TITLE

A genetic study based on PCNA-ubiquitin fusions reveals no requirement for PCNA polyubiquitylation in DNA damage tolerance

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ABSTRACT

Post-translational modifications of Proliferating Cell Nuclear Antigen (PCNA) play a key role in regulating the bypass of DNA lesions during DNA replication. PCNA can be monoubiquitylated at lysine 164 by the RAD6-RAD18 ubiquitin ligase complex. Through this modification, PCNA can interact with low fidelity Y family DNA polymerases to promote translesion synthesis. Monoubiquitylated PCNA can be polyubiquitylated on lysine 63 of ubiquitin by a further ubiquitin-conjugating complex. This modification promotes a template switching bypass process in yeast, while its role in higher eukaryotes is less clear.

We investigated the function of PCNA ubiquitylation using a $PCNA^{K164R}$ mutant DT40 chicken B lymphoblastoma cell line, which is hypersensitive to DNA damaging agents such as methyl methanesulfonate (MMS), cisplatin or ultraviolet radiation (UV) due to the loss of PCNA modifications. In the $PCNA^{K164R}$ mutant we also detected cell cycle arrest following UV treatment, a reduced rate of damage bypass through translesion DNA synthesis on synthetic UV photoproducts, and an increased rate of genomic mutagenesis following MMS treatment. PCNA-ubiquitin fusion proteins have been reported to mimic endogenous PCNA ubiquitylation. We found that the stable expression of a PCNA^{K164R}-ubiquitin fusion protein fully or partially rescued the observed defects of the $PCNA^{K164R}$ mutant. The expression of a PCNA^{K164R}-ubiquitin^{K63R} fusion protein, on which the formation of lysine 63-linked polyubiquitin chains is not possible, similarly rescued the cell cycle arrest, DNA damage sensitivity, reduction of translesion synthesis and increase of MMS-induced genomic mutagenesis. Template switching bypass was not affected by the genetic elimination of PCNA polyubiquitylation, but it was reduced in the absence of the recombination proteins BRCA1 or XRCC3. Our study found no requirement for PCNA polyubiquitylation to protect cells from replication-stalling DNA damage.

KEYWORDS

DNA damage bypass, translesion synthesis, template switching, PCNA, PCNA monoubiquitylation, PCNA polyubiquitylation

ABBREVIATIONS

PCNA, proliferating cell nuclear antigen; Ub, ubiquitin; TLS, translesion synthesis; TSw, template switching; MMS, methyl methanesulfonate; UV, ultraviolet radiation; SNV, single nucleotide variation; HR, homologous recombination

1. INTRODUCTION

The successful bypass of sites of DNA damage during the replication of chromosomal DNA is essential for cell survival and the maintenance of genome integrity. PCNA is a key protein in DNA replication, DNA repair and cell cycle regulation (Maga and Hubscher, 2003; Moldovan et al., 2007). Post-translational modifications (ubiquitylation or sumoylation) of PCNA play an important role in regulating its function in lesion bypass during DNA replication. The conserved lysine 164 side chain can be monoubiquitylated by the RAD6-RAD18 ubiquitin ligase complex in a variety of organisms from yeast to higher eukaryotes (Fox et al., 2011; Hoege et al., 2002; Simpson et al., 2006). Monoubiquitylated PCNA recruits low fidelity Y family DNA polymerases (Pol η, Pol κ, Pol ι) to promote TLS (Bienko et al., 2005; Kannouche et al., 2004; Plosky et al., 2006). Monoubiquitylated PCNA can also undergo further ubiquitylation catalysed by Ubc13-Mms2 and the Rad5 ubiquitin ligase in yeast, or UBC13-MMS2 and the Rad5 orthologues HLTF and SHPRH in human cells, resulting in the build-up of short lysine 63 (K63)-linked polyubiquitin chains (Branzei et al., 2004; Hoege et al., 2002; Unk et al., 2008; Unk et al., 2006). This modification is different from the K48-linked polyubiquitin chain that serves as a degradation signal. The K63-linked polyubiquitylation of PCNA was genetically tied to a Rad18- and Rad5-dependent error-free bypass mechanism in yeast that uses the sister chromatid as an alternative template to extend the stalled DNA strand past a lesion in the template (Zhang and Lawrence, 2005). Two alternative mechanisms have been proposed for such a template switching process. Two-dimensional gel analysis of replication intermediates demonstrated a process that operates through a recombinational mechanism, forming sister chromatid junctions behind the replication fork (Branzei et al., 2008). Indeed, PCNA polyubiquitlylation-dependent template switching in yeast is dependent on the key HR protein Rad51, demonstrating a functional overlap between template switching and HR (Branzei et al., 2008; Minca and Kowalski, 2010). An alternative mechanism for template switching through the reversal and regression of stalled replication forks (Atkinson and McGlynn, 2009; Higgins et al., 1976) is supported by the structure-specific helicase ability of Rad5 (Blastyak et al., 2007), though recently the helicase activity of Rad5 was shown to be dispensable for template switching (Ball et al., 2014).

Lesion bypass through the use of an alternative template has also been demonstrated in higher eukaryotes (Izhar et al., 2013; Szuts et al., 2008), and increased UV-induced mutagenesis upon overexpressing K63R mutant ubiquitin indirectly implicates PCNA polyubiquitylation in the process (Chiu et al., 2006). However, direct evidence for the connection between PCNA polyubiquitylation and damage bypass is lacking. SHPRH promotes mutagenic bypass on UV lesions, while HLTF also regulates PCNA monoubiquitylation (Lin et al., 2011), which likely contributes to its effect in DNA damage tolerance.

