USP7 Is a Suppressor of PCNA Ubiquitination and Oxidative-Stress-Induced Mutagenesis in Human Cells

Graphical Abstract

Highlights

- USP7 deubiquitinates mono-ubiquitinated PCNA in vitro
- USP7 suppresses UV- and oxidative-stress-induced PCNA mono-ubiquitination in cells
- USP1 acts in S-phase, whereas USP7 acts throughout interphase
- USP1 and USP7 suppress UV- and H₂O₂-induced mutagenesis, respectively

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In Brief

PCNA mono-ubiquitination regulation is crucial for a balance of DNA damage tolerance and mutagenesis. Kashiwaba et al. reveal that USP7 deubiquitinates mono-ubiquitinated PCNA. Different from USP1, which suppresses DNA-replication-coupled PCNA ubiquitination and mutagenesis after UV irradiation, USP7 suppresses oxidative-stress-induced PCNA ubiquitination and mutagenesis independently of the cell cycle.

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USP7 Is a Suppressor of PCNA Ubiquitination and Oxidative-Stress-Induced Mutagenesis in Human Cells

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SUMMARY

Mono-ubiquitinated PCNA activates error-prone DNA polymerases; therefore, strict regulation of PCNA mono-ubiquitination is crucial in avoiding undesirable mutagenesis. In this study, we used an in vitro assay system to identify USP7 as a deubiquitinating enzyme of mono-ubiquitinated PCNA. Suppression of USP1, a previously identified PCNA deubiquitinase, or USP7 increased UV- and H2O2-induced PCNA mono-ubiquitination in a distinct and additive manner, suggesting that USP1 and USP7 make different contributions to PCNA deubiquitination in human cells. Cell-cycle-synchronization analyses revealed that USP7 suppression increased H2O2-induced PCNA ubiquitination throughout interphase, whereas USP1 suppression specifically increased ubiquitination in S-phase cells. UV-induced mutagenesis was elevated in USP1-suppressed cells, whereas H2O2-induced mutagenesis was elevated in USP7-suppressed cells. These results suggest that USP1 suppresses UV-induced mutations produced in a manner involving DNA replication, whereas USP7 suppresses H2O2-induced mutagenesis involving cell-cycle-independent processes such as DNA repair.

INTRODUCTION

Post-translational mono-ubiquitination of PCNA (proliferating cell nuclear antigen) is an important event in the regulation of translesion DNA synthesis (TLS) pathways (Hoege et al., 2002). Human DNA polymerase η (Poliη), mutations that are responsible for the cancer-prone syndrome Xeroderma pigmentosum variant (Johnson et al., 1999; Masutani et al., 1999), and the other Y-family DNA polymerases—polymerase i (Poli), polymerase κ (Polek), and REV1—have PCNA- and ubiquitin-interacting domains and are recruited to stalled replication forks (Bienko et al., 2005; Garg and Burgers, 2005; Guo et al., 2006; Jones et al., 2012; Kannouche et al., 2004; Qin et al., 2013). These DNA polymerases are intrinsically mutagenic; therefore, mono-ubiquitination of PCNA must be strictly tuned in order to regulate these error-prone polymerases and maintain genome integrity. In human cells, the E2-ubiquitin-conjugating enzyme RAD6 and the E3 ubiquitin ligase RAD18 play crucial roles in PCNA ubiquitination in response to DNA damage induced by UV irradiation or agents including reactive oxygen species (Kannouche et al., 2004; Niimi et al., 2008; Zlatanou et al., 2011). Deubiquitinating enzymes (DUBs) also play crucial roles in regulating the ubiquitination levels of cellular proteins. Downregulation of USP1 increases the basal level of PCNA mono-ubiquitination, which is produced during DNA replication and after UV irradiation. Notably, USP1 is autocleaved and subsequently degraded by the proteasomal pathway several hours after UV irradiation at a rate that is inversely proportional to the increase in mono-ubiquitinated PCNA (Huang et al., 2006). By contrast, oxidative-stress-induced PCNA mono-ubiquitination is regulated independently of USP1 (Zlatanou et al., 2011). Furthermore, mono-ubiquitination of PCNA in response to DNA damage does not occur only in S-phase and facilitates the gap-filling step of DNA repair (Ogi et al., 2010; Yang et al., 2013). USP1 is suppressed by proteasomal degradation mediated by APC/Ccdc11 in G0/G1 phase (Cotto-Rios et al., 2011); therefore, other DUBs might be responsible for deubiquitinating the ubiquitinated PCNA outside of S-phase.

