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Regulation of monoubiquitinated PCNA by DUB autocleavage

Tony T. Huang[1], Sebastian M.B. Nijman[2], Kanchan D. Mirchandani[1], Paul J. Galardy[3], Martin A. Cohn[1], Wilhelm Haas[4], Steven P. Gygi[4], Hidde L. Ploegh[3], René Bernards[2] and Alan D. D'Andrea[1,5]

Monoubiquitination is a reversible post-translational protein modification that has an important regulatory function in many biological processes, including DNA repair. Deubiquitinating enzymes (DUBs) are proteases that are negative regulators of monoubiquitination, but little is known about their regulation and contribution to the control of conjugated-substrate levels. Here, we show that the DUB ubiquitin specific protease 1 (USP1) deubiquitinates the DNA replication processivity factor, PCNA, as a safeguard against error-prone translesion synthesis (TLS) of DNA. Ultraviolet (UV) irradiation inactivates USP1 through an autocleavage event, thus enabling monoubiquitinated PCNA to accumulate and to activate TLS. Significantly, the site of USP1 cleavage is immediately after a conserved internal ubiquitin-like diglycine (Gly–Gly) motif. This mechanism is reminiscent of the processing of precursors of ubiquitin and ubiquitin-like modifiers by DUBs. Our results define a regulatory mechanism for protein ubiquitination that involves the signal-induced degradation of an inhibitory DUB.

Monoubiquitination is a highly regulated process that is conserved in all eukaryotes[1,2] and controls a broad range of cellular functions, including DNA repair. Protein monoubiquitination is a reversible post-translational event that can be influenced by the opposing activities of a ubiquitin E3 ligase and a deubiquitinating enzyme (DUB), similar to the regulation of protein phosphorylation by kinases and phosphatases[3,4]. Protein monoubiquitination regulates the rescue of stalled DNA replication forks — an important cellular process required for cell survival[5]. The E2 ubiquitin conjugating enzyme RAD6 and the E3 ligase RAD18 are conserved in both yeast and human and they coordinate and activate the monoubiquitination of PCNA in response to UV damage or stalled replication forks[6–8]. Recent studies have shown that polη, a specialized TLS polymerase, is recruited to the replication fork through a specific interaction with monoubiquitinated PCNA[7]. The deployment of TLS polymerases during replication ensures timely bypass of the diverse DNA lesions encountered by the replication fork. Although many TLS polymerases are intrinsically mutagenic, polη allows replication past UV-damaged bases with high fidelity[9–11]. Thus, a model has emerged in which polη binds to monoubiquitinated PCNA and ensures accurate (error-free) replicative bypass of UV lesions. However, other (error-prone) TLS polymerases (such as polι and Rev1) have recently been shown to rely on monoubiquitinated PCNA for their function[12,13]. How cells limit PCNA monoubiquitination and the unwanted deployment of polη and/or other error-prone TLS polymerases in the absence or presence of extrinsic DNA damage during the synthesis of DNA in S phase is not known.

In humans, protein deubiquitination is controlled by a family of approximately 95 distinct DUB enzymes[14,15] but the function of most of these proteins is unknown. DUBs are cysteine proteases that cleave ubiquitin from specific mono- and poly-ubiquitinated substrates or from linear ubiquitin polypeptides. Recently, we identified the DUB USP1 as a negative regulator of the Fanconi anaemia pathway[16]. Inhibition of USP1 by siRNA knockdown increased the monoubiquitination of the Fanconi anaemia effector protein, FANCD2, and increased cellular resistance to DNA cross-linking agents, suggesting that USP1 deubiquitinates FANCD2. However, it is possible that USP1 may regulate the levels of other monoubiquitinated substrates in DNA repair pathways. Recent studies by our group and others have found that FANCD2 and PCNA colocalize at DNA repair foci during certain conditions of replication stress[17,18] (and data not shown). Therefore, we explored whether USP1 can deubiquitinate PCNA — a monoubiquitinated protein that localizes and functions at the DNA replication fork after UV damage or replication arrest. Here, we show that USP1 negatively regulates PCNA monoubiquitination *in vivo* and *in vitro*. Surprisingly, UV damage induces the degradation of USP1 to activate PCNA monoubiquitination. The mechanism of USP1 degradation involves an initial autocleavage event, followed by proteasomal degradation of the cleaved products. We also found that USP1 regulates TLS-induced mutagenesis.

[1]Department of Radiation Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA. [2]Division of Molecular Carcinogenesis and Center for Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam, Netherlands. [3]Department of Pathology, Harvard Medical School, Boston, MA 02115, USA. [4]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA.
[5]Correspondence should be addressed to A.D.A (e-mail: alan_dandrea#dfci.harvard.edu)

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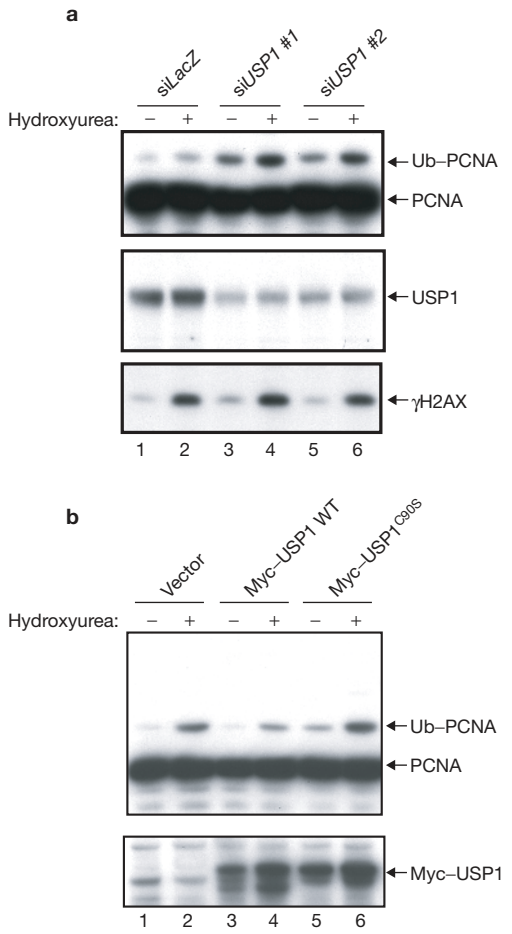

Figure 1 Knockdown of USP1 increases PCNA monoubiquitination. (**a**) HEK293 cells were transfected with the indicated siRNAs and treated with hydroxyurea (2 mM) for 12 h. Whole cell extracts were analysed by western blot with the indicated antibodies. All western blot analysis for USP1 used anti-USP1-C-term inal antibody unless otherwise indicated. (**b**) HEK293 cells were transiently transfected with the indicated expression plasmids and treated with hydroxyurea as in **a**. Samples were analysed by western blot using anti-PCNA or anti-Myc antibodies.

The implications of these findings is discussed in terms of general DUB regulation and the relationship between monoubiquitinated PCNA and TLS polymerases.

RESULTS

UV damage degrades USP1 and increases PCNA monoubiquitination

To determine whether USP1 regulates PCNA monoubiquitination, the effects of two USP1-specific siRNAs on PCNA were examined. The siRNA knockdown of USP1 increased the levels of monoubiquitinated PCNA both in the absence and presence of the replication-arresting agent, hydroxyurea (Fig. 1a). This increase in PCNA monoubiquitination was not an indirect result of an enhanced DNA-damage signal as no elevation in histone 2A.X phosphorylation (Fig. 1a) or p53 induction (data not shown) was observed. In addition, overexpression of wild-type USP1 in HEK293 cells inhibited hydroxyurea-induced monoubiquitination of PCNA, whereas the catalytically inactive form of USP1 slightly enhanced PCNA modification (Fig. 1b). These data suggest that different USP1 protein levels may modulate the levels of PCNA monoubiquitination.

