Regulation of translesion synthesis polymerases during somatic hypermutation and DNA damage tolerance
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HLTF and SHPRH are not essential for PCNA polyubiquitination, survival and somatic hypermutation: existence of an alternative E3 ligase

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ABSTRACT

DNA damage tolerance is regulated at least in part at the level of proliferating cell nuclear antigen (PCNA) ubiquitination. Monoubiquitination (PCNA-Ub) at lysine residue 164 (K164) stimulates error-prone translesion synthesis (TLS), Rad5-dependent polyubiquitination (PCNA-Ub\textsuperscript{n}) stimulates error-free template switching (TS). To generate high affinity antibodies by somatic hypermutation (SHM), B cells profit from error-prone TLS polymerases. Consistent with the role of PCNA-Ub in stimulating TLS, hypermutated B cells of PCNA\textsuperscript{K164R} mutant mice display a defect in generating selective point mutations. Two Rad5 orthologs, HLTf and SHPRH have been identified as alternative E3 ligases generating PCNA-Ub\textsuperscript{n} in mammals. As PCNA-Ub and PCNA-Ub\textsuperscript{n} both make use of K164, error-free PCNA-Ub\textsuperscript{n}-dependent TS may suppress error-prone PCNA-Ub-dependent TLS. To determine a regulatory role of Shprh and Hltf in SHM, we generated Shprh/Hltf double mutant mice. Interestingly, while the formation of PCNA-Ub and PCNA-Ub\textsuperscript{n} is prohibited in PCNA\textsuperscript{K164R} MEFs, the formation of PCNA-Ub\textsuperscript{n} is not abolished in Shprh/Hltf mutant MEFs. In line with these observations Shprh/Hltf double mutant B cells were not hypersensitive to DNA damage. Furthermore, SHM was normal in Shprh/Hltf mutant B cells. These data suggest the existence of an alternative E3 ligase in the generation of PCNA-Ub\textsuperscript{n}. 
1 INTRODUCTION

Damaged DNA can block high fidelity polymerases and consequently lead to replication stalling. If the ‘stalling’ lesion is not repaired, the replication fork may collapse, causing double strand breaks, which in turn can trigger cell death. To suppress these detrimental effects, cells are equipped with DNA damage tolerance (DDT) pathways, that allow cells to continue DNA synthesis without an a priori repair of the initial lesion [1, 2]. Studies in S. cerevisiae identified two alternative DDT pathways: 1) Template switching (TS) avoids the damage, i.e. the lesion is bypassed indirectly by making use of the undamaged sister chromatid, and 2) Translesion synthesis (TLS), which enables specialized DNA polymerases to replicate directly across a damaged template. In contrast to replicative DNA polymerases, TLS polymerases lack proofreading activity and can accommodate non Watson-Crick base pairs within their catalytic center. While beneficial regarding the accurate replication across modified bases, such as UV-C induced cyclobutane pyrimidine dimers by polymerase η, TLS polymerases can be highly mutagenic when replicating across undamaged DNA or other defined lesions [1-3]. Both modes of lesion bypass appear to be controlled by site-specific ubiquitination of the homotrimeric DNA sliding clamp PCNA [4, 5]. During DNA synthesis PCNA serves as a critical processivity factor by tethering DNA polymerases to their template. When high fidelity replication gets stalled by a DNA lesion, Rad6/Rad18-mediated site-specific monoubiquitination of PCNA (PCNA-Ub) at lysine residue 164 (PCNAK164) is thought to control polymerase switching and activation of TLS [4]. The alternative pathway of damage tolerance, TS requires further polyubiquitination of PCNA-Ub (PCNA-Ubn) [4]. In yeast the heterodimeric e2 ubiquitin conjugases Ubc13/Mms2 cooperate with the RING finger e3 ligase Rad5 in specific K63-linked polyubiquitination of PCNA-Ub. How PCNA-Ubn mechanistically activates the error-free branch of DDT is currently unknown.