USP7 (or HAUSP) is a regulator of the p53-Mdm2 ubiquitination pathway (Brooks and Gu, 2006). In addition, a number of recent reports suggested that USP7 contributes to DNA damage responses. Specifically, USP7 plays crucial roles in transcription-coupled nucleotide excision repair by interacting with UVSSA (Schwertman et al., 2012; Zhang et al., 2012), and it is also involved in global genome nucleotide excision repair by deubiquitinating XPC protein (He et al., 2014). In addition, USP7 is involved in the efficient repair of oxidative DNA lesions
by regulating chromatin structure (Khoronenkova et al., 2011). Furthermore, USP7 has been implicated in the regulation of PCNA ubiquitination systems. In particular, USP7 deubiquitinates one of the human orthologs of yeast Rad5, HLTF, and facilitates the poly-ubiquitination of PCNA (Qing et al., 2011). Recent work also showed that USP7 indirectly modulates UV-induced PCNA mono-ubiquitination by regulating the stability of Polh (Qian et al., 2015). During the review of this article, it was also reported that USP7 stabilizes RAD18 by deubiquitination and, therefore, upregulates PCNA ubiquitination (Zlatanou et al., 2015).

In this study, we identified USP7 as a deubiquitinating enzyme of mono-ubiquitinated PCNA in vitro. Downregulation of USP7 and USP1 increased the level of PCNA mono-ubiquitination induced by UV irradiation and oxidative stresses in different manners. Importantly, downregulation of USP1 and USP7 promoted UV-induced and H2O2-induced mutagenesis, respectively, and both effects were abolished by the downregulation of RAD18. These results indicate that USP7 regulates DNA-damage-induced mutagenesis in human cells via deubiquitination of mono-ubiquitinated PCNA.

RESULTS

USP7 Deubiquitinates Mono-ubiquitinated PCNA In Vitro
To elucidate the regulatory mechanisms of PCNA mono-ubiquitination, we investigated protein factors from HeLa cell extracts that can modulate in vitro ubiquitination reactions. A deubiquitinating activity was detected, and sequential column chromatography was performed to purify the responsible protein (Figures 1A–1D). Ultimately, we identified a polypeptide that migrated at ~140 kDa in SDS-PAGE gels; this peptide co-eluted with the active fraction isolated by column chromatography (Figures 1C and 1D). Mass spectrometry (MS) analyses revealed that the 140-kDa polypeptide was the ubiquitin-specific protease USP7. Recombinant-hexahistidine-tagged recombinant USP7, by itself, was capable of deubiquitinating the mono-ubiquitinated PCNA, which was prepared by the in vitro ubiquitination system and subsequently purified (Figure 1E). With observations that no stoichiometric protein co-purified with USP7 from HeLa cells (Figure 1D) and that recombinant USP7 behaved similarly to USP7 from HeLa cells in gel filtration chromatography (data not shown), we conclude that the deubiquitinating activity for mono-ubiquitinated PCNA is intrinsic to USP7 without co-factors in vitro. USP1 protein was detected in the side fractions of MonoQ column chromatography (data not shown).

