

# DNA-damage tolerance mediated by PCNA•Ub fusions in human cells is dependent on Rev1 but not Pol $\eta$

Zhoushuai Qin<sup>1</sup>, Mengxue Lu<sup>1</sup>, Xin Xu<sup>1</sup>, Michelle Hanna<sup>2</sup>, Naoko Shiomi<sup>3</sup> and Wei Xiao<sup>1,2,\*</sup>

<sup>1</sup>College of Life Sciences, Capital Normal University, Beijing 100048, China, <sup>2</sup>Department of Microbiology and Immunology, University of Saskatchewan, Saskatoon S7N 5E5, Canada and <sup>3</sup>Project for Environmental Dynamics and Radiation Effects, Fukushima Project Headquarters, National Institute of Radiological Sciences, Chiba 263-8555, Japan

Received December 5, 2012; Accepted May 23, 2013

## ABSTRACT

In response to replication-blocking lesions, proliferating cell nuclear antigen (PCNA) can be sequentially ubiquitinated at the K164 residue, leading to two modes of DNA-damage tolerance, namely, translesion DNA synthesis (TLS) and error-free lesion bypass. Although the majority of reported data support a model whereby monoubiquitinated PCNA enhances its affinity for TLS polymerases and hence recruits them to the damage sites, this model has also been challenged by several observations. In this study, we expressed the *PCNA-164R* and ubiquitin (*UB*) fusion genes in an inducible manner in an attempt to mimic PCNA monoubiquitination in cultured human cells. It was found that expression of both N- and C-terminal PCNA•Ub fusions conferred significant tolerance to ultraviolet (UV)-induced DNA damage. Surprisingly, depletion of Pol $\eta$ , a TLS polymerase dedicated to bypassing UV-induced pyrimidine dimers, did not alter tolerance conferred by PCNA•Ub. In contrast, depletion of Rev1, another TLS polymerase serving as a scaffold for the assembly of the TLS complex, completely abolished PCNA•Ub-mediated damage tolerance. Similar genetic interactions were confirmed when UV-induced monoubiquitination of endogenous PCNA is abolished by *RAD18* deletion. Hence, PCNA•Ub fusions bypass the requirement for PCNA monoubiquitination, and UV damage tolerance conferred by these fusions is dependent on Rev1 but independent of Pol $\eta$ .

## INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is an auxiliary factor of DNA polymerases and forms the eukaryotic DNA sliding clamp, consisting of three PCNA monomers forming a closed ring structure (1–5). PCNA plays important roles not only in DNA replication but also in several DNA damage-responsive pathways (6). DNA-damage tolerance, also known as DNA post-replication repair in budding yeasts, uses at least two mechanisms to tolerate DNA damage. Error-free lesion bypass or damage avoidance uses a newly synthesized sister chromatid as a template to replicate across DNA replication-blocking lesions. Alternatively, translesion DNA synthesis (TLS) uses a set of specialized non-essential DNA polymerases to synthesize across the damaged template DNA, which can be either error-free or error-prone depending on the type of lesion and the TLS polymerase used (7–10). In budding yeast and possibly other eukaryotic organisms as well, the aforementioned survival mechanisms are regulated through covalent modifications of PCNA by ubiquitin (Ub) (11). Hence, PCNA can be monoubiquitinated by the E2-E3 complex Rad6-Rad18 at the K164 residue and further modified with a K63-linked Ub chain by another E2-E3 complex, Mms2-Ubc13-Rad5 (11). The non-canonical K63-linked Ub chain plays crucial roles in regulating various cell-signaling pathways by altering the target protein activity, which is different from conventional K48-linked Ub chains that target proteins for degradation by the 26S proteasome (12,13). On the other hand, PCNA monoubiquitination appears to favor the TLS pathway. Recent studies suggest a model by which monoubiquitinated PCNA recruits TLS polymerases through an enhanced physical interaction. Indeed, most Y-family TLS polymerases contain separate

\*To whom correspondence should be addressed. Tel: +86 10 6890 3412; Fax: +86 10 6890 9617; Email: wei.xiao@usask.ca

PCNA-binding and Ub-binding motifs, and their affinity for monoubiquitinated PCNA is higher than for PCNA alone (14).

In mammalian cells, the four Y-family TLS polymerases that have been found are Polk, Polt, Pol $\eta$  and Rev1 (15,16). These enzymes do not contain a 3'-5' proofreading exonuclease activity, replicate undamaged DNA *in vitro* with low fidelity and poor processivity and are responsible for most spontaneous and induced mutations (17). However, some of the specialized TLS polymerases may replicate past cognate DNA lesions with unusually high efficiency and fidelity. For example, Pol $\eta$  is considered an error-free polymerase when bypassing ultraviolet (UV)-induced thymine dimers (18). A typical Y-family TLS polymerase contains one or two Ub-binding UBM or ubiquitin-binding zinc finger domain (UBZ) motifs and a PCNA-binding PIP box, which contribute to their affinity for ubiquitinated PCNA. Rev1 contains two UBM motifs but does not contain a classic PIP box; it interacts with PCNA via a BRCT domain in the N-terminus (19) and/or a polymerase-associated domain (20). In addition, the C-terminal region of Rev1 can interact with other TLS polymerases as well as the Rev7 subunit of Pol $\eta$  (21–23), whereas its catalytic activity does not appear to be essential for TLS of UV-induced DNA damage (24,25), suggesting that Rev1 serves as a scaffold for TLS.

The critical roles of Ub-binding and PCNA-binding domains of Y-family polymerases in their TLS activity have been extensively characterized (14,19,26–33); however, whether monoubiquitinated PCNA promotes Y-family polymerase activity *in vitro* has been a subject of debate (29,34–36). Furthermore, it remains unclear whether and how monoubiquitinated PCNA directly recruits certain Y-family polymerase(s) to promote TLS activity *in vivo*. Although lack of PCNA ubiquitination can be achieved by manipulating Rad18 or mutating the PCNA-K164 residue, one technical difficulty in the study of consequences of PCNA ubiquitination *in vivo* is that only a small portion of PCNA is ubiquitinated after DNA-damaging treatment, and that PCNA is deubiquitinated by the Ub-specific protease Usp1 (37). In the past few years, attempts have been made to create and express artificial Ub and PCNA fusion proteins to mimic native ubiquitinated PCNA with limited success in budding and fission yeasts (38–40). In contrast, such a systematic study in mammalian cells has been largely missing. Here, we report a carefully crafted system to express PCNA and Ub fusion proteins to mimic endogenous PCNA monoubiquitination and the utilization of this system to address its *in vivo* functions in TLS.

## MATERIALS AND METHODS

### Plasmids and plasmid construction

The PCNA-K164R mutation was created by a megaprimer PCR method (41), and the open reading frame (ORF) was cloned into plasmid vector pcDNA5.0FRT/TO (Invitrogen) as a *Hind*III-*Bam*HI fragment. The *UB* gene lacking the C-terminal Gly-Gly codons was cloned

into the aforementioned resulting plasmid to make either N-terminal (Ub-PCNA) or C-terminal (PCNA-Ub) fusion protein. A Ub-I44A mutation was created in the plasmid PCNA-Ub by site-directed mutagenesis for the co-immunoprecipitation experiment. Plasmids expressing GFP-mRev1 (42) was received from Dr C. Guo (Beijing Institute of Genomics, Chinese Academic of Sciences), in which the entire ORFs were cloned into a plasmid pEGFP (Clontech, USA). A similar GFP-Pol $\eta$  plasmid was constructed in this study.

### Cell culture and UV treatment

The FIP-InTM T-RexTM 293 cell line (T-Rex 293, Catalog no. R780-07, Invitrogen) was cultured in Dulbecco's modified Eagle's medium (DMEM) (SH30243.01B, Hyclone) plus 100  $\mu$ g/ml Zeocin, 15  $\mu$ g/ml blasticidin and 10% fetal bovine serum (FBS) (SH30256.01B, Hyclone) in a 37°C, 5% CO<sub>2</sub> humidified incubator. T-Rex 293 cells were transfected with a desired plasmid, incubated in the presence of 100  $\mu$ g/ml hygromycin B, and stably-transfected cell lines were selected after a 3-day incubation. To induce ectopic PCNA expression, 2  $\mu$ g/ml doxycycline (Dox) was added to the culture medium, and the incubation continued for 2 days or otherwise as indicated. It was found that under the aforementioned conditions, the ectopic PCNA was noticeably induced after as little as 2 h and remained induced for several days. To study the roles of Rad18 in TLS, both copies of the *RAD18* gene were disrupted by the neomycin and puromycin-resistance cassettes in HCT116 cells (43), and the resulting *RAD18*<sup>-/-</sup> cells were cultured in DMEM containing 0.35 mg/ml G418 and 0.35  $\mu$ g/ml puromycin, supplemented with 10% FBS. The primary fibroblast cell line GM1604 (Coriell Institute) was originally derived from human fetal lung tissue. The telomerase-immortalized cells (NF1604) were cultured in DMEM supplemented with 10% FBS. AS26 cells, a generous gift from Dr W.G. McGregor (University of Louisville, USA) were derived from NF1604 in which the *RAD18* gene expression was suppressed by antisense RNA (44) and the stably transfected cells were maintained in the presence of 200  $\mu$ g/ml G418.

For UV treatment, the culture medium was removed and cells in a dish were exposed to 254-nm UV irradiation at given doses. The culture medium was immediately replaced, and cells were returned to incubation for a given period as indicated before analysis. For a yeast UV sensitivity assay, 10-fold serial dilutions of overnight cultures were spotted onto YPD plates and exposed to various UV doses and then the plates were incubated at 30°C in the dark for an additional 60 h before photography.

### Experimental depletion of target TLS polymerases

The expression of TLS polymerases Pol $\eta$  or Rev1 was suppressed in T-Rex 293, HCT116 and NF1604 derivatives by transfection with 40  $\mu$ m of siRNA (custom-synthesized by Shanghai GenePharm) using Lipofectamine RNAiMAX (Invitrogen) or opti-MEM (Cat. 31985 from Gibco). The efficacy of target gene

suppression was assessed by qRT-PCR and/or western blotting 60 h after siRNA transfection. The following target sequences were used for siRNAs, Pol $\eta$ -1, 5'-CAG CCAAATGCCCATTCGCAA-3'; Pol $\eta$ -2, 5'-CTGGTTG TGAGCATTCGTGTA-3'; Rev1-1, 5'-AAGCATCAAA GCTGGACGACTTT-3'; Rev1-2, 5'-CAGCGCATCTG TGCCAAAGAA-3'; and Rev1-3, 5'-ATCGGTGGAAT CGGTTTGGAA-3'.

