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Modification of PCNA by ISG15 Plays a Crucial Role in Termination of Error-Prone Translesion DNA Synthesis

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SUMMARY

In response to DNA damage, PCNA is mono-ubiquitinated and triggers translesion DNA synthesis (TLS) by recruiting polymerase-η. However, it remained unknown how error-prone TLS is turned off after DNA lesion bypass to prevent mutagenesis. Here we showed that ISG15 modification (ISGylation) of PCNA plays a key role in TLS termination. Upon UV irradiation, EFP, an ISG15 E3 ligase, bound to mono-ubiquitinated PCNA and promoted its ISGylation. ISGylated PCNA then tethered USP10 for deubiquitination and in turn the release of polymerase-η from PCNA. Eventually, PCNA was deISGylated by UBP43 for reloading of replicative DNA polymerases and resuming normal DNA replication. However, ISGylation-defective Lys-to-Arg mutations in PCNA or knockdown of any of ISG15, EFP, or USP10 led to persistent recruitment of mono-ubiquitinated PCNA and polymerase-η to nuclear foci, causing an increase in mutation frequency. These findings establish a crucial role of PCNA ISGylation in termination of error-prone TLS for preventing excessive mutagenesis.

INTRODUCTION

For faithful transmission of the genetic information, cells must carry out highly processive, error-free replication of the genome and efficient repair of DNA damage or misincorporated nucleotides (Ciccia and Elledge, 2010; Mailand et al., 2013). The sliding clamp proliferating cell nuclear antigen (PCNA) plays an essential role as a processivity factor as well as a scaffold for recruiting replication machinery. In addition, PCNA plays a crucial role in DNA damage bypass and their repair by serving as a platform for recruiting essential components necessary for DNA damage response (Moldovan et al., 2007). Therefore, PCNA has been regarded as a key mediator for maintenance of genome stability and cell survival.

When replicating cells are exposed to DNA damage, PCNA undergoes numerous posttranslational modifications, such as ubiquitination and sumoylation, for the control of DNA damage response (Bergink and Jentsch, 2009; Jackson and Durocher, 2013; Mailand et al., 2013; Ulrich and Walden, 2010). UV, to which human epithelial cells are persistently exposed, induces mono-ubiquitination of a highly conserved Lys164 residue in PCNA by the RAD6-RAD18 complex (Hoege et al., 2002). This modification of PCNA triggers the exchange of replicative DNA polymerases, such as Polδ, with damage-tolerant, Y family of DNA polymerases, including Polη, for TLS (Bienko et al., 2005; Kannouche et al., 2004; Lehmann et al., 2007; Stelter and Ulrich, 2003). TLS polymerases bypass damaged DNA, and therefore, replication fork progression can occur without the need of removal of the damage and the risk of fork collapse (Sale et al., 2012). However, TLS polymerases, lacking proofreading activity, can introduce incorrect nucleotides and are thus potentially mutagenic (Loeb and Monnat, 2008; Matsuda et al., 2000; Sale et al., 2012). Thus, error-prone TLS polymerases need to be released from PCNA after DNA lesion bypass for preventing excessive mutagenesis.

While PCNA is mono-ubiquitinated under DNA damage conditions, Polη is mono-ubiquitinated in the UBZ domain under normal conditions, and this modification prevents its interaction with PCNA (Bienko et al., 2005, 2010). Upon DNA damage by UV, however, Polη is deubiquitinated by an unknown USP, binds to mono-ubiquitinated PCNA, and carries out TLS. PCNA could also normally be mono-ubiquitinated by the $CRL4^{cdt2}$ ubiquitin ligase complex (Terai et al., 2010), but this process is rapidly reversed by USP1 for preventing unnecessary TLS (Huang et al., 2006). Upon DNA damage, however, USP1 is degraded by autocleavage for abrogating the negative regulation of PCNA mono-ubiquitination, implicating the role of USP1 in prevention of TLS under normal, but not under DNA damage, conditions. Therefore, a key unanswered issue is how TLS is turned off after DNA lesion bypass to prevent TLS-mediated mutagenesis. It is unknown how replicative DNA synthesis can be resumed after TLS termination.

ISG15, the interferon-stimulated gene 15, is the first reported ubiquitin-like protein (Haas et al., 1987). ISG15 is robustly induced by type I interferon, lipopolysaccharides, and viral infection (Kim et al., 2002; Yuan and Krug, 2001). Like ubiquitination, protein ISGylation is catalyzed by a three step enzyme system: UBE1L as an ISG15-activating E1 enzyme, UBCH8 as an ISG15-conjugating E2 enzyme, and EFP and HERC5 as ISG15

E3 ligases (Dastur et al., 2006; Kim et al., 2004; Yuan and Krug, 2001; Zhao et al., 2004; Zou and Zhang, 2006). In addition, protein ISGylation can be reversed by a deISGylating enzyme, UBP43 (Malakhov et al., 2002). Although more than 300 cellular proteins were identified as targets for ISGylation, only a few cases of their functional significance have been uncovered (Jeon et al., 2010). We have recently shown that DNA-damaging agents, such as doxorubicin, induce the expression of ISG15, UBE1L, and UBCH8, leading to ISGylation and downregulation of ΔNp63α (Jeon et al., 2012), which acts as a mitotic and oncogenic protein by suppressing the transactivities of proapoptotic p53 family members, including p53 and TAp63 (Yang et al., 1998). Therefore, we have suggested that ISG15 serves as a tumor suppressor via its conjugation to ΔNp63α.

In this study, we showed that UV also induces the expression of ISG15, UBE1L, and UBCH8, and PCNA serves as a target for ISGylation. EFP interacted with mono-ubiquitinated PCNA and promoted its ISGylation. Moreover, ISGylation of PCNA was required for its binding to USP10. The bound USP10 then removed mono-ubiquitin from PCNA and in turn triggered the release of Polη from PCNA for TLS termination. We further showed that Lys-to-Arg mutations of the ISGylation sites in PCNA or knockdown of any of ISG15, UBE1L, EFP, or USP10 results in persistent recruitment of mono-ubiquitinated PCNA and Polη at DNA damage sites, causing a marked increase in mutation frequency. Eventually, ISG15 expression was downregulated and PCNA was deISGylated by UBP43, which would allow reloading of replicative DNA polymerases and resumption of normal DNA replication. Collectively, these results indicate that PCNA ISGylation plays a crucial role in TLS termination after lesion bypass for escaping from excessive mutagenesis.

RESULTS

UV Induces ISG15-Conjugating System

We have recently shown that DNA-damaging drugs, such as doxorubicin, induce the expression of ISG15, UBE1L, and UBCH8 (referred to as ISG15-conjugating system) (Jeon et al., 2012). Here we examined whether UV can also induce the expression of ISG15-conjugating system and thereby ISGylation of a different set of target proteins in HeLa cells. UV irradiation robustly elevated both the mRNA and protein levels of ISG15-conjugating system (Figures 1A and 1B, respectively). Consistently, UV treatment led to a gradual increase in the level of ISGylated cellular proteins, as analyzed by immunoblot with anti-ISG15 antibody, which detects ISG15 and its conjugated proteins, but not ubiquitin, other ubiquitin-like proteins, and their conjugates (Figure 1C).

To identify target proteins for ISGylation in response to UV, HeLa cells treated with and without UV were subjected to immuno-affinity purification using anti-ISG15 antibody-immobilized resins. Proteins bound to the resins were subjected to SDS-PAGE (Figure S1A available online) and mass spectrometry. Since the identified proteins included PCNA (Figure S1B) and since modifications of PCNA by ubiquitin and SUMO are known to regulate DNA damage response (Bergink and Jentsch, 2009; Jackson and Durocher, 2013; Ulrich and Walden, 2010), we chose PCNA for further studies.

PCNA Has Two ISG15 Acceptor Sites

To determine whether PCNA could indeed be ISGylated, ISG15-conjugating system was overexpressed with HisMax-PCNA in HeLa cells. Two anti-ISG15 antibody-reactive bands appeared, which could be eliminated by coexpression of UBP43 (Figure 1D). The size of the upper band was 69 kDa, which corresponds to a PCNA subunit modified by two ISG15 molecules (referred to as doubly ISGylated PCNA), while that of the lower band was 54 kDa, corresponding to mono-ISGylated PCNA. Note that PCNA behaved as a 35 kDa protein in SDS-PAGE gels, although its actual size is 29 kDa. These results suggest that two ISG15 molecules could be ligated at two different sites in the same subunit of a trimeric PCNA molecule. To exclude a possibility that a dimeric ISG15 chain might be ligated to a single site, we generated a Lys-less ISG15 variant by replacing all eight Lys residues in ISG15 by Arg. Overexpression of the variant, like that of wild-type ISG15, led to appearance of doubly ISGylated PCNA, which could also be eliminated by coexpression of UBP43 (Figure 1E), indicating that two ISG15 molecules are ligated at two different sites in the same subunit of a PCNA molecule.

