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Regulation of Proliferating Cell Nuclear Antigen ubiquitination in mammalian cells

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Abbreviations:

CPD, cyclobutane pyrimidine dimer; DUB, de-ubiquitinating enzyme; HU, hydroxyurea; MMC, mitomycin C, MMS, methyl methanesulfonate; 6-4 PP, pyrimidine(6-4)pyrimidone photoproduct; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; TLS, translesion synthesis; Ub-PCNA, ubiquitinated PCNA; UV, ultraviolet.

Abstract

Following exposure to DNA damaging agents that block the progress of the replication fork, mono-ubiquitination of PCNA mediates the switch from replicative to translesion synthesis DNA polymerases. We show that in human cells, PCNA is mono-ubiquitinated in response to methyl methanesulfonate and mitomycin C, as well as UV light, albeit with different kinetics, but not in response to bleomycin or camptothecin. Cyclobutane pyrimidine dimers are responsible for most of the PCNA ubiquitination events following UV-irradiation. Failure to ubiquitinate PCNA results in substantial sensitivity to UV and MMS, but not to camptothecin or bleomycin. PCNA ubiquitination is dependent on RPA, but independent of ATR-mediated checkpoint activation. After UV-irradiation, there is a temporal correlation between the disappearance of the de-ubiquitinating enzyme USP1 and the presence of PCNA ubiquitination, but this correlation was not found after chemical mutagen treatment. Using cells expressing photolyases, we are able to remove the UV lesions and we show that PCNA ubiquitination persists for many hours after the damage has been removed. We present a model of translesion synthesis behind the replication fork to explain the persistence of ubiquitinated PCNA.

Introduction

The replication of damaged DNA is a topic of much current interest following the discovery of the specialised Y-family of DNA polymerases, which are able to bypass lesions in DNA. There are four Y-family members in mammalian cells, DNA polymerase (pol) η , pol ι , pol κ and Rev1, each with a different substrate specificity (1-3).

Genetic studies using *Saccharomyces cerevisiae* have implicated ubiquitin-conjugating systems in the replication of damaged DNA, and the ubiquitination target is the DNA polymerase sliding clamp accessory protein, PCNA (4). In response to DNA damage, Rad6 and Rad18 mediate the mono-ubiquitination of PCNA on lysine-164, and subsequent poly-ubiquitination is brought about by Ubc13-Mms2 and Rad5. Mono-ubiquitination appears to trigger translesion synthesis (TLS) to bypass DNA lesions, whereas polyubiquitination channels the damage into a poorly understood error-free damage-avoidance mechanism (4, 5).

In human fibroblasts, mono-ubiquitination on lysine-164 is by far the major modification of PCNA and is easily detectable on exposure of replicating cells to DNA damage induced by ultraviolet light (UV) or to replication arrest by hydroxyurea (HU) (6). Polyubiquitination has recently been detected at much lower levels (7, 8). Monoubiquitination of PCNA increases its affinity for pol η , pol ι and Rev1 (6, 9-12). The increased affinity of mono-ubiquitinated PCNA (Ub-PCNA) for Y-family polymerases is mediated by ubiquitin-binding domains that have been identified in all the Y-family polymerases (10-13), and provides a mechanism for bringing about the polymerase switch, whereby the blocked replicative DNA

polymerase is replaced by a TLS polymerase that can bypass the blocking lesion (1). Ubiquitinated PCNA activates the in vitro damage-bypass activities of pol η and Rev1 (14).

Whereas ubiquitination of PCNA is brought about by the Rad6-Rad18 system, it is kept in check in human cells by the de-ubiquitinating enzyme (DUB) USP1 (15). Following high doses of UV, USP1 disappears from the cell (15, 16).

In this paper we examine the response of PCNA ubiquitination to different DNA damaging agents in human cells, we show that mutation of PCNA-K164 confers UV and MMS sensitivity to the cells, we demonstrate that PCNA ubiquitination and activation of cell cycle checkpoints are independent events and we show that PCNA ubiquitination persists even after removal of the lesions.