The fact that the RAD6 epistasis group has functional orthologs in higher eukaryotes suggests that these pathways of DDT are evolutionary conserved and of general importance. In support of this notion, UV-irradiation of mammalian cells was shown to lead to the monoubiquitination at the conserved K164 residue of PCNA. In addition, mammalian polymerase η specifically interacts with PCNA-Ub [6] and localizes to sites of DNA damage in a RAD18-dependent manner [7]. These data imply a conserved mechanism between yeast and mammals in the recruitment and activation of TLS polymerases. Furthermore, damage-inducible PCNA-Ubn has been observed in mammals [8], and was found to be mediated by the two known Rad5 orthologs, HLTf and SHPRH. Like yeast Rad5, both SHPRH and HLTf physically interact with the RAD6/RAD18 and UBC13/MMS2 complexes and promote PCNA polyubiquitination at K164 in a RAD18-dependent manner [9-12]. The role for PCNA-Ubn in mammals is currently unknown, however depletion of either SHPRH or HLTf in human cells increases the sensitivity to
methyl methanesulfonate (MMS) and enhances genomic instability. These data implicate a role for PCNA-Ub in mammalian DNA damage tolerance.

Paradoxically, while the above mentioned pathways of DDT normally serve to maintain genome integrity, B cells take advantage of the intrinsic error-prone nature of TLS polymerases to generate defined point mutations into the variable region of their rearranged immunoglobulin (Ig) genes, which eventually may encode antibodies of higher affinity. This process of somatic hypermutation (SHM) occurs at an astonishing rate of one per thousand bases per generation, six orders of magnitude greater than spontaneous mutagenesis [13]. The overall SHM frequency is one to three percent, and the mutations are equally distributed over G/C and A/T base pairs. SHM is initiated by the induction of the activation-induced cytidine deaminase (AID) in B cells of the germinal center [14]. AID generates ‘intentional’ DNA lesions by deaminating cytosine (C) to uracil (U), and targets both DNA strands in the variable regions of Ig genes. Three alternative mutagenic pathways can process this initial lesion: 1.) Replication across a U instructs a thymidine (T) to DNA polymerases and generates G/C to A/T transitions. 2.) Removal of U by uracil-DNA glycosylase (Ung2) generates a non-instructive apyrimidinic (AP) site. TLS across AP sites mainly generate G/C transversions and may also contribute to G/C transitions. 3.) Alternatively, the U can be recognized as a U:G mismatch by the mismatch repair complex Msh2-Msh6, resulting in exonuclease 1 (Exo-1) activation and the formation of a single-stranded gap. Interestingly, Msh2, Msh6 and Exo-1-deficient B cells lack 80-90% of all A/T mutations, suggesting that the gap-filling process involves TLS polymerases predominantly generating A/T mutations [13]. Since each polymerase displays its own mutagenic signature (error preference), alterations in the mutation spectrum can often be attributed retrospectively to the absence of, or failure in activating specific polymerases. The TLS polymerases η, Rev1 and to some degree polymerase κ are employed during SHM. While Rev1-deficient B cells display reduced frequencies of specific G/C transversions [15, 16], polymerase η-deficient B cells lack a significant fraction of A/T mutations, [17-19]. The deficiency of polymerase κ had no effect on SHM [20], but in the absence of polymerase η, polymerase κ appears to be activated instead to generate A/T mutations, albeit at lower frequency [21].