### Western blotting

Samples were collected and placed on ice in a lysis solution [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol] containing 0.5% SDS and 2  $\mu$ M PMSF with a protease inhibitor cocktail (Sigma P-8340, 1:100) and 10 mM N-ethylmaleimide (NEM). Cellular proteins were resolved on a 12% SDS-PAGE gel run at 4°C. Gels were blotted in 5% Carnation instant skim milk in PBS with 0.1% Tween-20 (PBST), and the membrane was probed with the anti-PCNA (1:2500 overnight), anti-tubulin (1:5000 1 h) and anti-actin (1:5000 1 h) antibodies. To prepare the soluble and chromatin-bound fractions, cultures were first rinsed with ice-cold PBS and exposed to PBS containing 0.4% NP-40, 10 mM NEM and protease inhibitors for 5 min. After centrifugation at 13 000 rpm for 10 min, the supernatant was collected as the soluble fraction. For the chromatin-bound fraction, the plate was rinsed three times with ice-cold PBST, and the pellet was resuspended in a 2 $\times$  loading buffer [250 mM Tris-HCl (pH 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol, 5%  $\beta$ -mercaptoethanol], boiled for 10 min before SDS-PAGE, and western blotting against anti-PCNA, anti-MDC1 (1:10,000 overnight) and anti-tubulin antibodies. The immunoreactivity was detected using a horseradish peroxidase-conjugated goat anti-mouse or rabbit antibody from Santa Cruz (1:5000, 1 h). To measure *RAD18* expression, a rabbit anti-Rad18 polyclonal antibody (45) was used. Western blots were scanned and band intensity measured using a Luminescent Image Analyzer (LAS-4000).

For co-immunoprecipitation of GFP-Rev1 or GFP-Pol $\eta$  with PCNA-Ub and wild-type PCNA, T-Rex 293/PCNA-Ub cells were transfected with GFP-Rev1 or GFP-Pol $\eta$  and incubated in the presence of Dox for 2 days to induce the PCNA-Ub expression. The cell lysates were incubated with GFP-Trap<sup>®</sup> A (gta-20, ChromoTek) for 4 h or overnight. Beads were boiled with a 5 $\times$  loading buffer for 5 min followed by three washes with the lysis buffer to remove unbound proteins. The input and the co-immunoprecipitated proteins were detected by western blotting with anti-PCNA (NA03, Calbiochem) and anti-GFP (7G9, Abmart) antibodies.

### RPA nuclear focus assay

Cultured cells were routinely seeded onto poly-lysine-coated cover slips, rinsed with ice-cold PBS and treated with 0.4% NP-40 in PBS for 20 min on ice before fixation in 4% formaldehyde for 30 min. Fixed cells were rinsed four times over 30 min with PBST before incubation with a mouse anti-replication protein A (RPA) antibody (Abcam, Ab2175, 1:500 for 1 h or overnight). Following

four washes with PBST, the Alexa 488 goat anti-mouse secondary antibody (A11001, 1:3000) and 1.5  $\mu$ g/ml 4',6-diamidino-2-phenylindole (DAPI) were added for 1 h and then washed again four times. Microscopy was performed with an inverted Olympus 10 $\times$  22 microscope equipped with a 40 $\times$  immersion lens, and images were acquired using the CCD RoHs (Q26053). Samples for comparison in each panel were always included in the same experiment and treated identically. Within each experiment, images containing at least 1000 cells for each treatment were captured and analyzed.

### Cell survival assay

Cultured human cells were treated with siRNA with or without Dox. Three days later, cells were irradiated with UV and incubated for an additional day before being fixed with 4% formaldehyde. Fixed cultures were stained with DAPI, and random fields of view from each culture dish were photographed. Cells with round and intact nuclei were counted as viable cells.

### *Saccharomyces cerevisiae* strains, plasmids and techniques

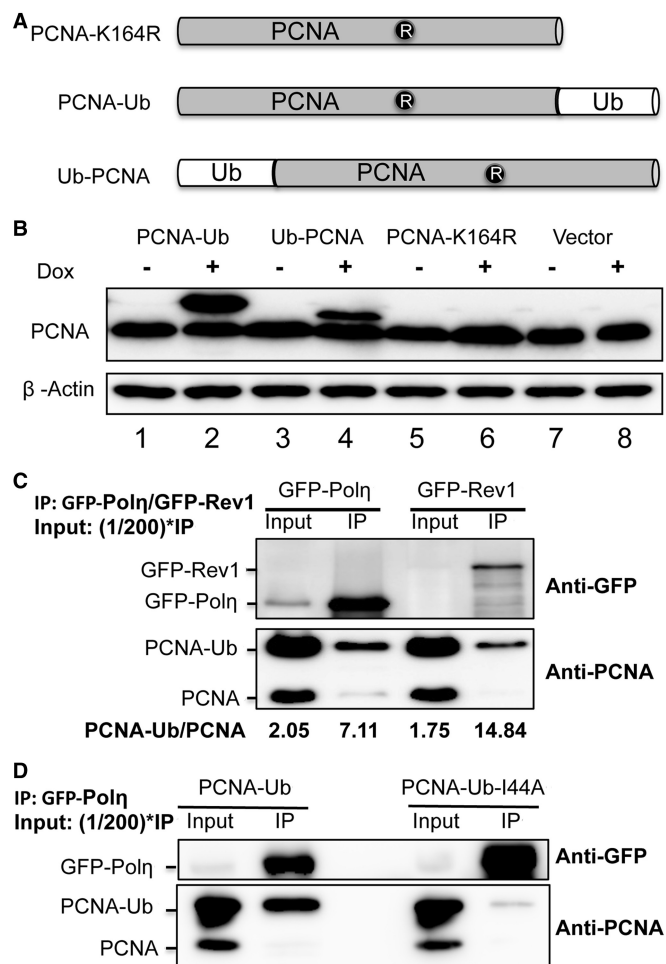
The haploid *Saccharomyces cerevisiae* strain HK578-10D was used as the wild-type source for the creation of all strains listed in Supplementary Table S1. Yeast cell cultures and plasmid transformations were performed as previously described (38). Disruption of genomic *POL30* and creation of YCpL plasmids carrying the *POL30* gene derivatives were as previously described (38). One-step targeted gene disruption (46) was used to delete *REV1* and/or *RAD30* genes, and the target gene deletion was confirmed by genomic PCR. YCpL plasmids were transformed into mutant strains relying on plasmid pBL211 (47) (YCp, *URA3*, *POL30*, from Dr P. Burgers) for survival, and subsequent loss of the pBL211 plasmid was confirmed by growth on 5-fluoroorotic acid.

A serial dilution assay was used to assess relative sensitivity of yeast cells to UV irradiation. Tenfold dilutions of overnight cultures were made and spotted onto YPD plates followed by UV exposure and incubation as previously described (48).

## RESULTS

### Experimental design and validation

Several considerations were taken into account when making the PCNA and Ub fusion (PCNA•Ub) constructs. First, the PCNA ORF used for fusions contains a K164R mutation to prevent *in vivo* PCNA ubiquitination at the K164 residue following UV irradiation. Second, we created both N- and C-terminal fusions (Figure 1A) in an attempt to mimic PCNA-K164 ubiquitination, as the PCNA N-terminus is situated on a separate but similar ridge to K164 and in a medial location between the faces, whereas the PCNA C-terminus and K164 reside on the same ridge, albeit on separate faces of the molecule (38). Hence, both fusions mimic distinct aspects of native K164-ubiquitinated PCNA. Third, the C-terminal two Gly residues were removed from Ub in



**Figure 1.** Establishment of stable cell lines expressing different forms of PCNA. (A) Schematic diagrams of PCNA•Ub fusion constructs. All ORFs contain a K164R mutation to prevent *in vivo* PCNA ubiquitination. In addition, the Ub-coding region is either fused to PCNA at the N-terminus (Ub-PCNA) or at the C-terminus (PCNA-Ub). All three ORFs are cloned into plasmid pcDNA5.0FRT/TO. (B) Ectopic expression of different forms of PCNA in stably transfected T-Rex-293 cells. Dox-induced expression of ectopic PCNA was analyzed by western blot using an anti-PCNA antibody, and the cellular β-actin serves as a loading control. PCNA-Ub and Ub-PCNA migrate differently, although they have the same molecular weight. (C) Polη and Rev1 preferentially bind to PCNA-Ub over PCNA. T-Rex-293/PCNA-Ub cells were transfected with GFP-Polη or GFP-mRev1 and incubated for 6 h followed by Dox treatment for 2 days. Immunoprecipitation was performed by GFP-Trap-A beads, and the products were analyzed by western blot. (D) The Ub-I44 residue is required for the enhanced affinity between PCNA-Ub and GFP-Polη. T-Rex-293/PCNA-Ub and T-Rex-293/PCNA-Ub-I44A cells were transfected with GFP-Polη, incubated for 6 h followed by Dox treatment for 2 days. Immunoprecipitation was performed by GFP-Trap-A beads, and the products were analyzed by western blot.

the fusion constructs to prevent protease cleavage (for Ub-PCNA) or further modifications (for PCNA-Ub). Fourth, no other tag is added to the fusion constructs to avoid unnecessary complications of the fusion proteins. Finally, an inducible FLP-FRT-mediated site-specific integration system was used in this study so that all stable transfectants are expected to be homogenous except for the genes of interest and so that target gene expression can

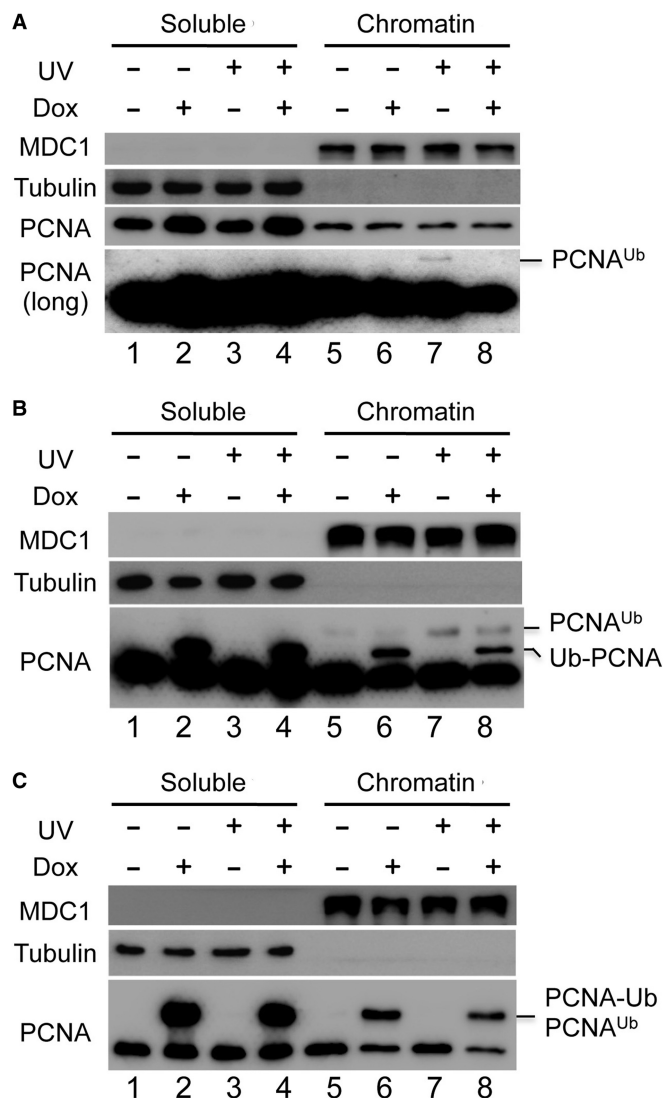
be experimentally induced by adding Dox to the culture medium. Indeed, stable cell lines obtained from the same plasmid transfection displayed indistinguishable levels of fusion gene expression, which was under tight control of Dox (Figure 1B). It was noticed that the ectopic PCNA-K164R migrates with the same speed as endogenous PCNA (cf. lanes 5 and 6). Interestingly, PCNA-Ub appears to migrate slower and express at a slightly higher level than Ub-PCNA (cf. lanes 2 and 4). Nevertheless, the levels of ectopic fusion proteins are comparable with the level of endogenous PCNA, which facilitates this study.