Results

PCNA ubiquitination following exposure to different damaging treatments.

We previously showed that PCNA was monoubiquitinated in response to UV-irradiation or treatment with HU, but not with ionising radiation (6). In our earlier work, PCNA ubiquitination remained elevated for at least 24 h after UV-irradiation, and our data shown in Figure 1A and B (upper panels) indicate that this elevated level persisted in MRC5V1 cells for more than 48 h after UV doses of 10 and 20 Jm⁻². In all experiments shown, we loaded the same proportion of the total cell population in each lane. Thus the intensity of the band corresponding to Ub-PCNA represents the absolute level of ubiquitinated PCNA in the culture rather than the amount relative to

unmodified PCNA or per μg protein. We have analysed the data in this way to avoid any apparent loss of PCNA ubiquitination by dilution when cells divide.

UV-irradiation generates two major photoproducts in DNA, cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP). To determine which of these lesions is responsible for the ubiquitination of PCNA, we used XP-A cells which express photolyases specific for CPD, 6-4PP or both (17). When UV-irradiated cells are exposed to visible light immediately after UV-irradiation, the photolyases reverse the cognate photoproducts in situ. More than 90% of the lesions disappear from the DNA on exposure to visible light for 90-120 min (Fig S6). Removal of just the CPDs results in a significant reduction of PCNA ubiquitination (Fig 1C, compare lanes 3 and 4). In contrast removal of 6-4PP has a barely detectable effect (lanes 7, 8), but this might be expected as 6-4PP form only 20-30% of total photoproducts. Removal of both photolesions prevents the ubiquitination completely (lanes 11 and 12). We conclude that both photoproducts are able to elicit ubiquitination of PCNA.

In an attempt to understand the triggering structure(s) for PCNA mono-ubiquitination, we have exposed cells to different agents and measured PCNA ubiquitination for extended periods of time after damaging treatments. The monofunctional methylating agent, methyl methanesulfonate (MMS) generates mainly 7-methylguanine and 3-methyladenine in DNA together with a small amount of O-6-methylguanine (18). Figure 1D (upper panel) shows that Ub-PCNA was detectable 3 h after a one-hour MMS treatment and increased in intensity at 24 h and later times. With the crosslinking agent mitomycin C, little Ub-PCNA was detectable in the first few hours

after treatment, but as with MMS treatment, a strong band appeared at 24 h and increased in intensity up to 48 h (Fig 1E, upper panel).

In contrast to these agents, which generate chemical alterations in DNA, bleomycin, like ionising radiation, produces double-strand breaks. With this agent, minimal Ub-PCNA was detected up to 48 h after treatment (Figure S7A). Similar results were obtained with camptothecin, an inhibitor of topoisomerase I that generates double-strand breaks in DNA during DNA replication (data not shown). No PCNA ubiquitination was detected following treatment with the microtubule spindle poison nocodazole (not shown), confirming that agents that disrupt cell cycle progression without affecting DNA replication do not induce the ubiquitination of PCNA.

Failure to ubiquitinate PCNA confers UV and MMS sensitivity

We have used SV40-transformed MRC5V1 human fibroblasts to generate cell lines expressing His-tagged PCNA, either wild-type or mutated at lysine-164, at levels similar to those of endogenous PCNA (Figure 2A, lanes 1 and 3). The cDNA for the exogenous PCNA contained silent mutations to make it refractory to targeting by siRNA directed against endogenous PCNA. Using siRNA, we were able to deplete the endogenous PCNA such that more than 80% of the PCNA is expressed from the transfected cDNA (Figure 2A, lanes 2 and 4). The transfected wild-type his-PCNA is ubiquitinated following UV-irradiation (Fig 2B, lane 2), whereas the K164R mutant his-PCNA is not (Fig 2B, lane 4). Depletion of the endogenous PCNA results in a substantial sensitisation of cells expressing mutant PCNA to UV-irradiation (Fig 2C, KR) compared to those expressing wild-type PCNA (Fig 2C, WT). Additional depletion of pol η in cells expressing wild-type PCNA results in only a modest

decrease in survival after UV-irradiation (Fig 2C, WT-h) and in cells expressing mutant PCNA there is no further decrease in survival (Fig 2C, KR-h). These data demonstrate the importance of PCNA ubiquitination for cell survival after UV irradiation.