To identify ISGylation sites, each of 16 Lys residues in PCNA was replaced by Arg. The mutation of Lys164 (K164R) or Lys168 (K168R), but not the others, prevented PCNA ISGylation (Figure 1F). Of note, however, was the finding that the mutation of one site prevented ISGylation of the other site as well. To confirm this finding, we repeated the experiment together with K164R/K168R double mutant. All of the single and double mutations led to disappearance of both mono-ISGylated and doubly ISGylated PCNA bands (Figure 1G). These results indicate that ISGylation of one site influences that of the other site. Since Lys164 of PCNA is known as the site for mono-ubiquitination, it appeared possible that ubiquitination of PCNA might influence its ISGylation (see below).

UV Induces Sequential Modification of PCNA by Ubiquitin and ISG15

To determine whether UV can induce ISGylation of PCNA in addition to mono-ubiquitination, HeLa cells exposed to UV were subjected to immunoprecipitation analysis. Mono-ubiquitinated PCNA appeared from 6 to 12 hr after UV treatment and disappeared at 24 hr (Figure 2A). In contrast, doubly ISGylated PCNA (the 69 kDa band) could be seen only at 24 hr. Similar results were obtained with other cell lines, including MCF10A, MRC5, and HCT116 cells (Figure S2A). However, we could not locate mono-ISGylated PCNA (54 kDa) due to nonspecific interaction of IgG heavy chain, which ran as a 55 kDa protein in the same SDS-PAGE gels. Therefore, we repeated the experiment but with ectopic expression of PCNA-V5-His. Both mono-ubiquitinated PCNA (43 kDa) and mono-ISGylated PCNA (54 kDa), but not doubly ISGylated PCNA (69 kDa), could be seen at 12 hr after UV treatment (Figure 2B). On the other hand, both mono-ISGylated and doubly ISGylated PCNA, but not mono-ubiquitinated PCNA, could be seen at 24 hr, suggesting that mono-ubiquitination of PCNA is switched by ISGylation in a time-dependent fashion. To confirm this finding, we examined the effect of ISG15 knockdown on mono-ubiquitination of PCNA. Remarkably, expression of an ISG15-specific shRNA (shISG15), but not a nonspecific shRNA (shNS), led to sustained mono-ubiquitination

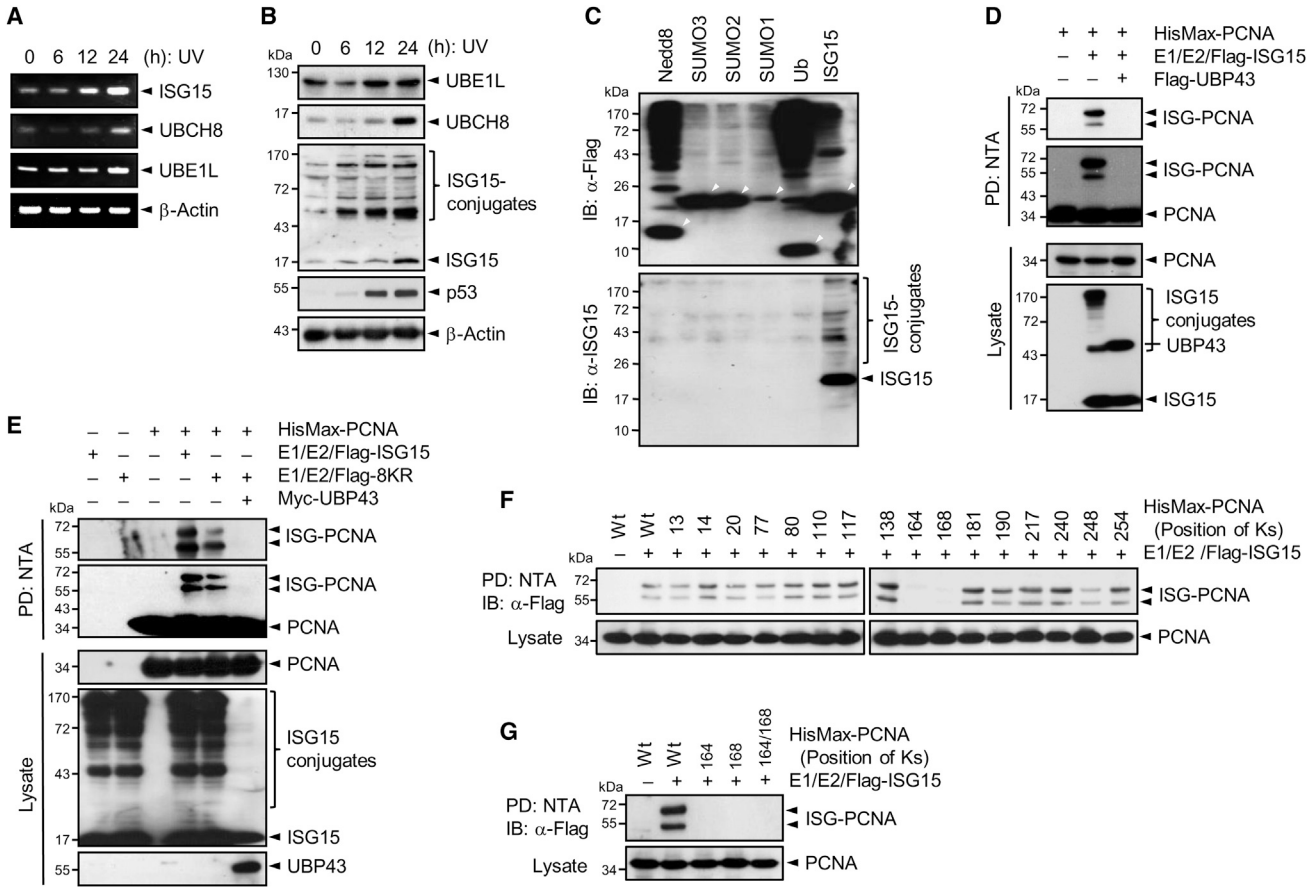


Figure 1. Identification of PCNA as a Target for ISGylation

(A and B) UV induces ISG15-conjugating system.

(A) HeLa cells that had been exposed to UV (20 J/m^2) were incubated for increasing periods. Total mRNAs were prepared from their lysates and subjected to RT-PCR.

(B) Cell lysates were subjected to immunoblot analysis. Induction of p53 expression indicates that cells properly respond to UV.

(C) Specificity of anti-ISG15 antibody. Cells expressing Flag-tagged ubiquitin and ubiquitin-like proteins (UBLs) were subjected to immunoblot with anti-Flag and anti-ISG15 antibodies. The white arrowheads indicate ubiquitin and UBLs.

(D) PCNA has two ISGylation sites. HisMax-PCNA and ISG15-conjugating system (E1/E2/Flag-ISG15) were overexpressed in cells with and without Flag-UBP43. Cell lysates were subjected to pulldown (PD) with NTA resins followed by immunoblot with anti-Flag or anti-Xpress antibody. The lysates were also directly probed with the same antibodies.

(E) ISG15 conjugated to PCNA does not form a dimeric ISG15 chain. Flag-tagged ISG15 or its Lys-less variant (8KR) was overexpressed in cells with HisMax-PCNA in the presence and absence of Myc-UBP43. Cell lysates were subjected to pulldown with NTA resins followed by immunoblot with anti-Flag and anti-Xpress antibodies.

(F) Identification of ISG15 acceptor sites in PCNA. HisMax-PCNA was subjected to mutagenesis for substituting each of 16 Lys residues with Arg, followed by expression in cells with ISG15-conjugating system. Cell lysates were subjected to pulldown with NTA resins followed by immunoblot with anti-Flag antibody.

(G) K164 and K168 in PCNA are the ISGylation sites. HisMax-tagged PCNA or its K-to-R mutants were expressed in cells with ISG15-conjugating system. Cell lysates were then treated as in (F).

of PCNA at 24 hr (Figure 2C, top). Note that mono-ISGylated PCNA can be seen at 12 and 24 hr when PCNA was ectopically expressed (Figure 2C, bottom). Similar results were obtained when ISG15 was knocked down by an additional shRNA (shISG15-2), and this effect on PCNA mono-ubiquitination could be reversed by complementation of wild-type ISG15, but not by its conjugation-deficient mutant (ISG15-G/A), of which the C-terminal Gly was replaced by Ala (Figure S2B). These results suggest that ISGylation of PCNA is required for downregulation of its mono-ubiquitination.

We next examined whether PCNA ISGylation can be induced by other types of DNA damage. Mono-ubiquitinated PCNA appeared 6 hr after treatment with methyl methane sulfonate (MMS) or hydroxyurea (HU), and disappeared at 24 hr (Figures 2D and 2E). On the other hand, doubly ISGylated PCNA (69 kDa) could be seen at 24 hr. These results indicate that PCNA ISGylation can be induced not only by UV but also by other DNA-damaging agents that can induce PCNA mono-ubiquitination.

