by immunoblotting with an anti-HA antibody and anti-PCNA antibody, respectively (Figure 1E, lanes 2 and 3). (ii) Unmodified PCNA was converted to a 44 kDa band of monoubiquitinated PCNA *in vitro* by purified Rad18 and Rad6B of human origin plus ubiquitin (Figure 1E, lanes 7, 11, and 12). When ubiquitin was replaced with FLAG-tagged ubiquitin in this system, a 45 kDa band appeared (Figure 1E, lane 13). These results indicate that Rad18 is a ubiquitin ligase for the monoubiquitination of PCNA.

In budding yeast, Rad18 binds to Rad6 through its Rad6-binding domain (R6BD) (Bailly *et al*, 1997b). This domain is

highly conserved among various species. To confirm that the putative R6BD in hRad18 (amino-acid residues 340–395; Figure 2A) was a binding site for Rad6A/B, we transfected a Rad18 plasmid lacking R6BD together with a Rad6A/B plasmid into COS-7 cells, and performed co-immunoprecipitation experiments. Rad18 protein lacking R6BD localized in the nuclei like WT Rad18 (data not shown), but did not interact with human Rad6A/B (Figure 2A, lanes 1, 2, 5, and 6). To confirm whether the failure of PCNA ubiquitination in *RAD18*^{-/-} cells was really due to a defect in Rad18, we established multiple *RAD18*^{-/-} cell clones stably expressing WT human Rad18 (hRad18) (Figure 2B). PCNA ubiquitination following UV irradiation was restored to the WT level, whereas control *RAD18*^{-/-} cells transfected with an empty vector did not show such recovery (Figure 2B). To examine whether PCNA ubiquitination required Rad6A/B together with Rad18, we established *RAD18*^{-/-} cells stably expressing Rad18 but lacking R6BD (hRad18DR6). In these cells, PCNA was not ubiquitinated after DNA damage (Figure 2B). Furthermore, to confirm the requirement of Rad6A/B for PCNA monoubiquitination directly, Rad6 siRNA corresponding to both Rad6A and Rad6B was transfected into human cells, and reduced levels of Rad6A/B protein levels were confirmed by Western blot. In these cells, PCNA monoubiquitination was substantially reduced (Figure 2C). These results clearly indicate that in UV-irradiated mammalian cells, PCNA is monoubiquitinated in a Rad18- and Rad6A/B-dependent manner. To evaluate the significance of the monoubiquitination activity of Rad18, we determined the UV sensitivity of *RAD18*^{-/-} mouse cells stably expressing hRad18. These cells showed almost normal UV sensitivity, while stable transformants with hRad18 lacking R6BD, or with the vector alone, remained sensitive to UV at the parent cell levels (Figure 2D). These results suggest that the UV sensitivity of *RAD18*^{-/-} cells is caused at least in part by defects in the monoubiquitination of PCNA and subsequent foci formation of pol η .

Relocalization of Rad18 at stalling sites with PCNA

In mammalian cells fixed with formaldehyde, a substantial fraction of Rad18 was homogeneously localized in the nucleus, while the remaining fraction existed as dots of irregular shapes and sizes (Figure 3A, left). Notably, most of the nuclear dots of Rad18 dispersed throughout the nucleus within 15 min with UV doses as low as 5 J/m² (Figure 3A, middle). Rad18 dispersion occurred in the presence of cycloheximide (data not shown), suggesting that direct or indirect post-translational modification of Rad18 is involved in this process. Within a few hours after UV irradiation, nuclear foci of Rad18 with uniform sizes appeared (Figure 3A, right). Such dynamic intranuclear translocation of Rad18 was much more clearly detected in cells fixed with methanol (Figure 3B). To investigate the relationship between Rad18 and PCNA, we performed double immunostaining on methanol-fixed cells. Under normal conditions, partial colocalization of Rad18 with PCNA was observed (Figure 3B, upper). Within 1 h after UV irradiation, almost all of Rad18 became colocalized with PCNA (Figure 3B, lower) and such colocalization was observed at least up to 4 h, suggesting that Rad18 translocates to the replication stalling sites. To confirm directly this assumption, UV-irradiated cells were labeled with BrdU and stained for Rad18 and incorporated BrdU. Before UV

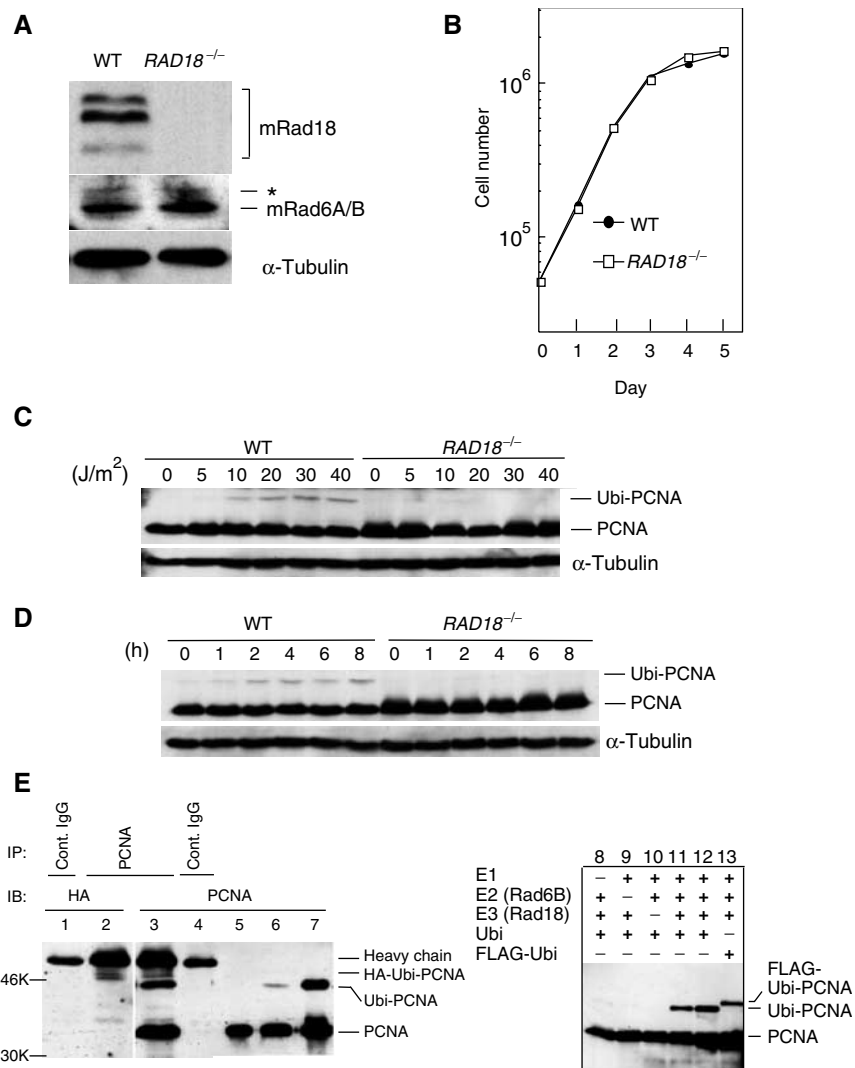


Figure 1 Rad18 dependent monoubiquitination of PCNA by Rad18 and Rad6A/B *in vivo* and *in vitro*. (A) Western blot of Rad18 and Rad6A/B in *RAD18*^{-/-} cells. α -Tubulin was included as a control. An asterisk shows nonspecific bands. (B) Growth curves of *RAD18*^{-/-} cells. (C, D) Monoubiquitination of PCNA as determined by Western blot. Cells were harvested 5 h later following various doses of UV irradiation (C). In (D), cells were irradiated at 30 J/m² and harvested at the indicated times. (E) *In vivo* (left, lanes 1–6) and *in vitro* (right, lanes 7–13) monoubiquitination of PCNA. GM637 cells were transfected with HA-ubiquitin (lanes 1–4) and irradiated with UV (13 J/m², 6 h). Lysates were immunoprecipitated and blotted as indicated. In lanes 5 and 6, GM637 cells without transfection were irradiated at 0 and 13 J/m² (6 h), respectively. Lane 7 represents an *in vitro* ubiquitination product. In lane 12, two-fold amounts of E2 and E3 were included in the reaction.