To study the effect of UV light, a more physiological and potent inducer of PCNA monoubiquitination than hydroxyurea, the expression of USP1 under different UV dosages and exposures was examined (Fig. 2a). UV activated the degradation of USP1 and this decrease in USP1 was directly correlated with an increase in monoubiquitinated PCNA (Fig. 2a). USP1 knockdown by siRNA also increased PCNA monoubiquitination after shorter exposures to UV (Fig. 2a). After longer exposures, both the degradation of USP1 and the monoubiquitination of PCNA were maximal. It is unclear why treatment with hydroxyurea did not affect USP1 protein levels as dramatically as UV. This may be due to the nature of the DNA lesions generated by different types of genotoxic insults. UV treatment was found to be a more potent inducer of PCNA monoubiquitination than hydroxyurea. Consistent with this finding, it was found that ionizing radiation, which primarily causes DNA double-strand breaks through oxidative damage, did not induce PCNA monoubiquitination[7] or USP1 degradation (data not shown).

In yeast and mammalian cells, monoubiquitination of PCNA occurs on the conserved Lys 164 residue and is required for TLS function[6–8]. Accordingly, the increase in monoubiquitinated PCNA observed after knockdown of USP1 by siRNA requires Lys 164, as the mutation of this residue to arginine abrogated PCNA ubiquitin conjugation (Fig. 2b). This suggests that, *in vivo*, USP1 regulates a specific monoubiquitination site on PCNA. We next considered whether or not USP1 can deubiquitinate PCNA *in vitro*. Wild-type GFP–USP1 immunoprecipitated from transfected HEK293 cells deubiquitinated recombinant PCNA (Fig. 2c). The deubiquitination of PCNA was inhibited by N-ethylmaleimide (NEM) or by using the catalytic inactive USP1 mutant (GFP–USP1^{C90S}). An unrelated DUB enzyme (GFP–VDU1) failed to deubiquitinate PCNA (Fig. 2c), suggesting that not all DUBs share this activity.

The UV-dependent monoubiquitination of PCNA requires both the ubiquitin-conjugating enzyme (E2) RAD6 and the ubiquitin (E3) ligase RAD18[6–8,19]. Overexpression of RAD18 in HEK293 cells resulted in elevated levels of monoubiquitinated PCNA (Fig. 2d) but did not affect the levels of monoubiquitinated FANCD2 (data not shown). Conversely, siRNA knockdown of endogenous RAD18 decreased the levels of monoubiquitinated PCNA (Fig. 2d), but had little effect on FANCD2 ubiquitin conjugation (data not shown). The UV-induced degradation of USP1 was also not affected by altering RAD18 levels (Fig. 2d), suggesting that RAD18 is not the E3 ligase for USP1 degradation. Previous studies have indicated that the UV-dependent monoubiquitination of FANCD2 requires an intact Fanconi anaemia core complex[20,21] — a multisubunit E3 ligase containing at least eight Fanconi anaemia protein subunits (A, B, C, E, F, G, L and M)[22,23]. The Fanconi anaemia core complex was systematically excluded from the degradation of USP1, as analysis of patient-derived Fanconi anaemia fibroblasts from different Fanconi anaemia subtypes (FA-A, FA-C, FA-G and FA-L) showed that they remained competent for USP1 degradation and for the monoubiquitination of PCNA (Fig. 3a and data not shown). Other DNA repair and checkpoint pathways were also analysed for involvement in the UV-induced degradation of USP1. Interestingly, the pathways involving nucleotide excision repair (NER), BRCA1 and ATR–ATM do not regulate USP1 degradation (Fig. 3b–d).

Degradation of USP1 requires its own catalytic activity

To study the mechanism of UV-induced USP1 degradation USP1 protein levels were tracked using antibodies directed against opposite termini