USP7 Contributes to the Suppression of UV-Induced PCNA Mono-ubiquitination in a Mode Distinct from that of USP1
To determine whether USP7 contributes to UV-induced PCNA mono-ubiquitination in addition to USP1, we performed siRNA-mediated suppression of these USPs in human cells. After irradiation with UVC, the level of mono-ubiquitinated PCNA gradually increased for 6–12 hr and then decreased at 24 hr in human fibroblast WI38VA13 cells transfected with control siRNA (Figure 2A, lanes 1–5). The increase in PCNA mono-ubiquitination was inversely proportional to the USP1 protein level, which decreased several hours after UV irradiation, as reported previously (Huang et al., 2006). Suppression of USP1 increased the basal level of PCNA mono-ubiquitination in unirradiated cells, which is produced during DNA replication (Huang et al., 2006). In USP7-suppressed cells, the level of PCNA mono-ubiquitination was...
significantly higher than in control cells; however, in contrast to USP1-suppressed cells, the alteration in kinetic profile was not as evident (Figure 2A, lanes 6–10). In cells in which both USP7 and USP1 were suppressed, the levels of basal and UV-induced mono-ubiquitination were elevated, and the kinetic profile after UV irradiation was similar to that of USP1-suppressed cells (Figure 2A, lanes 16–20). These results suggest that USP1 plays a prominent role in suppressing PCNA mono-ubiquitination in the relatively early period after UV irradiation, as well as in the unirradiated state, and that USP7 contributes to the quantitative regulation. Mono-ubiquitinated PCNA induced by UV irradiation decreased at 24 hr, even in the absence of both USP1 and USP7 (Figure 2A, lane 20), suggesting that cells have an additional mechanism for scavenging mono-ubiquitinated PCNA. Thus, PCNA mono-ubiquitination is regulated by multiple DUBs, including USP7.

**USP7 Contributes Prominently to the Suppression of H2O2-Induced in Addition to UV-Induced PCNA Mono-ubiquitination**

In contrast to UV-induced PCNA mono-ubiquitination, which is a relatively slow and prolonged response, oxidative-stress-induced mono-ubiquitination occurs more quickly and transiently. The level of mono-ubiquitinated PCNA increased immediately after H2O2 treatment, reached a maximum after 15–30 min, and then almost vanished within 2 hr (Figure S1A). H2O2 promoted mono-ubiquitination of PCNA in a dose-dependent manner, but the kinetics were barely affected by the H2O2 dose (Figure S1B). No significant changes in the levels of USP1 and USP7 were observed for 2 hr following H2O2 treatment (Figure S1B). Notably, suppression of USP7 increased the level of mono-ubiquitinated PCNA after H2O2 treatment (Figure S1B). Independent siRNAs targeting USP7 increased the level of H2O2-induced PCNA mono-ubiquitination (Figures S1C and S1D). Ectopic expression of FLAG-USP7 resistant to the siRNA cancelled the effect, but expression of the catalytically dead USP7 mutant (Faesen et al., 2011) did not (Figure S1E). This confirmed the USP7 dependence of the observed phenomena and ruled out an off-target effect of the siRNA. Suppression of USP7 increased H2O2-induced PCNA mono-ubiquitination not only in WI38VA13 cells but also in the other cell lines tested (Figures S1F and S1G), and also in HeLa cells (as shown later). From these results, we conclude that USP7 contributes to the regulation of H2O2-induced PCNA mono-ubiquitination in human cells. Suppression of neither USP1 nor USP7 affected cell-cycle distribution (Figure S1H); therefore, the observed differences in H2O2-induced PCNA mono-ubiquitination between USP1- or USP7-suppressed cells were not due to differences in the populations of cells at various stages of the cell cycle. H2O2 treatment inactivates DUBs, including USP1, resulting in the elevation of ubiquitinated PCNA (Lee et al., 2013). However, suppression...
Cell-Cycle-Independent Contribution of USP7 to the Suppression of PCNA Mono-ubiquitination