irradiation, BrdU sites were partially colocalized with Rad18 (Figure 3C, upper), but after UV irradiation most of the BrdU sites were colocalized with translocated Rad18 (Figure 3C, lower). Since colocalization of Rad18 with PCNA was observed in XPV cells with a similar time course, it was inferred that translocation of Rad18 does not require functional pol η (data not shown). To determine the subnuclear localization of PCNA, chromatin fractions were separated from UV-irradiated cells. Almost equal amounts of unmodified PCNA were obtained in the soluble and chromatin fractions irrespective of UV irradiation. In contrast, monoubiquitinated PCNA was exclusively recovered in the chromatin fraction of UV-irradiated cells, and it moved to the solubilized nuclear fraction after treatment with micrococcal nuclease (Figure 3D), suggesting that monoubiquitinated PCNA is tightly associated with chromatin. We could not detect any apparent physical interaction between Rad18 and PCNA before or after UV irradiation by co-immunoprecipitation,

suggesting that the interaction is weak or transient (data not shown).

Requirement of Rad18 for pol η focus formation

Using pol η fused to enhanced green fluorescent protein (eGFP-pol η), Kannouche *et al* (2001) found that pol η , which localizes uniformly in the nucleus under normal conditions, formed distinct nuclear foci at the replication stalling sites after treatment with DNA-damaging agents including UV and MMS. This pol η focus formation is essential for UV survival, because mutant pol η , which is defective in focus formation, could not complement UV survival of XPV cells (Kannouche *et al*, 2001). To investigate whether Rad18 is required for pol η focus formation, we introduced eGFP-hpol η into either *RAD18*^{-/-} or *RAD18*^{+/+} mouse cells. While pol η focus formation was clearly observed in the UV-irradiated WT cells, pol η remained uniformly dispersed in the nucleus of UV-irradiated *RAD18*^{-/-} cells (Figure 4A). The

formation of pol η foci proceeded gradually and reached a plateau at 6 h after UV irradiation at least with dosages ranging 10–20 J/m² (Figure 4B). Defective focus formation in *RAD18*^{-/-} cells could be restored by concomitant introduction of WT hRad18 with or without a FLAG tag in its N-terminal region (Figure 4A and C, data not shown). However, hRad18 lacking R6BD did not restore the focus formation (Figure 4C). Furthermore, the formation of pol η foci was significantly inhibited in cells treated with Rad6A/B siRNA (Figure 4D). These results indicate that UV-induced pol η focus formation is dependent on both Rad18 and Rad6A/B.

Association of Rad18 with pol η

Immunostaining for Rad18 clearly demonstrated that Rad18 colocalized with eGFP-pol η at the foci following UV irradiation

(Figure 5A, lower). To investigate the interaction between Rad18 and pol η , HA-tagged pol η was transfected into GM637 cells, and co-immunoprecipitation experiments were performed. Rad18 was consistently associated with pol η , irrespective of UV irradiation (Figure 5B). Furthermore, purified pol η bound to purified Rad18 in an immunoprecipitation assay, but pol δ did not (Figure 5C, lanes 2 and 4), indicating that at least a part of Rad18 is directly associated with pol η in a UV-independent manner. To determine the binding site of pol η to Rad18, we overexpressed a series of deletion mutants of pol η fused with GST at their N-terminal regions (Figure 6A) in insect cells, and purified them with glutathione beads (Figure 6B). GM637 cell lysates were pulled down with these beads. Rad18 interacted with full-length pol η and a C-terminal fragment of pol η (GST-pol η 158c) spanning amino-acid residues 556–713 (Figure 6C). We also determined the binding site of Rad18 to pol η in a similar way. In this assay, Myc-tagged WT and deleted Rad18 proteins were overexpressed in COS-7 cells (Figure 6D and E), and cell lysates were pulled down with glutathione beads associated with GST-pol η 158c. Among the deletion mutants, only Rad18 lacking a region spanning amino-acid residues 402–444 could not interact with pol η (Figure 6F). To evaluate the biological significance of the interaction between Rad18 and pol η , hRad18 lacking the pol η -binding domain (hRad18DC2) was transiently expressed in *RAD18*^{-/-} mouse cells together with eGFP-pol η . Formation of eGFP-pol η foci was not restored following UV irradiation (Supplementary Figure S1). Furthermore, *RAD18*^{-/-} cells stably expressing Rad18 lacking the pol η -binding domain showed high UV sensitivity like cells transformed with an empty vector (Supplementary Figures S2 and S3), while they had normal levels of monoubiquitination of PCNA after UV irradiation. These results suggest that Rad18 recruits pol η to replication stalling sites through direct interaction. Since eGFP-pol η localized uniformly in the nucleus with Rad18 under normal conditions (Figure 5A), the nuclear dots of Rad18 in nonirradiated cells might be reservoirs of free Rad18.