The cells expressing only PCNA-K164R are also sensitive to MMS (Fig 2D), but not to camptothecin (Fig 2E) or bleomycin (Fig S7B), consistent with the patterns of ubiquitination of PCNA (Fig 1).

PCNA ubiquitination and cell cycle checkpoints

The spectrum of damaging agents giving rise to PCNA ubiquitination, its dependence on Rad18 and the single-stranded DNA-binding properties of Rad18 lead to the hypothesis that single-stranded DNA exposed at the site of stalled forks can trigger Rad18-mediated ubiquitination of PCNA. Single-stranded regions are also the trigger for cell-cycle checkpoints mediated by the ATR protein kinase. To trigger the checkpoint, single-stranded DNA needs to be coated with the single-strand DNA binding protein, RPA (19). We depleted cells of RPA using the same conditions as Zou and Elledge (19). In our initial experiments, we obtained substantial depletion of RPA without affecting the ubiquitination of PCNA following UV treatment (data not shown). However, when we altered our transfection conditions, we were able to reduce RPA to levels that were undetectable on Western blotting (Figure 3A, panel a). Under these conditions phosphorylation of Chk1 was reduced as described previously (19) (Figure 3A, panel b) and we found that Ub-PCNA formation was also reduced substantially (Figure 3A, panel c). We were concerned that depletion of RPA might deplete the S phase population of the cells, and that the decreased ubiquitination of

PCNA might be a secondary consequence of a lack of S phase cells. We therefore analysed the cell cycle status of the RPA- depleted population. Flow cytometry showed that the cell cycle distribution of cells depleted of RPA was very similar to that of undepleted cells (Table S1), suggesting that, although undetectable by immunoblotting, residual RPA is sufficient to permit DNA replication to continue. Our data suggest, therefore, that ubiquitination of PCNA is dependent on RPA.

To examine if Ub-PCNA formation depends on checkpoint activation, we depleted MRC5V1 cells of ATR using siRNA. (Figure 3B, panel a). This prevented the UV-induced phosphorylation of Chk1 (Fig 3B, panel b), demonstrating that checkpoint activation had been abrogated. However, 6 h after exposure to 20 Jm^{-2} UV-irradiation, depletion of ATR had no effect on the levels of Ub-PCNA (Figure 3B, panel c). We also showed that the ubiquitination of PCNA in cells from a normal individual and from a child with Seckel Syndrome caused by a mutation in the *ATR* gene and deficient in ATR signalling (20), were very similar (Figure 3C). We conclude that ubiquitination of PCNA is not dependent on a checkpoint response mediated by ATR.

Persistence of Ub-PCNA after removal of the damage.

The results of Figure 1 demonstrate that Ub-PCNA persists for a long time after formation of the DNA damage. However many types of damage are known to persist for long periods, and the apparent persistence of Ub-PCNA may represent a dynamic equilibrium between cycles of ubiquitination and de-ubiquitination as the replication machinery encounters successive lesions. To test if this is the case, we again used the XP-A cells that express both photolyases (17). These cells were UV-irradiated (20 Jm^{-2}) and then incubated for 6 h in the dark to permit replication forks to stall at damaged

sites and Ub-PCNA to accumulate. The cells were then exposed to visible light for two hours. Samples were taken at various times after photoreactivation and analysed for Ub-PCNA. Figure 4A (odd lanes) shows that despite the removal of nearly all the damage, Ub-PCNA persisted for many hours. We obtained similar results after a lower dose of 5 Jm^{-2} (not shown). We considered the possibility that, after the damage has been removed, the Ub-PCNA is released from the chromatin into the PCNA pool. In the photoreactivated cells, however, although much of the unmodified PCNA was extracted by triton, most of the Ub-PCNA was refractory to triton extraction (Figure 4A, compare even with odd lanes), indicating that it remained associated with chromatin for many hours after removal of the damage.