We sought to establish a clear genetic assay for the effect of PCNA polyubiquitylation in DNA damage tolerance. As it is not clear which enzymes are capable of polyubiquitylation in higher eukaryotes (Krijger et al., 2011; Motegi et al., 2008), we opted for the use of PCNA-ubiquitin (PCNA-Ub) fusion proteins in which the ligation of ubiquitin units can be controlled by altering the target lysines. The N- or C-terminal linear fusion of one ubiquitin molecule to PCNA has been shown to partially substitute for the endogenous ubiquitylation of PCNA in damage tolerance in *S. cerevisiae* (Parker et al., 2007; Pastushok et al., 2010), *S. pombe* (Ramasubramanyan et al., 2010) and cultured human cells (Qin et al., 2013). The fusion proteins in these studies allowed the potential formation of polyubiquitin chains on the fused ubiquitin moiety. In our study we used combinations of mutations of the target lysines in the endogenous PCNA gene and in expressed C-terminal PCNA-Ub fusion proteins to genetically allow or inhibit PCNA polyubiquitylation. Our results suggest that PCNA polyubiquitylation is not required for DNA damage tolerance or the prevention of mutagenesis in chicken DT40 cells.

2. MATERIALS AND METHODS

2.1. Transgenic expression constructs

For the non-fusion PCNA expression constructs, the full length chicken PCNA coding sequence was amplified from the cDNA of the wild type DT40 cell line using primers CAGCTAGC(NheI)CAGTCGAC(SalI)ATGTTCGAGGCGCGGCTTGTG (primer P1) and CAGGATCC(BamHI)TTAAGAGCCTTCTTGCTGGTCTTCAATC, and

cloned into the multiple cloning site of pIRES2-EGFP with SalI and BamHI. PCNA-IRES-EGFP was amplified with primers P1 and CAGCGGCCGC(NotI)TTACTTGTACAGCTCGTCCATGC, cloned into the pExpress-derived pXpsn2 (Arakawa et al., 2001; Ross et al., 2005) using SalI and NotI, cut with SpeI and cloned into NheI-cut pLoxBsr or pLoxPuro (Arakawa et al., 2001). For the PCNA-Ub fusion expression constructs, the PCNA coding sequence was amplified using primers P1 and CACAATTG(MfeI)CTCGGCAGTGCCAGAGCCTTCTTGCTGGTCTTCAATCT, thereby removing the stop codon and inserting a C-terminal GTAEQL peptide linker in analogy with the GTAEIQ linker used in *S. cerevisiae* (Parker et al., 2007). A ubiquitin coding sequence without the C-terminal two glycines was also amplified from cDNA using primers GTCACAATTG(MfeI)ATGCAGATCTTCGTGAAAACGTTGACTGG and GTCAGGATCC(BamHI)CTA(stop)CCTCAGGCGCAGCACCAGATG. The PCNA and ubiquitin sequences were assembled in a vector with a purpose-made multiple cloning site. The PCNA K164R modification and the ubiquitin K63R modification were generated by site directed mutagenesis. The PCNA-Ub fusion sequences were subcloned using MluI into modified, non-loxable pLoxBsr/Puro plasmids, which were prepared by amplifying the IRES-EGFP sequence from pIRES2-EGFP using primers CAGTCGAC(SalI)AGCAATTG(MfeI)TCACGCGT(MluI)CGCCCCTCTCCCTCCCC and CAGCGGCCGC(NotI)TTACTTGTACAGCTCGTCCATGCC, cloning into pXpsn2 using SalI and NotI, excising the transgene with SpeI and cloning into SpeI-cut pLoxBsr/Puro.

2.2 In vitro ubiquitylation assay

Human UBA1 was purchased from Sigma Aldrich. The complex of UBC13-MMS2 was expressed in baculovirus expression system. Plasmids containg the human UBC13 and MMS2 cDNA were purchased from Addgene and recloned into a modified pFL baculovirus vector (Fitzgerald et al., 2006). The expression of MMS2 was under control of the p10 promoter, while the expression of UBC13 in frame with and N-terminal His_6-tag was controlled under the polh promoter of pFL. The complex was expressed in Sf9 insect cells and purified via the N-terminal histidine tag of UBC13 on a Ni-NTA (GE Healthcare) affinity column, followed by Q-Sepharose Hi-

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Trap 1 mL (GE Healthcare) and Superdex 200 Increase columns pre-equilibrated with 20 mM Na-HEPES pH 7.5, 100 mM NaCl, 5% glycerol. Human ubiquitin was amplified from human cDNA library and cloned into the pETM-11 vector. The protein was expressed in the Rosetta II *E.coli* strain and affinity purified on a 1 mL Ni-NTA (GE Healthcare) column, then further purified on Superdex 75 16/60 size exclusion chromatography column (GE Healthcare) pre-equilibrated with 20 mM Na-HEPES pH 7.5, 100 mM NaCl, 5% glycerol. The full length human HLTF cDNA (Unk et al., 2008) was cloned into pFL under the polh promoter. The protein was expressed in Sf9 insect cells and was affinity purified in one step on amylose resin through its maltose binding protein (MBP) tag in buffer containing 40 mM Na-HEPES pH 7.5, 10% glycerol, 2 mM 2-mercaptoethanol and 100 mM NaCl. PCNA constructs were subcloned into the pET-15b vector (Novagen) and expressed in *E. coli*. The N-terminal his-tagged fusion proteins were affinity purified on Ni-NTA Superflow resin (QIAGEN), followed by gel filtration on a Superose 12 column (GE Healthcare). Ubiquitylation reactions all contained 40 mM Na-HEPES pH 7.5, 8 mM $MgCl_2$, 50 mM NaCl, 1 mM ATP, 250 nM ubiquitin and 100 nM PCNA substrate, plus 100 nM UBA1, 250 nM UBC13-MMS2 complex and 250 nM HLTF as indicated. Reactions were incubated at 30°C for 60 minutes and stopped with the addition of SDS sample buffer.