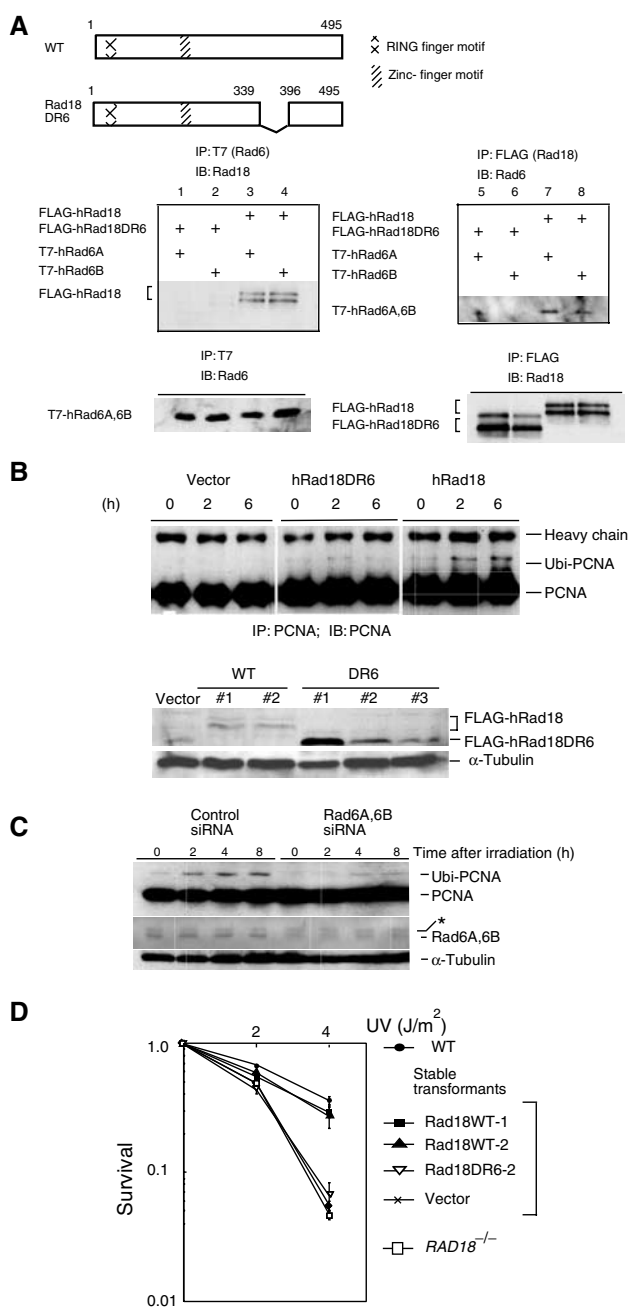


Figure 2 Requirement of Rad6A/B for monoubiquitination of PCNA in UV-irradiated cells. (A) Interaction of WT and mutant hRad18 with hRad6A/B. Full-length WT and mutant Rad18 proteins are schematically shown on the top panel. Plasmids were transfected into COS-7 cells with different combinations indicated on the left of the middle panels, and immunoprecipitation was performed. Similar levels of expression of hRad18 and hRad6A/B proteins in the transformed cells were confirmed in the lower panel. (B) Restoration of PCNA monoubiquitination in *RAD18*^{-/-} cells by expression of WT hRad18 but not of mutant hRad18. Cells were incubated for 6 h following UV irradiation at 20 J/m². Cell lysates were immunoprecipitated and blotted with an anti-PCNA antibody (upper panel). Expression of FLAG-hRad18 or FLAG-Rad18DR6 was confirmed in individual clones of stable transformants of *RAD18*^{-/-} mouse fibroblasts by Western blot with an anti-Rad18 rabbit antibody (lower panel). α -Tubulin was indicated as a volume control. (C) Inhibition of PCNA monoubiquitination by siRNA for Rad6A/B. WI38VA13 cells were transfected with Rad6A and Rad6B siRNA, incubated for 4 days, and then irradiated with 10 J/m² of UV light. At the indicated times, protein levels of monoubiquitinated PCNA were determined by Western blot. An asterisk shows a nonspecific band that remained constant following the siRNA treatment. (D) Restoration of UV sensitivity of *RAD18*^{-/-} mouse cells by introduction of human Rad18 as determined by a colony-forming assay. Two independent clones of stable transformants (WT#1 and WT#2 in (B)) were tested.

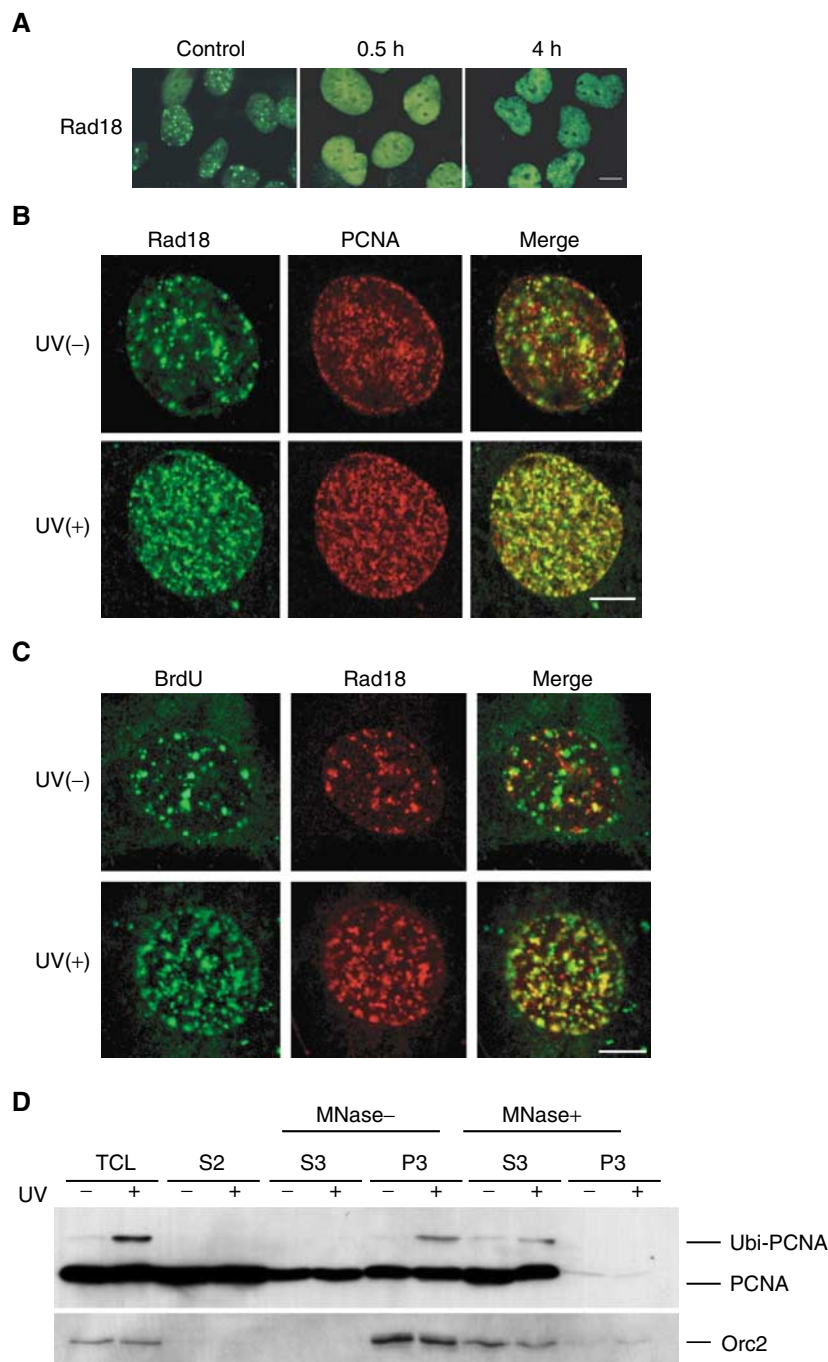


Figure 3 Colocalization of Rad18 with PCNA on chromatin following UV irradiation. (A) Dispersion and relocation of Rad18. GM637 cells irradiated at 15 J/m^2 were fixed with formaldehyde and stained for Rad18. Bar = $20\ \mu\text{m}$. (B) UV-induced colocalization of Rad18 with PCNA. GM637 cells irradiated at 15 J/m^2 were fixed with methanol 4 h after UV irradiation and processed for double staining for Rad18 (green) and PCNA (red). Bar = $10\ \mu\text{m}$. (C) Accumulation of Rad18 at the replication stalling sites. UV-irradiated (15 J/m^2) GM637 cells were labeled for 2 h with BrdU, fixed with methanol, and processed for double staining for Rad18 (red) and BrdU (green). Bar = $10\ \mu\text{m}$. (D) Binding of monoubiquitinated PCNA to chromatin. Chromatin fractions were isolated from UV-irradiated (15 J/m^2 , 6 h) or nonirradiated HeLa cells, and then treated with micrococcal nuclease (MNase). The distributions of PCNA in the total cell lysate (TCL), soluble nuclear fraction (S2), solubilized nuclear fraction (S3), and chromatin-enriched fraction (P3) are shown. Orc2 is shown as a chromatin fraction marker.