To investigate the contributions of USP7 to the regulation of PCNA ubiquitination in different cell-cycle phases, HeLa cells were transfected with control, USP7-targeting, or USP1-targeting siRNA and synchronized at M-phase by nocodazole treatment. Mitotic cells were collected and released into the cell cycle, and then they were treated with H$_2$O$_2$ or irradiated with UV at 8, 16, or 20 hr after release (Figure 3A). Suppression of USP7 or USP1 did not alter the efficiency of synchronization and cell-cycle progression after release (Figure 3B): about 80% of the cells were synchronized in M-phase at the time of release (0 hr). At 8 hr after release, the majority of the cells (>60%) were in G1, and only a trace population had entered S-phase. The percentage of S-phase cells reached a maximum (about 40%) at 16 hr after release. These results suggest that H$_2$O$_2$-induced mono-ubiquitination of PCNA regulated by USP7 occurred on lysine 164 in an RAD18-dependent manner. It was recently reported that knockout or complete suppression of USP7 destabilizes RAD18, resulting in compromised PCNA mono-ubiquitination (Zlatanou et al., 2015). We also observed decreases in the RAD18 protein levels in some experiments (Figures S1D and S1G). Fortunately, however, cells still had sufficient RAD18 protein to produce mono-ubiquitinated PCNA under our experimental conditions, allowing us to examine the roles of USP7 in PCNA deubiquitination.

Cell-Cycle-Independent Contribution of USP7 to the Suppression of PCNA Mono-ubiquitination

of USP7 and also USP1 clearly increased H$_2$O$_2$-induced PCNA mono-ubiquitination, indicating that some fraction of these enzymes is still active in H$_2$O$_2$-treated cells and capable of suppressing H$_2$O$_2$-induced PCNA ubiquitination. The increase in H$_2$O$_2$-induced mono-ubiquitination following USP7 suppression was not observed in cells expressing hemagglutinin (HA)-tagged PCNA mutated at K164R (Figure 2C), and H$_2$O$_2$-induced PCNA mono-ubiquitination promoted by the suppression of USP7 was significantly reduced upon co-suppression of RAD18 (Figure 2D). These results suggest that H$_2$O$_2$-induced mono-ubiquitination of PCNA regulated by USP7 occurred on lysine 164 in an RAD18-dependent manner. It was recently reported that knockout or complete suppression of USP7 destabilizes RAD18, resulting in compromised PCNA mono-ubiquitination (Zlatanou et al., 2015). We also observed decreases in the RAD18 protein levels in some experiments (Figures S1D and S1G). Fortunately, however, cells still had sufficient RAD18 protein to produce mono-ubiquitinated PCNA under our experimental conditions, allowing us to examine the roles of USP7 in PCNA deubiquitination.

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16 hr and then decreased to about 10% by 20 hr after release. The low populations of S-phase cells at 8 hr were confirmed by the absence of cyclin A, which was present at 16 and 20 hr after release (Figures 3C and 3D). Thus, at 8 and 16 hr after release, the most prevalent populations were G1- and S-phase cells, respectively, and at 20 hr, substantial populations were S/G2-phase cells, although the major population was in G1. Consistent with a previous report that USP1 is downregulated in G0/G1-phase (Cotto-Rios et al., 2011), USP1 protein levels tended to be lower in control and USP7-suppressed cells at 8 hr after release than at 16 or 20 hr, regardless of UV irradiation or H2O2 treatment (Figures 3C and 3D, lanes 1–12). On the other hand, USP7 protein levels did not change throughout the cell cycle in control or USP1-suppressed cells (Figures 3C and 3D, lanes 1–6 and 13–18).