Recently USP1 was identified as a DUB that de-ubiquitinates Ub-PCNA. Following high doses of UV-irradiation, USP1 was cleaved and this permitted Ub-PCNA to accumulate (15). These data suggested that USP1 might regulate the level of PCNA ubiquitination. Given the existence of a DUB for Ub-PCNA, it seemed curious that Ub-PCNA persisted in our experiments. We therefore measured the level of USP1 and Ub-PCNA in the same cell pellet under different conditions. In agreement with the report of Huang et al (15), we observed that USP1 disappeared from cell extracts after UV-irradiation of the cells. We found that this occurred even after relatively low UV doses (Figure 1A, B, lower panels). There was an approximately 70% reduction 8 h after 10 Jm^{-2} and recovery at 72 h, whereas, after 20 Jm^{-2} , as might be expected, the response was more severe. USP1 became barely detectable after 8 h and did not recover within the time of the experiment. After both doses, the ubiquitination of PCNA (upper panels) correlated well with the disappearance of USP1. Furthermore in the photolyase experiments described above, we found that USP1 remained at

undetectable levels for at least 24 hours after reversal of the damage by photoreactivation (Figure 4B, top panel). The levels of USP1 in these experiments therefore show a good inverse correlation with those of Ub-PCNA, consistent with the idea that USP1 is an important regulator of Ub-PCNA (15).

We also measured USP1 levels following treatments with MMS and MMC. In striking contrast to the results with UV-irradiation, we were unable to detect any significant loss of USP1 after treatment with these chemicals (Figs 1D and E, lower panels). Indeed there was an increase in USP1 at later times, more or less concomitant with the increase in PCNA ubiquitination.

Discussion

Ubiquitination of PCNA is a central control point for mediating the replication of damaged DNA, but many questions remain concerning the ubiquitination process. What is the trigger that turns it on and what is the mechanism for turning it off? We and others have shown that PCNA is efficiently ubiquitinated following exposure to UV, MMS, MMC and HU, but not by ionising radiation, bleomycin or camptothecin (this paper and (21)) nor by daunomycin, actinomycin D and neocarzinostatin (22). The former agents all cause stalling of the replication fork. A likely result of fork stalling is either the dissociation of the replicative helicase from the stalled replication machinery and exposure of single-stranded DNA ahead of the replication fork, or uncoupling of the synthesis on leading and lagging strands, exposing single-stranded regions on the leading strand (23). This single-stranded DNA likely binds Rad18, which together with either or both of the Rad6 orthologs carries out the ubiquitination process (24, 25). Ionising radiation, bleomycin, neocarzinostatin and camptothecin

generate double strand breaks either directly or during replication and would not therefore be expected to generate regions of single-stranded DNA at the forks. MMC produces interstrand DNA crosslinks. These will result in stalling of the fork, but the cross-links are likely to provide physical barriers to unwinding of the DNA ahead of the stalled forks. This may account for the lack of ubiquitination of PCNA at early times after treatment. The accumulation of Ub-PCNA at much later times is likely to be a result of secondary processes involved in the repair of the cross-links.

When we replaced PCNA with the K164R mutant form that cannot be ubiquitinated, the viability of the cells was unaffected. Consistent with this observation, Langerak et al recently generated a knock-in PCNA-K164R mouse, which was viable (26). These mice were infertile and had an altered somatic hypermutation spectrum, but were otherwise healthy. As in budding and fission yeasts (4, 27) and DT40 chicken cells (28), therefore, the inability to ubiquitinate PCNA is compatible with life in mammals. When treated with PCNA-specific siRNA, our “K164R cells” are, like DT40 cells expressing human PCNA-K164R as the sole source of PCNA (28), sensitive to UV-irradiation. At first sight this may appear not unexpected. However, in response to UV, Ub-PCNA has been hypothesized to facilitate the switch from replicative to TLS polymerase to enable TLS past UV lesions (6). Interestingly, cells in which pol η is depleted (Figure 2C), like XP variant cells defective in pol η (29), are barely sensitive to killing by UV light. The PCNA-K164R cells, in contrast, show much more pronounced UV sensitivity. This suggests that modification of PCNA has roles other than recruitment of pol η in response to UV-irradiation. These roles could include recruitment of other polymerases involved in TLS past 6-4 photoproducts. For example, Rev1 also binds to ubiquitinated PCNA (12) as well as to other Y-family