Preferential binding of pol η to monoubiquitinated PCNA

To investigate the molecular mechanism of how UV-induced monoubiquitination of PCNA functions in polymerase switching to pol η , the physical interaction between PCNA and pol η was determined by a pull-down assay. GST-pol η bound to glutathione beads was mixed with lysates prepared from UV-irradiated HeLa cells, and PCNA associated with the

GST-pol η beads was revealed by Western blot. While monoubiquitinated PCNA was a minor fraction of the total PCNA in the lysates, it was recovered predominantly from the precipitated beads in a time-dependent manner (Figure 7A, right). In contrast, monoubiquitinated PCNA was not associated with GST-pol δ in the same assay (Figure 7A, middle). The affinity of monoubiquitinated PCNA for pol η was much

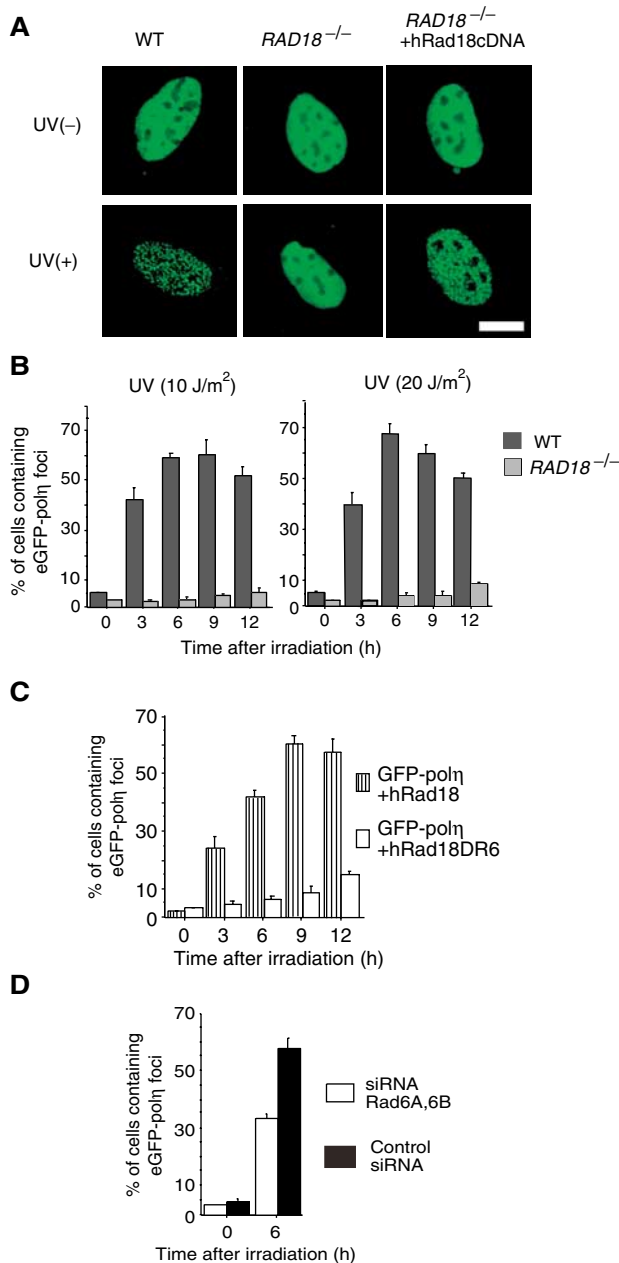


Figure 4 Rad18- and Rad6-dependent formation of pol η foci. (A) Focus formation of eGFP-pol η following UV irradiation in WT cells but not in *RAD18*^{-/-} cells. Cells were irradiated at 15 J/m². After 6 h, the distribution of eGFP-pol η was examined after fixation. Defective focus formation of pol η was recovered by concomitant expression of Rad18. Bar = 10 μ m. (B) Time course of eGFP-pol η focus formation in UV-irradiated cells. *RAD18*^{-/-} mouse cells and WT cells were transfected with eGFP-pol η . After 20 h, cells were irradiated with UV at the indicated doses. (C) Restoration of eGFP-pol η focus formation in UV-irradiated (20 J/m²) *RAD18*^{-/-} cells by expression of WT hRad18 but not of mutant hRad18 lacking the Rad6-binding domain. (D) Inhibition of pol η focus formation by siRNA for Rad6. WI38VA13 cells were transfected with Rad6A and Rad6B siRNA, cultured for 3 days, and then transfected again with an eGFP-pol η plasmid. After 20 h, cells were irradiated with UV (10 J/m²), and 6 h later cells containing eGFP-pol η foci were counted.

higher than that of unmodified PCNA, because even at higher salt concentrations, monoubiquitinated PCNA remained bound to pol η (Figure 7B, left). Monoubiquitinated PCNA bound to pol η was much more refractory to elution by high

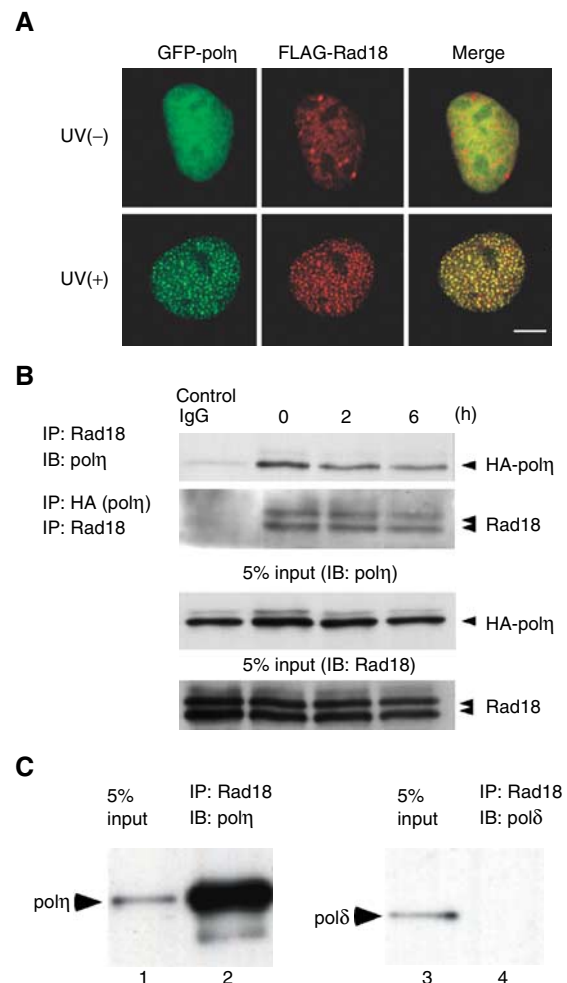
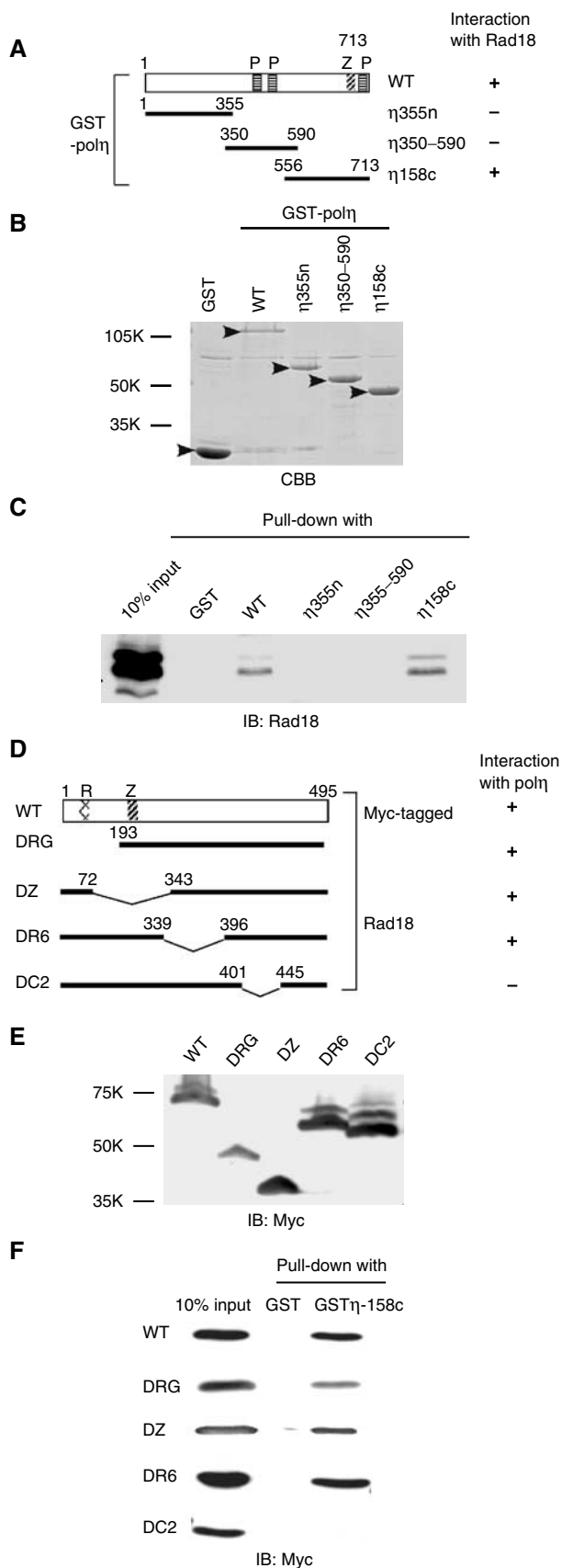