2.2. Cell culture and colony survival assay

This study used a wild type DT40 cell line (Molnár et al., 2014), a *PCNA^{K164R/K164R}* line referred to in the text as *PCNA^{K164R}* (Arakawa et al., 2006), *XPA⁻* and *XPA⁻ PCNA^{K164R/K164R}* cells (Szuts et al., 2008). *XPA⁻ BRCA1⁻/⁻* and *XPA⁻ XRCC3⁻/⁻* cell lines were generated by disrupting the single-copy *XPA* gene in the respective heterozygous HR mutants, then disrupting the second allele of *BRCA1* (Vandenberg et al., 2003) and *XRCC3* (Yonetani et al., 2005).

Cells were grown at 37°C under 5% CO_2 in RPMI-1640 medium supplemented with 7% fetal bovine serum, 3% chicken serum, 50 μM 2-mercaptoethanol and penicillin/streptomycin.

MMS, cisplatin and UV sensitivities were measured using colony survival assays; treated cells were plated in culture medium containing 1% methylcellulose (Sigma) using a tenfold dilution series, and surviving colonies were counted 10 days later.

2.3. Shuttle plasmid lesion bypass assay

600,000 cells were transfected with 0.1 μg pQ-CPDs shuttle plasmid containing two synthetic T-T cyclobutyl pyrimidine dimers in a staggered arrangement (Varga et al., 2012). The transfections were performed using a Nucleofector device (Lonza) as described previously (Varga et al., 2012). The transfected cells were cultured at 37°C for 24 hours, then the plasmid was extracted using the Hirt protocol and digested with 10 U DpnI restriction endonuclease in 10 μl at 37°C for 30 minutes to destroy unreplicated *dam* methylated plasmids. The efficiency of plasmid replication was quantified from qPCR measurements as the amount of a recovered DpnI-susceptible plasmid fragment divided by the amount of a control fragment (Varga et al., 2012). The digested DNA was purified and used as template for PCR amplification with Pfu Turbo (Agilent) of a 1369 bp section of DpnI resistant replicated pQ-CPDs using primers CAGTCGACTTTTGATTTAGAATTGTCCAC and CAGCGGCCGCTTTGCAAGCAGCAGATTACG. To determine in bulk the proportion of each common sequence outcome of the replication of the CPD lesion containing DNA section, allele specific qPCR primers were used on the PCR product. The primer pairs used to distinguish double stranded DNA templates after CPD lesion bypass were: GATTTAGAATTGTCCACCTC<u>GCC</u> and CCTTACTGCATGGATCGA<u>GCT</u> for GC at the site of both lesions, GATTTAGAATTGTCCACCTC<u>GCC</u> and TCCCTTACTGCATGGATCGA<u>TTT</u> for AA insertion by TLS at the downstream lesion or TTTTGATTTAGAATTGTCCACCTC<u>TT</u> and CCTTACTGCATGGATCGA<u>GC</u> for AA insertion by TLS at the upstream lesion (the allele specific bases are underlined). These primers provide 1.000-100.000 fold enrichment over templates with the alternative sequence variants.

2.4. Flow cytometry, cell cycle analysis and Western blotting

DT40 cells were UV-irradiated, labelled with bromodeoxyuridine, fixed and prepared for cell cycle analysis exactly as described (Varga et al., 2012), and analysed using a FACSCanto II instrument and FACSDiva 6.1 software (BD Biosciences). EGFP expression was measured in live cells using the same instrument.

For Western blot analysis, whole cell extracts of DT40 cells were fractionated by SDS-PAGE and transferred to a PVDF membrane using wet-transfer. After blocking in Tris-buffered saline (TBS) containing 5% dry nonfat milk for 1 h, the membrane was incubated with anti-PCNA (PC10, Abcam ab29, dilution 1:20000) at 4°C overnight. Blots were washed and incubated with anti-mouse (Sigma A9044, dilution 1:20000) secondary antibody for 1 h, and developed with the ECL system (Clarity Western ECL Blotting Substrate, Bio-Rad Laboratories). Chemiluminescent imaging was performed with a ChemiDoc MP system and the band intensities were quantified using the ImageLab software (Bio-Rad Laboratories). For the analysis of *in vitro* ubiquitylation, blots were blocked in TBS complemented with 5% bovine serum albumin, then incubated with anti-ubiquitin primary antibody (Abcam ab7780, dilution 1:1000) in TBS with 3% bovine serum albumin for 3.5 hours at room temperature.

2.5. Whole genome sequencing and mutation calling

For the mutagenesis experiment, a single cell starting clone was isolated from each cell line and expanded, then four rounds of MMS (Sigma) treatments were performed in weekly intervals. One million cells were treated each time for one hour. Following the last treatment, single cell clones were isolated and expanded to two million cells prior to genomic DNA preparation using the Gentra Puregene Cell Kit (Qiagen).