polymerases and to pol ζ , suggesting that it might act as a platform for recruiting other polymerases (30, 31). In addition polyubiquitination of PCNA might facilitate an error-free recombination-mediated process for bypassing lesions, as found in yeast (4, 5). A further possibility is that ubiquitination of PCNA has a role outside of S phase. We have shown in *S pombe* that PCNA is ubiquitinated in G2 cells in response to DNA damage (27), and work by N Zlatanou and PLK, to be published elsewhere, has revealed that PCNA is ubiquitinated in response to DNA damage in quiescent human fibroblasts. The PCNA-K164R cells are also sensitive to MMS, implying an important role for ubiquitinated PCNA in recovery from MMS-induced damage. Further studies will be required to determine if this role involves TLS, recombination, or both.

Stalling of the replication fork also activates ATR-mediated cell cycle checkpoints and mono-ubiquitination of FANCD2 (32). This raises the question as to whether these processes are co-ordinated and interdependent. We found that depletion of RPA resulted in a reduction in PCNA ubiquitination, in agreement with findings of Bi et al (33), and with recent observations in *S. cerevisiae* (34). However, we found that reduced levels of ATR had little effect on PCNA ubiquitination. These results are somewhat at variance with those of Bi et al, who reported some reduction of ubiquitination in ATR deficient cells (33), but agree with a recent report showing no effect of reduction of ATR (35). They are also consistent with our earlier findings in *S. pombe*, in which deletion of the checkpoint kinase genes *rad3* and *tell* (ATR and ATM orthologs) had no effect on PCNA ubiquitination (27) and with similar results in *S. cerevisiae* (34) and *Xenopus laevis* (24). We envisage therefore that PCNA ubiquitination and checkpoint activation are independently and automatically

triggered by a “state of emergency” indicated by exposed single stranded DNA at the replication fork.

Ubiquitination of PCNA is also regulated by the DUB USP1, which is able to remove ubiquitin from Ub-PCNA (15). This is at first sight difficult to reconcile with our finding that Ub-PCNA persisted for many hours even after replication blocks were removed. However, we have extended the original observations of Huang et al (15) to demonstrate that, after UV-irradiation of MRC5V1 cells, USP1 disappears and is barely detectable during the periods when PCNA ubiquitination persists, even when the damage has been removed. In contrast, ubiquitination of PCNA following MMS or MMC treatment was not accompanied by a loss of USP1. This suggests that USP1 is an important regulator of PCNA ubiquitination in response to UV, whereas after chemical treatments, the ubiquitinated PCNA is refractory to USP1. One possible explanation is that USP1 is sequestered away from the Ub-PCNA. Alternatively, USP1 is itself regulated and activated by association with a partner protein, UAF1 (16), and this activation might be differentially affected by different DNA damaging treatments.

What is the explanation for the persistence of Ub-PCNA following UV-irradiation, even after UV damage has been removed? In *E. coli*, DNA synthesised in UV-irradiated cells contains gaps opposite UV lesions and these gaps are subsequently sealed (36). This led to a model in which the gaps were sealed behind the replication fork so that the bypass past the lesion was independent of replication fork progression. More recent models have however assumed that TLS occurs at the stalled forks and that fork progression and TLS are co-ordinated. This may not be the case and recent