To determine whether PCNA ISGylation is regulated by DNA damage-related kinases, HeLa cells were treated with caffeine

(an ATR/ATM inhibitor) and SB302580 (a p38 inhibitor) immediately after exposure to UV. SB302580 inhibited neither ISGylation nor mono-ubiquitination of PCNA, indicating that p38 is not involved in UV-induced PCNA ISGylation (Figure S2E). On the other hand, caffeine inhibited both mono-ubiquitination and ISGylation of PCNA (Figure S2F). However, it showed little or no effect on ISGylation of cellular proteins and induction of ISG15 expression, indicating that the inhibitory effect of caffeine on ISGylation of PCNA is mediated by that on mono-ubiquitination. These results suggest that ATR kinase indirectly regulates ISGylation of PCNA by promoting its mono-ubiquitination.

EFP Serves as an ISG15 E3 Ligase of PCNA

EFP and HERC5 are known as ISG15 E3 ligases (Dastur et al., 2006; Zou and Zhang, 2006). To determine whether any of them is responsible for PCNA ISGylation, we first examined their ability to interact with PCNA. EFP, but not HERC5, bound to PCNA (Figures 2F and 2G, respectively). Moreover, PCNA ISGylation was markedly enhanced by overexpression of EFP, but not by that of its mutant (C13/16S), of which the conserved Cys13 and Cys16 residues in the RING domain were replaced by Ser (Figure 2H). On the other hand, HERC5 was unable to promote PCNA ISGylation (Figure 2I). Furthermore, expression of an EFP-specific shRNA (shEFP), but not shNS, prevented PCNA ISGylation (Figure 2J, top). Again, note that mono-ISGylated PCNA can be seen at 12 and 24 hr after UV treatment upon ectopic expression of PCNA (Figure 2J, bottom). EFP knockdown also led to sustained mono-ubiquitination of PCNA at 24 hr. Similar results were obtained when EFP was knocked down by an additional shRNA (shEFP-2), and this effect on PCNA mono-ubiquitination could be reversed by complementation of wild-type EFP, but not by its C13/16S mutant (Figure S2C), indicating that ISGylation of PCNA downregulates its mono-ubiquitination. Notably, EFP expression was induced by UV, like that of ISG15 (Figure 2J, top). Thus, EFP appears to serve as an ISG15 E3 ligase for PCNA under UV-induced DNA damage conditions. To locate the regions for binding between EFP and PCNA, their deletions were generated and expressed in HeLa cells. PCNA bound to the N-terminal region having the RING domain of EFP, and the ligase bound to the C-terminal half of PCNA (Figure S3).

Mono-Ubiquitination of PCNA Is Required for Its Interaction with EFP

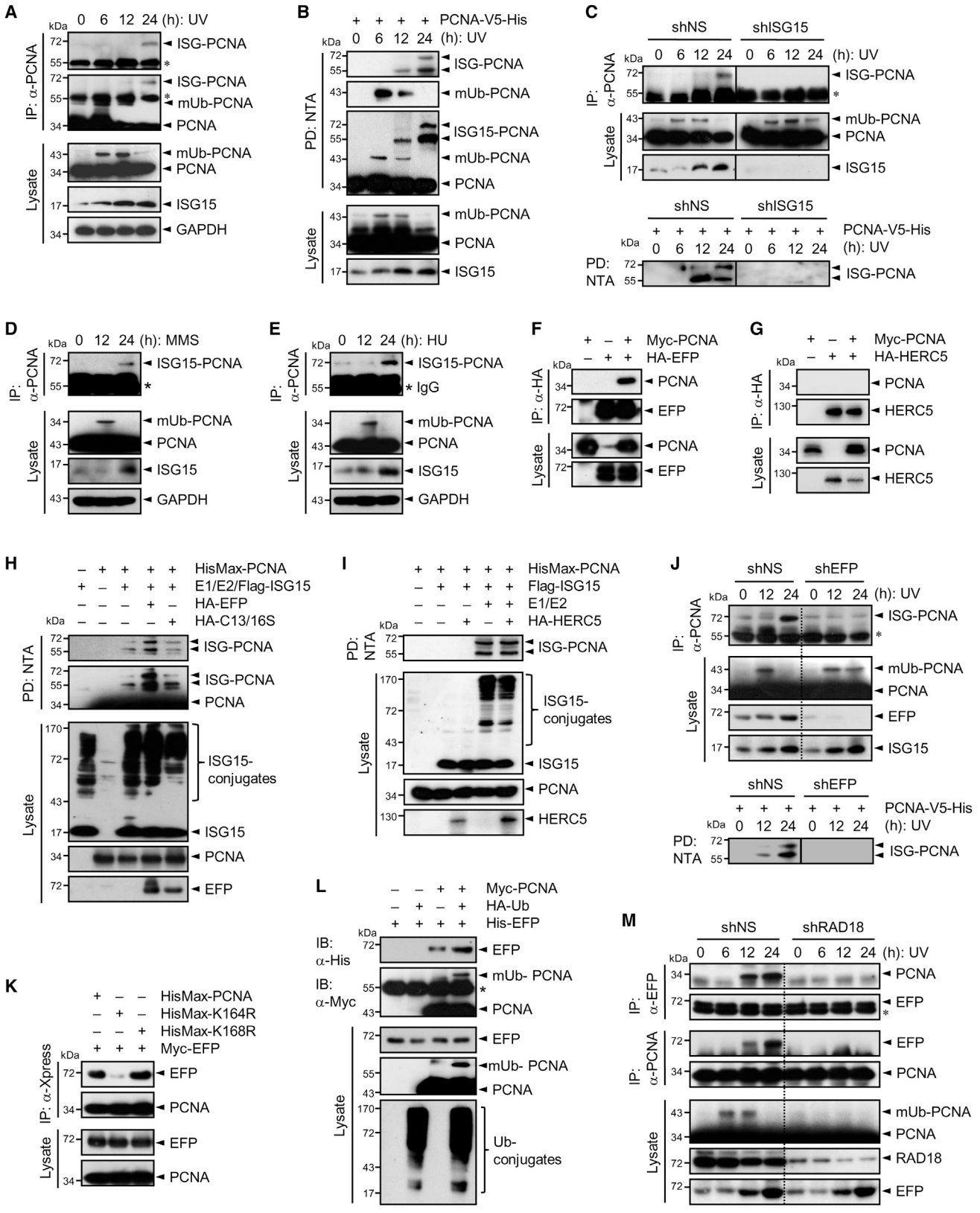
Of note was the finding that the K-to-R mutation of one site in PCNA prevents ISGylation of the other site as well (Figure 1G). To clarify this unexpected finding, we first examined whether the mutations affect the binding of PCNA to EFP. Surprisingly, K164R mutation dramatically reduced the interaction between PCNA and EFP (Figure 2K), suggesting that unmodified Lys164 might be required for the binding of EFP to PCNA. However, mono-ubiquitination of PCNA can occur even under normal conditions (Terai et al., 2010), although it is rapidly reversed by USP1 (Huang et al., 2006). Thus, it appeared also possible that EFP preferentially binds to mono-ubiquitinated PCNA over its unmodified form. To test this possibility, we performed an in vitro binding assay by using purified EFP. Figure 2L shows that EFP has a much higher affinity to mono-ubiquitinated PCNA than to its unmodified form. To confirm this finding, we examined the effect of knockdown of RAD18, a PCNA-specific ubiquitin E3 ligase (Hoege et al., 2002), on the interaction between PCNA and EFP. Expression of a RAD18-specific shRNA (shRAD18), but not shNS, abolished the interaction of PCNA with EFP in addition to its mono-ubiquitination (Figure 2M). These results indicate that mono-ubiquitination of Lys164 is required for the binding of EFP to PCNA, leading to PCNA ISGylation. This finding explains why Lys168 of K164R mutant cannot be ISGylated. However, it remains unclear why Lys164 of K168R mutant could not be ISGylated.

USP10 Has a PIP Box and Removes Mono-Ubiquitin from PCNA

Since mono-ubiquitinated PCNA disappeared at 24 hr after UV treatment (Figure 2), it should have been deubiquitinated before the time point. Yeast Ubp3, an ortholog of human USP10, has a conserved (QXXL/I) PIP box variant (Gallego-Sánchez et al., 2010). Moreover, UV induces the expression of USP10 and its translocation from the cytoplasm to the nucleus (Yuan et al., 2010). Figure 3A shows that USP10 also has a noncanonical PIP box (Moldovan et al., 2007; Xu et al., 2001). To determine whether the PIP box of USP10 is required for its interaction with PCNA, the conserved Leu and two Phe residues were substituted by Ala. Unlike wild-type USP10, the PIP mutant could neither interact with (Figure 3B) nor deubiquitinate PCNA (Figure 3C). On the other hand, PCNA could interact with an inactive USP10 (C424A), of which the active site Cys424 was substituted by Ala (Figure 3D), indicating that the activity of USP10 is not required for its binding to PCNA. Figure 3E confirms the inability of C424A mutant to deubiquitinate PCNA. Furthermore, knockdown of USP10 by a USP10-specific shRNA (shUSP10) led to persistent mono-ubiquitination of PCNA (Figure 3F), and this effect was independent of USP1, as its level gradually declined after UV treatment as reported (Huang et al., 2006). Similar results were obtained when USP10 was knocked down by an additional shRNA (shUSP10-2), and this effect on PCNA mono-ubiquitination could be reversed by complementation of wild-type USP10, but not by its C424A mutant (Figure S2D). These results indicate that USP10 serves as a PCNA-specific deubiquitinating enzyme under UV-induced DNA damage conditions.