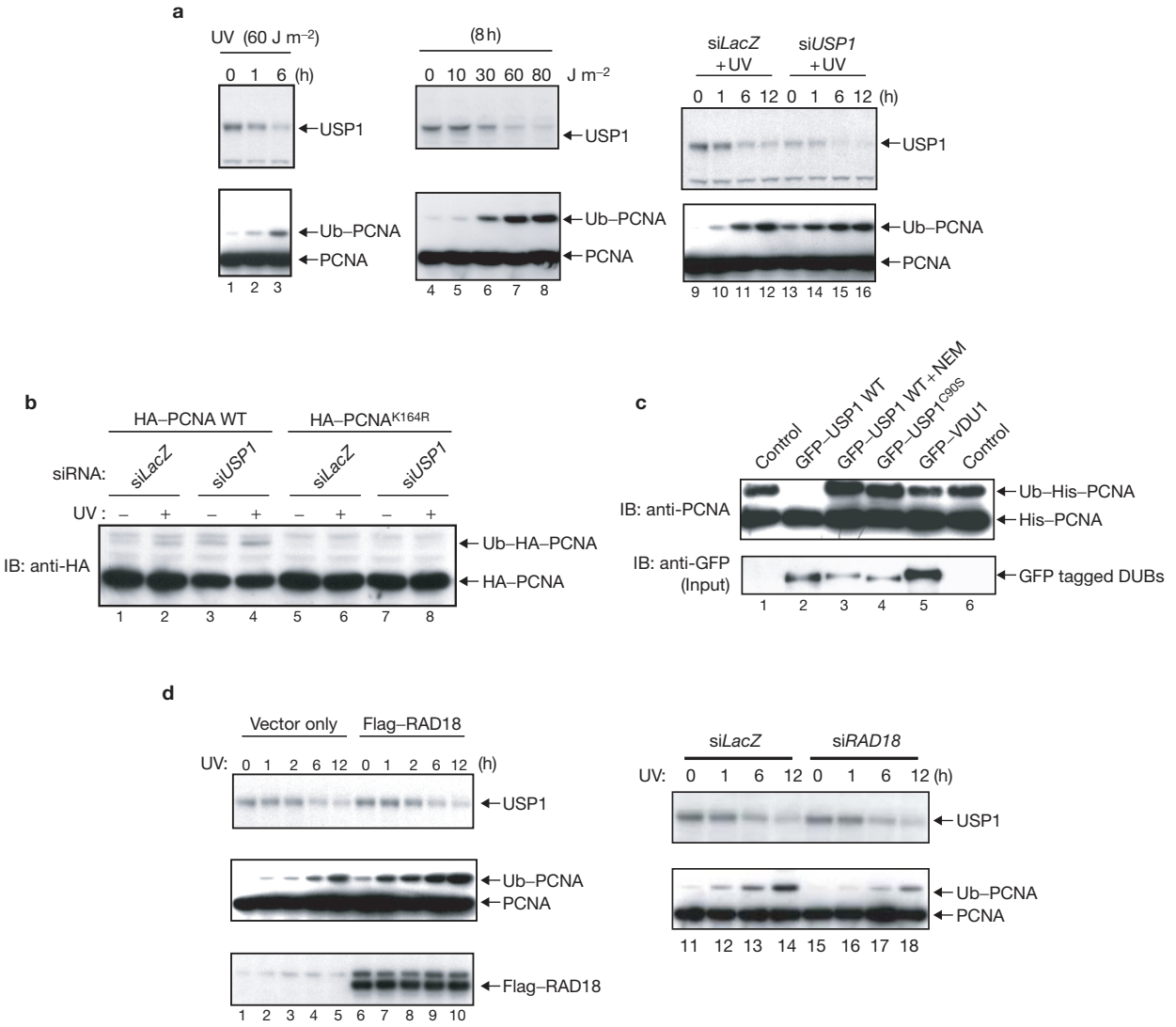

Figure 2 UV damage degrades USP1 and increases PCNA monoubiquitination. (**a**) HEK293 cells were exposed to UV in a time-course and dose-dependent manner or transfected with the indicated siRNAs as indicated. A UV dose of 60 J m^{-2} was used for lanes 9–16 . Whole cell extracts were analysed by western blot with indicated antibodies. (**b**) HEK293 cells were stably transfected with either HA–PCNA (wild type) WT or HA–$PCNA^{K164R}$ expression constructs and then subsequently transfected with the indicated siRNAs. Cells were then treated with UV (60 J m^{-2}) for 2 h and analysed by western blot with anti-HA antibody. (**c**) HEK293 cells were transfected with the indicated plasmids (control is untransfected). Post-transfection (48 h), cells were lysed and extracts were made and prepared for immunoprecipitation (IP) with anti-GFP antibody. IP samples (on beads) were then incubated with *in vitro* monoubiquitinated PCNA with or without NEM (1.0 mM). Supernatants from the reactions were then analysed by western blot (anti-PCNA). The remaining beads were also analysed by western blot (anti-GFP) for total imput of immunoprecipitated DUBs. (**d**) HEK293 cells stably overexpressing either vector control or Flag-tagged *RAD18* cDNA (left) or transfected with siRNAs against *LacZ* control or *RAD18* (right). Cells were then exposed to UV (60 J m^{-2}) and whole cell extracts were analysed by western blot with the indicated antibodies.

of USP1 (anti-USP1-amino (N)-terminal and anti-USP1-carboxy (C)-terminal antibodies). Interestingly, cellular exposure to a range of UV doses resulted in an internal cleavage of the full-length USP1 polypeptide (molecular weight (M_r) of approximately 90 k), leaving an approximately 75 k amino-terminal fragment that was only detectable with the anti-USP1-N-terminal antibody (Fig. 4a). This fragment, referred to as USP1-Trunc or p75, was stabilized by lactacystin (an irreversible proteasome inhibitor), suggesting that this labile fragment is degraded by the proteasome. These results suggest that USP1 degradation occurs through a process of at least two steps: an internal cleavage step followed by the proteasomal degradation of the liberated amino-terminal fragment.

We next addressed whether or not the USP1-Trunc fragment was enzymatically active and if catalytically inactive forms of USP1 undergo autocleavage. A series of mutant and C-terminal truncations of USP1 were expressed in HEK293 cells and tested for DUB activity using an HA-tagged ubiquitin vinyl methylester (HA–UbVME) DUB activity probe. This probe covalently captures active DUB enzymes, as previously described[24]. Full-length exogenously expressed wild-type USP1 was labelled with the HA–UbVME DUB probe (Fig. 4b, lanes 1 and 2) and was degraded after UV treatment (Fig. 4c, lanes 5–8). The USP1-Trunc fragment failed to form conjugates when labelled with the HA–UbVME DUB probe (Fig. 4b, lanes 1 and 2) suggesting that USP1-Trunc was not enzymatically active. As expected, the loss of conserved regions of the His domain (constructs 1–523, 1–575 and 1–746) and the catalytic mutant ($USP1^{C90S}$) disrupted the DUB activity of USP1 (Fig. 4b, lanes 3–10). The USP1-Trunc fragment is a longer C-terminal

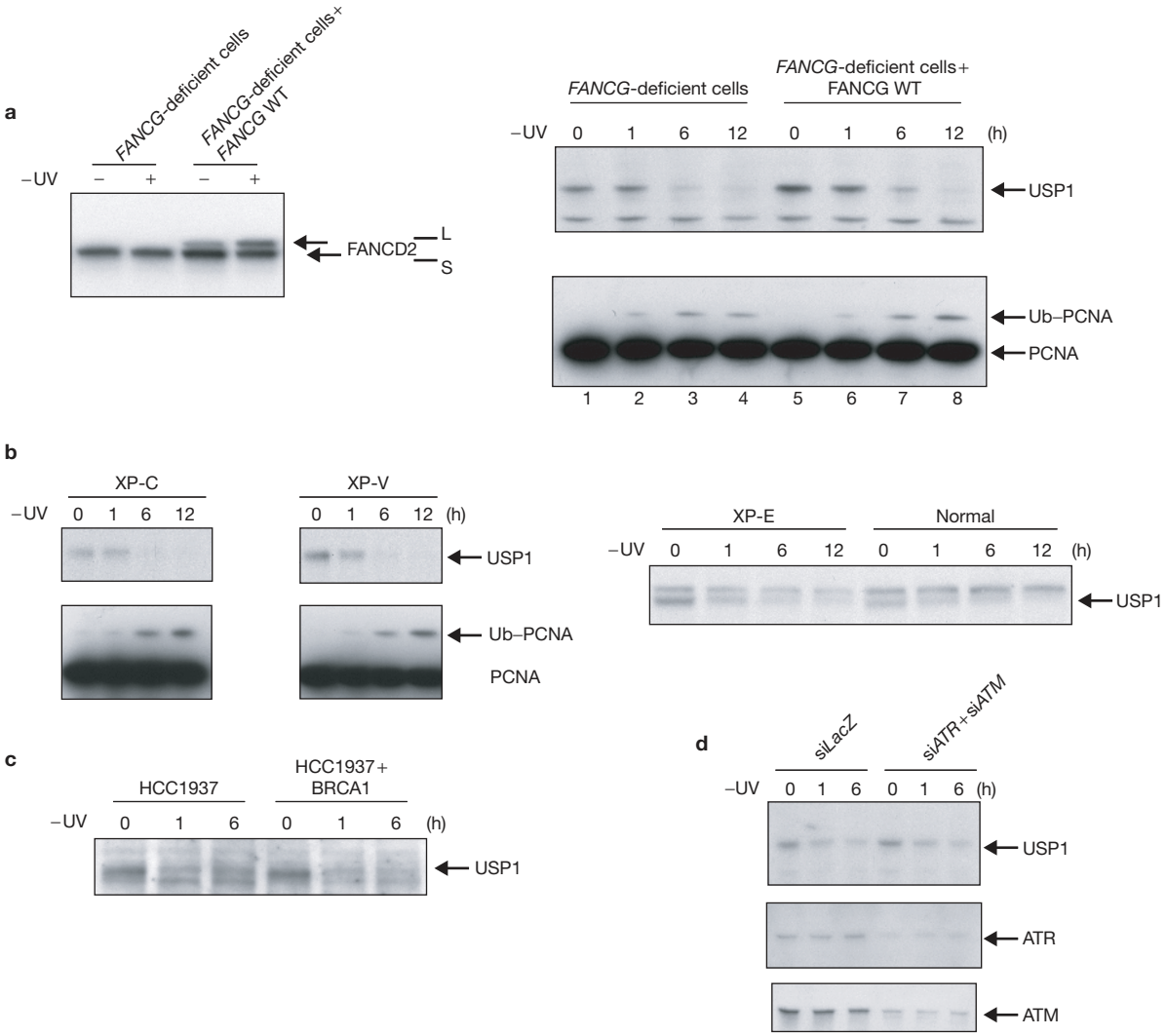

Figure 3 Inhibition of diverse DNA repair pathways does not affect UV-induced USP1 degradation. **(a)** Patient-derived *FANCG*-deficient (PD316) fibroblasts retrovirally complemented with either vector or wild-type *FANCG* cDNA were exposed to UV (60 J m^{-2}) and analysed by western blot with the indicated antibodies. L (monoubiquitinated and S (unmodified) represent long and short forms of FANCD2, respectively. **(b)** SV40-transformed fibroblasts from different patient-derived lines were exposed to UV (60 J m^{-2}) and analysed by western blot with the indicated antibodies. Xeroderma pigmentosa (XP) complemenation group C (XP-C) and E (XP-E) and the variant form (XP-V) are indicated. **(c)** HCC1937 patient-derived BRCA1-mutant cells were transfected with full-length wild-type BRCA1 as described in a previous study [20]. **(d)** HEK293 cells transfected with either *lacZ* or *ATM* and *ATR* siRNAs were exposed to UV (60 J m^{-2}) and analysed by western blot with the indicated antibodies as described in a previous study [38]. All anti-USP1 blots were probed with the anti-USP1- C-terminal antibody.

deletion of USP1 than the inactive 1–746 constuct. Therefore, it is likely that the initial autocleavage event on USP1 is inhibitory. Surprisingly, only wild-type USP1 could undergo an internal cleavage event to form USP1-Trunc as the catalytically inactive mutants did not form additional truncated fragments of USP1 (Fig. 4b). The degradation of USP1 by UV was also linked to its own catalytic activity. $USP1^{C90S}$ was refractory to UV-induced degradation (Fig. 4c, lanes 9–12). Thus, the generation of USP1-Trunc is dependent on the protease activity of USP1 itself.