work in yeast has provided direct support for the older model of gaps behind the replication fork (23). Furthermore Heller and Marians have shown, using a bacterial in vitro system, that replication can restart downstream of a replication block, even on the leading strand (37). In addition, Waters and Walker found high levels of the Y-family polymerase Rev1 in G2 cells in yeast and inferred that this was an indication of a postreplicative gap-filling step (38). We develop this model to explain the persistence of Ub-PCNA in UV-irradiated cells (Figure 5). We propose that when the replication fork stalls at a lesion, PCNA becomes ubiquitinated (Figure 5A) and shortly afterwards a new replication apparatus is assembled beyond the lesion, with a new molecule of PCNA (Figure 5B). Synthesis continues up to the next lesion, where another Ub-PCNA molecule is deposited and replication restarts again beyond the lesion (Figure 5C). At some later time, the gaps are sealed by pol η and/or maybe other Y-family polymerases, depending on the nature of the lesion (Figure 5D), and the ubiquitinated PCNA is left on the DNA, perhaps because there are no RFC molecules in the vicinity to unload it. The net result is that Ub-PCNA molecules remain on the DNA until they are disassembled at the next round of replication or, at least in the case of UV damage, de-ubiquitinated when USP1 levels are restored. Figure 5 displays the proposed situation on the leading strand, but a similar process could occur on the lagging strand.

Might the persistence of Ub-PCNA after the damage has been removed, together with its affinity for error-prone Y-family polymerases, result in inappropriate recruitment of these polymerases to the replication fork and an elevated mutation rate? This is unlikely for two reasons. First, if the model proposed above is correct, the Ub-PCNA remaining on the chromatin will be behind the replication fork on DNA that has

already been replicated, and will therefore be harmless. Second, the replicative polymerases are much more efficient and processive than the Y-family members. Once the replicative polymerase is engaged and replicating an undamaged stretch of DNA, it is unlikely that a relatively inefficient Y-family polymerase will be able to compete effectively (39). It is only when passage of the replication fork is blocked and the replicative polymerases cannot proceed, that engagement of the Y-family polymerases becomes an issue.

Is the above model of TLS behind the forks compatible with the findings in several reports that pol η and ι and Rev1 are localised in replication foci (40-42)? There is a widely held misconception that forks and foci are one and the same entity. In fact, foci are quite large structures that are thought to contain 5-20 replication forks. It is therefore perfectly plausible that the gapped structure, though behind the fork, remains associated with the focus.

Materials and Methods

SV40-transformed human fibroblast line MRC5V1, grown in Eagle's MEM with 10% or 15% fetal calf serum, was used in most experiments. For generation of cells expressing exogenous PCNA, His-PCNA constructs were used that contained mutations rendering them refractory to siRNA knock-down. Transfection and siRNA treatment used standard procedures. For photoreactivation, XP-A cells expressing photolyases were exposed to visible light at different times following UVC irradiation. Further details of Materials and Methods are presented as supplementary information.

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Figure legends

Figure 1 PCNA ubiquitination after DNA damaging agents.

MRC5V1 cells were UV-irradiated with (A) 10 or (B) 20 Jm⁻², incubated for the indicated times and analysed by immunoblotting with anti-PCNA (upper panels) or anti-USP1 antibody (lower panels). - UV, mock-treated cells incubated for 6h; (C) XP-A cells expressing the indicated photolyase were UV-irradiated (10 Jm⁻²), exposed or not to photoreactivating light for 2 h (PR), incubated for a further 6 h and analysed as in (A). Cells were treated with (D) 1 mM MMS for 1h, (E) 8 µg/ml mitomycin C for 30 min, followed by incubation for the indicated times prior to harvesting and analysis by immunoblotting.

Figure 2 UV sensitivity of cells expressing PCNA-K164R

A, MRC5V1 cell clones expressing his-tagged wild-type PCNA or PCNA-K164R were either mock-transfected or treated with PCNA-specific siRNA and PCNA levels measured after 72h; B, After transfection with PCNA siRNA, the cells were UV-irradiated (20 Jm⁻²), incubated for 5 h and analysed for PCNA ubiquitination. C, D, E: UV, MMS, and camptothecin survival curves of cells depleted for endogenous PCNA and expressing wild-type (WT) or mutant (KR) his-PCNA. Where indicated (-h), cells were also depleted for polη. Error bars: SEM of 3-4 experiments.