It has been previously reported that ubiquitinated PCNA has an increased affinity for Polη (14) and Rev1 (27). To ask whether Polη and Rev1 also preferentially bind to PCNA•Ub fusions over native PCNA, we transiently expressed GFP-Polη or GFP-Rev1 in Dox-induced T-Rex-293/PCNA-Ub cells followed by co-IP using GFP-Trap-A beads. As shown in Figure 1C, compared with the 'input' ratio, GFP-Polη and GFP-Rev1 pulled down more PCNA-Ub than PCNA, suggesting that the PCNA-Ub fusion protein has higher affinity for Polη and Rev1 than the native PCNA. We noticed that the optimal PCNA-Ub:PCNA precipitation ratio for GFP-Rev1 is 8.5:1, whereas that for GFP-Polη, it is 3.5:1. However, we are unable to rule out the possibility that the difference was partly due to different levels of GFP-Polη and GFP-Rev1 expression in the two cell lines (Figure 1C, top panel) and/or potential experimental variations.

A UBZ near the C terminus of Polη is responsible for an interaction with monoubiquitinated PCNA (14) and a Ub-I44A mutation abolishes its interaction with Polη UBZ (49). To ask whether the increased affinity of PCNA-Ub fusion for Polη is mediated through the Ub-I44 residue, we transiently expressed GFP-polη in Dox-induced T-Rex-293/PCNA-Ub and T-Rex-293/PCNA-Ub-I44A cells followed by co-IP and western blot analysis. As shown in Figure 1D, the Ub-I44A mutation in the PCNA-Ub fusion protein dramatically reduced its interaction with GFP-Polη, reinforcing the structural and functional similarity between PCNA•Ub fusions and the native PCNA-K164 monoubiquitination.

### PCNA•Ub fusion proteins are associated with chromatin

PCNA may exist in two forms: soluble or chromatin-bound in the nucleus (50). As only chromatin-bound PCNA is likely to be ubiquitinated (Supplementary Figure S1), we first asked whether the PCNA•Ub fusion proteins could exist in the chromatin-bound form. As shown in Figure 2A, monoubiquitinated PCNA (PCNA<sup>Ub</sup>) was only detected after UV irradiation and in the chromatin-bound fraction (lane 7). Interestingly, induced expression of PCNA-K164R markedly reduced the PCNA<sup>Ub</sup> level (cf. lanes 7 and 8), indicating that this mutated PCNA can form DNA clamps and compete with endogenous PCNA. Similarly, Figure 2B and C reveal that PCNA•Ub fusions can also be associated with chromatin even in the absence of DNA damage (lane 6). By taking advantage of the fact that Ub-PCNA migrates differently from PCNA<sup>Ub</sup>, one can see that they appear to



**Figure 2.** Endogenous and ectopic expression of different forms of PCNA in soluble and chromatin-bound nuclear fractions. The stably transfected T-Rex 293 cell lines were induced with Dox for 2 days, treated with  $10\text{J/m}^2$  UV or left untreated and then subjected to fractionation after a 6 h incubation. Subcellular PCNA distributions were analyzed by western blot using an anti-PCNA antibody. MDC1 serves as a reference of the chromatin-bound fraction, whereas Tubulin serves as a reference for cytoplasmic fractions. The PCNA blots were exposed for different times to reveal monoubiquitinated PCNA. (A) PCNA-K164R cells, (B) Ub-PCNA cells and (C) PCNA-Ub cells.

compete with each other in the chromatin-bound fraction (Figure 2B, cf. lanes 6, 7 and 8). In contrast, PCNA-Ub and PCNA<sup>Ub</sup> migrate indistinguishably (Figure 2C). Interestingly, despite the fact that expression of ectopic PCNA•Ub increases total cellular levels of PCNA, the chromatin-bound PCNA of different forms appears to be constant (Figure 2B and C, lanes 5–8).

Compared with non-induced cells, expression of PCNA-K164R or PCNA•Ub fusions alone did not affect cell cycle progression (Supplementary Figure S2) or proliferation (Figure 3F–H) in the absence of DNA damage, indicating that PCNA•Ub fusion proteins do

not affect normal DNA replication. The aforementioned observations set a desired platform for the investigation of PCNA•Ub fusion functions.

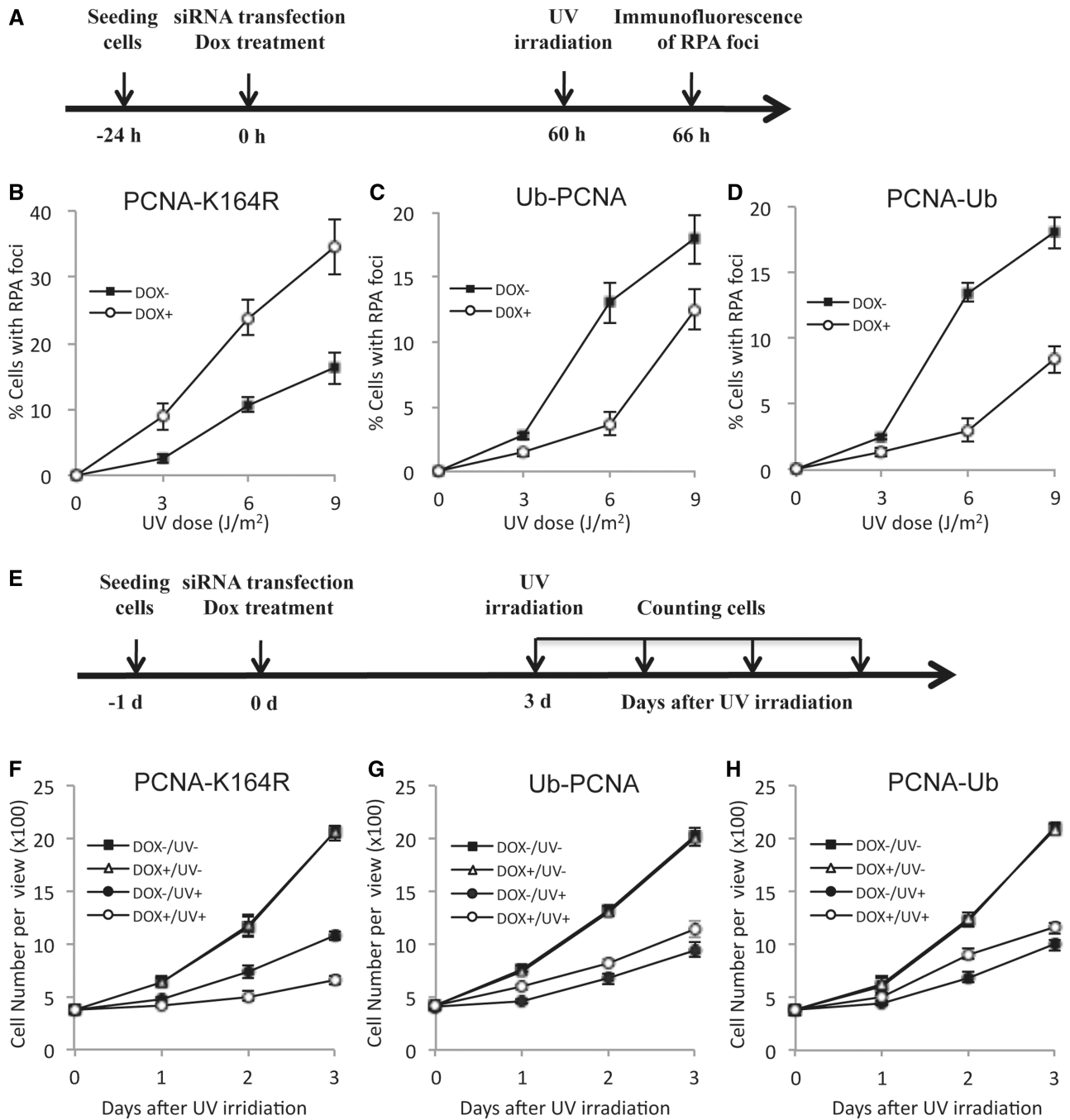
### Expression of PCNA-K164R sensitizes cells, whereas expression of PCNA•Ub fusions confers resistance to UV damage

It has been well established that in budding yeast monoubiquitination of PCNA at the K164 residue facilitates TLS (11), and similar effects have also been reported in mammalian cells (14,26). However, most assessments of TLS have been through indirect assays including nuclear focus formation and the cell survival assay. We attempted to measure TLS activity by monitoring single-strand DNA (ssDNA) in cultured human cells. It was reasoned that as RPA is a trimeric protein that specifically coats ssDNA and protects ssDNA gaps at stalled replication forks (51,52), the extent of RPA nuclear foci may be correlated with TLS activity. Preliminary experimental data as shown in Supplementary Figure S3 indicate that the percentage of cells with RPA foci (Supplementary Figure S3A) and cell survival (Supplementary Figure S3B) are in a linear relationship with UV dose. After treatment of T-Rex 293 cells with  $6\text{J/m}^2$  UV, the number of cells with RPA-positive foci plateaued between 2 and 8 h and gradually disappeared over 24 h (Supplementary Figure S3C). When this work was in progress, Livneh *et al.* (53) reported the use of RPA foci to monitor TLS activity, which validates this approach.