ISGylation of PCNA Is Required for Its Interaction with USP10

Since mono-ISGylated PCNA appeared before the removal of mono-ubiquitin from PCNA (Figure 2B) and since the action of USP10 needs to be timely regulated for termination of TLS after DNA lesion bypass, we suspected if PCNA ISGylation might be involved in the control of USP10 action. Overexpression of ISG15-conjugating system significantly enhanced the interaction between PCNA and USP10, and this increase was abrogated by coexpression of UBP43 (Figure 3G), suggesting that ISGylated PCNA has a much higher affinity to USP10 than its unmodified form. To confirm this finding, we performed in vitro binding assay by using purified USP10. Figure 3H shows that USP10 binds to ISGylated PCNA much more tightly than to its unmodified form. Moreover, PCNA binding to USP10 was abrogated by K168R



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mutation or ISG15 knockdown (Figures 3I and 3J, respectively), both of which block PCNA ISGylation. These results indicate that USP10 preferentially binds to ISGylated PCNA to cleave off mono-ubiquitin for TLS termination.

Significantly, USP10 interacted with PCNA when doubly ISGylated PCNA appeared (i.e., at 24 hr after UV treatment), but not when PCNA was mono-ISGylated (i.e., at 12 hr) (Figure 3J). Note that the majority of ISGylated PCNA is mono-ISGylated form at 12 hr (Figure 2C). Thus, it appears that endogenous USP10 interacts only with doubly ISGylated PCNA, although under overexpression conditions, USP10 can weakly bind to unmodified PCNA via its PIP box (Figures 3G and 3H).

PCNA ISGylation Causes the Release of Polη from PCNA

Since mono-ubiquitination of PCNA was switched by ISGylation in a time-dependent fashion after UV treatment (Figures 2A–2C), we examined whether PCNA ISGylation influences the recruitment of Polη to PCNA. HeLa cells overexpressing PCNA or its K168R mutant were exposed to UV and subjected to chromatin fractionation. In cells expressing PCNA, incubation time-dependent rise-and-fall of the Polη level in chromatin fraction was tightly correlated with that of mono-ubiquitinated PCNA (Figure 4A). However, overexpression of ISGylation-deficient K168R mutant led to sustained recruitment of Polη to mono-ubiquitinated PCNA in chromatin fraction. Furthermore, knockdown of any of ISG15, EFP, or USP10 also resulted in sustained recruitment of Polη to mono-ubiquitinated PCNA in the same fraction (Figures 4B–4D). These results indicate that ISGylation of PCNA plays a critical role in the release of Polη from PCNA for TLS termination.

PCNA ISGylation Downregulates Nuclear Foci Formation

We next examined whether PCNA ISGylation affects UV-induced formation of nuclear foci at DNA damage sites. Nuclear foci were formed with overexpressed PCNA and Polη at 12 hr after UV treatment and disappeared at 24 hr (Figure 5A). However, overexpression of K168R mutant led to persistent appearance nuclear foci at 24 hr. Furthermore, knockdown of any of ISG15, EFP, or USP10 also resulted in sustained foci formation at 24 hr (Figures 5B–5D). The images of multiple cells per field were also shown, and the fractions of cells with Polη foci were quantified (Figure S4). On the other hand, ISG15, UBE1L, and UbcH8 did not form damage-induced foci, although they are present in the nucleus (data not shown). These results indicate that PCNA ISGylation plays a crucial role in downregulation of foci formation by promoting USP10-mediated deubiquitination of PCNA and the release of Polη from PCNA.

PCNA ISGylation Blocks TLS-Mediated Mutagenesis

TLS polymerases can introduce incorrect nucleotides (Matsuda et al., 2000; Loeb and Monnat, 2008; Sale et al., 2012). Using a *supF* plasmid-based mutation assay (Parris and Seidman, 1992), we examined whether PCNA ISGylation is involved in prevention of TLS-mediated mutagenesis. All the approaches used for blocking PCNA ISGylation (i.e., overexpression of K168R mutant and knockdown of ISG15, EFP, and USP10) led to a dramatic increase in mutation frequency in response to UV (Figure 6A). Similar results were obtained when each of them was knocked down by additional shRNAs, and this effect on mutation frequency could be reversed by complementation of wild-type proteins, but not by their inactive mutants (Figure S5A). Moreover, prevention of PCNA ISGylation led to a marked reduction in the viability of UV-irradiated cells (Figure 6B). Note that to minimize the effect of endogenous PCNA in the mutation assay, cells that stably express both shPCNA and the shRNA-insensitive PCNA or its K168R mutant were generated (Figure 6C). Figure 6D shows that the shRNAs used in Figure 6 effectively deplete corresponding proteins. These results indicate that PCNA ISGylation play a key role in preventing the accumulation of mutations and thereby in cell survival.

Figure 2. UV Induces Sequential Modification of PCNA by Ubiquitin and ISG15

(A and B) UV induces ISGylation of PCNA.

(A) HeLa cells were treated with UV, and their lysates were subjected to immunoprecipitation (IP) with anti-PCNA antibody followed by immunoblot with anti-ISG15 and anti-PCNA antibodies.

(B) Cells were treated as in (A), but with overexpression of PCNA-V5-His. They were then subjected to pulldown with NTA resins followed by immunoblot with anti-ISG15, anti-ubiquitin, and anti-V5 antibodies. The asterisk in (A) and elsewhere indicates IgG heavy chain.

(C) ISG15 knockdown leads to sustained mono-ubiquitination of PCNA. Cells expressing shNS or shISG15 were treated with UV. They were then subjected to immunoprecipitation with anti-PCNA antibody followed by immunoblot with anti-ISG15 antibody (top). Cells were treated above but with overexpression of PCNA-V5-His. They were then subjected to pulldown with NTA resins followed by immunoblot with anti-ISG15 antibody (bottom).

(D and E) MMS and HU also induce PCNA ISGylation. Cells were treated with 0.01% MMS (D) and 4 mM HU (E). They were then subjected to immunoprecipitation with anti-PCNA antibody followed by immunoblot with anti-ISG15 antibody.

(F and G) EFP, but not HERC5, interacts with PCNA. Myc-PCNA was expressed in cells with HA-EFP (F) or HA-HERC5 (G). Cell lysates were subjected to immunoprecipitation with anti-HA antibody followed by immunoblot with anti-Myc or anti-HA antibody.

(H and I) EFP, but not HERC5, promotes PCNA ISGylation. HisMax-PCNA was expressed in cell with ISG15-conjugating system in the presence and absence of HA-tagged EFP and its C13/16S mutant (H) or HA-HERC5 (I). Cell lysates were subjected to pulldown with NTA resins followed by immunoblot with anti-Flag and/or anti-Xpress antibodies.

(J) EFP knockdown leads to sustained mono-ubiquitination of PCNA. Experiments were performed as in (C), except the use of shEFP in place of shISG15.

(K) K164R mutation prevents the interaction of PCNA with EFP. Myc-EFP was expressed in cells with HisMax-tagged PCNA or its K-to-R mutants. Cell lysates were subjected to immunoprecipitation with anti-Xpress antibody followed by immunoblot with anti-Myc or anti-Xpress antibody.

(L) EFP preferentially binds to mono-ubiquitinated PCNA in vitro. Myc-PCNA was expressed in cells with and without HA-ubiquitin. After exposure to UV, cells were subjected to incubation with anti-Myc antibody followed by pulldown with protein-A-conjugated resins. Purified EFP was then incubated with the resins, washed, and subjected to immunoblot with anti-His and anti-Myc antibodies.

(M) Interaction of PCNA with EFP requires its mono-ubiquitination. Cells expressing shNS or shRAD18 were treated with UV. They were then subjected to immunoprecipitation with anti-PCNA and anti-EFP antibodies followed by immunoblot with the same antibodies.