Figure 5 Direct interaction of pol η with Rad18. (A) UV-induced colocalization of Rad18 with eGFP-pol η in GM637 cells. Cells transfected with eGFP-pol η and FLAG-Rad18 plasmids were irradiated at 15 J/m² and incubated for 6 h. After fixation, cells were stained for Rad18 with an antibody against FLAG. Bar = 10 μ m. (B) Interaction of Rad18 with pol η . HA-pol η was transiently expressed in GM637 cells. Immunoprecipitation was performed at various times after UV irradiation (12.5 J/m²). As a control, UV-irradiated cell lysates (6 h) were immunoprecipitated with control IgG. (C) Direct binding of Rad18 with pol η . Recombinant Rad18 and pol η were purified from insect cells. After incubation of the mixture, Rad18 was immunoprecipitated and pol η bound to Rad18 was detected by Western blot. Pol δ was used as a control.

salt concentrations than unmodified PCNA (Figure 7B, right). To investigate whether pol η interacted with monoubiquitinated PCNA in UV-irradiated cells, HA-pol η was transiently expressed in GM637 cells, and co-immunoprecipitation assay was performed. In this experiment, cells were treated with 0.1% NP-40 before preparation of cell lysates. This treatment allowed specific crosslinking between chromatin-bound pol η and monoubiquitinated PCNA probably by excluding unmodified PCNA and a diffused form of pol η from nuclei. Monoubiquitinated PCNA was preferentially immunoprecipitated with HA-pol η in the UV-irradiated cells (Figure 7C, lanes 5 and 6). In contrast, monoubiquitinated PCNA was not immunoprecipitated in nonirradiated cells (Figure 7C, lanes 2 and 3). Taken together, these results indicate that pol η preferentially binds to monoubiquitinated PCNA both *in vitro* and *in vivo*. To prove that the interaction between pol η and

monoubiquitinated PCNA is direct, PCNA was monoubiquitinated in the *in vitro* PCNA ubiquitination reaction (Figure 1E). Rad18 and Rad6B were then removed from the

in vitro PCNA ubiquitination reaction mixture (Figure 1E) by multiple cycles of immunodepletion with an anti-Rad18 antibody (Figure 7D, upper). Immunodepletion of Rad18 and Rad6B was confirmed by Western blot. Monoubiquitinated PCNA still bound to pol η in a pull-down assay (Figure 7D, lane 3). In contrast, pol η lacking the three putative PCNA-binding sites on the C-terminus (Kannouche *et al*, 2001) showed no interaction with PCNA (Figure 7D, lane 2). Immunostaining demonstrated that more than 50% of the transfected eGFP-pol η colocalized with endogenous pol δ 5 h after UV irradiation (Figure 7E). Furthermore, endogenous pol δ colocalized with PCNA in UV-irradiated cells (Figure 7F), suggesting that both polymerases and Rad18 localize at the same stalling sites.



Discussion

Tolerance to UV-induced DNA damage involves damage-induced PCNA monoubiquitination (Hoegge *et al*, 2002) and the formation of pol η foci at the replication stalling sites, thereby inducing TLS by pol η . We have addressed two important questions: How is pol η recruited to replication stalling sites after UV irradiation? And, what is the function of monoubiquitinated PCNA in the PRR process? In the work presented here, we analyzed these processes using mammalian cells, and found that Rad18 plays pivotal roles as a coordinator in both PCNA monoubiquitination and the formation of pol η foci.

Pol η synthesizes DNA with a low fidelity, misincorporating nucleotides with a frequency of 10^{-2} – 10^{-3} (Washington *et al*, 1999). Therefore, to prevent incidental mutagenesis, it is critical to restrict accession of pol η to DNA replication sites under normal conditions. This task seemed to be performed by regulation of intranuclear localization of pol η . Pol η localizes uniformly in the nucleus under normal conditions, but relocates at discrete nuclear foci following UV irradiation (Kannouche *et al*, 2001). We found that Rad18 interacts directly with pol η in cells irrespective of genotoxic stresses (Figure 5B). Purified pol η bound to Rad18, while pol δ did not (Figure 5C). Rad18 relocated to the replication stalling sites in response to UV irradiation, where pol η and PCNA colocalized (Figures 3B and 5A). These results support the view that Rad18 and pol η relocate to replication stalling sites simultaneously as a complex and form nuclear foci. Furthermore, pol η focus formation requires Rad6A/B, because *RAD18*^{-/-} cells expressing mutant Rad18 lacking the Rad6A/B-binding domain or normal cells suppressed expression of Rad6A/B by siRNA show little or reduced focus formation, respectively.

Figure 6 Determination of binding sites. (A) Structural domains of GST-pol η fusion proteins. P: putative PCNA-binding domain; Z: zinc-finger domain. (B) Purification of GST-pol η fusion proteins by glutathione beads. Proteins bound to the beads were stained with Coomassie brilliant blue (CBB, arrowheads). (C) Pull-down assay. GM637 cell lysates were pulled down with GST-pol η fusion proteins bound to glutathione beads. Interaction with Rad18 was analyzed by Western blot. (D) Structural domains of Myc-tagged Rad18 proteins. R: RING finger domain; Z: zinc-finger domain. (E) Deletion mutant proteins were overexpressed in COS-7 cells and their expression was confirmed by Western blot. (F) COS-7 cell lysates containing Myc-tagged mutant Rad18 proteins were pulled down with GST-pol η 158c bound to glutathione beads. Association of WT and mutant Rad18 proteins with pol η was analyzed by Western blot using an anti-Myc antibody.