Figure 3 PCNA ubiquitination in RPA or ATR knock down cells

A, MRC5V1 cells were transfected with RPA70 siRNA. After 72h incubation, cells were either irradiated or not with 10 Jm⁻² UV and incubated for 0.5 or 6 h. Cell extracts were analysed by immunoblotting with (top to bottom) anti-RPA70, anti-Chk1-P-Ser317, PC10 and anti-vimentin (loading control) antibody. Lanes 1, 3, 5;

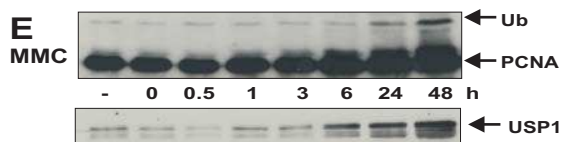
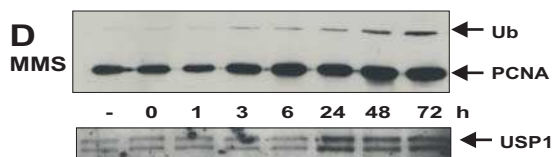
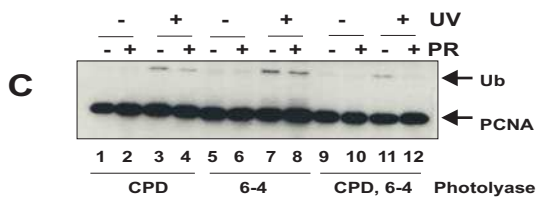
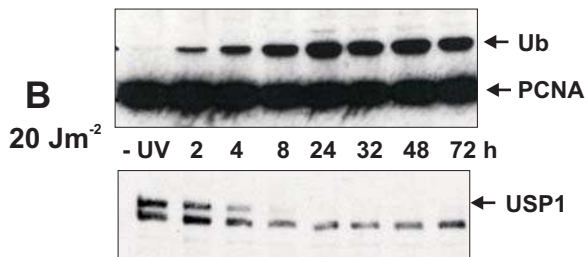
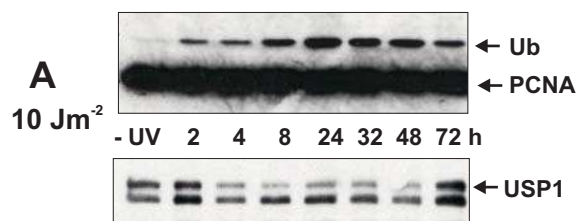
non-targeting siRNA control. Lanes 2, 4, 6; RPA siRNA transfected samples. B, MRC5V1 cells were transfected with non-targeting or ATR siRNA, UV irradiated (20 Jm^{-2}) 72 h later, incubated for 6 h and analysed as in A. C, Normal or Seckel Syndrome lymphoblastoid cells were UV-irradiated with the indicated doses and incubated for 6 h prior to lysis and analysis.

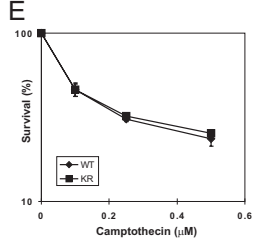
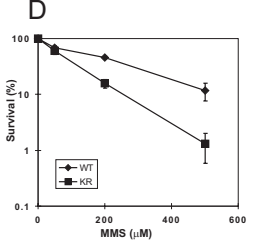
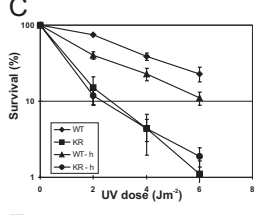
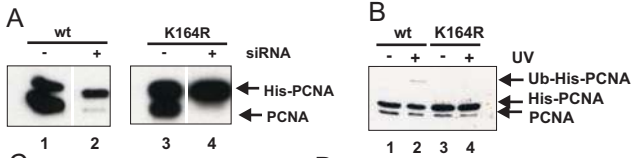
Figure 4 Persistence of PCNA ubiquitination