As expected, expression of PCNA-K164R significantly increased UV-induced RPA focus-positive cells, for example, from 10 to 24% with  $6\text{J/m}^2$  irradiation (Figure 3B). Similarly, expression of PCNA-K164R sensitized cells to UV damage, whereas in the absence of UV irradiation, expression of PCNA-K164R had no growth effect (Figure 3F). In sharp contrast, expression of Ub-PCNA (Figure 3C) or PCNA-Ub (Figure 3D) significantly reduced the percentage of cells with RPA foci. For instance, after  $6\text{J/m}^2$  UV irradiation, 13–14% of cells that did not express fusion proteins contain RPA foci, whereas when the same cells expressed the PCNA•Ub fusion proteins, RPA focus-positive cells were reduced to ~3%. If the expression of PCNA-K164R is used as a reference, its Ub fusions result in an 8-fold reduction in RPA focus-positive cells. Consequently, expression of Ub-PCNA (Figure 3G) or PCNA-Ub (Figure 3H) actually protected ‘wild-type’ cells from UV-induced growth inhibition, albeit to a small extent.

### Polη is dispensable for DNA-damage tolerance conferred by PCNA•Ub fusions

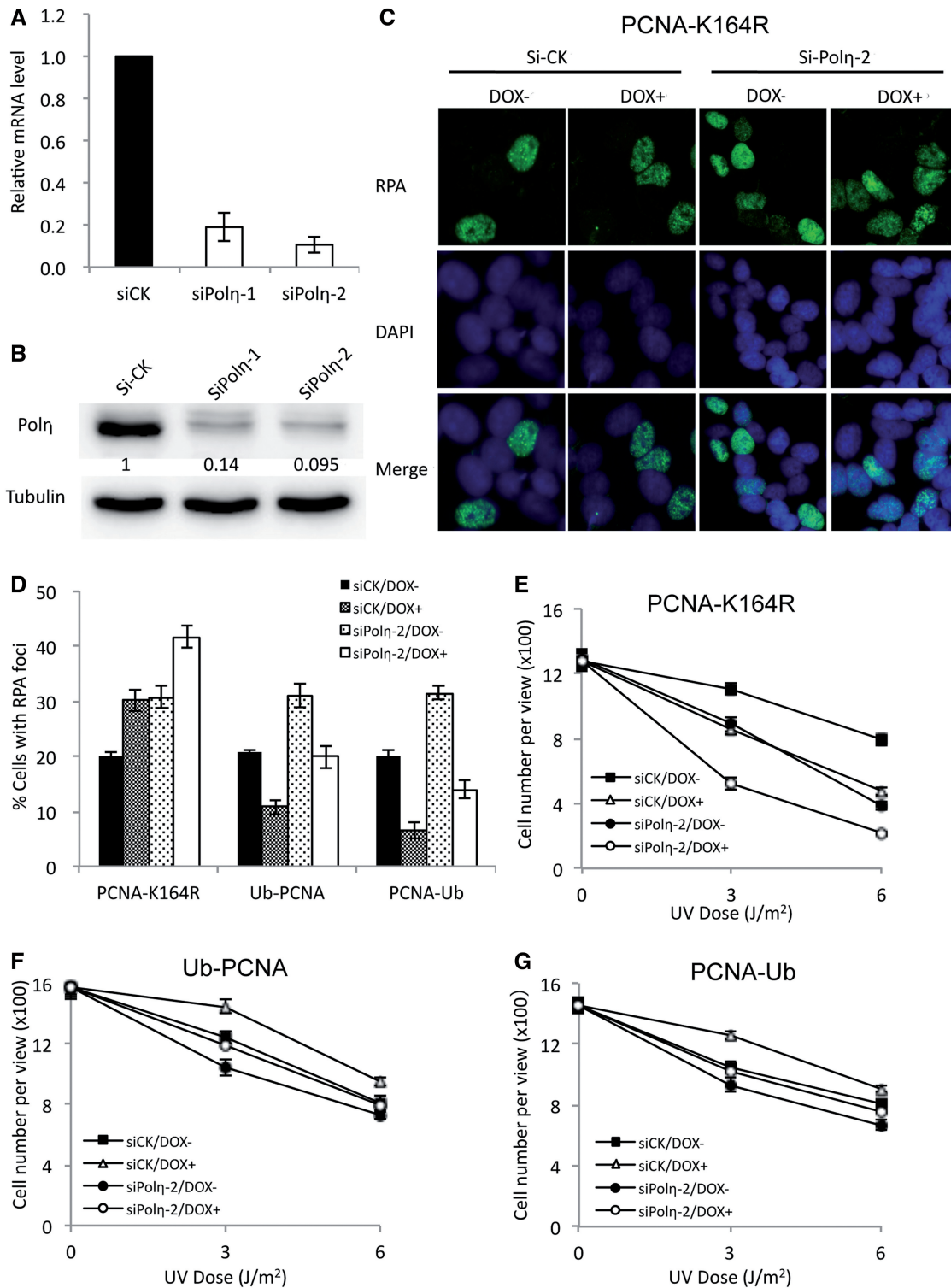
As monoubiquitinated PCNA is thought to mediate TLS through an enhanced physical association with TLS polymerases, an immediate prediction is that inactivation of cognate TLS polymerase(s) will reverse the tolerance effect conferred by the PCNA•Ub fusions. To test this hypothesis, we depleted cellular Polη by using two different siRNAs, both of which effectively reduced cellular Polη RNA (Figure 4A) and protein (Figure 4B) to 10–20% of



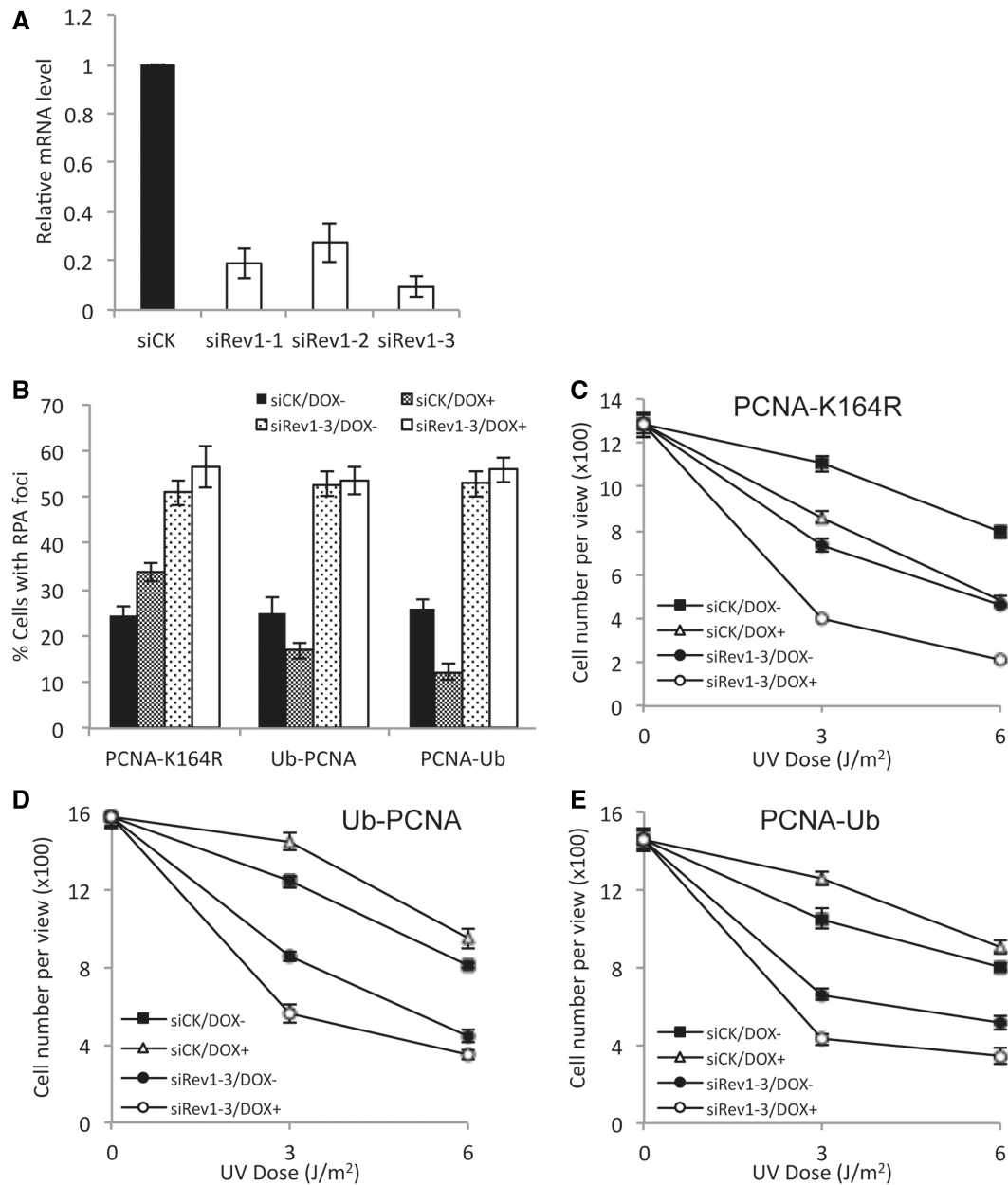
**Figure 3.** Phenotypic evaluation of *in vivo* PCNA•Ub fusions. (A) A schematic timeline for the RPA focus assay. (B–D) Percentage of RPA foci positive cells after UV irradiation. (E) A schematic timeline for the cell survival assay. (F–H) Relative sensitivity of T-Rex-293 transfectants to UV-induced growth inhibition. (B and F) The PCNA-K164R transfectants. (C and G) The Ub-PCNA transfectants. (D and H) The PCNA-Ub transfectants. The results are the average of at least three independent experiments with standard deviations.

normal levels. Depletion of Polη led to an enhanced UV sensitivity, regardless of strain background or assays used (Figure 4D–G). Quantitative analysis indicates that depletion of Polη causes an effect similar to that caused by PCNA-K164R expression, and the two treatments appear to be additive (Figure 4C–E). Depletion of Polη in Ub-PCNA and PCNA-Ub transfected cells also increased RPA focus-positive cells, regardless of whether the fusion proteins were produced (Figure 4D and

Supplementary Figure S4A). Quantitative analysis indicates that the tolerant effect conferred by PCNA•Ub fusions and the sensitive effect caused by Polη depletion are two independent events, and the net results are the simple sum of two separate treatments. The survival assay results also indicate that the two treatments appear to offset each other so that cells returned to the wild-type state (Figure 4F and G), which reinforces the conclusions drawn from the RPA nuclear focus assay.