These results indicate that both Rad18 and Rad6A/B are essential for pol η focus formation. When cells are exposed to UV irradiation, CPDs form on both the leading and the

lagging strands. In either case, single-stranded gap regions are formed on the 3' side of the lesion by uncoupling replication (Svoboda and Vos, 1995). Because Rad18 can bind to single-stranded DNA (Bailey *et al*, 1994), Rad18 together with pol η might be located at the gaps where replicative machinery containing PCNA and pol δ stalled on the template DNA. In the initial step of this pathway, Rad18 might be activated through a mechanism sensing stalling of replication. We observed that some fractions of Rad18 exist as nuclear dots under normal conditions, and that, following UV irradiation, these dots disperse throughout the nucleoplasm in a short time. Rad18 dispersion is also observed in cells treated with hydroxyurea (data not shown), suggesting that it is triggered not by DNA damage but by stalling of replication. In both cases, Rad18 dispersion takes place even under conditions where protein synthesis is inhibited. It is possible that post-translational modification (e.g. phosphorylation) of Rad18 is involved in the reaction.

The binding site of pol η to Rad18 is located on the C-terminal region, which contains one putative PCNA-binding site and a zinc-finger domain (Figure 6A). It is reported that pol η lacking a C-terminal region, which overlaps with the Rad18-binding domain, does not show pol η focus formation following UV irradiation, and that a fragment of pol η containing the C-terminal 120 amino-acid residues is able to form UV-induced foci (Kannouche *et al*, 2001). Furthermore, clinical manifestations of XPV patients with large deletions in the C-terminal region of pol η (class I) are similar to those of XPV patients with small deletions corresponding to the Rad18-binding domain (class III) (Broughton *et al*, 2002). Probably, inability of pol η to form a complex with Rad18 may be a primary cause of dysfunction of pol η in these cases.

As in the case of yeast (Hoegge *et al*, 2002), we showed that PCNA is monoubiquitinated in UV-irradiated mammalian cells in a Rad18- and Rad6-dependent manner. The PCNA monoubiquitination reaction is reconstituted *in vitro* by the presence of human Rad18 and Rad6B proteins (Figure 1E),

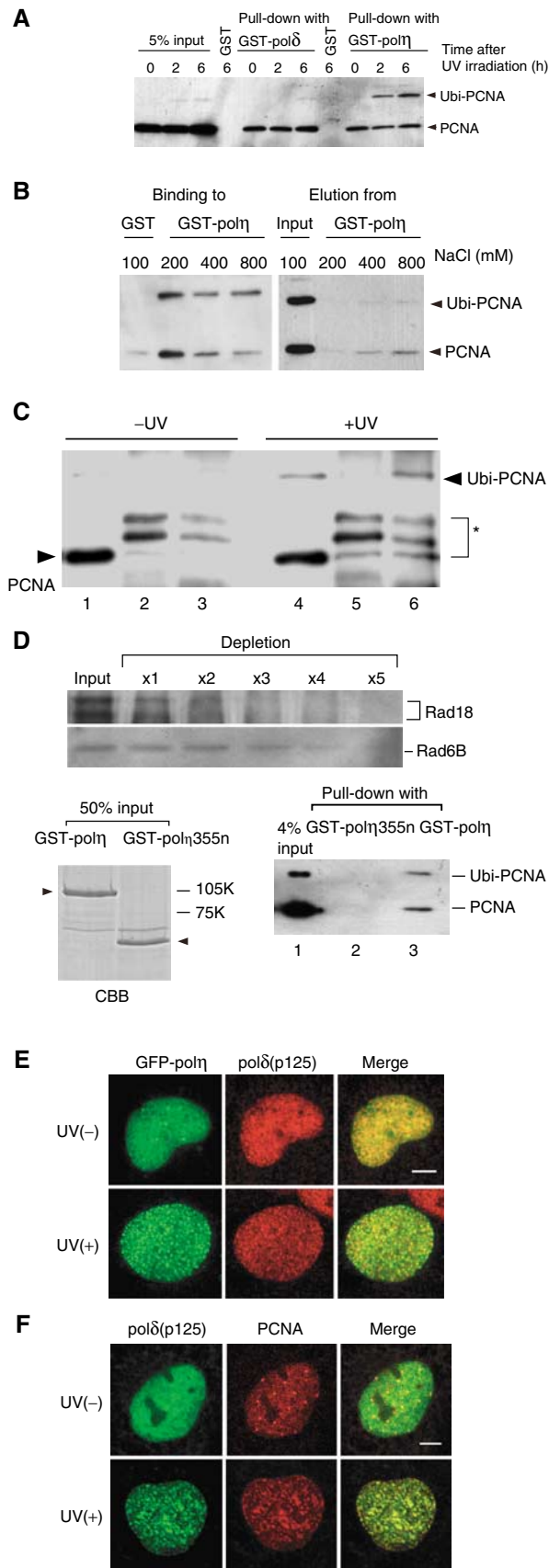


Figure 7 Preferential binding of pol η to monoubiquitinated PCNA. (A) Binding of pol η to ubiquitinated PCNA. HeLa cells were irradiated with UV at 20 J/m². PCNA in the cell lysates was pulled down with either GST-pol η beads or pol δ beads, and analyzed by Western blot using an anti-PCNA antibody. (B) Effects of different salt concentrations on the binding of PCNA to GST-pol η (left) and on PCNA elution from GST-pol η (right). PCNA pulled down was washed with buffer containing various concentrations of NaCl. PCNA in bound or eluted fractions was analyzed as in (A). (C) Preferential binding of pol η to monoubiquitinated PCNA in living cells. GM637 cells were transfected with an HA-pol η plasmid. After 2 days, these cells were irradiated with 20 J/m² of UV (lanes 4–6), or remained untreated (lanes 1–3), and incubated for 5 h. After immunoprecipitation with an anti-pol η antibody (lanes 3 and 6) or control IgG (lanes 2 and 5), binding PCNA was detected by Western blot with an anti-PCNA antibody. Lanes 1 and 4 represent 5% samples of the whole-cell lysate. An asterisk shows nonspecific bands. (D) Direct binding of pol η to monoubiquitinated PCNA. Rad18 was removed from the *in vitro* PCNA ubiquitination reaction mixture by immunoprecipitation with an anti-Rad18 antibody (upper panel). Note that Rad6B was also depleted, probably due to direct interaction with Rad18 (upper). Remaining PCNA and monoubiquitinated PCNA were pulled down with purified GST-pol η bound to glutathione beads (lower, right). GST-pol η 355n was used as a control. Purity of the polymerase samples is shown on the left panel (arrowheads) by the Coomassie brilliant blue (CBB) staining. (E) Colocalization of eGFP-pol η with pol δ in UV-irradiated (10 J/m², 5 h) GM637 cells. Bar = 5 μ m. (F) Colocalization of pol δ with PCNA in UV-irradiated (10 J/m², 5 h) WI38VA13 cells. Bar = 5 μ m.

indicating that Rad18 protein is definitely a ubiquitin ligase (E3) specific for PCNA. At present, it is not clear whether PCNA monoubiquitination occurs at the stalling sites, or to PCNA free in the nucleoplasm. Because Rad6 interacts not only with Rad18 but also other E3s such as Ubr1 and Bre1 under normal conditions (Dohmen *et al*, 1991; Watkins *et al*, 1993), dispersion and relocalization of Rad18 might increase the chance of interaction of Rad18 with Rad6A/B, thereby channeling Rad6A/B to a DNA damage tolerance pathway. In the case of pol α -pol δ switching on regular replication, loading of PCNA onto DNA is critical because pol δ binds PCNA while pol α does not (Tsurimoto and Stillman, 1991). However, in the case of the switch from pol δ to pol η , loading of unmodified PCNA would not be sufficient to promote the switch because both pol δ and pol η interact with PCNA (Tsurimoto and Stillman, 1991; Haracska *et al*, 2001b). We found that pol η preferentially binds to mono-ubiquitinated PCNA while pol δ does not (Figure 7A). We assume that the preferential binding is the motive force for the polymerase switch. Immunostaining results revealed that pol δ remains associated with the Rad18-pol η complex at the foci, suggesting that both of the polymerases are retained at the replication stalling sites.