Library preparation used the TruSeq DNA Nano Library Preparation Kit (Illumina) or the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs). Sequencing was done on Illumina HiSeq 2500 v4 instruments (2x125bp PE). Library preparation and DNA sequencing was done at the Research Technology Support Facility of Michigan State University, USA, and at Novogene, Beijing, China. The reads were aligned to the chicken (Gallus gallus) reference sequence Galgal4.73, duplicate reads were removed and the aligned reads were realigned near indels as described (Zamborszky et al., 2017).

Independently arising SNVs and short indels were identified using the IsoMut method developed for multiple isogenic samples (Pipek et al., 2017). Data from 40 different sequenced DT40 clones was used at this step, which included the samples presented

in this article. Briefly, after applying a base quality filter of 30, data from all samples were compared at each genomic position, and filtered using optimised parameters of minimum mutated allele frequency (0.2), minimum coverage of the mutated sample (5), and minimum reference allele frequency of all the other samples (0.93), and also filtered using a probability-based quality score calculated from the mutated sample and one other sample with the lowest reference allele frequency. The quality score threshold was set such that no more than five false positive SNVs would be detected in pre-treatment starting clones. Raw sequence data has been deposited with the European Nucleotide Archive under study accession number ERP019364.

3. RESULTS

3.1. Model for mimicking the monoubiquitylated form of PCNA

To analyse and separate the phenotype of mono- and K63-linked polyubiquitylated PCNA *in vivo*, we fused wild type or K63R mutant versions of ubiquitin to the carboxy terminus of the non-ubiquitylable K164R mutant form of chicken PCNA. The two C-terminal glycine residues were removed from ubiquitin in the fusion constructs to prevent further modifications (Parker et al., 2007). Alongside these PCNA^{K164R}-Ub and PCNA^{K164R}-Ub^{K63R} fusion constructs two further expression constructs were made that contained the wild type or K164R mutant PCNA as positive and negative controls (Fig. 1A). These constructs also express EGFP as part of a bicistronic message with an internal ribosome entry site (IRES) to indicate the expression level.

The PCNA fusion constructs were stably transfected into the DT40 *PCNA^{K164R}* cell line in which the endogenous ubiquitylation/sumoylation site of PCNA is eliminated (Arakawa et al., 2006), therefore the effect of ubiquitylation is only from the fusion. Unlike the wild type ubiquitin fusion, the fused Ub^{K63R} cannot be polyubiquitylated through lysine 63, allowing us to genetically test the requirement for K63-linked polyubiquitylation. The expression levels of the constructs were carefully matched via EGFP expression and anti-PCNA Western blots (Fig. 1B, C). The amounts of the fusion proteins in these cell lines were approximately similar to the endogenous expression level of PCNA, indicating a 2-fold overexpression of PCNA in the

transfected lines. We were unable to detect polyubiquitylated forms of either the endogenous PCNA or the fusion constructs in cell extracts by Western blot, therefore our studies must be considered primarily genetic, correlating phenotypic changes with the losses of potential PCNA post-translational modifications. To explore this issue further, we set up an *in vitro* ubiquitylation assay with human E1 (UBA1), E2 (UBC13-MMS2) and E3 (HLTF) enzymes, which have been shown to ubiquitylate PCNA that is monoubiquitylated by RAD6 and RAD18 (Unk et al., 2008). We detected a band consistent with the ubiquitylation of PCNA^{K164R}-Ub, which was absent when we used the PCNA^{K164R}-Ub^{K63R} substrate (Fig. 1D). The formation of this modification was dependent on the E1 and E2 enzymes, and slightly enhanced by the presence of HLTF. Interestingly, we did not detect longer ubiquitin chains, possibly suggesting that the HLTF preparation had low activity. Although it has been suggested that PCNA polyubiquitylation takes place through the addition of pre-assembled polyubiquitin chains (Masuda et al., 2012), we conclude from these results that the PCNA^{K164R}-Ub fusion protein can be further ubiquitylated by UBC13-MMS2 in the absence of RAD18 in a manner dependent on lysine 63 of ubiquitin. This suggests that the PCNA-Ub fusion protein is a suitable substrate for K63-linked polyubiquitylation, and provides support for the argument that polyubiquitin chains cannot form on the PCNA^{K164R}-Ub^{K63R} protein.

3.2. The role of PCNA modifications in UV damage tolerance

PCNA modifications on K164 are required for the unhindered bypass of the two most common UV photoproducts (Szuts et al., 2008; Varga et al., 2012). To test whether the forced ubiquitylation of PCNA in the cell lines that express PCNA-Ub fusions can substitute for these modifications, we first measured cell cycle progression without or with UV irradiation (Fig. 2). Untreated populations had similar cell cycle distribution, so without DNA damaging treatment, there appear to be no differences between the newly generated cell lines (Fig. 2B). After low-dose UV irradiation and 6 h recovery there was a marked increase in cells arrested in S phase in all cell lines (Fig. 2C), but after 24 h recovery the cell cycle profile of the wild type cells was very similar to that of the untreated cells, with a slight increase in the proportion of cells arrested in S phase (Fig. 2D). In contrast, there was a marked increase in G2/M phase cells and a reduction of G1 and S phase cells in the *PCNA^{K164R}* cell line. The expression of wild

type PCNA rescued this defect, while the expression of the mutated PCNA^{K164R} protein did not. There was a clear, though partial rescue of the UV-induced cell cycle delay in the cell lines expressing the PCNA^{K164R}-Ub or PCNA^{K164R}-Ub^{K63R} fusion proteins, in which 38-39% of the cells were in S phase 24 hours after UV exposure, with no significant difference between them (Fig. 2D). The proportion of S phase cells in the PCNA^{K164R}-Ub^{K63R} expressing line was not significantly different from the wild type cell line (p=0.09, t-test), but significantly lower than the rescue line that expresses wild type PCNA (p=0.0002) and significantly higher than the *PCNA^{K164R}* and *PCNA^{K164R}*:PCNA^{K164R} negative controls (p<0.0001 and p=0.0010, respectively; due to multiple comparisons, the significance limit should be lowered to p=0.01 in this set of tests). This indicates that the single C-terminally fused ubiquitin with or without further potential K63-linked ubiquitin modifications can equally substitute for PCNA modifications on K164.