**Figure 4.** Effects of Polη depletion on DNA-damage tolerance provided by PCNA•Ub fusions. (A and B) Efficacy of the two anti-Polη siRNAs assessed by qRT-PCR (A) and western blot (B) in T-Rex-293 cells. The relative Polη level is indicated underneath the Polη image. (C) Sample images of UV-induced RPA nuclear foci. (D) Percentage of cells with RPA foci after 8 J/m<sup>2</sup> UV irradiation, with or without Dox induction and siPolη treatment. The experimental timeline is as in Figure 3A. (E–G) Survival assays of T-Rex 293 stable transfectants in response to UV damage. Cells were transfected with siPolη and treated with Dox for 3 days before UV irradiation. Two days after UV irradiation, viable cells were counted as a measure of UV tolerance. (E) PCNA-K164R, (F) Ub-PCNA and (G) PCNA-Ub.



**Figure 5.** Effects of Rev1 depletion on DNA-damage tolerance provided by PCNA•Ub fusions. (A) Efficacy of three siRNAs by qRT-PCR in T-Rex 293 cells. (B) Percentage of cells with RPA foci after 8 J/m<sup>2</sup> UV irradiation, with or without Dox induction and siRev1 treatment. The experimental timeline is as in Figure 3A. (C–E) Cell survival assays. Cells were transfected with siRev1 and treated with Dox for 3 days before UV irradiation. Two days after UV irradiation, viable cells were counted as a measure of UV tolerance. (C) PCNA-K164R, (D) Ub-PCNA and (E) PCNA-Ub.

From the aforementioned analyses, we conclude that the DNA-damage tolerance conferred by PCNA•Ub fusions does not rely on functional Pol $\eta$ .

#### DNA-damage tolerance conferred by PCNA•Ub fusions is dependent on Rev1

Having ruled out the requirement for Pol $\eta$  in PCNA•Ub-mediated damage tolerance, we turned our attention to Rev1, another major player in TLS of UV-induced damage. The involvement of Rev1 in UV-induced TLS does not appear to be due to its polymerase activity

(24,25), although its catalytic activity is required for bypass of some other lesions (25,54). Instead, Rev1 interacts with PCNA and Ub as well as other TLS polymerases, and these interaction domains are all required for TLS, making it a likely candidate of the PCNA monoubiquitination signal.

The three siRev1s used in this study resulted in the depletion of the *REV1* transcript by ~70–90%, with siRev13 appearing to be the most effective (Figure 5A). Depletion of Rev1 alone by siRev13 in the absence of ectopic proteins resulted in a massive increase in RPA focus-positive cells; expression of ectopic proteins, whether it



was PCNA-K164R, Ub-PCNA or PCNA-Ub, did not further impact the RPA focus formation (Figure 5B). Hence, in the absence of functional Rev1, cellular UV sensitivity conferred by PCNA-K164R or tolerance conferred by PCNA•Ub is irrelevant (Figure 5B and Supplementary Figure S4B and C). These results are consistent with a prediction of PCNA monoubiquitination being completely dependent on Rev1 for TLS activity. Experimental data from the cell survival assay also indicate that inactivation of Rev1 makes cells equally sensitive to UV-induced DNA damage, regardless of whether cells express PCNA-K164R (Figure 5C) or PCNA•Ub (Figure 5D and E), which is in sharp contrast to the inactivation of Pol $\eta$  (Figure 4). Furthermore, it appears that when Rev1 is inactivated, expression of PCNA•Ub becomes detrimental to cells (Figure 5D and E, and Supplementary Figure S5), making them similar in sensitivity to those expressing PCNA-K164R (Figure 5C).

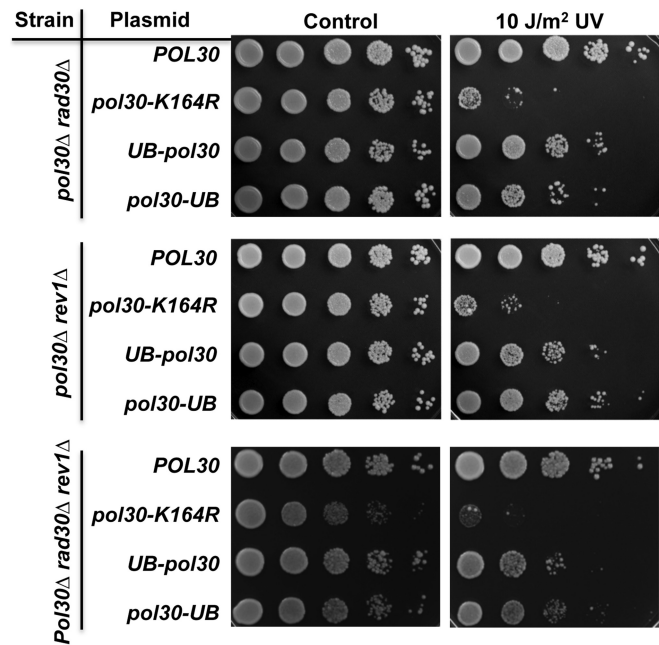
It was noticed that inactivation of Rev1 by siRev1-1 or siRev1-2, which were less effective than siRev1-3 (Figure 5A), also resulted in fewer cells with RPA-positive foci; in this case, expression of PCNA-K164R further increased RPA focus-positive cells to a level comparable with that of siRev1-3 (Figure 5B and Supplementary Figure S4B and C).

#### Neither Rev1 nor Pol $\eta$ is required for DNA-damage tolerance conferred by PCNA•Ub fusions in yeast cells

The aforementioned observation that DNA-damage tolerance conferred by PCNA•Ub fusions is independent of Pol $\eta$  but dependent on Rev1 is surprising, as we (38) and others (39) have previously reported that such tolerance in budding and fission yeasts is independent of Rev3, a polymerase thought to be required for all aspects of TLS (55). To directly compare between yeast and human cells, we examined UV damage response of yeast cells in various genetic backgrounds. As shown in Figure 6, expression of PCNA•Ub fusions made host cells much more tolerant to UV irradiation, which is not compromised by the deletion of either *RAD30* (Pol $\eta$ ) or *REV1* gene. Furthermore, simultaneous deletion of both *REV1* and *RAD30* genes still does not affect UV damage tolerance conferred by the expression of PCNA•Ub fusions.

#### Rev1 but not Pol $\eta$ depletion is epistatic to the lack of PCNA ubiquitination

The aforementioned conclusions were primarily obtained by expressing PCNA•Ub fusions. To address whether the function of native PCNA monoubiquitination is also dependent on Rev1 but independent of Pol $\eta$ , we used *RAD18*-null HCT116 cells, in which both copies of *RAD18* were deleted (43,56). Compared with parental cells, the *RAD18* expression was undetectable in *RAD18*<sup>-/-</sup> cells (Figure 7A), which resulted in a complete abolishment of native PCNA monoubiquitination in response to UV irradiation (Figure 7B). Depletion of Rev1 by three siRNAs (Figure 7C) and Pol $\eta$  by two siRNAs (Figure 7D) was effective in HCT116 cells and comparable with that in T-Rex 293 cells (Figures 4A and 5A). Depletion of Rev1 resulted in

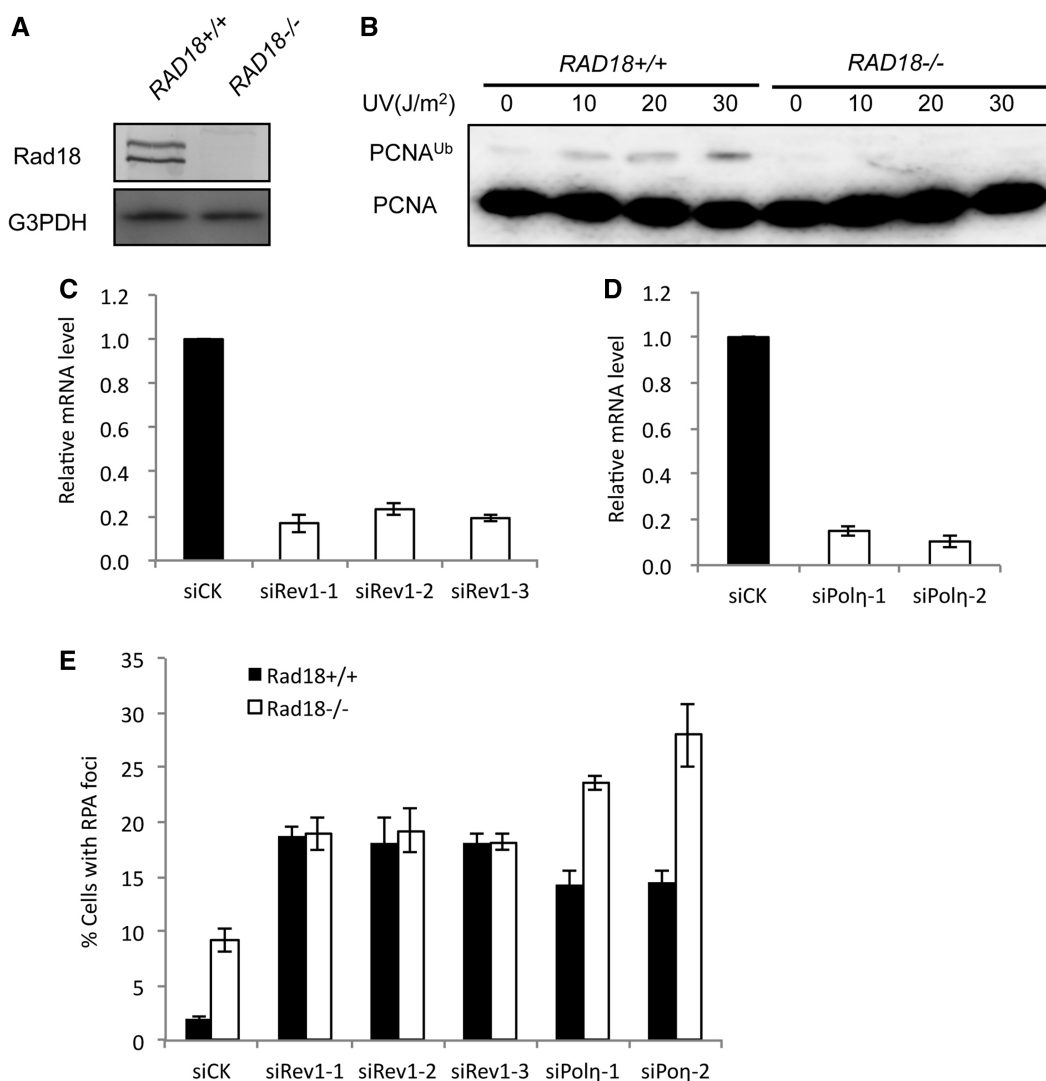


**Figure 6.** Expression of PCNA•Ub fusions in *S. cerevisiae* confers DNA damage tolerance that is independent of both Rad30 and Rev1. Yeast deletion strains carrying single-copy plasmids with the native *POL30* (PCNA) promoter were spotted on plates for a serial dilution assay. Plates were treated with different doses of UV irradiation in a UV cross-linker, and then incubated in the dark at 30°C for 48 h before photography. Only one UV dose is shown. Cells carrying PCNA•Ub fusion constructs (bottom two rows) display UV tolerance compared with cells carrying a *pol30-K164R* plasmid (second row). If this resistance were dependent on functional *RAD30* (Pol $\eta$ ) and/or *REV1*, it would be diminished in the corresponding mutant strains.

an increase in cells with RPA foci, and the effects were indistinguishable between wild-type and *RAD18*<sup>-/-</sup> cells (Figure 7C). In sharp contrast, in *RAD18*<sup>-/-</sup> cells, depletion of Pol $\eta$  further increased RPA focus-positive cells compared with *RAD18*<sup>-/-</sup> cells alone or Pol $\eta$  depletion in the wild-type cells (Figure 7E). Similar results were also observed in NF1604 and its *RAD18*-suppressed cell line AS26 (Supplementary Figure S5). These observations collectively support a notion that in mammalian cells, with respect to RPA nuclear focus formation, depletion of Rad18 is epistatic to the Rev1 depletion while additive to the Pol $\eta$  depletion.