Rad18 performs two roles in pol η foci formation in UV-irradiated cells: targeting Rad6A/B and pol η to stalling sites as a guide, and directing monoubiquitination of PCNA as a ubiquitin ligase. Although Rad18 associates constitutively with pol η , Rad18 lacking the Rad6A/B-binding domain did not induce the formation of pol η foci in UV-irradiated *RAD18*^{-/-} cells. It is probable that translocation of a Rad18/pol η complex to stalled replication sites is not sufficient to form stable pol η foci, and this may require monoubiquitinated PCNA. In the budding yeast, monoubiquitinated PCNA is further ubiquitinated by an Mms2/Ubc13/Rad5 complex (Hoege *et al*, 2002). Following the appearance of monoubiquitinated PCNA, we detected a new band corresponding to approximately 50 kDa by Western blot using an anti-PCNA antibody (data not shown). Judging from its size, this band could be either a multiubiquitinated or sumoylated form of PCNA. The role of multiubiquitination of PCNA is largely unknown, but it probably stimulates polymerase switching in the process of TLS. Our finding that Rad18 interacts with pol η and is involved in the monoubiquitination of PCNA accounts well for the requirement for Rad18 for pol η focus formation after UV irradiation.

Materials and methods

Establishment of *RAD18*^{-/-} cells

RAD18^{+/-} ES cells were injected into C57BL/6 blastocysts to generate chimeric mice (Tateishi *et al*, 2003), which transmitted the mutation into gametes. *RAD18*^{+/-} mice were intercrossed to generate *RAD18*^{-/-} mice. *RAD18*^{-/-} fibroblasts were obtained from the lungs of the mice and immortalized with SV40. *RAD18*^{+/+} cells were similarly established from WT mice. Stable transformants of *RAD18*^{-/-} cells expressing FLAG-hRad18 were selected with hygromycin B, and clones expressing high levels of the transgenes were used.

Protein purification

Human Rad18 and Rad6B proteins were expressed simultaneously in Sf9 insect cells by using a recombinant baculovirus. Rad6B protein had a polyhistidine tag at the N-terminal region. A Rad18

and His-Rad6B protein complex was purified with an Ni ion-loaded HiTrap Chelating HP column (Amersham).

Immunostaining

To examine the colocalization of PCNA with Rad18, GM637 cells were fixed with 100% methanol and processed for indirect double staining. First, cells were stained with an anti-Rad18 rabbit antibody (Tateishi *et al*, 2000) and an anti-PCNA human serum (Toschi and Bravo, 1988), and then stained with FITC-anti-rabbit IgG (goat, Cappel) and rhodamine-anti-mouse IgG (goat, Cappel). To address the site of Rad18 relocalized after dispersion, UV-irradiated (15 J/m²) GM637 cells were cultured for 30 min, and then labeled with BrdU (10 μ M) for 2 h. As a control, nonirradiated cells were labeled with BrdU (80 μ M) for 20 min. After fixation with 100% cold methanol, these cells were stained for Rad18 with an anti-Rad18 rabbit antibody and a rhodamine-anti-rabbit antibody (goat, Cappel). After fixing again with 3.7% formaldehyde for 10 min, the cells were treated for 20 min with 2.5 N HCl, and then stained for BrdU with an FITC-anti-BrdU mouse monoclonal antibody (Progen). GM637 purchased from NIGMS (USA) is a normal human fibroblast cell line immortalized with SV40. Colocalization of pol δ with PCNA was examined similarly by using WI38VA13 cells and an anti-pol δ goat antibody (A-9, Santa Cruz). To examine the colocalization of eGFP-pol η with FLAG-Rad18, GM637 cells were cotransfected with pEGFP-pol η and pcDNA3 FLAG-Rad18 using Fugene 6 (Roche). Cells were pre-fixed with 3.7% formaldehyde, washed with phosphate-buffered saline (PBS), and fixed with 80% methanol. Cells were stained for FLAG with an anti-FLAG antibody (M2, Sigma), and a rhodamine-anti-mouse IgG antibody (Cappel) consecutively. To examine the colocalization of pol δ with eGFP-pol η , GM637 cells were transfected with eGFP-pol η and stained for pol δ with an anti-pol δ antibody (A-9, Santa Cruz). Stained cells were observed with a confocal laser-scanning microscope (FV300, Olympus).

Immunoprecipitation

COS-7 cells were transfected with plasmids using Fugene 6, and incubated for 48 h. Cells were harvested in a lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% NP-40, 8% glycerol, 0.5 mM dithiothreitol (DTT), 50 mM NaF, and protease inhibitors). Cell lysates were mixed with an anti-T7 monoclonal antibody (Novagen) or an anti-FLAG antibody for 1 h at 4°C, and immunoprecipitated with Protein G Sepharose beads (Amersham Pharmacia) for another 1 h at 4°C. Precipitated proteins were separated by SDS-PAGE and analyzed by Western blot. To determine the interaction of HA-pol η and Rad18 *in vivo*, GM637 cells were transfected with pCAGGS HA-pol η , incubated for 48 h, and irradiated with UV at 12.5 J/m². These cells were treated with 5 mM dimethyl 3,3'-dithiobispropionimidate 2HCl (DTBP (Pierce) for protein cross-linking before preparation of cell lysates (Pearson *et al*, 2000). Cell lysates were prepared in a buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0% NP-40, 50 mM NaF, and protease inhibitors). After sonication, the supernatants were immunoprecipitated with either a monoclonal anti-HA antibody (HA.11, BABCO) or an anti-hRad18 rabbit antibody. Precipitates were analyzed by SDS-PAGE and Western blot. To investigate the interaction between HA-pol η and monoubiquitinated PCNA *in vivo*, GM637 cells were transfected with pCAGGS HA-pol η and incubated for 48 h. After UV irradiation at 20 J/m², cells were cultured for 5 h and treated with 0.1% NP-40 for 5 min on ice. These cells were washed once with PBS, and then treated with DTBP for crosslinking. Cells were collected in a lysis buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1.0% NP-40, 0.1% SDS, 0.1% Na-deoxycholic acid, and protease inhibitors) and disrupted by sonication. After centrifugation, cleared supernatants were immunoprecipitated with an anti-pol η antibody. PCNA binding to pol η was detected by SDS-PAGE and Western blot using an anti-PCNA antibody.

To examine the interaction between Rad18 and pol η *in vitro*, His-Rad18 and His-pol η were overexpressed in Sf9 insect cells and purified with Ni-NTA agarose beads (Invitrogen). Purified Rad18 protein was incubated in buffer A (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, and 0.5 mM DTT) for 30 min at 25°C either with purified pol η or with pol δ . Final concentrations of the proteins were set at 0.1 μ M. Rad18 protein was then immunoprecipitated with an anti-Rad18 antibody. Polymerases bound to Rad18 were detected by Western blot using an anti-pol η antibody (B-7, Santa Cruz) or an anti-pol δ antibody.