To complement the cell cycle analysis, we measured the UV sensitivity of the cell lines using colony formation assays. Compared to the wild type, the *PCNA^{K164R}* and *PCNA^{K164R}*:PCNA^{K164R} negative control cells were hypersensitive to UV, while the cell lines that express the PCNA^{K164R}-Ub and PCNA^{K164R}-Ub^{K63R} fusion proteins showed similar UV sensitivity to the wild type and the *PCNA^{K164R}*:PCNA rescue cell line (Fig. 3A). This suggests that the potential polyubiquitylation in the PCNA^{K164R}-Ub expressing cell line is not required for cell survival following UV irradiation.

3.3. PCNA monoubiquitylation is required for efficient translesion synthesis over a UV lesion

We next investigated the influence of PCNA modifications on the choice of damage bypass mechanism using a shuttle plasmid assay that can measure the ratio of TLS and error-free bypass that uses the complementary strand as template (Szuts et al., 2008; Varga et al., 2012). In order to investigate DNA damage bypass only, we eliminated the primary repair system of UV lesions by creating a nucleotide excision repair deficient, *XPA$^-$* knock-out genetic background in each cell line. We investigated the replication products of pQ1-CPDs plasmids that contained one T-T CPD lesion on each strand in a staggered arrangement, separated by 28 bp, and opposed by a mismatched GC dinucleotide (Varga et al., 2012). The overall

proportion of replicated plasmids, calculated from the DpnI resistance of short plasmid fragments, was in the 2.2-4.9% range across all the samples, with no significant differences found by ANOVA (Fig. 2B). Allele-specific primers were used in quantitative PCR reactions to measure the relative quantities of the main sequence variants at the lesions among replication products. The insertion of AA opposite either lesion indicates TLS, while the presence of GC at the site of both lesions in the replication product was taken as evidence of error-free bypass by template switching (Fig. 4A). The rare mutagenic TLS outcomes such as TA were not measured, which is expected to exclude 11-17% of all TLS events from our analysis (Varga et al., 2012). In XPA^- cells 61% of successful bypass products bore evidence of TLS, whereas in XPA^- $PCNA^{K164R}$ we observed a modest but significant reduction (p=0.0284, t-test) in the proportion of TLS to a mean of 52% (Fig. 4C). These data, obtained with allele-specific qPCR, are in very good agreement with earlier data derived from the sequencing of individual replication products of the same experiment (Varga et al., 2012). The reduced proportion of TLS confirms a role for PCNA ubiquitylation in the process, and the remaining PCNA ubiquitylation independent TLS is likely due to TLS polymerase recruitment through REV1 (Hendel et al., 2011; Szuts et al., 2008).

The expression of the non-ubiquitylable mutant $PCNA^{K164R}$ did not rescue the TLS defect (p=0.0166), whereas the transgenic expression of wild type PCNA fully restored the use of TLS for CPD bypass. Importantly, the expression of either PCNA-Ub fusion construct ($PCNA^{K164R}$-Ub and $PCNA^{K164R}$-Ub^{K63R}) also restored the proportion of TLS to near the 60% level (Fig. 4C). Thus the C-terminally fused ubiquitin can substitute for the requirement for PCNA ubiquitylation in the regulation of TLS. Moreover, the fusion of K63R-mutant ubiquitin provides the same rescue effect, even though it does not allow the formation of polyubiquitin chains. We can conclude that PCNA monoubiquitylation is the only PCNA modification that is required for the bypass of CPD lesions, and potential polyubiquitylation of PCNA does not influence the outcome through either TLS or the use of the sister chromatid as template.

3.4. The role of PCNA modifications in the bypass of cisplatin- and MMS-induced DNA damage

UV photoproducts comprise a specific set of well-studied DNA lesions. To assay the role of PCNA polyubiquitylation in the bypass of a wider variety of DNA lesions, we treated cells with MMS, an alkylating agent (Wyatt and Pittman, 2006), or cisplatin, which generates intrastrand and interstrand crosslinks (Eastman, 1986). The $PCNA^{K164R}$ cell line and the PCNA^{K164R}-expressing negative control were hypersensitive to both MMS and cisplatin, and the expression of wild type PCNA in the $PCNA^{K164R}$ background fully restored sensitivity to the wild type level (Fig. 3B, C). In case of MMS, the expression of either the PCNA^{K164R}-Ub or the PCNA^{K164R}-Ub^{K63R} fusion proteins also fully rescued the hypersensitivity, thus the monoubiquitin modification was sufficient to support a wild type MMS response. The expression of PCNA^{K164R}-Ub or PCNA^{K164R}-Ub^{K63R} resulted in similar partial rescue of the cisplatin sensitivity of the $PCNA^{K164R}$ cell line (see discussion).

To further test whether the reduced sensitivity upon the expression of PCNA-ubiquitin fusions is not due to the increase in the overall amounts of PCNA in the cell, we created cell lines expressing matched levels of PCNA^{K164R}-Ub or PCNA^{K164R}-Ub^{K63R} in a wild type background. We found that the presence of endogenous and transgenic PCNA together (doubling the amount of endogenous PCNA) did not change MMS sensitivity (Fig. 3D), demonstrating the specificity of rescuing effect of the fusion proteins in the $PCNA^{K164R}$ background.