#### Rev1 and Pol $\eta$ do not act in the same pathway during TLS

As the resistance to UV-induced DNA damage provided by Ub and PCNA fusions is dependent on Rev1, but not Pol $\eta$ , an immediate implication is that these two proteins do not function in the same TLS pathway. Surprisingly, despite extensive characterization of TLS polymerases in the past decade, the genetic interaction between Rev1 and Pol $\eta$  has not been well established. It was reported that the recruitment of Rev1 to UV-induced damage sites is dependent on Pol $\eta$  (57), and the physical interaction between these two proteins (21,23) appears to support this notion. On the other hand, the initial observation



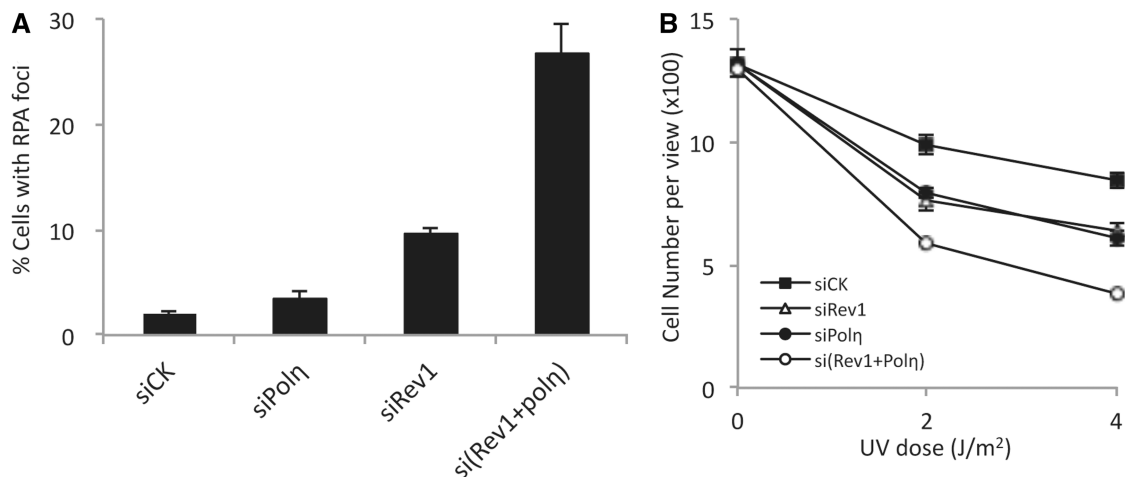
**Figure 7.** Genetic interactions between Rev1 or Pol $\eta$  deletion and *RAD18* deletion in HCT116 cells by western blot analysis. **(B)** UV-induced PCNA monoubiquitination in wild-type and *RAD18*<sup>-/-</sup> cells. Experimental conditions were as described in 'Materials and Methods' section and Supplementary Figure S1. **(C)** Efficacy of the three siRNAs against Rev1 (siRev1) as assessed by qRT-PCR in HCT116 cells. **(D)** Efficacy of the two siRNAs against Pol $\eta$  (siPol $\eta$ ) as assessed by qRT-PCR in HCT116 cells. **(E)** Percentage of cells with RPA foci after 2J/m<sup>2</sup> UV irradiation in HCT116 and *RAD18*<sup>-/-</sup> cells treated with either siPol $\eta$  or siRev1. The results are the average of at least three independent experiments with standard deviations.

that Rev1 and Pol $\eta$  are independently recruited to the damage site (58) also gained support from some recent studies (59,60). We argued that if our PCNA•Ub fusions functionally replace endogenous PCNA monoubiquitination, Rev1 and Pol $\eta$  would be expected to function in different TLS pathways in response to UV irradiation. To test this hypothesis, T-Rex 293 cells were treated with either siPol $\eta$  or siRev1, or both, followed with UV irradiation and analysis of the RPA nuclear focus formation and cell survival. With 2J/m<sup>2</sup> UV exposure, depletion of Rev1 appears to cause more cells with RPA nuclear foci than depletion of Pol $\eta$  (Figure 8A), which is consistent with previous observations (Figures 4D and 5B), suggesting that Rev1 plays a more predominant role than Pol $\eta$  in UV tolerance. More importantly, simultaneous depletion of both proteins had a strong additive effect (Figure 8A), suggesting that they are

involved in separate TLS pathways. Interestingly, at various UV doses, siPol $\eta$  and siRev1 displayed a similar level of cell growth inhibition, whereas the combined effects were clearly additive (Figure 8B). Similar additive effects between *rad30* $\Delta$  and *rev1* $\Delta$  have also been reported in budding yeast (61), which at least partially rules out the possibility that the additive effect was solely due to a lack of clear genetic approach that completely inactivates each protein.

## DISCUSSION

In this report, we used a carefully designed system to express PCNA•Ub fusion proteins and found that they were loaded onto DNA and able to protect host cells from UV-induced DNA damage with characteristic TLS activities. We further demonstrated that such a protective



**Figure 8.** siRev1 and siPol $\eta$  are additive in response to UV damage. (A) The RPA nuclear focus assay. Following siRNA treatment for 3 days, T-Rex 293 cells were exposed to 2J/m<sup>2</sup> UV, and the RPA nuclear focus formation was measured. (B) Cell survival assay. Following siRNA treatment for 3 days, cells were exposed to the predetermined UV dose as indicated, and viable cells were counted. Results shown in (A) and (B) are the average of at least three experiments with standard deviations.

role does not rely on Pol $\eta$ , but absolutely requires Rev1. As this finding is rather surprising, one concern is whether the artificial PCNA•Ub fusions functionally imitate native PCNA monoubiquitination. The following observations argue in favor of their similarities. First, our previous structural modeling (38) indicates that N- and C-terminal PCNA•Ub fusions closely resemble two different aspects of monoubiquitinated PCNA; yet, both fusions provide similar levels of protection, suggesting that the critical element of PCNA ubiquitination is to bring the two molecules in close contact, which can be achieved by the artificial fusion. Second, artificial PCNA•Ub fusions partially rescue the *rad18* mutants in yeast cells (38–40), suggesting that they fulfill cellular function(s) owing to lack of endogenous PCNA ubiquitination. Third, like monoubiquitinated PCNA, PCNA•Ub fusion proteins also display an enhanced affinity for Pol $\eta$  and Rev1 in a Ub-UBM/UBZ interaction-dependent manner, which is a prerequisite for TLS signaling (14,19,27,62). Fourth, we demonstrated that PCNA•Ub fusion proteins bind to the chromatin, which is also required for monoubiquitinated PCNA to function in TLS (63). Fifth, to validate that what we observed with the PCNA•Ub fusion proteins is also true for PCNA monoubiquitination at K164, we found that *RAD18* deletion or depletion is epistatic to the Rev1 depletion, whereas it is additive to the Pol $\eta$  depletion under the same experimental conditions. Finally, our observation that Rev1 depletion and Pol $\eta$  depletion are additive is consistent with previous reports (58–60), supporting a notion that these two proteins could function in separate TLS pathways in response to UV-induced DNA damage. The aforementioned observations collectively imply that PCNA•Ub fusions made in this study are able to mimic PCNA-K164 monoubiquitination and hence encourage us to use this system to explore roles of PCNA monoubiquitination in TLS signaling.

DNA-damage tolerance and particularly TLS appears to be evolutionarily conserved from prokaryotes to

eukaryotes (64); however, their regulatory mechanisms are apparently different. In eukaryotes, DNA-damage tolerance is achieved through sequential ubiquitination of the DNA clamp PCNA (11), within which monoubiquitination leads to TLS perhaps through PCNA's enhanced affinity for Y-family polymerases (14). Bacterial cells do not have the ubiquitination system although the  $\beta$  clamp is also thought to be involved in the polymerase switch (65). Although numerous previous studies have well established that both PCNA monoubiquitination and the Ub- and PCNA-binding motifs of Y-family polymerases play critical roles in TLS signaling, molecular details of this signal transduction are still largely obscure. By using an inducible expression of PCNA•Ub fusion proteins in this study, we were able to show that the UV damage tolerance conferred by these fusions is completely dependent on functional Rev1, providing direct evidence that recruitment of Rev1 is essential for monoubiquitinated PCNA. This finding is significant because in the budding yeast, removal of Pol $\eta$  (38,39) or Rev1 does not reverse DNA-damage tolerance conferred by the expression of PCNA•Ub fusions. Another surprising finding in this study is that depletion of Pol $\eta$  did not impact the function of PCNA•Ub fusions, and that the two treatments appear to independently affect UV-induced DNA damage. There are at least three possibilities to explain why Pol $\eta$  is dispensable for TLS mediated by PCNA monoubiquitination. One is that in the absence of Pol $\eta$ , another Y-family polymerase may step in, albeit with an increased chance of making mutations. A likely candidate polymerase is Pol $\zeta$ , which colocalizes with Pol $\eta$  in response to UV irradiation (66) and, along with Pol $\eta$ , has been shown to back up Pol $\eta$  for TLS of UV-induced lesions (67). However, budding yeast does not contain Pol $\zeta$  and Pol $\zeta$  homologs; yet, Pol $\zeta$  is still dispensable for the UV-damage tolerance conferred by PCNA•Ub fusions. Alternatively, Rev1 may be recruited to the UV-induced damage site independently of Pol $\eta$

(58,59) and use its polymerase domain to bypass UV-induced lesions, perhaps with a poor efficiency. Unfortunately, this model does not explain why the tolerance conferred by PCNA•Ub fusions is independent of Rev1 in yeast cells. Finally, it was recently shown that the budding yeast Rev1 is able to facilitate the replacement of the Pol $\eta$  catalytic subunit with the Pol $\eta$  catalytic subunit Rev3 (68–71), which may complete the lesion bypass in the absence of Pol $\eta$ . To date, the aforementioned Rev1-mediated Pol $\eta$ -Pol $\eta$  switch has not been demonstrated in mammalian cells. On the other hand, the additive effect of Pol $\eta$  and Rev1 depletions in response to UV-induced damage indicates that Pol $\eta$  must play a role independent of Rev1 and PCNA monoubiquitination, which is consistent with a recent report (72), although how this is achieved remains unclear.