Analysis of eGFP-pol η foci after UV irradiation

Cells were transfected with eGFP-pol η , or cotransfected with eGFP-pol η and RAD18 cDNA, and cultured for 20 h before UV irradiation. For observation, cells were fixed with 3.7% formaldehyde. At least 200 cells were counted for each time point, and the experiments were repeated three times.

In vitro ubiquitination of PCNA

HHR6B (hRad6B) and hRad18 were prepared by using a baculovirus, and purified with Ni-NTA agarose beads. E1 enzyme was purchased from Boston Biochem. Recombinant PCNA purified from *E. coli* (Tsurimoto and Stillman, 1991) was incubated for 1 h at 25°C with other components in a buffer containing 50 mM HEPES (pH 7.6), 0.05 mM DTT, 1 mM MgCl₂, 1 mM ATP, and an ATP-regenerating system together with either 5 mM ubiquitin (Boston Biochem) or FLAG-ubiquitin (Sigma). Samples were analyzed for PCNA by Western blot.

Physical interaction between Rad18 and pol η

GST-pol η cDNA was generated by PCR by using pEGFP-pol η as a template with the following primers: gccaattcATGGCTACTGGACAGATCG and gcgaattcCTAATGTGTAAATGGCTTAAAAAATGATTC. A fragment digested with *EcoRI* was ligated into the *EcoRI* site of pAcGHLT-A vector (PharMingen). GST-pol η mutant cDNA was subcloned by PCR by using pEGFP-pol η as a template. To generate pol η 355n, gccaattcATGGCTACTGGACAGATCG and cgggatcCTAGTCTTTAGTCAGTCTCTCTC were used as primers. After digestion with *EcoRI* and *BamHI*, the product was inserted into the pAcGHLT-A vector digested with *EcoRI*-*BglII*. To obtain a fragment of pol η 350–590, ggaattcGAGAGACTGACTAAAGACCG and gca gatcGCTTTAGAGGATTCTTCTAGC were used as primers. The product was digested with *EcoRI* and *BglII* and inserted into the pAcGHLT-A vector digested with *EcoRI*-*BglII*. To generate pol η 158c, pAcGHLT-A-pol η was digested with *NcoI*, and the 3' 477 bp fragment was inserted into the pAcGHLT-A vector. Full-length Rad18 cDNA tagged with Myc at its C-terminal region was generated by PCR with the following primers: cccaattcATGGAC TCCCTGCC and cccaattcCTTACAAGTCTCTTTCAGAAATGAGCT TTTGCTCATTCTATTACGCT. A fragment digested with *EcoRI* was ligated into the *EcoRI* site of the pCAGGS vector. Myc-tagged Rad18 DRG cDNA containing amino-acid residues 193–495 was subcloned by PCR. Myc-tagged Rad18 DZ cDNA was produced by ligation of the two fragments corresponding to amino-acid residues 1–72 and 343–495. The product was inserted into the *EcoRI* site of the pCAGGS vector. DR6 and DC2 plasmids were generated by inverse PCR using pCAGGS containing Myc-tagged full-length Rad18 as a template by using the primers GTGGATTTCATCTATTTCTTTCTG/ACCTCAGTAACAAACCAC and GTGGTTGTACTGAGGTC/GACAT CATAAGATCTTTTAGAAG, respectively. Final plasmids were obtained by self-ligation of the PCR products. To investigate the binding domain of Rad18 to pol η , COS-7 cells were transfected with pCAGGS plasmids containing Myc-tagged full-length Rad18 or deletion mutant Rad18, and cultured for 48 h. Cells were harvested in a lysis buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.25% NP-40, and protease inhibitors). Cell lysates were mixed with GST-pol η 158c beads for 1 h at 4°C. Precipitated proteins were separated by SDS-PAGE and analyzed by Western blot using an anti-c-Myc antibody (9E10, Santa Cruz). To investigate the binding domain of pol η with Rad18, GM637 cell lysates were mixed with GST-pol η or deletion mutants of pol η beads for 1 h at 4°C. Precipitated proteins were separated by SDS-PAGE and analyzed by Western blot using an anti-Rad18 antibody.

Pull-down assay

GST-pol η overexpressed in Sf9 insect cells was harvested with glutathione Sepharose 4B (Amersham). His-pol δ (p125) over-

expressed in insect cells together with GST-p66 and His-p50 was harvested with glutathione beads as a complex (Shikata *et al*, 2001). After washing with PBS, the beads were suspended in a lysis buffer (20 mM sodium phosphate pH 7.3, 10% glycerol, 10 μ M β -mercaptoethanol, 300 mM NaCl, 1% NP-40, and protease inhibitors). Cell lysates were prepared from UV-irradiated (20 J/m²) HeLa cells with a lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM DTT, 100–800 mM NaCl, 1% NP-40, 50 mM NaF, and protease inhibitors), and mixed with either GST-pol η beads or GST-pol δ beads for 1 h at 4°C. As a control, GST beads were used. Precipitated proteins were analyzed for PCNA by Western blot. To examine elution of PCNA from GST-pol η beads, the beads were washed with a lysis buffer containing 200–800 mM NaCl, and eluted PCNA was analyzed by Western blot.

Immunodepletion

PCNA was monoubiquitinated *in vitro*. Rad18 was removed from the reaction mixture by five cycles of immunodepletion using an anti-Rad18 antibody and protein G Sepharose. After immunodepletion, PCNA was pulled down with either GST-pol η or GST-pol η 355n in a buffer containing 50 mM NaCl, 20 mM HEPES-KOH (pH 7.4), 1 mM MgCl₂, 2 mM DTT, and 0.005% NP-40. Binding was revealed by Western blot using an anti-PCNA monoclonal antibody (PC10, Santa Cruz). GST-pol η 355n is an N-terminal fragment of pol η (amino-acid residues 1–355) fused with GST at the N-terminus.

Chromatin isolation

Chromatin fractions were isolated from UV-irradiated (15 J/m², 6 h culture) or nonirradiated HeLa cells as described elsewhere (Mendez and Stillman, 2000). A part of chromatin fractions was further treated with 0.2 U micrococcal nuclease (MNase) for 1 min at 37°C, and separated into solubilized fractions as described (Mendez and Stillman, 2000).

Transfection of siRNA duplexes

WI38VA13 is a normal human lung fibroblast cell line immortalized with SV40 (purchased from the American Type Culture Collection). WI38VA13 cells were transfected with Oligofectamine (Invitrogen) with a mixture of siRNA specific for HHR6A and HHR6B, the coding strands of which were CGGAAUUAUGAAAAGCGU(TT) and GAGUUUCGGCAUUGUUGA(TT), respectively. These cells were cultured for 72–96 h before the second transfection. For the control transfection, the negative control siRNA (QIAGEN) was used.

UV survival assay

Appropriate numbers of cells were inoculated onto 60-mm dishes and left to attach for 8–9 h. Subsequently, cells were washed with PBS, exposed to UV light (254 nm) at a fluence rate of 0.63 J/m²/s, and cultured for 6–7 days. Colonies were fixed with 80% methanol and stained with 5% Giemsa solution. For each UV dose, at least three dishes were used.

Supplementary data

Supplementary data are available at *The EMBO Journal Online*.

Acknowledgements

This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, and by a grant of the Princess Takamatsu Cancer Research Fund. We thank A Lehmann for eGFP-pol η , Y Takasaki for anti-PCNA human serum, S Watanabe for fluorescent pictures, H Niwa, J Miyazaki, K Araki, and K Yamamura for establishing *RAD18*^{-/-} knockout mice, and D Catcheside for critical reading of the manuscript.

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