3.5. Monoubiquitylated PCNA is sufficient to restore the mutation spectrum

An important consequence of TLS is the potential generation of somatic mutations, in contrast with error-free lesion bypass using an alternative template. Interference with PCNA ubiquitylation-dependent template switching is therefore expected to increase the rate of mutagenesis. Whole genome sequencing allows the detection of all genomic mutations that arise during the course of an experiment, and we recently employed this strategy to demonstrate a high rate of mutagenesis in response to MMS treatment, with the predominance of T>A and C>A changes amongst base substitution mutations (Zamborszky et al., 2017). To use MMS-induced mutations as a background for measuring the mutagenic impact of PCNA modifications, we repeatedly subjected single cell clones to one-hour treatments with 20 ppm (236 μM) MMS, followed by the isolation of post-treatment single cell clones. The

identification of unique mutations in cell clones isolated from the treated populations indicated increased base substitution mutagenesis in the *PCNA^{K164R}* cell line compared to the wild type (Fig. 5A), with an especially noticeable 2.6-fold increase in the number of T>A mutations, primarily in the context of further pyrimidine bases (Fig. 5A, B). The expression of wild type PCNA nearly rescued this phenotype, reducing both the overall number of SNVs and specifically the T>A mutations. This reduction in mutagenesis in the presence of wild type PCNA cannot be the consequence of PCNA polyubiquitylation-dependent template switching, as the expression of the PCNA^{K164R}-Ub^{K63R} fusion protein similarly reduced SNV numbers and nearly restored the wild type mutation spectrum (Fig. 5A, B). If, based on their identical MMS sensitivity we considered results from the wild type and the two rescued cell line samples as belonging to the same distribution, the *PCNA^{K164R}* sample would fall outside the 95% confidence interval for both the overall number of SNVs and the T>A mutations. The increased mutagenesis in the *PCNA^{K164R}* cell line may be due to mutagenic REV1-dependent TLS, as shown in the case of UV CPD lesions (Varga et al., 2012).

3.6. Template switching bypass uses a recombinational mechanism

The proportion of bypass events with a template switch outcome was unaffected in the *PCNA^{K164R}*:PCNA^{K164R}-Ub^{K63R} mutant (Fig. 4C), arguing against an involvement of PCNA polyubiquitylation in template switching bypass. To test whether the observed use of the sister chromatid as template requires HR factors, we created *XPA^− BRCA^{−/−}* and *XPA^− XRCC3^{−/−}* cell lines. BRCA1 is a critical factor of HR, playing roles in controlling the resection of broken double stranded DNA ends (Bunting et al., 2010) and in the recruitment of the essential HR protein RAD51 (Qing et al., 2011; Zhang et al., 2009), while XRCC3 is a RAD51 paralogue that is also required for RAD51 recruitment (Bishop et al., 1998). When comparing to the *XPA^−* cell line, allele-specific qPCR analysis of replicated pQ1-CPDs plasmids revealed a significant increase in the proportional use of TLS from 61% to 74% and 72% due to the disruption of *XRCC3* and *BRCA1* respectively, with a corresponding decrease of template switching outcomes (Fig. 4C, p=0.006 and p=0.0428, *t*-test). The disappearance of the apparent template switch events suggests that the use of the

sister chromatid as a template during lesion bypass is in large part a BRCA1-dependent, HR-related mechanism.

4. DISCUSSION

In this study we made use of genetic experiments in the chicken DT40 cell line to probe the function of PCNA modifications in DNA damage tolerance and lesion bypass. The fusion of a single ubiquitin to PCNA rescued the damage sensitivity of a non-ubiquitylable PCNA mutant, and also restored the rate of TLS over CPD UV photoproducts. The expression of a PCNA-ubiquitin fusion variant that prevented the addition of further ubiquitin units showed identical rescuing phenotypes, demonstrating a lack of requirement for PCNA polyubiquitylation in UV and MMS-induced DNA damage tolerance.

The conserved K164 residue of PCNA is a platform for different modifications: mono- and polyubiquitylation, sumoylation. Researchers have been able to specifically test the function of PCNA ubiquitylation using PCNA-ubiquitin fusion proteins, which partially rescued damage sensitivity in *S. cerevisiae* Rad18 mutants (Parker et al., 2007; Pastushok et al., 2010), or TLS in *S. pombe pcn1-K164R* mutants (Ramasubramanyan et al., 2010). In contrast, we observed a full rescue of UV and MMS damage sensitivity and TLS in *PCNA^K164R* mutant vertebrate cells which offered the opportunity to specifically test the requirement for PCNA mono- or polyubiquitylation. The full rescue suggests that TLS polymerases are able to bind C-terminal PCNA-Ub fusion proteins. Though siRNA silencing based experiments in human cells suggested that Pol η was not required for DNA damage tolerance conferred by PCNA-Ub fusions (Qin et al., 2013), we previously observed an identical requirement for PCNA ubiquitylation and Pol η in the bypass of CPD UV photoproducts (Varga et al., 2012), and in this study the same wild type level of TLS was achieved by the monoubiquitylation-equivalent Ub^K63R fusion, suggesting that at CPD lesions the main function of PCNA ubiquitylation is the recruitment of Pol η.