To examine whether USP1 autocleavage can occur in an *in vitro* extract, USP1 and its catalytic mutant were transcribed and translated and their protein levels were tracked after treatment with cycloheximide (Fig. 4d). Only wild-type USP1 was capable of generating its truncated fragment. $USP1^{C90S}$ was not cleaved, even though it contains an intact internal cleavage site (see below). This further suggested that the protease activity of USP1 is likely to be responsible for its autocleavage. As the cleavage of USP1 can still occur in the absence of *in vivo* UV damage, it is possible that the extracts used for this assay contain rate-limiting factors that are normally required for the DNA-damage-induced cleavage of USP1.

The conserved diglycine motif of USP1 is required for its autocleavage

To determine the cleavage site of USP1, an epitope-tagged USP1 was stably expressed in HeLa cells and the isolated full-length and USP1-Trunc forms were digested with trypsin as previously described[25]. Mass spectrometric analysis of the full-length USP1 protein revealed peptides spanning its entire length (Fig. 5a). In contrast, peptides originating from the truncated form spanned almost all of USP1, with the exception of the last approximately 100 amino acids, and also

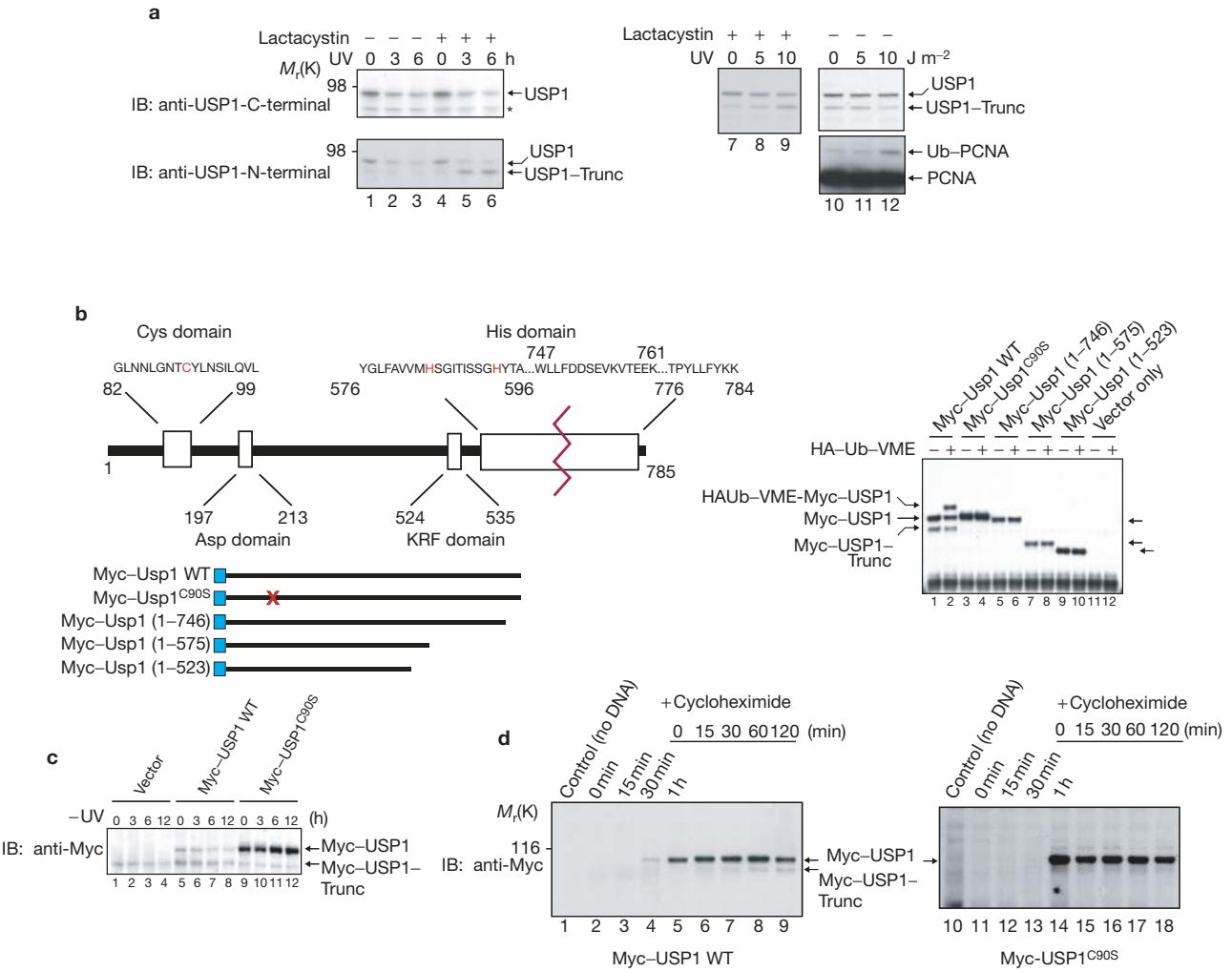

Figure 4 Degradation of USP1 requires its own catalytic activity. (**a**) HEK293 cells were treated with or without UV (60 J m^{-2}) and/or lactacystin (5 μM) for 3 h (lanes 1–6). HEK293 cells were also treated or not as in lanes 1–6 and exposed to UV in a dose-dependent manner for 3 h (lanes 7–12). Whole cell extracts were analysed by western blot with indicated antibodies. The asterisk indicates a non-specific band. (**b**) Schematic representation of the USP1 catalytic domains with corresponding Myc-tagged USP1 truncation and point mutants as indicated. HEK293 cells were transfected with vector or the indicated Myc-tagged USP1 expression constructs. Extracts were incubated with or without HA–UbVME DUB probe and then analysed by western blot with anti-Myc antibody. (**c**) HEK293 cells were transfected with the indicated plasmids and treated with UV (60 J m^{-2}) for the indicated times. Whole cell extracts were analysed by western blot. (**d**) *In vitro* translated (1 h) Myc–USP1 and USP1^{C90S} were incubated at 30 °C for the indicated times in the presence of cycloheximide (1 mM) to stop further protein translation. Protein expression was analysed by western blot with anti-Myc antibody.

contained non-tryptic peptides ending at Gly 671 (Fig. 5b, c). Thus, the cleavage site of USP1 occurs immediately after a well-conserved Gly–Gly region within its His domain (Fig. 5d). The protease cleavage after the Gly–Gly residues is reminiscent of the cleavage and processing of poly-ubiquitin and other ubiquitin-like modifiers such as Nedd8, SUMO1 and ISG15 (refs 26, 27). Therefore, the sequence upstream of the USP1 Gly–Gly region was examined for homology to ubiquitin. Using the T-COFFEE (version 1.41) sequence alignment/homology program, a ubiquitin-like domain was identified immediately upstream of the Gly–Gly region (data not shown). The C-terminal fragment (approximately 15 K) that resulted from USP1 autocleavage was not detected. Interestingly, this fragment has an N-terminal glutamine residue (Fig. 5d), suggesting that it may be rapidly degraded as an N-end rule substrate[28].