In undamaged cells, suppression of USP1 significantly promoted mono-ubiquitination of PCNA at 16 and 20 hr, but not at 8 hr, after release (Figures 3C and 3D, lanes 13–15). Suppression of USP7 only faintly increased the level of mono-ubiquitinated PCNA at 16 and 20 hr after release (Figures 3C and 3D, lanes 8–9). These findings suggest that USP1 plays a prominent role in the suppression of basal levels of PCNA mono-ubiquitination during S-phase. We could detect a tiny amount of mono-ubiquitinated PCNA at 30 min after UV irradiation, regardless of the cell-cycle phase (Figure 3C, lanes 4–6, for a long exposure). Suppression of USP1 promoted UV-induced PCNA mono-ubiquitination at 16 and 20 hr, but not at 8 hr, after release (Figure 3C; compare lanes 16–18 with lanes 4–6), again suggesting that USP1 suppresses UV-induced PCNA mono-ubiquitination during DNA replication. No contribution of USP7 to PCNA mono-ubiquitination 30 min after UV irradiation was evident at 8, 16, or 20 hr after release (Figure 3C, lanes 10–12).

After H2O2 treatment for 20 min, mono-ubiquitination of PCNA was induced more effectively at 8 and 20 hr than at 16 hr after release in cells treated with control siRNA (Figure 3D, lanes 4–6). Suppression of USP1 increased H2O2-induced PCNA mono-ubiquitination to a greater extent at 16 and 20 hr than at 8 hr after release (Figure 3D, lanes 16–18), relative to control cells (lanes 4–6). In contrast to USP1 suppression, suppression of USP7 promoted H2O2-induced mono-ubiquitination of PCNA at all three time points relative to control cells (Figure 3D; compare lanes 10–12 with lanes 4–6). These results suggest that USP1 is crucial for suppressing replication-coupled PCNA mono-ubiquitination, whereas USP7 contributes to the suppression of PCNA mono-ubiquitination during cell-cycle-independent events.

Exclusive Contributions of USP1 and USP7 to the Suppression of UV- and H2O2-Induced Mutagenesis

To investigate the physiological relevance of PCNA mono-ubiquitination regulated by USP7, we compared the impacts of USP7 and USP1 suppression on cellular sensitivity and mutagenesis following UV irradiation and H2O2 treatment. USP7 and USP1 contribute to multiple pathways by regulating the ubiquitination status of several target proteins (Garcia-Santisteban et al., 2013; Nicholson and Suresh Kumar, 2011). Therefore, we also targeted RAD18 to evaluate the effects on the regulation of PCNA ubiquitination (Figure S2). Given that suppression of RAD18 would diminish PCNA ubiquitination, we hypothesized that co-suppression of RAD18 would abolish the effect of single USP suppression if that effect was brought about by elevated PCNA ubiquitination.

No effect of USP1 suppression on cellular sensitivity to UV irradiation was evident, but USP7-suppressed cells exhibited a mild reduction in survival relative to cells treated with control siRNA (Figure 4A; Figure S2D). This is consistent with previous reports that USP7 contributes to transcription-coupled nucleotide excision repair of UV-induced DNA lesions by stabilizing UVSSA and that depletion of USP7 increases UV sensitivity similarly to the depletion of UVSSA (Schwertman et al., 2012; Zhang et al., 2012). Suppression of USP7 and/or USP1 had no effect on cellular sensitivity to H2O2 treatment (Figures 4B and S2E).

Suppression of USP1 promotes UV-induced mutagenesis in supF mutation analyses (Huang et al., 2006). Consistent with this, the mutation frequency (determined by counting 6-thioguanine-resistant colonies) increased in USP1-suppressed cells following UV irradiation (p < 0.01), indicating that USP1 suppression promoted UV-induced mutations in the HPRT gene (Figure 4C; Table S1). However, we could not exclude the possibility that USP1 suppressed spontaneous mutations because USP1 suppression tended to increase mutations in mock-treated cells. Co-suppression of RAD18 decreased the mutation frequency of USP1-suppressed cells (p < 0.1), suggesting that the mutations were induced in a manner that depended upon PCNA ubiquitination. USP7 suppression might also have the potential to increase UV-induced mutations, but not significantly so (Figure 4C; Table S1). Therefore, we conclude that USP1, but not USP7, plays a prominent role in suppressing UV-induced mutations by controlling PCNA ubiquitination.