TLS is not fully dependent on PCNA ubiquitylation (Hendel et al., 2011; Szuts et al., 2008), which is concordant with the increased rate of mutagenesis we observed in the genome of MMS-treated *PCNA^K164R* cells, which is likely due to mutagenic TLS in

the absence of PCNA ubiquitylation. The reduction of the rate of mutagenesis and the restoration of the wild type mutagenic spectrum by the expression of the PCNA^{K164R}-Ub^{K63R} fusion may then be explained by the recruitment of alternative, less mutagenic TLS polymerases. The only contexts in which we did not observe full rescue by 'monoubiquitylated' PCNA were sensitivity to cisplatin, and recovery from UV-induced cell cycle arrest. Here, the similar partial rescue by wild type or K63R mutant ubiquitin suggests that some further PCNA modification may be needed for damage tolerance. A candidate for such modification is PCNA SUMOylation, which is not possible at residue K164 in the cell lines that express the PCNA-Ub fusion proteins, though it may be necessary to regulate HR during the repair or bypass of cisplatin lesions (Gali et al., 2012; Pfander et al., 2005). An alternative explanation one must consider for the observed imperfect rescue is that the PCNA^{K164R}-Ub fusion may not be a perfect mimic for PCNA monoubiquitylated on K164 with regards to cisplatin survival, or, if the PCNA^{K164R}-Ub fusion cannot be properly polyubiquitylated, this might demonstrate a requirement for polyubiquitylation under these circumstances.

The main conclusion from our results is the lack of a detectable requirement for PCNA polyubiquitylation. We did not physically detect polyubiquitylation of the expressed PCNA^{K164R}-Ub fusion protein *in vivo*. However, regardless of whether this can take place, the full rescue of several PCNA^{K164R} phenotypes by both PCNA-Ub fusions provides a genetic demonstration that monoubiquitylation was sufficient for tolerance to UV and MMS, bypass of CPD UV photoproducts, and the avoidance of MMS-induced genomic mutations.

Post-translational modification of PCNA with two or more ubiquitin units has been observed in multiple experimental systems, including the DT40 cells used in this study (Tomi et al., 2014), even though HLTF is absent from the chicken genome. What may then be the function of PCNA polyubiquitylation? The structure specific helicase activity of Rad5 and its orthologues HLTF and SHPRH provided the best lead for a function in replication fork reversal, which is a potential mechanism for non-mutagenic template switching bypass. However, HLTF and SHPRH have damage-specific and rather complex effects on point mutations, mostly due to their influence on TLS (Lin et al., 2011), while a template switching role would predict a clear anti-mutagenic function. The ZRANB3 protein can bind polyubiquitylated PCNA and promote replication fork remodelling and restart (Ciccia et al., 2012), and

emerging evidence suggests a distinct role for fork reversal in the protection of stalled replication forks (Neelsen and Lopes, 2015; Schlacher et al., 2011). A role for PCNA polyubiquitylation in fork reversal and stabilisation would explain the increased rate of chromosome breakage due to the loss of SHPRH or HLTF (Motegi et al., 2008; Motegi et al., 2006), and could also explain the incomplete rescue of late S/G2 phase arrest we observed with the PCNA^{K164R}-Ub^{K63R} fusion.

In conclusion, our dissection of PCNA post-translational modifications in the DT40 genetic model suggests there is no requirement for PCNA polyubiquitylation in DNA damage tolerance and error-free damage bypass.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. PCNA fusion constructs and their expression levels

(A) Schematic representation of PCNA (positive control), PCNA^{K164R} (negative control) and PCNA^{K164R}-Ub fusion protein constructs expressed in the DT40 *PCNA^{K164R}* cell line. PCNA mutation at K164 prevents *in vivo* PCNA ubiquitylation, ubiquitin mutation at K63 prevents *in vivo* polyubiquitylation. (B) Histograms of the EGFP fluorescence level of each cell line expressing the respective PCNA construct as aligned with panel (A), measured by flow cytometry. (C) The levels of unmodified and modified PCNA detected by anti-PCNA blot in whole cell DT40 extracts. The percentage of total PCNA that is present as ubiquitin fusion, as measured by

densitometry, is indicated above the last two lanes. Ponceau S staining of the membrane is shown as loading control. WT stands for wild type in all figures. (D) *In vitro* ubiquitylation assay with the indicated purified components, presented using Coomassie Brilliant Blue staining (top panel) and anti-Ub Western blot (bottom panel). MMS2, UBC13 and ubiquitin have migrated beyond the bottom of the gel. The arrow indicates a ubiquitylated form of the PCNA^{K164R}-Ub fusion protein. Size markers are in kDa.

Figure 2. Cell cycle analysis

The DNA content of DT40 cells of the indicated genotypes were analysed using propidium iodide staining (horizontal axis), and the rate of DNA replication using anti-BrdU-FITC antibody staining (vertical axis). (A) The rationale for assigning cell populations to different cell cycle phases, an apoptotic category with sub-G1 DNA content, and an arrested S phase category for non-replicating cells with an S phase DNA content. (B, C, D) The percentage of cells in each category. Cell cycle phases were measured in untreated cells (B) or following 1 J/m^2 UV irradiation and 6 h (C) or 24 h recovery (D). The mean and S.E.M of five (three for the 6 h time point) independent experiments is shown.

Figure 3. UV, MMS and cisplatin sensitivity

Colony survival assays measuring the sensitivity of the indicated cell lines to UV (A), MMS (B, D) and cisplatin (C). The top legend refers to panels (A-C). The mean of three independent experiments is shown, error bars indicate S.E.M.