In summary, we have created and validated a controlled expression and artificial fusion system to investigate roles of PCNA monoubiquitination in mammalian cells. Our initial observations answered some questions that have not been previously addressed. This study focused on UV-induced DNA damage as UV irradiation is the most investigated damaging agent in TLS studies. As PCNA monoubiquitination can be induced by various other DNA-damaging agents including methyl methanesulfonate, mitomycin C, hydroxyurea and bulky adduct-forming genotoxins like benzo[*a*]pyrene dihydrodiol epoxide (73,74), the system developed in this study can be readily applied to these studies. Furthermore, one can also address why PCNA monoubiquitination relies on Rev1 for DNA-damage tolerance, despite the fact that it enhances the affinity for both Rev1 and Pol $\eta$ .

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–5.

## ACKNOWLEDGEMENTS

The authors thank Drs C. Guo, W.G. McGregor, P. Burgers, S. Nakajima and A. Yasui for invaluable reagents, and other laboratory members for helpful discussion.

## FUNDING

Chinese National 973 Project [2013CB911003]; Capital Normal University 211 Special Fund [10531182313]; Canadian Institutes of Health Research operating [MOP-93612]. Funding for open access charge: Capital Normal University 211 Special Fund [10531182313].

*Conflict of interest statement.* None declared.

## REFERENCES

- Bravo,R., Fey,S.J., Bellatin,J., Larsen,P.M. and Celis,J.E. (1982) Identification of a nuclear polypeptide ("cyclin") whose relative proportion is sensitive to changes in the rate of cell proliferation and to transformation. *Prog. Clin. Biol. Res.*, **85(Pt A)**, 235–248.
- Bravo,R. and Celis,J.E. (1980) A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. *J. Cell Biol.*, **84**, 795–802.
- Mathews,M.B., Bernstein,R.M., Franza,B.R. Jr and Garrels,J.I. (1984) Identity of the proliferating cell nuclear antigen and cyclin. *Nature*, **309**, 374–376.
- Krishna,T.S., Kong,X.P., Gary,S., Burgers,P.M. and Kuriyan,J. (1994) Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. *Cell*, **79**, 1233–1243.
- Waga,S. and Stillman,B. (1998) The DNA replication fork in eukaryotic cells. *Annu. Rev. Biochem.*, **67**, 721–751.
- Moldovan,G.L., Pfander,B. and Jentsch,S. (2007) PCNA, the maestro of the replication fork. *Cell*, **129**, 665–679.
- Andersen,P.L., Xu,F. and Xiao,W. (2008) Eukaryotic DNA damage tolerance and translesion synthesis through covalent modifications of PCNA. *Cell Res.*, **18**, 162–173.
- Lehmann,A.R., Niimi,A., Ogi,T., Brown,S., Sabbioneda,S., Wing,J.F., Kannouche,P.L. and Green,C.M. (2007) Translesion synthesis: Y-family polymerases and the polymerase switch. *DNA Rep.*, **6**, 891–899.
- Friedberg,E.C. and Gerlach,V.L. (1999) Novel DNA polymerases offer clues to the molecular basis of mutagenesis. *Cell*, **98**, 413–416.
- Zhang,W., Qin,Z., Zhang,X. and Xiao,W. (2011) Roles of sequential ubiquitination of PCNA in DNA-damage tolerance. *FEBS Lett.*, **585**, 2786–2794.
- Hoegge,C., Pfander,B., Moldovan,G.L., Pyrowolakis,G. and Jentsch,S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*, **419**, 135–141.
- Pickart,C.M. and Fushman,D. (2004) Polyubiquitin chains: polymeric protein signals. *Curr. Opin. Chem. Biol.*, **8**, 610–616.
- Hochstrasser,M. (1996) Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.*, **30**, 405–439.
- Bienko,M., Green,C.M., Crosetto,N., Rudolf,F., Zapart,G., Coull,B., Kannouche,P., Wider,G., Peter,M., Lehmann,A.R. *et al.* (2005) Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. *Science*, **310**, 1821–1824.
- Prakash,S., Johnson,R.E. and Prakash,L. (2005) Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu. Rev. Biochem.*, **74**, 317–353.
- Ohmori,H., Friedberg,E.C., Fuchs,R.P., Goodman,M.F., Hanaoka,F., Hinkle,D., Kunkel,T.A., Lawrence,C.W., Livneh,Z., Nohmi,T. *et al.* (2001) The Y-family of DNA polymerases. *Mol. Cell*, **8**, 7–8.
- Guo,C., Kosarek-Stancel,J.N., Tang,T.S. and Friedberg,E.C. (2009) Y-family DNA polymerases in mammalian cells. *Cell. Mol. Life Sci.*, **66**, 2363–2381.
- Johnson,R.E., Washington,M.T., Prakash,S. and Prakash,L. (2000) Fidelity of human DNA polymerase  $\epsilon$ . *J. Biol. Chem.*, **275**, 7447–7450.
- Guo,C., Sonoda,E., Tang,T.S., Parker,J.L., Bielen,A.B., Takeda,S., Ulrich,H.D. and Friedberg,E.C. (2006) REV1 protein interacts with PCNA: significance of the REV1 BRCT domain *in vitro* and *in vivo*. *Mol. Cell*, **23**, 265–271.
- Sharma,N.M., Kochenova,O.V. and Shcherbakova,P.V. (2011) The non-canonical protein binding site at the monomer-monomer interface of yeast proliferating cell nuclear antigen (PCNA) regulates the Rev1-PCNA interaction and Polzeta/Rev1-dependent translesion DNA synthesis. *J. Biol. Chem.*, **286**, 33557–33566.
- Guo,C., Fischhaber,P.L., Luk-Paszyc,M.J., Masuda,Y., Zhou,J., Kamiya,K., Kisker,C. and Friedberg,E.C. (2003) Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis. *EMBO J.*, **22**, 6621–6630.
- Murakumo,Y., Ogura,Y., Ishii,H., Numata,S., Ichihara,M., Croce,C.M., Fishel,R. and Takahashi,M. (2001) Interactions in the error-prone postreplication repair proteins hREV1, hREV3, and hREV7. *J. Biol. Chem.*, **276**, 35644–35651.
- Ohashi,E., Murakumo,Y., Kanjo,N., Akagi,J., Masutani,C., Hanaoka,F. and Ohmori,H. (2004) Interaction of hREV1 with three human Y-family DNA polymerases. *Genes Cells*, **9**, 523–531.
- Otsuka,C., Kunitomi,N., Iwai,S., Loakes,D. and Negishi,K. (2005) Roles of the polymerase and BRCT domains of Rev1