By contrast, suppression of USP7 by two independent siRNAs significantly increased the rate of H2O2-induced HPRT mutation (p < 0.01) (Figure 4D; Table S2). Co-suppression of RAD18 suppressed these increases in mutation frequency (p < 0.05), suggesting that the mutations were induced in a manner that depended upon PCNA ubiquitination. Suppression of USP1 did not increase the frequency of H2O2-induced mutations, regardless of the USP7 status (Figure 4D; Table S2), confirming its minor role in H2O2-induced mutagenesis. USP1 is reportedly inactivated after H2O2 treatment (Cotto-Rios et al., 2012). Therefore, one possible explanation is that USP7 has a general back-up role in targeting mono-ubiquitinated PCNA and that this is more noticeable when USP1 is inactivated. If this was the case, it is expected that UV-induced mutagenesis of USP1-suppressed cells would be increased by co-suppression of USP7. However, co-suppression of USP7 had little effect on UV-induced mutagenesis (Figure 4C; Table S1). Together with the findings that USP1 and USP7 had different contributions during cell-cycle stages (Figure 3), it is likely that USP7 suppresses H2O2-induced mutagenesis during cell-cycle-independent processes by controlling PCNA ubiquitination independently of USP1.

**DISCUSSION**

In this study, we identified the ubiquitin-specific protease USP7 as a regulator of PCNA ubiquitination and oxidative-stress-induced mutagenesis in human cells. Our findings suggest that...
Figure 4. Exclusive Contributions of USP7 and USP1 to Cellular Responses to H2O2 Treatment and UV Irradiation

(A and B) Cellular sensitivities. WI38VA13 cells transfected with the indicated siRNAs were treated with indicated doses of UVC (A) or H2O2 (B). Data represent the means ± SD of at least three independent experiments. si, small interfering; Ctrl, control.

(C and D) Mutagenesis. HAT (hypoxanthine, aminopterin, and thymidine) pre-selected WI38VA13 cells transfected with the indicated siRNAs were treated with 8 J/m² UVC (C) or 250 μM H2O2 for 1 hr (D). Data represent the means ± SD of at least three independent experiments.

See also Figure S2, Table S1, and Table S2.
USP7 and USP1 exclusively regulate PCNA ubiquitination, depending on lesion type and cell-cycle state, to maintain genome integrity.

In addition to UV irradiation or treatment with DNA-damaging agents, PCNA mono-ubiquitination is also induced by treatment with excess thymidine or hydroxyurea (Yusa et al., 2006), suggesting that DNA replication stresses, rather than DNA lesions themselves, induce PCNA ubiquitination. However, recent reports also demonstrate that DNA-replication-independent mono-ubiquitination of PCNA results in the recruitment of TLS polymerases and facilitates repair synthesis (Ogi et al., 2010; Yang et al., 2013; Zlatanou et al., 2011). These results suggest that at least two distinguishable PCNA mono-ubiquitination systems exist, one coupled with DNA replication and the other coupled with DNA repair. Our observation that USP1 and USP7 contribute to the suppression of PCNA mono-ubiquitination provides additional support for the existence of two exclusive systems. Furthermore, inactivation of USP1 in S-phase is crucial for the activation of TLS past UV-induced lesions (Huang et al., 2006), whereas USP7 acts throughout interphase, suggesting that USP1 and USP7 regulate DNA-replication-coupled and DNA-repair-coupled PCNA mono-ubiquitination, respectively. In this scenario, if USP1 is suppressed, replication-coupled PCNA mono-ubiquitination would be disordered, excess error-prone DNA polymerases would be included in the DNA replication process, and the rate of replication-coupled mutation would be elevated. On the other hand, in the absence of USP7, the level of repair-coupled PCNA mono-ubiquitination would be elevated, excess error-prone DNA polymerases would be included in the DNA repair process, and the rate of repair-coupled mutation would increase.