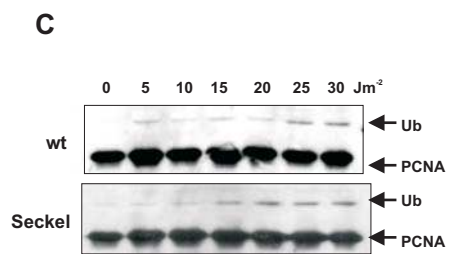
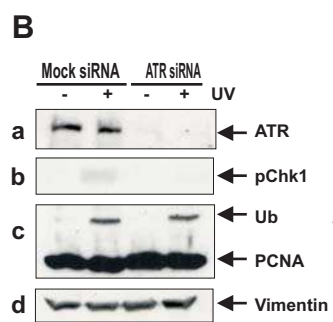
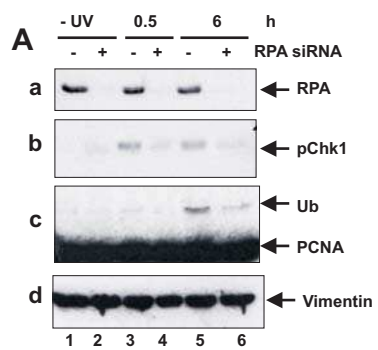
PH-XPA cells were irradiated with 20 Jm^{-2} UV, incubated for 6h, and then photoreactivated for 2h. After further incubation for the indicated times, PCNA in cell lysates was detected by immunoblotting. In A, duplicate samples were analysed either with or without prior extraction with triton, as indicated. In B, lysates were analysed for both USP1 and PCNA ubiquitination.

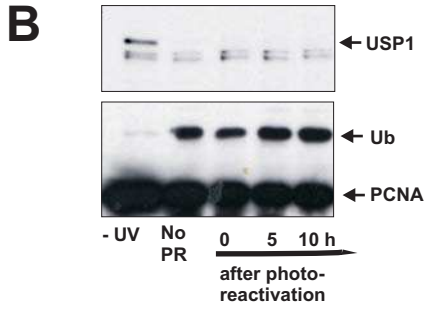
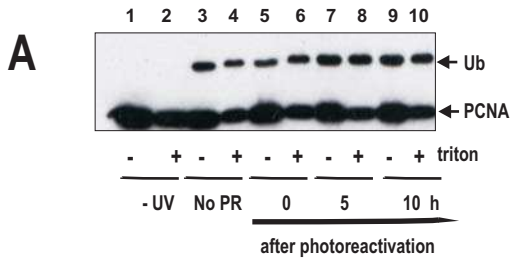
Figure 5 Model for persistence of Ub-PCNA

(A) On blocking of the replication fork at a lesion (X), PCNA becomes ubiquitinated (U) (Note that only one ubiquitin molecule is shown for simplicity, but it is likely that all three monomers of the homotrimeric ring become ubiquitinated). (B) A new replication apparatus is assembled beyond the lesion, leaving a gap. (C) The process is repeated at the next lesion. (D) Some time later the gap opposite the first lesion is filled, as indicated by the thick line.









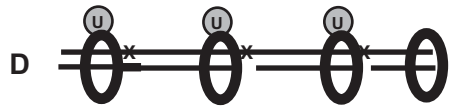
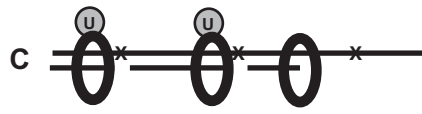
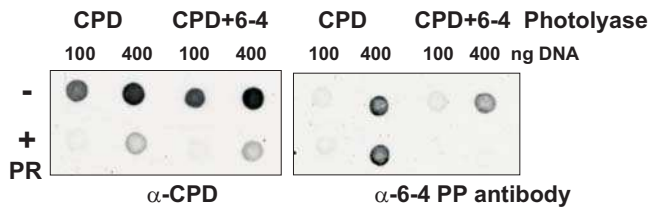
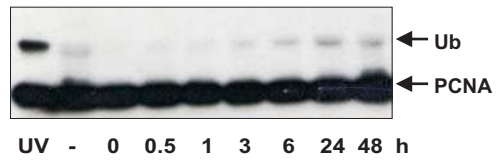


Figure S6



A**B**