To determine whether the cleavage of USP1 requires the diglycine motif, the Gly–Gly sequence was mutated to Ala–Ala (USP1$^{GG670/671AA}$). This mutation inhibited both the cleavage and degradation of USP1 (Fig. 5e, f). This hyperstable mutant also bound to the HA–UbVME DUB probe, suggesting that the USP1 autocleavage event is not required for DUB activity (data not shown).

Previous studies have indicated that RAD18-dependent monoubiquitination of PCNA results in the recruitment of the TLS polymerase polη to PCNA. PCNA monoubiquitination allows the proper localization of polη into DNA repair foci and is indicative of proper TLS function[8,29]. To test the function of this active, non-degradable form of USP1, USP1$^{GG670/671AA}$ was expressed in HEK293 cells and it was found to inhibit PCNA monoubiquitination and reduce the UV-dependent interaction of PCNA with polη (Fig. 5f). As expected, the reduction of monoubiquitinated PCNA also decreased UV-induced polη foci formation (Fig. 5g). USP1 autocleavage is therefore not necessary for substrate recognition as the USP1 cleavage mutant can still affect the monoubiquitination status of PCNA. These data provide evidence that UV-induced degradation of USP1 is critical to maintain proper localization and TLS function of polη.

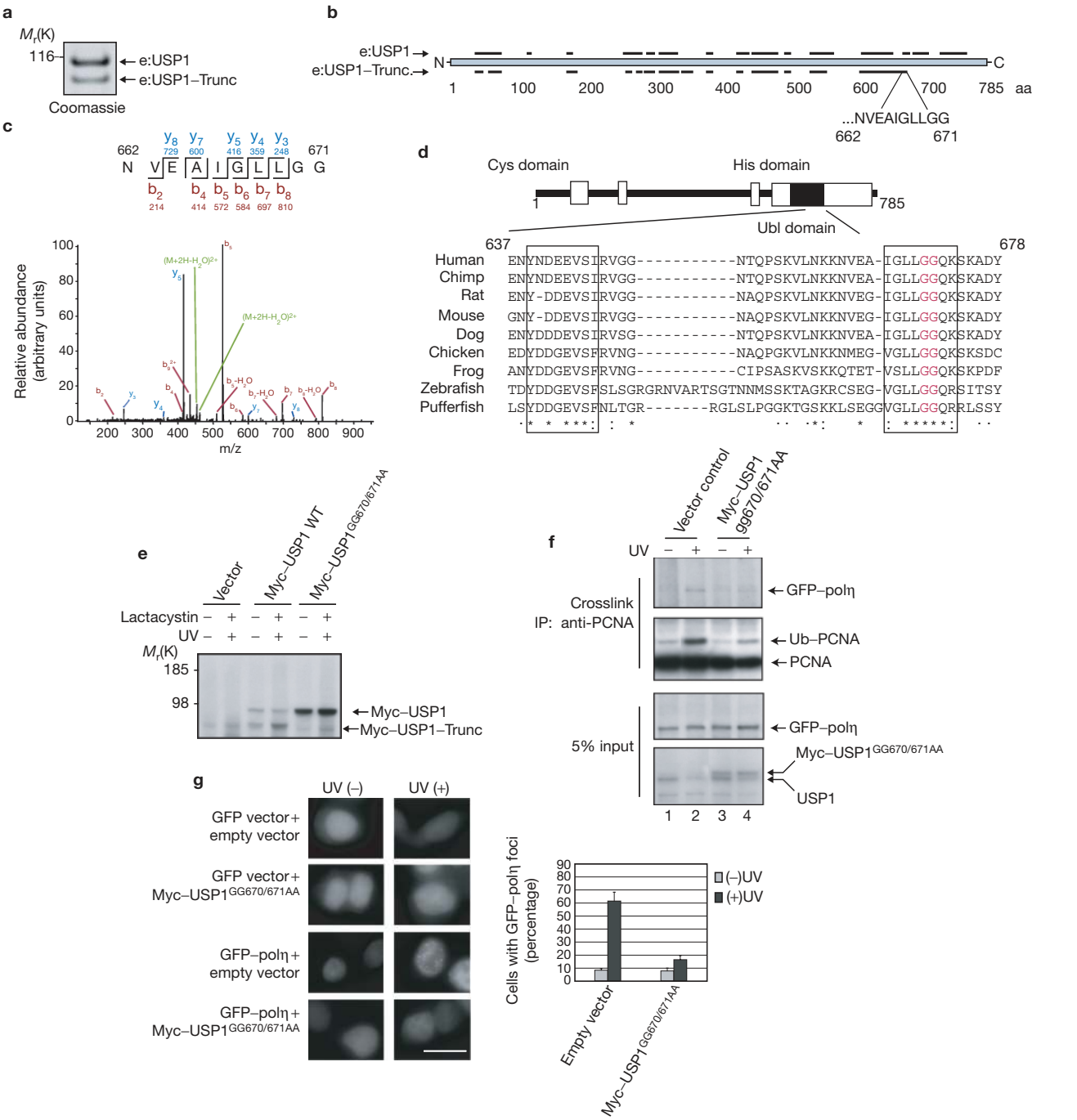

Figure 5 The conserved diglycine motif of USP1 is required for its autocleavage. (**a**) Coomassie blue-stained SDS–PAGE of Flag–HA-tagged USP1 (e:USP1) purified from HeLa cell nuclear extracts. (**b**) Tryptic peptides were identified from full-length (e:USP1) and truncated (e:USP1–Trunc) USP1 by mass spectrometry and aligned above (peptide coverage from full-length) or below (truncated) the schematic representation of USP1 (drawn to scale). (**c**) MS/MS spectrum of the doubly charged C-terminal peptide NVEAIGLLGG (m/z 471.76688, $m/z_{calcd.}$ 471.76637, mass accuracy 1.1 ppm) of USP1–Trunc. Observed b- and y-type ions are shown above and below the peptide sequence in blue and red, respectively. The coordinates of the N- and C-terminal amino acids are shown above the sequence in black. (**d**) Sequence alignment (ClustalW) and comparison of USP1 from different organisms spanning from residues 637–678 within the variable His domain. Red letters indicate the conserved glycine residues. Boxed residues signify conserved regions of the USP1 putative ubiquitin-like domain. (**e**) HEK293 cells were transfected with the indicated plasmids, including the cleavage mutant (Myc-USP1$^{GG670/671AA}$) and co-treated with lactacystin (5 μM) and UV (60 J m^{-2}) or not. Whole cell extracts were prepared and analysed by western blot (anti-Myc). (**f**) HEK293 cells transfected with GFP–polη and with vector control or Myc–USP1$^{GG670/671AA}$ mutant were stimulated with UV (60 J m^{-2}) or not. Cells were lysed and crosslinked as described in the Methods. Samples that were not crosslinked (input) were analysed by western blot with either anti-GFP or anti-USP1-C-terminal antibody. Extracts that were crosslinked and solubilized were also immunoprecipitated with anti-PCNA and analysed with anti-GFP or anti-PCNA by western blot. (**g**) HEK293 cells were transfected with either GFP vector and empty vector or Myc–USP1$^{GG670/671AA}$ or GFP–polη and empty vector or Myc–USP1$^{GG670/671AA}$ at a 1:3 plasmid DNA ratio to ensure that all GFP-containing cells have excess Myc–USP1 expression plasmids. Cells were then irradiated or not with UV (20 J m^{-2}) for 6 h. Cells were prepared for viewing and counting of foci (cell with 5 or more foci was counted as positive) as described in the Methods. The scale bar represents 10 μm. The error bars indicate standard deviations of three independent experiments.