However, despite the elevation of UV-induced PCNA ubiquitination in either USP1- or USP7-suppressed cells, we detected a higher rate of mutation after UV irradiation in USP1-suppressed cells, but this was not significant in USP7-suppressed cells. The converse was also true: H2O2-induced mono-ubiquitination of PCNA was increased by suppression of either USP1 or USP7, but elevation of the H2O2-induced mutation frequency was observed only in USP7-suppressed cells. This complexity might be explained by differences in the processes involved in the repair of UV- and H2O2-induced DNA lesions.

Since UV-induced PCNA ubiquitination is replication coupled, UV-induced mutagenesis stimulated by suppression of USP1 associated with enhanced PCNA ubiquitination could be a consequence of inaccurate DNA replication. The cyclobutane pyrimidine dimer (CPD), the most prominent DNA lesion induced by UV irradiation, is repaired very slowly. By contrast, another major type of UV-induced DNA damage, 6-4 photoproduct (6-4PP), is repaired within a few hours after UV irradiation (Sugasawa, 2010). Therefore, 6-4PP persists only early after irradiation, whereas CPD persists for several hours. Importantly, Polh can efficiently and accurately catalyze TLS past CPD lesions but is inefficient and inaccurate for TLS past 6-4PP (Masutani et al., 2000). In this context, slow accumulation of mono-ubiquitinated PCNA caused by USP1 degradation, which occurs several hours after UV irradiation (Huang et al., 2006), could contribute to the suppression of UV-induced mutagenesis caused by 6-4PP lesions. When USP1 gene expression is suppressed, the level of PCNA mono-ubiquitination immediately increases after UV irradiation, which would induce aberrant activation of TLS past 6-4PP lesions and result in UV-induced mutations. On the other hand, the suppression of USP7 gene expression simply increased the level of PCNA mono-ubiquitination without altering the kinetic profile at which mono-ubiquitinated PCNA is produced; therefore, accurate TLS past CPD lesions was activated after the 6-4PP lesions had been repaired. Thus, USP1 (but not USP7) contributes significantly to the suppression of UV-induced mutagenesis. By contrast, H2O2-induced PCNA mono-ubiquitination occurred transiently and independently of the cell-cycle stage, suggesting that replication stress was not the major cause of this ubiquitination. Consistent with this idea, H2O2-induced PCNA mono-ubiquitination is involved in the alternative repair of clustered oxidative DNA lesions, in which Msh2–Msh6 and Polh also participate (Zlatanou et al., 2011). Our results suggest that the repair pathway for oxidative lesions is potentially mutagenic if the PCNA ubiquitination level is inappropriately elevated by the suppression of USP7. Although the detailed mechanisms remain unknown, it is likely that USP7 plays a crucial role in maintaining the fidelity of repair-coupled DNA synthesis at oxidative DNA lesions.

Interestingly, during review of this paper, it was reported that knockdown or complete suppression of USP7 destabilizes RAD18, resulting in compromised PCNA ubiquitination (Zlatanou et al., 2015). Actually, we observed a reduction in the RAD18 protein level by relatively strong suppression of USP7 (Figures S1D and S1G). We realized that the siRNAs used in this study moderately suppressed USP7, the residual level of which was fortunately sufficient to maintain RAD18 protein in order to produce mono-ubiquitinated PCNA but not sufficient to scavenge mono-ubiquitinated PCNA, leading to the findings of this study. These results indicate that USP7 regulates PCNA ubiquitination in two modes: one is upregulation by stabilizing RAD18, and the other is downregulation by direct deubiquitination of mono-ubiquitinated PCNA. This could be a mechanism for the sharp response of cellular levels of mono-ubiquitinated PCNA under exposure to oxidative DNA damage. It is interesting that USP7 regulates p53 stability in two ways: one is via direct deubiquitination of p53, which increases p53 stability, and the other is via increased stabilization of Mdm2, a critical ubiquitin ligase of p53, which decreases p53 stability (Brooks and Gu, 2006). These two modes of regulation could be a common feature of the regulation of physiological systems by USP7.