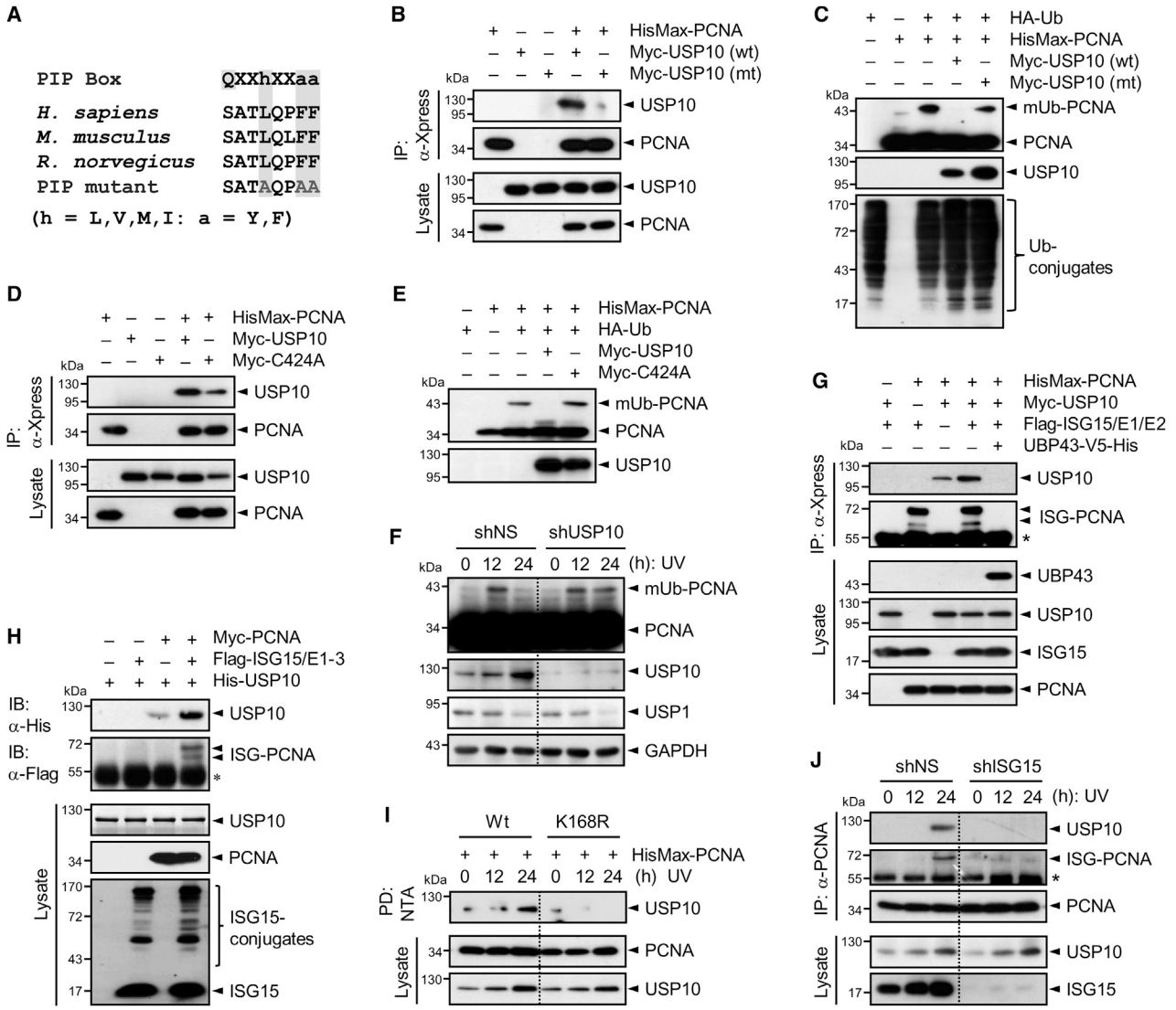

Figure 3. USP10 Has a PIP Box and Removes Mono-ubiquitin from PCNA

(A) USP10 has a PIP box. The conserved residues shaded by gray color in human USP10 were substituted by Ala. "h" and "a" represent hydrophobic and aromatic amino acids, respectively.

(B) PIP mutant cannot bind to PCNA. HisMax-PCNA was expressed in HeLa cells with USP10 (wt) or its PIP mutant (mt). Cell lysates were subjected to immunoprecipitation with anti-Xpress antibody followed by immunoblot with anti-Myc and anti-Xpress antibodies.

(C) PIP mutant cannot deubiquitinate PCNA. Cells were treated as in (B), but with overexpression of HA-ubiquitin. They were then subjected to immunoblot with anti-Xpress, anti-Myc, and anti-HA antibodies.

(D) USP10 binds to PCNA. HisMax-PCNA was expressed in cells with USP10 or its C424A mutant. Cell lysates were subjected to immunoprecipitation with anti-Xpress antibody followed by immunoblot with anti-Myc and anti-Xpress antibodies.

(E) USP10 deubiquitinates PCNA. Cells were treated as in (D), but with overexpression of HA-ubiquitin. They were then subjected to immunoblot with anti-Xpress and anti-Myc antibodies.

(F) USP10 knockdown leads to sustained mono-ubiquitination of PCNA. Cells expressing shNS or shUSP10 were treated with UV. They were then subjected to immunoblot with anti-PCNA, anti-USP10, and anti-USP1 antibodies.

(G) USP10 preferentially binds to ISGylated PCNA in vivo. HisMax-PCNA, Myc-USP10, and ISG15-conjugating system were expressed in cells with and without UBP43-V5-His. Cell lysates were subjected to immunoprecipitation with anti-Xpress antibody followed by immunoblot with anti-Myc and anti-Flag antibodies.

(H) USP10 preferentially binds to ISGylated PCNA in vitro. Myc-PCNA was overexpressed in cells with and without ISG15-conjugating system and EFP. Cell lysates were subjected to incubation with anti-Myc antibody, followed by pulldown with protein-A-conjugated resins. Purified USP10 was then incubated with the resins, washed, and subjected to immunoblot with anti-His and anti-Flag antibodies.

(I) K168R mutation prevents the interaction of PCNA with USP10. HeLa cells overexpressing HisMax-tagged PCNA (Wt) or its K168R mutant were irradiated with UV. They were then subjected to pulldown with NTA resins followed by immunoblot with anti-USP10 antibody.

(J) ISG15 knockdown blocks the interaction of PCNA with USP10. Cells expressing shNS or shISG15 were treated with UV. They were then subjected to immunoprecipitation with anti-PCNA antibody, followed by immunoblot with anti-USP10 and anti-ISG15 antibodies.

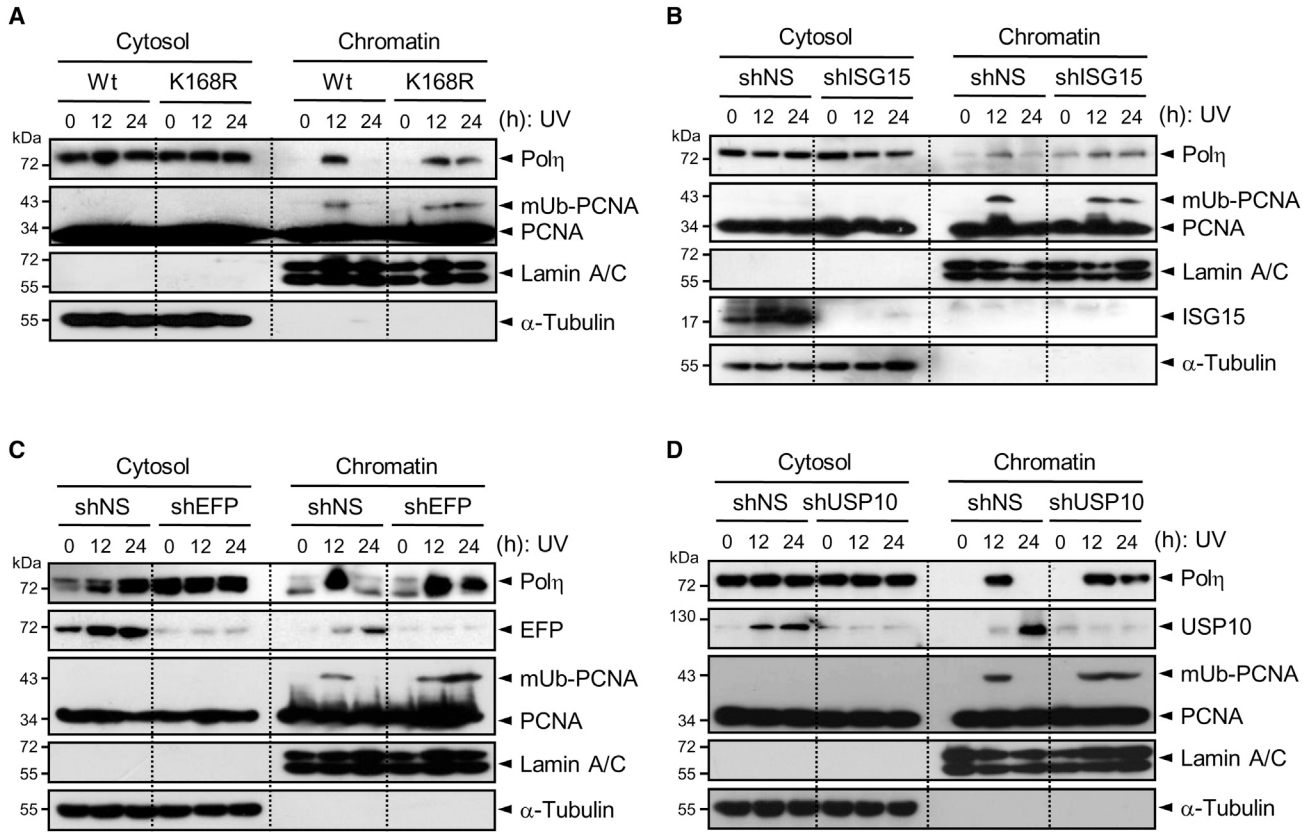

Figure 4. PCNA ISGylation Causes the Release of Polη from PCNA

(A–D) HisMax-tagged PCNA or its K168R mutant (A), shISG15 (B), shEFP (C), or shUSP10 (D) was expressed in HeLa cells. After UV treatment, cells were subjected to chromatin fractionation followed by immunoblot analysis. Lamin A/C was used as a maker for chromatic fraction and α-tubulin was as that for cytosolic fraction.