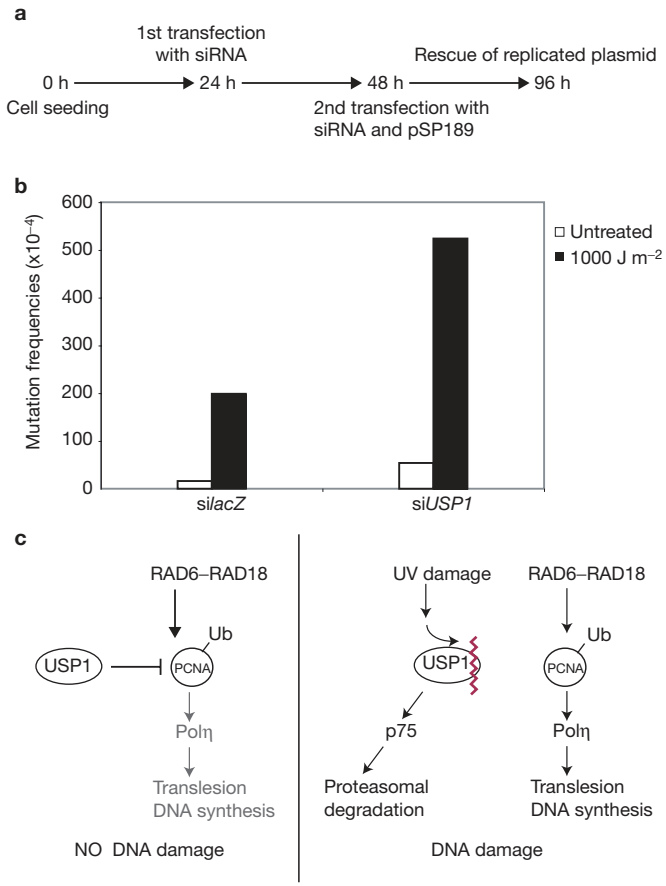

Figure 6 Increased mutation frequency in cells depleted of USP1. (**a**) Schematic representation of the experimental approach used to study UV mutagenesis in siRNA treated cells. (**b**) *SupF* mutation frequencies for untreated and UV-irradiated plasmids are indicated for *lacZ* (control) and *USP1* siRNA treated cells. The results indicated are representative of three separate experiments. **(c)** Schematic representation of USP1 autocleavage and promotion of PCNA monoubiquitination and TLS in the absence or presence of UV damage. Ub, ubiquitination.

Increased mutation frequency in cells depleted of USP1

To determine the role of USP1 in UV-induced mutagenesis — as a measure of TLS — an siRNA knockdown approach was used in combination with a *supF* shuttle vector[30,31], which replicates in mammalian cells (Fig. 6a). UV irradiation predominantly generates cyclobutane–pyrimidine dimers (CPDs) and 6–4 photoproducts in DNA. *In vivo*, CPDs are normally repaired by nucleotide excision repair or accurately bypassed by a polη-dependent TLS process. As previously demonstrated [30], siRNA knockdown of polη increased the mutation frequency in UV-irradiated *supF* plasmids (see Supplementary Information, Fig. S1). In the absence of polη, it is likely that other (error-prone) TLS polymerases are utilized to bypass UV lesions and this accounts for the increase in mutation frequency. siRNA knockdown of USP1 resulted in an approximately twofold increase in the mutation frequency in both untreated and UV-irradiated *supF* plasmids (Fig. 6b and see Supplementary Information, Fig. S2). The majority of *supF* mutants analyzed from *USP1* siRNA treated cells were point mutants (see Supplementary Information, Fig. S3) indicative of TLS events. This suggests that upregulation of monoubiquitinated PCNA by USP1 knockdown may involve increased polη-dependent and/or polη-independent replication of damaged DNA. The influence of the Fanconi anaemia pathway or another unidentified USP1 substrate on TLS has not been excluded.

DISCUSSION

The cellular levels of monoubiquitinated PCNA are regulated by two counteracting processes — monoubiquitination by RAD6 or RAD18 and deubiquitination by USP1. Our data indicate that cellular exposure to UV results in USP1 degradation which leads to the accumulation of ubiquitinated PCNA (Fig. 6c). According to this model, in the absence of DNA damage, monoubiquitinated PCNA is subjected to constitutive deubiquitination by USP1, resulting in low steady-state levels of modified PCNA. However, in the presence of UV damage, USP1 switches its substrate target from ubiquitinated PCNA to its own C-terminal Gly–Gly motif, resulting in an increase in monoubiquitinated PCNA levels and TLS (Fig. 6c). The mechanism for this switch is not known. Our data suggest that many known DNA-damage-response pathways (such as those regulated by BRCA1, ATR, ATM and NER proteins or by Fanconi anaemia proteins) are not involved in USP1 autocleavage. It will be interesting to determine whether the activation of USP1 autocleavage is similar to activation of caspases by autocleavage (caspase homodimerization induces *trans*-cleavage[32]). Also, USP1 may be part of a larger DNA-damage sensor complex, where the recognition of UV lesions can trigger a direct conformational change in USP1 that allows autocleavage.

The increase in both spontaneous and damage-induced mutagenesis in cells depleted of USP1 can be explained by the following models: First, increased levels of monoubiquitinated PCNA may result in the dysregulated function of polη. Although polη can accurately replicate DNA past CPDs, its overuse, particularly in regions of undamaged DNA, may result in an overall increase in the frequency of mutations in a USP1-depleted cell. This has been observed *in vitro* where human polη copies undamaged DNA template with much lower fidelity than replicative polymerases[33] and indicates that the function of polη must be tightly controlled to prevent potentially mutagenic DNA synthesis. Second, the high levels of monoubiquitinated PCNA (due to the loss of USP1) may result in the recruitment and competition of other error-prone TLS polymerases (such as Rev3–Rev7, Rev1, polκ and polι) with polη for undamaged and damaged DNA templates. A recent study has identified two ubiquitin-binding domains (UBM and UBZ) that are evolutionarily conserved in all Y-family TLS polymerases (Rev1, polκ, polι and polη)[12]. These domains are required by polη and polι (and possibly other Y-family TLS polymerases) for interaction with monoubiquitinated PCNA. Also, *in vitro* studies have shown that monoubiquitinated PCNA, but not unmodified PCNA, is required for the activation of Rev1 to promote mutagenic DNA replication[13]. Thus, a condition that upregulates PCNA monoubiquitination (such as USP1 knockdown) is likely to increase replication-coupled mutagenesis through the recruitment and activation of multiple TLS polymerases. We speculate that one physiological role of USP1 is to limit or suppress mutagenesis by restraining the TLS polymerase activity both in the absence and presence of UV-induced DNA damage. Whether or not other DUB enzymes regulate the activity of TLS polymerases has yet to be explored.