Figure 4. The influence of PCNA modifications and HR proteins on the choice of lesion bypass mechanism

(A) A schematic representation of the possible bypass outcomes on two T-T CPD photoproducts arranged on separate strands of a lesion containing plasmid, each opposite a GC dinucleotide. GC at the site of both lesions bears evidence of a template switch (TSw), while AA opposite either photoproduct indicates TLS. (B) Replication efficiency of the pQ-CPDs plasmid in the indicated cell lines, as

measured by qPCR (see Materials and Methods). The mean and S.E.M. of three to eight independent measurements is shown. (C) The percentage of template switching and TLS outcomes in replicated lesion-containing plasmids recovered from the indicated cell lines, measured using allele-specific qPCR. The mean and S.E.M. of four to six independent measurements is shown.

Figure 5. Number and mutation spectrum of SNVs

(A) The number of SNVs and base substitution spectrum of mutations detected following four rounds of weekly 1 h treatments with 20 ppm MMS of cell lines of indicated genotypes. (B) Triplet mutation spectrum of MMS treatment of the indicated cell lines. Each mutation class, as indicated at the top of the panel, is separated into 16 categories based on the identity of the preceding and following nucleotide as shown at the bottom.

Highlights (revised)

- PCNA monoubiquitylation is necessary for efficient translesion synthesis
- PCNA polyubiquitylation is not required for bypass of UV and MMS-induced DNA damage
- Monoubiquitylated PCNA fusion is sufficient to restore the mutation spectrum
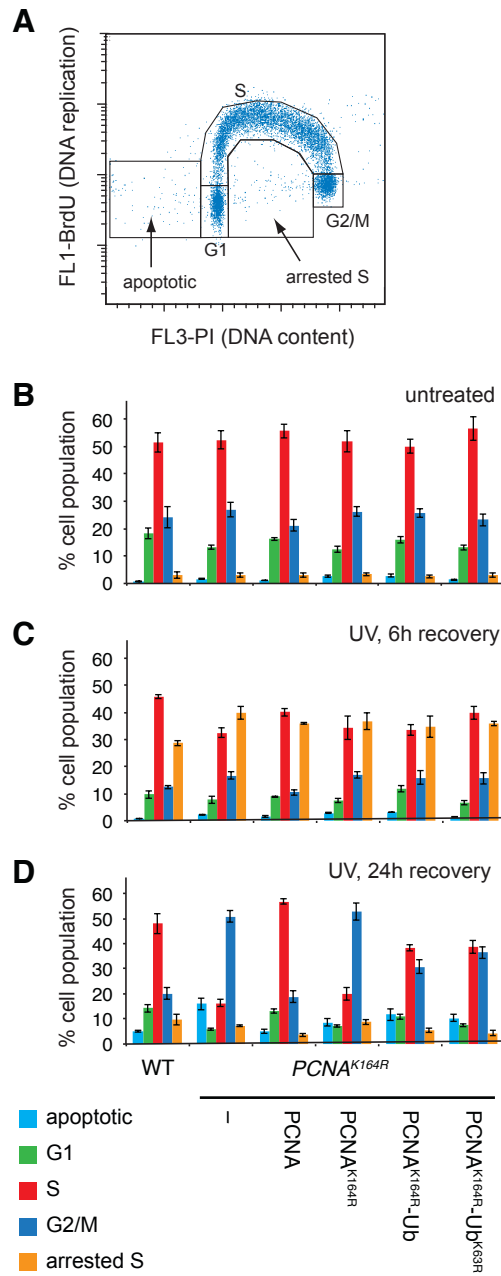- Template switching bypass uses a recombinational mechanism

Figure 1

A

PCNA^{K164R} genetic background

no transgene

PCNA

| PCNA |

PCNA^{K164R}

| PCNA |
K164R

PCNA^{K164R}-Ub

| PCNA | Ub |
K164R

PCNA^{K164R}-Ub^{K63R}

| PCNA | Ub |
K164R   K63R

B

FL1 (EGFP)

C

56% 53%

anti-PCNA

55
42
26

Ponceau

M   WT   *PCNA^{K164R}*

I   PCNA   PCNA^{K164R}   PCNA^{K164R}-Ub   PCNA^{K164R}-Ub^{K63R}

D

Coomassie

150 — UBA1
100 — HLTF
75
50 — PCNA^{K164R}-Ub, PCNA^{K164R}-Ub^{K63R}
37 — PCNA^{K164R}

anti-Ub

150
100
75
50 ← PCNA^{K164R}-Ub, PCNA^{K164R}-Ub^{K63R}
37

−	+	+	−	+	+	−	+	+	UBA1
−	+	+	−	+	+	−	+	+	UBC13-MMS2
−	−	+	−	−	+	−	−	+	HLTF

PCNA^{K164R}   PCNA^{K164R}   PCNA^{K164R}   PCNA^{K164R}-Ub   PCNA^{K164R}-Ub   PCNA^{K164R}-Ub   PCNA^{K164R}-Ub^{K63R}   PCNA^{K164R}-Ub^{K63R}   PCNA^{K164R}-Ub^{K63R}   substrate

Figure 2

A

FL1-BrdU (DNA replication)

S

G1   arrested S   G2/M

apoptotic

FL3-PI (DNA content)

B   untreated

% cell population

C   UV, 6h recovery

% cell population

D   UV, 24h recovery

% cell population

WT $PCNA^{K164R}$

apoptotic
G1
S
G2/M
arrested S

PCNA
$PCNA^{K164R}$
$PCNA^{K164R}$-Ub
$PCNA^{K164R}$-Ub^{K63R}

Figure 3

Figure 4

Figure 5