In this study, the levels of H2O2 observed during the cellular responses were much higher than those observed under normal physiological conditions. However, in some specific biological processes, such as inflammation, H2O2 can reach concentrations in the low-to-middle 10−14M range (Thomas and Fishman, 1986). One possibility is that the same mechanism contributes to the prevention of low levels of genetic instability under physiological levels of oxidative stress, not only under specific pathological conditions, although, in our experiments, we think that the responses would have been difficult to detect at low concentrations of H2O2.

Our finding that USP1 and USP7 contributed prominently to the suppression of PCNA mono-ubiquitination and mutagenesis induced by UV and H2O2, respectively, suggests that cells have
mechanisms for choosing the appropriate DUB(s) depending on the type of DNA lesion and cell-cycle stage. In addition, H$_2$O$_2$- and UV-induced mono-ubiquitination of PCNA ultimately disappeared, even in cells in which both USP1 and USP7 were suppressed, suggesting that cells also have other mechanisms for scavenging ubiquitinated PCNA. Future studies should address how multiple DUBs are concertedly regulated in DNA damage tolerance.

**EXPERIMENTAL PROCEDURES**

**Cells**

W138VA13 cells expressing HA-PCNA wild-type (WT) or HA-PCNA K164R were prepared as described previously (Kanao et al., 2015). W138VA13, HeLa, 293, and MRCS cells were used in this study. Transfections of siRNAs and plasmids into human cells were performed by electroporation using the Neon transfection system (Life Technologies). Detailed procedures are described in the Supplemental Information.

**Plasmids and siRNAs**

USP7 cDNA was obtained from a HeLa cDNA library by PCR. Site-directed mutagenesis was performed by PCR. siRNAs were purchased. The sequences of primers and siRNAs are described in the Supplemental Information.

**Antibodies**

Polyclonal antibodies against USP1 and USP7 were produced by immunizing rabbits with synthesized polypeptides corresponding to amino acids 40–57 of human USP1 and 1,084–1,102 of human USP7, respectively. Other antibodies used were purchased. Detailed procedures are indicated in the Supplemental Information.

**Proteins**

USP7 was purified from HeLa cells as summarized in Figure 1A. Detailed procedures are described in the Supplemental Information. The 140-kDa polypeptide was subjected to MALDI-TOF MS analysis, which was outsourced to Aproscience.

**Deubiquitination Assays**

Assays for the purification of the deubiquitinating activity from HeLa cell extracts (Figure 1B and 1C) were performed using ubiquitin, E1, His-RAD6B/RAD18, PCNA, and the crude HeLa replication factor C (RFC) fraction. Deubiquitinating reactions were performed with mono-ubiquitinated PCNA (Figure 1D). Detailed procedures are indicated in the Supplemental Information.

**Flow Cytometry**

Cells were trypsinized, washed with PBS, and fixed in 70% ethanol overnight at −20°C. Fixed cells were treated with 0.1 mg/ml RNase A for 30 min at 37°C and then stained with 50 μg/ml propidium iodide for 10 min on ice. Flow cytometric analyses were performed using a CytoFacs FC 500 (Beckman Coulter).

**Cell Survival**

Two days after siRNA transfection, 6 × 10$^4$ cells were replated in six-well plates. Cells were cultured for 20 hr and then irradiated with UVC or treated with H$_2$O$_2$. Four days after treatment, cell viability was measured by the MTS assay (Promega).

**Mutagenesis**

HPRT mutants were selected, and mutation frequencies were determined according to published protocols (Akagi et al., 2009), with minor modifications. Detailed procedures are described in the Supplemental Information.