To determine whether prevention of PCNA ISGylation influences the types of TLS-mediated mutation, we sequenced the *supF* plasmids originated from cells overexpressing PCNA or its K168 mutant or that had been transfected with shISG15, shEFP, or shUSP10. Analysis of the major UV hotspot (^{152}CTTCGAAG^{159}) sequence (Levy et al., 1996) revealed that the TT sequence shows much lower mutation frequency than the remaining sequence (Figure S5B), suggesting that the errors are likely introduced by Polη or another TLS polymerase. Therefore, the increase in mutation frequency by preventing PCNA ISGylation is due to sustained association of Polη or another TLS polymerase to mono-ubiquitinated PCNA (since USP10 cannot be recruited for deubiqutination without ISGylation of PCNA). Collectively, these results indicate that PCNA ISGylation plays a crucial role in maintenance of genome stability by preventing TLS-mediated mutagenesis.

Since TLS is mostly an S phase event, we examined whether overexpression of ISG15-conjugating system or knockdown of ISG15, EFP, USP10, or UBP43 (see below) might give any influence on cell-cycle progression and, in turn, indirectly on TLS-mediated mutagenesis. None of them showed any significant effect on cell-cycle progression or BrdU incorporation, except USP10 knockdown, which slightly increased the fraction of S phase cells (Figure S6). However, the mutation frequency seen by USP10 knockdown with UV treatment was much higher than that without UV (Figure 6A), indicating that the increase in TLS-mediated mutation frequency is not due to an indirect effect of shUSP10 on cell-cycle progression. In addition, overexpression of K164R and K168R mutants and ISG15 knockdown with their overexpression showed no effect on the cell cycle (data not shown). Collectively, these results indicate that the data obtained in this study are not due to any indirect effect of PCNA ISGylation on cell-cycle progression and DNA replication.

PCNA Is Eventually deISGylated by UBP43

To determine the fate of ISGylated PCNA that appeared 12–24 hr after UV treatment (Figures 2B and 2C), we first examined whether PCNA could interact with UBP43. Overexpressed PCNA could bind to UBP43 (Figure 7A, top). On the other hand, endogenous UBP43 interacted with PCNA only at 36 hr (i.e., when the level of UBP43 was dramatically increased) (Figure 7A, bottom). Furthermore, knockdown of UBP43 by shUBP43 led to sustained PCNA ISGylation (Figure 7B), indicating that UBP43 is responsible for deISGylation of PCNA at 36 hr after UV treatment.

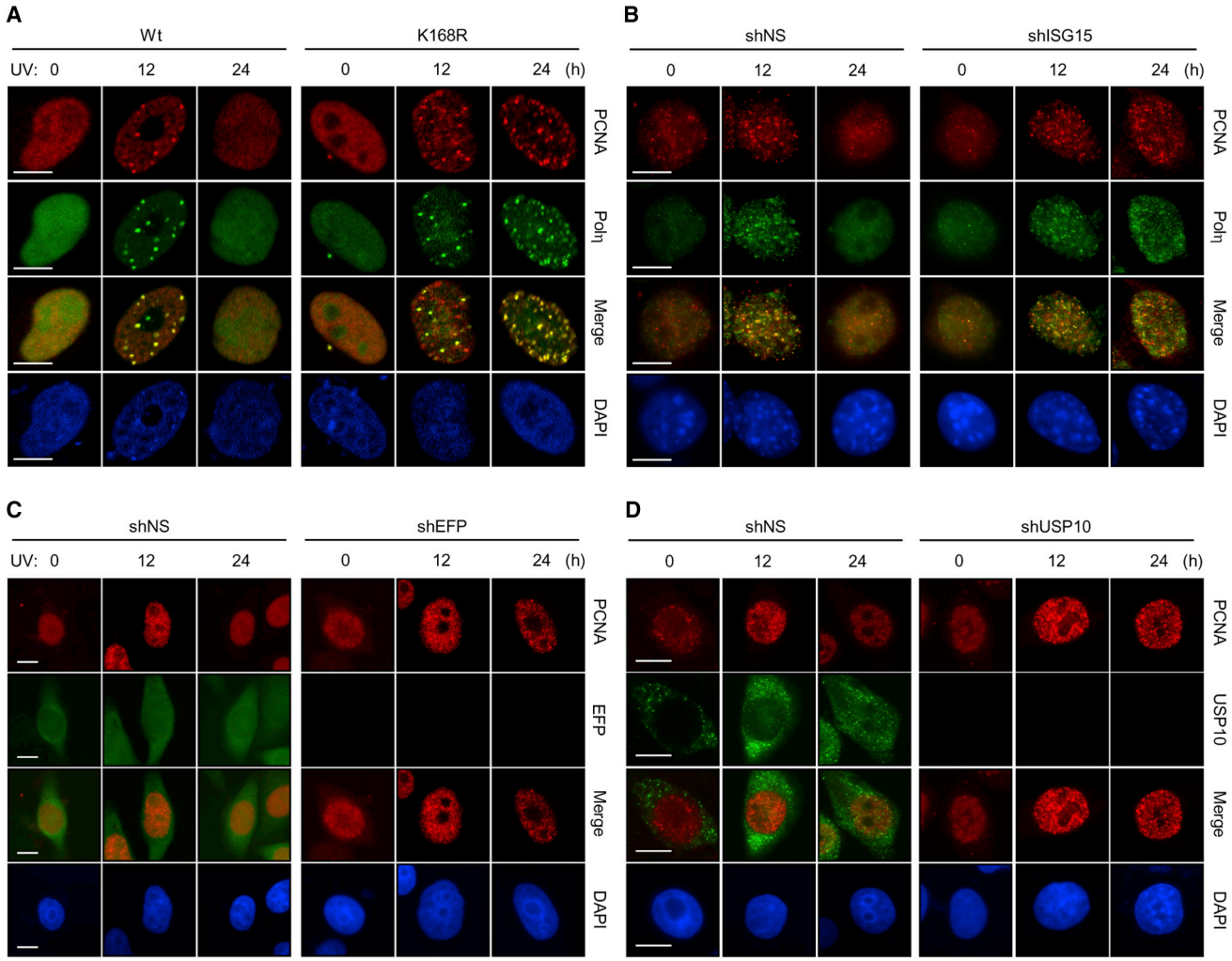

Figure 5. PCNA ISGylation Downregulates Nuclear Foci Formation

(A–D) PCNA or its K168R mutant (A), shISG15 (B), shEFP (C), or shUSP10 (D) was expressed in HeLa cells. After UV treatment, cells were stained with respective antibodies followed by confocal microscopy. Note that cells with more than four foci having both Polη and PCNA were selected for determining their colocalization and for determination of damage sites. Bars, 10 μm.

Of note was the finding that unlike UBP43, ISG15 level was markedly decreased at 36 hr after UV treatment as compared to that at 24 hr. To determine whether the changes in the levels of ISG15 and UBP43 correlate with the time-dependent alterations in the level of ISGylated PCNA, immunoblot analysis was performed using cell lysates obtained at various time points after UV treatment. The level of UBP43 began to increase from 30 hr and remained elevated up to 48 hr (Figure 7C). Consistently, the levels of both mono-ISGylated and doubly ISGylated PCNA began to fall from 30 hr and could no longer be detected after 36 hr. In contrast to UBP43, the levels of EFP and ISG15, which peaked at 24 hr for PCNA ISGylation, declined from 30 hr and could no longer be detected at 36 and 42 hr, respectively. In addition, the level of mono-ubiquitinated PCNA began to fall at 18 hr, concurrently with the increase in the levels of mono-ISGylated PCNA and USP10. Figure 7D summarizes the rise-and-fall of mono-ubiquitinated PCNA, ISG15, EFP, mono-ISGylated and doubly ISGylated PCNA, USP10, and UBP43 occurs in a time-dependent and sequential manner after UV irradiation. These results indicate that the changes in the levels of both mono-ubiquitinated and ISGylated PCNA are tightly regulated by the alterations in the expression of its modifying enzymes as well as of ISG15.