It will be interesting to determine whether DUB autocleavage-induced degradation or inactivation is a general mechanism for upregulating the ubiquitination state of substrates. Preliminary inspection of the primary amino-acid sequences of DUBs[34] and other non-ubiquitin

related proteins revealed the presence of Gly–Gly motifs, suggesting the possibility of other DUB autocleavage sites (data not shown). Whether or not the residues adjacent to the Gly–Gly motif also contribute to the auto-cleavage consensus site has not been determined. DUB inactivation may also result from other molecular mechanisms. In one case, genotoxic stress promoted the cleavage of HAUSP (also known as USP7, the negative regulator of p53 and Hdm2) by caspase-3 (ref. 35). In another case, the deubiqutination of TRAF2 by the cylindromatosis tumour suppressor, CYLD, was inhibited by phosphorylation[36], leading to the activation of the transcription factor NF-κB[37]. Thus, the emerging complexity involved in DUB regulation underscores the importance of DUBs in regulating numerous fundamental biological processes. □

METHODS

Materials, antibodies and plasmid construction. Fanconi anaemica patient-derived lines were obtained from the Coriell Cell Repository (Camden, NJ). GFP–USP1 constructs and GFP–VDU1 plasmids were generated as previously described[16]. To generate human Myc-tagged USP1, *USP1* cDNA was PCR amplified and subcloned into a modified pcDNA3 (Invitrogen, Carlsbad, CA) plasmid containing 5′ sequence coding for 2× Myc epitope. The point mutations C90S and GG670/671AA were made by two-step PCR mutagenesis from the original *USP1* template and verified by DNA sequencing. Truncation mutants were obtained by PCR amplification using specific 3′ *USP1* sequences. Retroviral Flag–RAD18, wild-type HA–PCNA and HA–PCNA^{K164R} plasmids was generated by PCR amplification from a human cDNA library and subcloned into the PMMPuro retroviral vector at *Nco*I and *BamH*I sites. Synthetic siRNA oligonucleotide sequences are as follows: human *USP1* #1, 5′-TCGGCAATACTTGCTATCTTA-3′; human *USP1* #2, 5′-TTGGCAAGTTATGAATTGATA-3′ and human *RAD18,* 5′-CCCGAGGTTAATGTAGTTGTT-3′. All *USP1* siRNA knockdown experiments were done using *USP1* #1 except where indicated. Anti-Myc (9E10), p53 (DO-1) and PCNA (PC10) antibodies were acquired from Santa Cruz Biotech (Santa Cruz, CA). Anti-GFP (JL-8) was from BD Biosciences (San Jose, CA) and phospho H2AX (JBW301) was from Upstate (Charlottesville, VA). Flag (M5) antibody and N-ethylmaleimide were purchased from Sigma (St Louis, MO). Anti-FAND2 antibody (FI17) was purchased from Santa Cruz. Anti-USP1-N-terminal and anti-USP1-C-terminal antibodies were generated using either the N-terminal eptitope corresponding to TDSQENEEKASEYRASEIC or the C-terminal epitope GTHESDRNKESSDQTC, respectively (Biosource, Camarillo, CA). MG132 and lactacystin were purchased from Calbiochem (San Diego, CA). All UV-C (254 nm) irradiation experiments were performed using a 2400 UV Stratalinker (Stratagene, La Jolla, CA).

Cell culture and transfection. All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal calf serum, glutamine, and penicillin–streptomycin. SiRNA tranfections were done using Lipofectamine 2000 (Invitrogen) as previously described[38]. Plasmid transfections used Fugene6 (Roche, Basel, Switzerland) according to the manufacturer's protocol.

Triton extraction, crosslinking, immunoprecipitation and immunoblotting. Isolation of crosslinked Triton-insoluble and soluble fractions is as previously described[7] with some modifications. Briefly, cells were washed in cold PBS, incubated in buffer A (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM Pipes at pH 6.8, 1 mM EGTA and 0.2% Triton X-100) and protease inhibitors. The incubated buffer was removed and collected (Triton-soluble fraction). The remaining cell extracts were then rinsed in PBS and crosslinked with 1% formaldehyde in PBS. The reaction was stopped with 0.1 M glycine base then rinsed with PBS and collected (Triton-insoluble fraction). Crosslinked samples were then extracted using standard RIPA buffer containing benzonase nuclease (Novagen, San Diego, CA). Immunoprecipitations were performed in RIPA buffer with specific antibodies and protein G beads and incubated at 4 °C overnight with gentle rotation. Flag–HA tagged USP1 was purfied from HeLa nuclear extract as previously described[25]. Western blots were performed with whole-cell extracts and separated on Nupage 3–12% Tris–Acetate or 4–12% Bis-Tris gradient gels (Invitrogen).

Deubiquitination and DUB activity and cleavage assays. HEK293 (1×10^7)cells were transfected with the cDNAs encoding the indicated GFP-tagged DUBs and lysed 48 h later in TNT buffer (0.1 M Tris at pH 7.5, 150 mM NaCl, 0.05% Tween-20, 1.0 mM EDTA) and sonicated. The immunoprecipitations were washed twice with TNT and then twice with DUB buffer (60 mM HEPES at pH 7.6, 5 mM $MgCl_2$, 4% glycerol). Beads were incubated overnight at 30 °C with *in vitro* monoubiquitinated PCNA substrate (using recombinant His-tagged PCNA, E1, RAD6, RAD18 and free ubiquitin in a ubiquitination reaction) as previously described[8]. The HA–UbVME probe reaction was performed as previously described[24]. *In vitro* transcription and translation was done according to the manufacturer's instructions (Promega, Madison, WI).

Fluorescence microscopy. Fluorescence microscopy on adherent cells was performed as previously described[39]. Briefly, transfected cells were split into either 4-well chamber slides or 60-mm dishes for foci counting. GFP-expressing cells were fixed and permeabilized in a staining solution (4% paraformaldehyde, 0.5% Triton X-100 and DAPI) for 15 min at room temperature.

SupF mutation assay. HEK293T (2×10^6) cells were transfected with *lacZ*, *pol*η or *USP1*-specific siRNAs using Lipofectamine 2000 as previously described[30] with modifications. Forty-eight hours after transfection, the pSP189 plasmid[31] was either left unirradiated or irradiated with 1,000 J m^{-2} of UV-C in 30 μl droplets and transfected into the siRNA-treated cells using Lipofectamine 2000. After a 48 hour incubation, plasmids were retrieved using a plasmid mini-prep kit (Promega), ethanol precipitated and *Dpn*I digested to remove unreplicated plasmids. The plasmid DNA was then electroporated into the MBM7070 bacterial strain, which carries an amber mutation in the *lacZ* gene. Transformed bacteria were plated on agar plates with 50 μg ml^{-1} ampicillin, 1 mM isopropyl-1-thio-β-D-galactopyranoside and 100 μg ml^{-1} X-gal. Mutant (white) and wild-type (blue) colonies were counted to determine the mutation frequency (number of white colonies over total colonies). The *lacZ* phenotype of the mutant colonies was verified by replating and mini-prepped DNA was analysed by *Eco*RI digestion followed by electrophoresis on an 0.8% agarose gel. Plasmids of the same size as wild type were classified as point mutants and the sequence was confirmed using an automated DNA sequencer.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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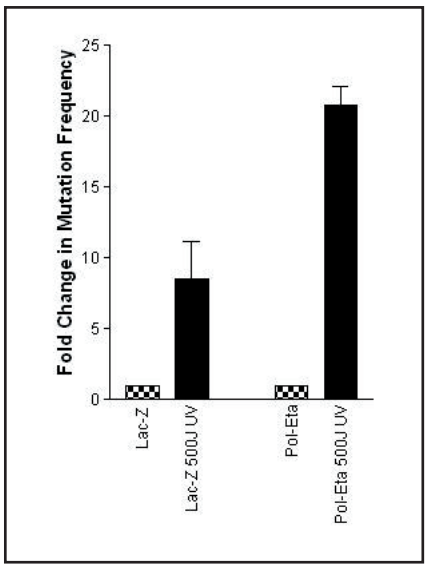


Figure S1 UV-induced hypermutability in POL(knockdown cells. Fold change in *supF* mutation frequency after replication of the UV-irradiated plasmid in HEK293T cells transfected with either control lacZ or POL(siRNAs according to Methods. The numbers were derived from three independent transfections on separate days (± S. D.).