- protein in translesion DNA synthesis in yeast *in vivo*. *Mutat. Res.*, **578**, 79–87.
25. Wiltrott, M.E. and Walker, G.C. (2011) The DNA polymerase activity of *Saccharomyces cerevisiae* Rev1 is biologically significant. *Genetics*, **187**, 21–35.
  26. Kannouche, P.L., Wing, J. and Lehmann, A.R. (2004) Interaction of human DNA polymerase  $\eta$  with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol. Cell*, **14**, 491–500.
  27. Guo, C., Tang, T.S., Bienko, M., Parker, J.L., Bielen, A.B., Sonoda, E., Takeda, S., Ulrich, H.D., Dikic, I. and Friedberg, E.C. (2006) Ubiquitin-binding motifs in REV1 protein are required for its role in the tolerance of DNA damage. *Mol. Cell Biol.*, **26**, 8892–8900.
  28. Acharya, N., Yoon, J.H., Gali, H., Unk, I., Haracska, L., Johnson, R.E., Hurwitz, J., Prakash, L. and Prakash, S. (2008) Roles of PCNA-binding and ubiquitin-binding domains in human DNA polymerase  $\eta$  in translesion DNA synthesis. *Proc. Natl Acad. Sci. USA*, **105**, 17724–17729.
  29. Acharya, N., Brahma, A., Haracska, L., Prakash, L. and Prakash, S. (2007) Mutations in the ubiquitin binding UBZ Motif of DNA polymerase  $\eta$  do not impair its function in translesion synthesis during replication. *Mol. Cell Biol.*, **27**, 7266–7272.
  30. Wood, A., Garg, P. and Burgers, P.M. (2007) A ubiquitin-binding motif in the translesion DNA polymerase Rev1 mediates its essential functional interaction with ubiquitinated proliferating cell nuclear antigen in response to DNA damage. *J. Biol. Chem.*, **282**, 20256–20263.
  31. Haracska, L., Kondratik, C.M., Unk, I., Prakash, S. and Prakash, L. (2001) Interaction with PCNA is essential for yeast DNA polymerase  $\eta$  function. *Mol. Cell*, **8**, 407–415.
  32. Haracska, L., Johnson, R.E., Unk, I., Phillips, B., Hurwitz, J., Prakash, L. and Prakash, S. (2001) Physical and functional interactions of human DNA polymerase  $\eta$  with PCNA. *Mol. Cell Biol.*, **21**, 7199–7206.
  33. Haracska, L., Johnson, R.E., Unk, I., Phillips, B.B., Hurwitz, J., Prakash, L. and Prakash, S. (2001) Targeting of human DNA polymerase  $\iota$  to the replication machinery via interaction with PCNA. *Proc. Natl Acad. Sci. USA*, **98**, 14256–14261.
  34. Garg, P., Stith, C.M., Majka, J. and Burgers, P.M. (2005) Proliferating cell nuclear antigen promotes translesion synthesis by DNA polymerase  $\zeta$ . *J. Biol. Chem.*, **280**, 23446–23450.
  35. Haracska, L., Prakash, L. and Prakash, S. (2002) Role of human DNA polymerase  $\kappa$  as an extender in translesion synthesis. *Proc. Natl Acad. Sci. USA*, **99**, 16000–16005.
  36. Haracska, L., Unk, I., Prakash, L. and Prakash, S. (2006) Ubiquitylation of yeast proliferating cell nuclear antigen and its implications for translesion DNA synthesis. *Proc. Natl Acad. Sci. USA*, **103**, 6477–6482.
  37. Huang, T.T., Nijman, S.M., Mirchandani, K.D., Galardy, P.J., Cohn, M.A., Haas, W., Gygi, S.P., Ploegh, H.L., Bernards, R. and D'Andrea, A.D. (2006) Regulation of monoubiquitinated PCNA by DUB autocleavage. *Nat. Cell Biol.*, **8**, 339–347.
  38. Pastushok, L., Hanna, M. and Xiao, W. (2010) Constitutive fusion of ubiquitin to PCNA provides DNA damage tolerance independent of translesion polymerase activities. *Nucleic Acids Res.*, **38**, 5047–5058.
  39. Ramasubramanian, S., Coulon, S., Fuchs, R.P., Lehmann, A.R. and Green, C.M. (2010) Ubiquitin-PCNA fusion as a mimic for monoubiquitinated PCNA in *Schizosaccharomyces pombe*. *DNA Rep.*, **9**, 777–784.
  40. Parker, J.L., Bielen, A.B., Dikic, I. and Ulrich, H.D. (2007) Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase  $\eta$  in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **35**, 881–889.
  41. Picard, V., Ersdal-Badju, E., Lu, A. and Bock, S.C. (1994) A rapid and efficient one-tube PCR-based mutagenesis technique using Pfu DNA polymerase. *Nucleic Acids Res.*, **22**, 2587–2591.
  42. Guo, C., Sonoda, E., Tang, T.S., Parker, J.L., Bielen, A.B., Takeda, S., Ulrich, H.D. and Friedberg, E.C. (2006) REV1 protein interacts with PCNA: significance of the REV1 BRCT domain *in vitro* and *in vivo*. *Mol. Cell*, **23**, 265–271.
  43. Shiomi, N., Mori, M., Tsuji, H., Imai, T., Inoue, H., Tateishi, S., Yamaizumi, M. and Shiomi, T. (2007) Human RAD18 is involved in S phase-specific single-strand break repair without PCNA monoubiquitination. *Nucleic Acids Res.*, **35**, e9.
  44. Watson, N.B., Nelson, E., Digman, M., Thornburg, J.A., Alphenaar, B.W. and McGregor, W.G. (2008) RAD18 and associated proteins are immobilized in nuclear foci in human cells entering S-phase with ultraviolet light-induced damage. *Mutat. Res.*, **648**, 23–31.
  45. Tateishi, S., Sakuraba, Y., Masuyama, S., Inoue, H. and Yamaizumi, M. (2000) Dysfunction of human Rad18 results in defective postreplication repair and hypersensitivity to multiple mutagens. *Proc. Natl Acad. Sci. USA*, **97**, 7927–7932.
  46. Rothstein, R.J. (1983) One-step gene disruption in yeast. *Methods Enzy.*, **101**, 202–211.
  47. Ayyagari, R., Impellizzeri, K.J., Yoder, B.L., Gary, S.L. and Burgers, P.M. (1995) A mutational analysis of the yeast proliferating cell nuclear antigen indicates distinct roles in DNA replication and DNA repair. *Mol. Cell Biol.*, **15**, 4420–4429.
  48. Barbour, L., Ball, L.G., Zhang, K. and Xiao, W. (2006) DNA damage checkpoints are involved in postreplication repair. *Genetics*, **174**, 1789–1800.
  49. Bomar, M.G., Pai, M.T., Tzeng, S.R., Li, S.S. and Zhou, P. (2007) Structure of the ubiquitin-binding zinc finger domain of human DNA Y-polymerase  $\eta$ . *EMBO Rep.*, **8**, 247–251.
  50. Waseem, N.H. and Lane, D.P. (1990) Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J. Cell Sci.*, **96**, 121–129.
  51. Lehmann, A.R. and Fuchs, R.P. (2006) Gaps and forks in DNA replication: rediscovering old models. *DNA Rep.*, **5**, 1495–1498.
  52. Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G.C., Lukas, J. and Jackson, S.P. (2006) ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat. Cell Biol.*, **8**, 37–45.
  53. Diamant, N., Hendel, A., Vered, I., Carell, T., Reissner, T., de Wind, N., Geaciov, N. and Livneh, Z. (2012) DNA damage bypass operates in the S and G2 phases of the cell cycle and exhibits differential mutagenicity. *Nucleic Acids Res.*, **40**, 170–180.
  54. Kim, N., Mudrak, S.V. and Jinks-Robertson, S. (2011) The dCMP transferase activity of yeast Rev1 is biologically relevant during the bypass of endogenously generated AP sites. *DNA Rep.*, **10**, 1262–1271.
  55. Lawrence, C.W. (2004) Cellular functions of DNA polymerase  $\zeta$  and Rev1 protein. *Adv. Protein Chem.*, **69**, 167–203.
  56. Nakajima, S., Lan, L., Kanno, S., Usami, N., Kobayashi, K., Mori, M., Shiomi, T. and Yasui, A. (2006) Replication-dependent and -independent responses of RAD18 to DNA damage in human cells. *J. Biol. Chem.*, **281**, 34687–34695.
  57. Akagi, J.I., Masutani, C., Kataoka, Y., Kan, T., Ohashi, E., Mori, T., Ohmori, H. and Hanaoka, F. (2009) Interaction with DNA polymerase  $\eta$  is required for nuclear accumulation of REV1 and suppression of spontaneous mutations in human cells. *DNA Rep.*, **8**, 585–599.
  58. Tissier, A., Kannouche, P., Reck, M.P., Lehmann, A.R., Fuchs, R.P. and Cordonnier, A. (2004) Co-localization in replication foci and interaction of human Y-family members, DNA polymerase  $\rho$   $\eta$  and REV1 protein. *DNA Rep.*, **3**, 1503–1514.
  59. Andersen, P.L., Xu, F., Ziola, B., McGregor, W.G. and Xiao, W. (2011) Sequential assembly of translesion DNA polymerases at UV-induced DNA damage sites. *Mol. Biol. Cell*, **22**, 2373–2383.
  60. Hashimoto, K., Cho, Y., Yang, I.Y., Akagi, J., Ohashi, E., Tateishi, S., de Wind, N., Hanaoka, F., Ohmori, H. and Moriya, M. (2012) The vital role of polymerase  $\zeta$  and REV1 in mutagenic, but not correct, DNA synthesis across benzo[a]pyrene-dG and recruitment of polymerase  $\zeta$  by REV1 to replication-stalled site. *J. Biol. Chem.*, **287**, 9613–9622.
  61. McDonald, J.P., Levine, A.S. and Woodgate, R. (1997) The *Saccharomyces cerevisiae* RAD30 gene, a homologue of *Escherichia coli* dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism. *Genetics*, **147**, 1557–1568.
  62. Watanabe, K., Tateishi, S., Kawasuji, M., Tsurimoto, T., Inoue, H. and Yamaizumi, M. (2004) Rad18 guides pol  $\eta$  to replication stalling sites through physical interaction and PCNA monoubiquitination. *EMBO J.*, **23**, 3886–3896.

63. Kannouche,P., Broughton,B.C., Volker,M., Hanaoka,F., Mullenders,L.H. and Lehmann,A.R. (2001) Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells. *Genes Dev.*, **15**, 158–172.
64. Waters,L.S., Minesinger,B.K., Wiltout,M.E., D'Souza,S., Woodruff,R.V. and Walker,G.C. (2009) Eukaryotic translesion polymerases and their roles and regulation in DNA damage tolerance. *Microbiol. Mol. Biol. Rev.*, **73**, 134–154.
65. Indiani,C., McInerney,P., Georgescu,R., Goodman,M.F. and O'Donnell,M. (2005) A sliding-clamp toolbelt binds high- and low-fidelity DNA polymerases simultaneously. *Mol. Cell*, **19**, 805–815.
66. Kannouche,P., Fernandez de Henestrosa,A.R., Coull,B., Vidal,A.E., Gray,C., Zicha,D., Woodgate,R. and Lehmann,A.R. (2002) Localization of DNA polymerases eta and iota to the replication machinery is tightly co-ordinated in human cells. *EMBO J.*, **21**, 6246–6256.
67. Ziv,O., Geacintov,N., Nakajima,S., Yasui,A. and Livneh,Z. (2009) DNA polymerase zeta cooperates with polymerases kappa and iota in translesion DNA synthesis across pyrimidine photodimers in cells from XPV patients. *Proc. Natl Acad. Sci. USA*, **106**, 11552–11557.
68. Acharya,N., Johnson,R.E., Pages,V., Prakash,L. and Prakash,S. (2009) Yeast Rev1 protein promotes complex formation of DNA polymerase zeta with Pol32 subunit of DNA polymerase delta. *Proc. Natl Acad. Sci. USA*, **106**, 9631–9636.
69. Baranovskiy,A.G., Lada,A.G., Siebler,H.M., Zhang,Y., Pavlov,Y.I. and Tahirov,T.H. (2012) DNA polymerase delta and zeta switch by sharing accessory subunits of DNA polymerase delta. *J. Biol. Chem.*, **287**, 17281–17287.
70. Johnson,R.E., Prakash,L. and Prakash,S. (2012) Pol31 and Pol32 subunits of yeast DNA polymerase delta are also essential subunits of DNA polymerase zeta. *Proc. Natl Acad. Sci. USA*, **109**, 12455–12460.
71. Makarova,A.V., Stodola,J.L. and Burgers,P.M. (2012) A four-subunit DNA polymerase zeta complex containing Pol delta accessory subunits is essential for PCNA-mediated mutagenesis. *Nucleic Acids Res.*, **40**, 11618–11626.
72. Hendel,A., Krijger,P.H., Diamant,N., Goren,Z., Langerak,P., Kim,J., Reissner,T., Lee,K.Y., Geacintov,N.E., Carell,T. *et al.* (2011) PCNA ubiquitination is important, but not essential for translesion DNA synthesis in mammalian cells. *PLoS Genet.*, **7**, e1002262.
73. Bi,X., Barkley,L.R., Slater,D.M., Tateishi,S., Yamaizumi,M., Ohmori,H. and Vaziri,C. (2006) Rad18 regulates DNA polymerase kappa and is required for recovery from S-phase checkpoint-mediated arrest. *Mol. Cell Biol.*, **26**, 3527–3540.
74. Niimi,A., Brown,S., Sabbioneda,S., Kannouche,P.L., Scott,A., Yasui,A., Green,C.M. and Lehmann,A.R. (2008) Regulation of proliferating cell nuclear antigen ubiquitination in mammalian cells. *Proc. Natl Acad. Sci. USA*, **105**, 16125–16130.