Since mono-ubiquitination and double ISGylation of PCNA are required for its interaction with EFP and USP10, respectively, UBP43-mediated deISGylation likely plays a role in dissociation of EFP and USP10 from PCNA and, in turn, in reloading of replicative DNA polymerases for resumption of normal DNA replication. To test this possibility, we performed in vitro binding assay by using purified PolD1 (the catalytic subunit of Polδ). ISGylation of PCNA, like mono-ubiquitination, strongly inhibited its interaction with PolD1, and this inhibition could be reversed by coexpression of UBP43 (Figure 7E). These results indicate that PolD1 preferentially binds to unmodified form of PCNA for DNA

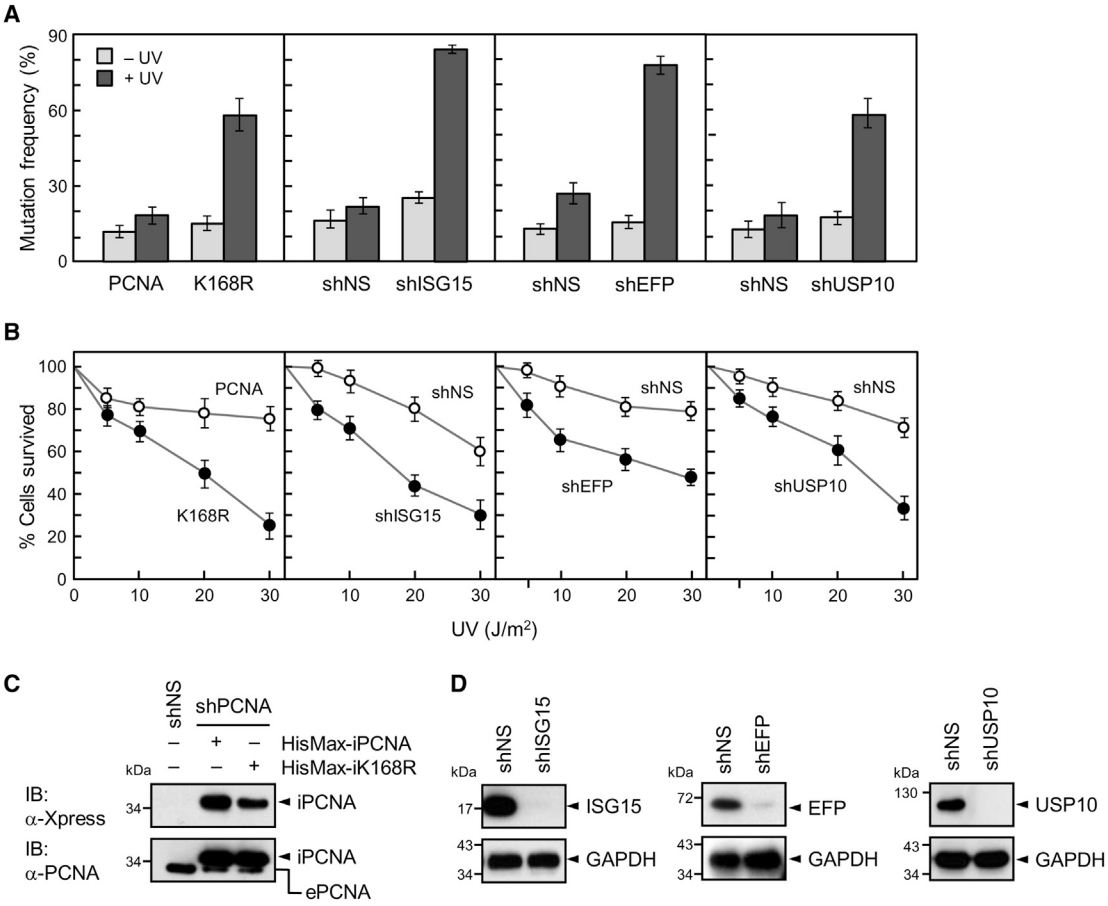

Figure 6. PCNA ISGylation Blocks TLS-Mediated Mutagenesis

(A) Prevention of PCNA ISGylation leads to an increase in mutation frequency. HeLa cells were subjected to *supF*-based mutation assay as described under Experimental Procedures. The data were obtained from three independent experiments (±SD).

(B) Prevention of PCNA ISGylation leads to an increase in cell death. Cells expressing PCNA or its K168R mutant, shISG15, shEFP, or shUSP10 were treated with UV. After incubation for 24 hr, their viability was determined by staining with trypan blue. Error bar, ±SD.

(C) HisMax-tagged, shRNA-insensitive PCNA or its K168R mutant was overexpressed in cells that had been transfected with shPCNA. Cells that stably express both shPCNA and the PCNA proteins were then selected by incubation with puromycin (2 μg/ml) and G418 (200 μg/ml) for 5 days. Cell lysates were subjected to immunoblot analysis. "i" and "e" denote insensitive and endogenous, respectively.

(D) Cells expressing shISG15, shEFP, or shUSP10 were subjected to immunoblot analysis.

synthesis. Collectively, these results indicate that reversible modification of PCNA by ISG15 plays a crucial role in TLS termination and subsequent resumption of normal DNA replication.

DISCUSSION

Based on the present findings, we propose a model for the role of reversible PCNA ISGylation in TLS termination and resumption of normal DNA replication (Figure S7). Upon DNA damage by UV, PCNA is mono-ubiquitinated for recruitment of Polη and initiation of TLS, as previously documented (Bienko et al., 2005; Kannouche et al., 2004; Lehmann et al., 2007; Stelter and Ulrich, 2003). After DNA lesion bypass, EFP is tethered to mono-ubiquitinated PCNA and generates mono-ISGylated PCNA and then doubly ISGylated PCNA. Doubly ISGylated PCNA subsequently recruits PIP-box-containing USP10 for deubiquitination of PCNA and, in turn, for the release of Polη from PCNA for termination of TLS. Finally, UBP43 is induced timely and cleaves off ISG15 from PCNA for reloading of replicative DNA polymerases and resuming of DNA replication. Collectively, these results indicate that mono-ubiquitination, ISGylation, deubiquitination, and deISGyation of PCNA occur in order after UV irradiation for initiation and termination of TLS and then for resumption of replicative DNA synthesis.

Of particular interest was the finding that the expression of PCNA-modifying enzymes (i.e., EFP, USP10, and UBP43) is timely and sequentially induced for step-by-step modification of PCNA after UV treatment (Figure 7). A dramatic example is the expression of UBP43, which occurred at last when PCNA ISGylation was no longer required (i.e., when TLS was turned off). Of also interest was the finding that EFP and ISG15 were rapidly eliminated from cells when they are no longer necessary, indicating that proteolysis is involved in this process. Note that the levels of USP10 and UBP43 also declined from 48 hr after