	Untreated supF silacZ (control)	**Untreated supF siUSP1**		**UV-irradiated supF silacZ (control)**	**UV-irradiated supF siUSP1**	
		Fold change in mutation frequency (above untreated control)	Relative fold increase in mutation frequency (above untreated control)	Fold change in mutation frequency (above untreated control)	Fold change in mutation frequency (above untreated control)	Relative fold increase in mutation frequency (above UV-irradiated control)
Experiment #1	1	1.6	1.6	5.8	9.2	1.59
Experiment #2	1	3.1	3.1	11.9	32.5	2.73
Experiment #3	1	2.5	2.5	5.0	13.0	2.6
		Relative mean fold increase in mutation frequency	2.4 (± 0.4426)		Relative mean fold increase in mutation frequency	2.3 (± 0.3636)

Figure S2 Increased spontaneous and UV-induced mutation frequencies in USP1 depleted cells. *SupF* mutation frequencies analyzed are displayed as fold change above untreated control as indicated in column (LacZ siRNA with no UV). Relative mean fold increase in mutation frequency compared between either untreated or irradiated *supF* plasmids are shown with Std. Error. Each set (#1-3) represents individual transfection experiments from different days.

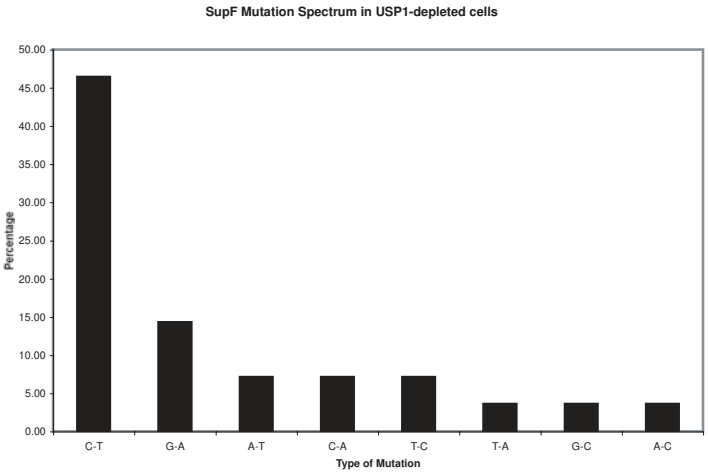
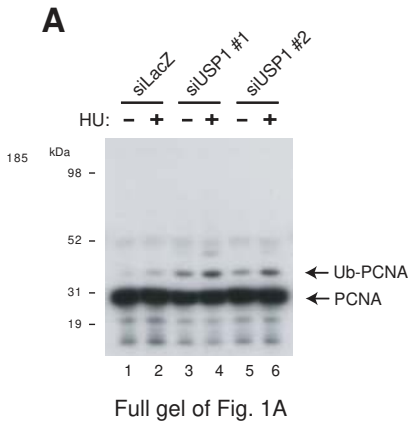


Figure S3 Mutation spectrum of *supF* mutants retrieved from USP1 depleted cells. Types of mutations in the *supF* gene after replication of the UV-irradiated plasmid in HEK293T cells depleted of USP1. The types of mutations induced were determined by DNA sequencing (Methods).



Full gel of Fig. 1A

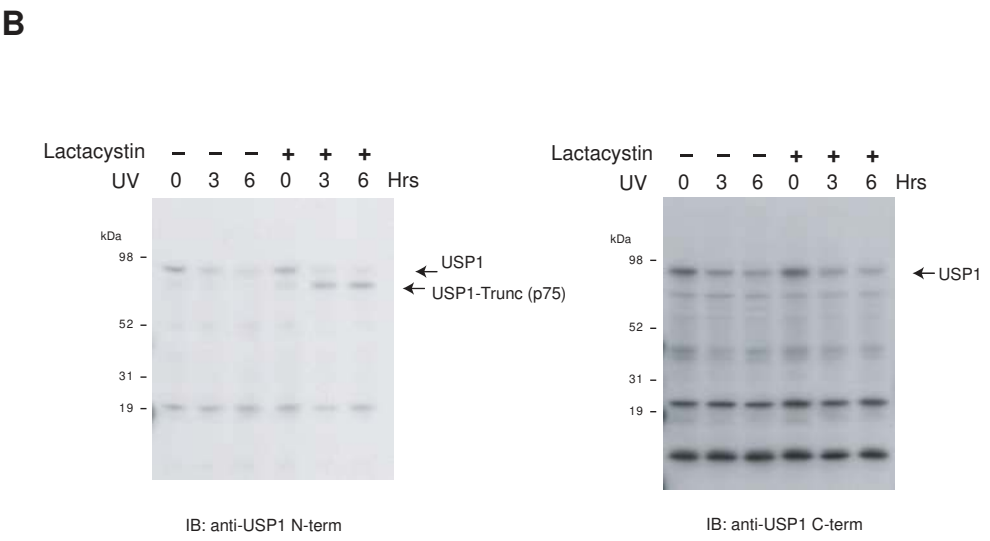
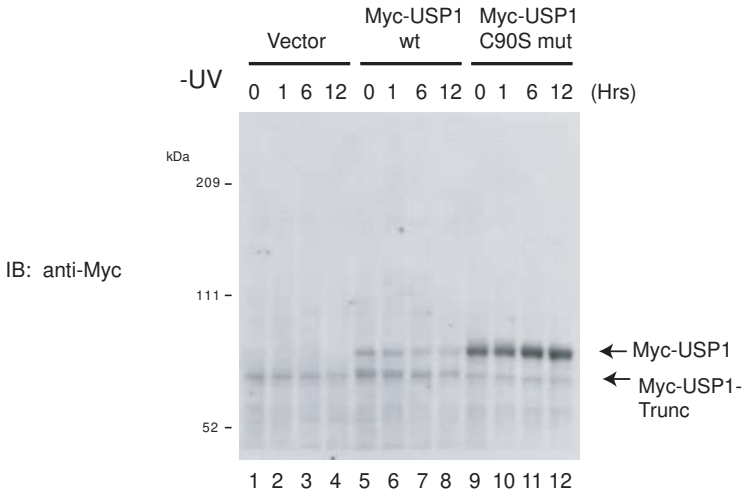


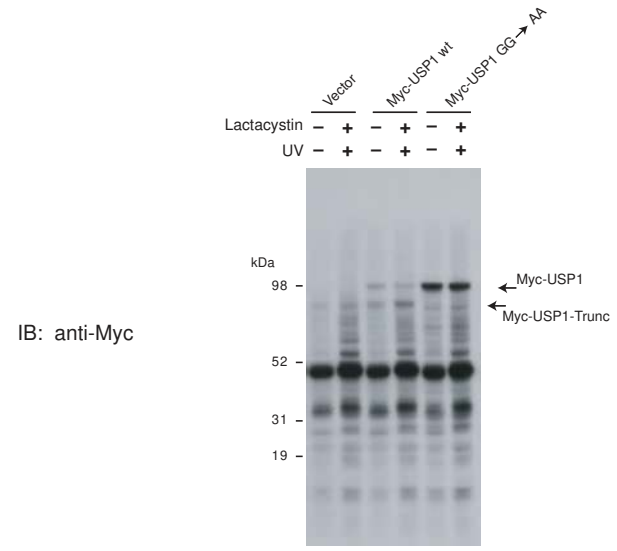
Figure S4 Full gels of Fig. 1A and Fig. 4A

A



Full gel of Fig. 4C

B



Full gel of Fig. 5E

Figure S5 Full gels of Fig. 4C and Fig. 5E

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ERRATUM

In the advance online publication of Huang *et al.* (*Nature Cell Biol.* 2006; DOI: 10.1038/ncb1378), the formulas in Fig. 5c were incorrect. This error has now been corrected online.