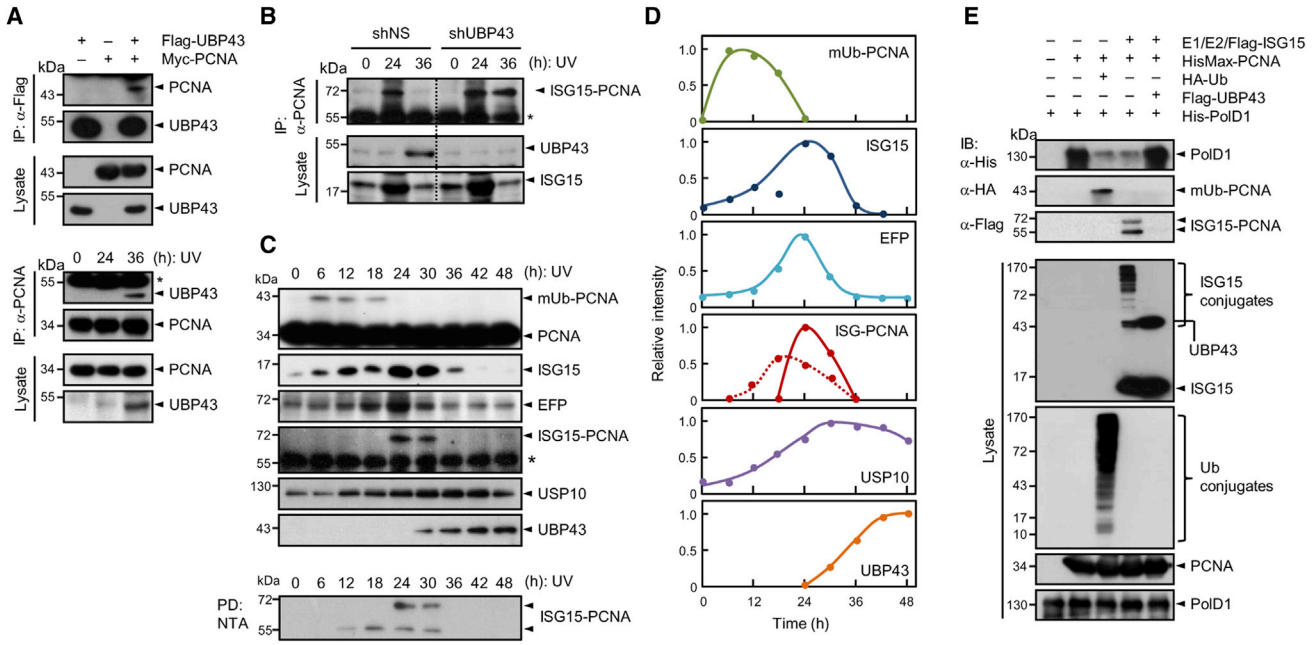

Figure 7. PCNA Is Eventually DeISGylated by UBP43 for Reloading of Polδ

(A) UBP43 interacts with PCNA. Flag-UBP43 was expressed in HeLa cells with Myc-PCNA. Cell lysates were subjected to immunoprecipitation with ant-Flag antibody followed by immunoblot with anti-Myc and anti-Flag antibodies (top). Cells treated with UV were subjected to immunoprecipitation with anti-PCNA antibody followed by immunoblot with anti-UBP43 and anti-PCNA antibodies (bottom).

(B) UBP43 knockdown leads to sustained PCNA ISGylation. Cells transfected with shNS and shUBP43 were treated with UV. They were then subjected to immunoprecipitation with anti-PCNA antibody, followed by immunoblot with anti-ISG15 antibody.

(C and D) Sequential modification of PCNA by ubiquitin and ISG15 and expression of ISG15, EFP, USP10, and UBP43.

(C) After UV treatment, cells were incubated for increasing periods. They were then subjected to immunoblot analysis (top). Cells were treated as above but with ectopic expression of HisMax-PCNA. Cell lysates were subjected to pulldown with NTA resins, followed by immunoblot with anti-ISG15 antibody (bottom).

(D) The gels in (C) were scanned using a densitometer, and the band intensities were determined using "Image J" software. The peak intensity of each protein was set as 1.0, and the intensities of the others were as its relative values. In the "ISG-PCNA" panel, the dotted and solid lines indicate the changes in the levels of mono-ISGylated and doubly ISGylated PCNA, respectively. These data were obtained from the bottom panel of (C).

(E) PolD1 preferentially interacts with unmodified PCNA. HisMax-PCNA was expressed in HeLa cells with and without ISG15-conjugating system, HA-ubiquitin, or Flag-UBP43. Cell lysates were subjected to incubation with anti-Xpress antibody, followed by pulldown with protein-A-conjugated resins, and incubated with purified His-PolD1. The resins were then washed and subjected to immunoblot with anti-His, anti-HA, and anti-Flag antibodies.

UV treatment (data not shown). Therefore, it would be of interest to see if ubiquitin-proteasome pathway is involved in the degradation of ISG15 and the PCNA-modifying enzymes. However, an important unanswered question is how the timely expression and degradation of the proteins is regulated in response to UV. This important question awaits further studies.

Surprisingly, the binding of USP10 to unmodified endogenous PCNA could not be detected (Figure 3J), despite the fact that it has a noncanonical PIP box. Recently, Kim et al. (2012) showed that the affinity of noncanonical PIP box in Srs2 to PCNA is much lower than that of classical PIP box, but it could dramatically be enhanced upon sumoylation of PCNA. They also observed a conformational change in sumoylated PCNA upon binding of Srs2. Based on this finding, they suggested that the binding affinity enhancement would give the ability of Srs2 to distinguish the sumoylated PCNA from its unmodified form and to compete with other PCNA-binding proteins. Likewise, the binding of USP10 to PCNA via its noncanonical PIP box might be very weak, but it could be markedly enhanced by PCNA ISGylation, and this enhancement might provide the ability of USP10 to distinguish ISGylated PCNA from its unmodified form and to compete with other PCNA-binding protein.

Other mechanisms for the replacement of Polη by replicative DNA polymerases have been proposed. Upon DNA damage, human DVC1 (also called SPARTAN), having both UBZ domain and PIP box, accumulates at stalled replication forks and recruits the ubiquitin-selective chaperone p97 independently of RAD18-mediated PCNA mono-ubiquitination (Mosbech et al., 2012). Moreover, DVC1 knockdown enhances TLS-mediated mutagenesis and causes hypersensitivity to replication stress-inducing agents. In addition, UV-induced PCNA mono-ubiquitination in MRC5V1 cells was shown to persist long after clearance of the damage and TLS polymerase foci (Niimi et al., 2008). Therefore, it was suggested that DVC1-bound p97, by means of its ATPase activity, may promote the exchange of Polη with replicative DNA polymerases after lesion bypass (Mosbech et al., 2012). Interestingly, we found that in certain cells, such as HEK293T cells, ISG15-conjugating system is not induced upon DNA damage by UV, HU, or MMS (data not shown). Moreover, as in MRC5V1 cells, the level mono-ubiquitinated in HEK293T cells

remained elevated for a much longer period than that in HeLa and MCF10A cells. Thus, it appears that cells lacking the ability to induce ISG15-conjugating system employ the DVC1-p97 system for replacement of Polη by replicative DNA polymerase after lesion bypass, thereby mitigating TLS-mediated mutagenesis.

An alternative mechanism for the exchange of Polη with replicative DNA Pols might involve PAF15 (Povlsen et al., 2012). PAF15, normally mono-ubiquitinated at two different sites, binds to PCNA but is rapidly degraded via ubiquitin-proteasome pathways in response to UV. Interestingly, mono-ubiquitination-deficient K-to-R mutations abrogate the recruitment of Polη to DNA damage-induced nuclear foci, while PAF15 knockdown results in its sustained recruitment. Therefore, it was suggested that PAF15 might play a role in the release of Polη from PCNA, although the underlying mechanism for the polymerase exchange remains unknown.

PCNA is subjected to a multitude of posttranslational modification for the control of diverse PCNA-mediated processes, including phosphorylation, ubiquitination, and sumoylation. Here, we introduced an additional type of PCNA modification by ISG15 in response to UV and provided evidence how this modification orchestrates with mono-ubiquitination for the control of error-prone TLS. Since numerous PCNA-associated proteins cooperate to ensure proper response to DNA damage, it will be of interest to see if other proteins are also modified by ISG15 and how this modification contributes to safeguarding genome stability.

EXPERIMENTAL PROCEDURES

Chromatin Fractionation

Chromatin fractionation was performed as described (Méndez and Stillman, 2000) with minor modifications. Briefly, cells were suspended in 10 mM HEPES (pH 7.8), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, and 1× protease inhibitor cocktail. After incubation for 5 min on ice, the samples were subjected to centrifugation for 5 min at 3,000 × *g*. The supernatants were again centrifuged at 15,000 × *g* for 15 min, and the soluble fractions were used as the cytosol. The pellets from the low-speed centrifugation were resuspended in 3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and 1× protease inhibitor cocktail; incubated on ice for 30 min; and centrifuged for 5 min at 3,000 × *g*. The insoluble fraction was used as the chromatin.

supF Plasmid-Based Mutation Assay

The pSP189 shuttle vector and MBM7070 *E. coli* strain used for the *supF* assay have been described (Parris and Seidman, 1992). The pSP189 plasmids were irradiated with UV (1,000 J/m^2) and transfected into HeLa cells that had been transfected with shRNAs. After incubation for 48 hr, the plasmids were purified from the cells using DNA mini-prep kit (Promega). Purified plasmids were digested with DpnI, ethanol-precipitated, and introduced into the *E. coli* MBM7070 strain by electroporation. Cells were then plated on X-gal/IPTG/Amp LB plates. The mutation frequency in the *supF*-coding region was calculated as the percentage of white colonies in total colonies.

Cell Cycle Analysis

HeLa cells were labeled with bromodeoxyuridine (BrdU) for 1 hr prior to harvesting. Cells were trypsinized and fixed in ice-cold 70% ethanol. They were then stained with anti-BrdU-conjugated fluorescein isothiocyanate (Becton Dickinson, San Jose). Cells were washed, centrifuged, and resuspended in 50 μg/ml propidium iodide, and cell-cycle progression was analyzed using a FACSCalibur flow cytometer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.03.031.

AUTHOR CONTRIBUTIONS

J.M.P., S.W.Y., J.H.S., Y.J.J., and C.H.C. conceived the experiments, analysed the results, and wrote the paper. J.M.P., S.W.Y., K.R.Y., S.H.K., and S.W.L. performed the experiments.

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