To test whether the Rad6-dependent polymerase switch is operative in mammalian cells, PCNA mutant mice that contain a lysine 164 to arginine mutation (PCNA<sup>K164R</sup>) were generated. This point mutation prohibits PCNA<sup>K164</sup> modifications without interfering with other pivotal functions of the protein. Analysis of the mutation spectra of mutated Ig genes in B cells from PCNA<sup>K164R</sup> knock-in mice revealed a selective 90% reduction of A/T mutations [22, 23]. In agreement, PCNA knock-out mice reconstituted with a PCNA<sup>K164R</sup> transgene showed a reduction of A/T mutations in Ig genes [24], suggesting that during SHM PCNA-Ub is required to recruit polymerase κ and η to introduce mutations at template A/T.
As PCNA-Ub and PCNA-Ub\(^n\) make use of the same lysine residue, the error-free pathway of DDT, i.e. TS may suppress error-prone TLS, and thereby balance the mutagenic outcome of DDT. In support of this, yeast strains deficient in TS show an increased TLS-mediated spontaneous and damage-induced mutagenesis [25-27]. Furthermore, in human fibroblasts the reduced expression of MMS2 and the inhibition of K63 polyubiquitination have been shown to increase the frequency of UV-induced mutations [8, 28]. In contrast to the overall suppressive function of PCNA-Ub\(^n\) on TLS-mediated mutagenesis, recent studies in *S. pombe* suggest that more complex TLS pathways may actually depend on PCNA-Ub\(^n\) to coordinate a serial activation of lesion bypass and extender TLS polymerases [29]. In addition to its catalytic function in generating PCNA-Ub\(^n\), Rad5 was previously shown to play a minor non-catalytic role in TLS of *S. cerevisiae*. [30, 31]. In summary, these data suggest that Rad5 may have a role in both activating and suppressing TLS. Given the fact that SHM depends on TLS and K164-specific PCNA modification, we questioned whether Rad5-deficiency in mammals may affect the outcome of SHM (Fig. 1). To determine the role of HLTf and SHPRH in PCNA polyubiquitination, survival and somatic hypermutation we analyzed mice deficient for *Shprh* and *Hltf*.

**Figure 1. Role of the Rad6 epistasis group in SHM.** The ring-shaped PCNA homo-trimer encircles DNA, and tethers DNA polymerases to the DNA template. Rad6/Rad18 monoubiquitinates PCNA at lysine 164 (K164). This modification is thought to recruit the TLS polymerase \(\eta\) to generate mutations at template A/T. Further K63-linked polyubiquitination of PCNA-Ub by Ubc13/Mms2 and SHPRH or HLTf activates template switching (TS) and may (?) suppress PCNA-Ub-dependent TLS, i.e. A/T mutagenesis during SHM. Furthermore, PCNA-Ub\(^n\) may stimulate TLS polymerases and affect SHM.
MATERIALS AND METHODS

2.1 Generation and maintenance of mice

To inactivate \textit{Shprh} in the mouse germ line, the embryonic stem cell clone RRJ453 was obtained from himeric mice were generated and crossed onto a 129/Sv background. The following primers were used for PCR genotyping: \textit{Shprh} wild type allele 5’-GCC TGG TGA TCT GTT TGC AGA TAG G-3’ and 5’-CAG TAT GCT GCT CAG AGT AGA TGT G-3’; \textit{Shprh} mutant allele 5’-CAT TCG CCA TTC AGG CTG CGC AAC TG-3’ and 5’-GTT AGC CAC CCC ATT CCA CAG TAT C-3’. RT-PCR using SuperscriptIII (Invitrogen) was performed to check wild-type and mutant \textit{Shprh} transcript using the following primers sets: P1: 5’-GAC TGC ATG CCA CAC ATA ACT TAA TGG-3’ and 5’-CCT AAT GGA CTG CTG CAC AGG CTG C-3’. P2: 5’-GAC TGC ATG CCA CAC ATA ACT TAA TGG-3’ and 5’-GAC AGT ATC GGC CTC AGG AAG ATC G-3’; P3: 5’-CTT TGA GGC ATG GAA GAA GG-3’ and 5’-TTG ATG TGG GGA TCG TAT TT-3’. Western Blot for SHPRH was performed using a rabbit polyclonal antibody raised against amino acids 515–535 of human Shprh [32], which locates N-terminal of the genetrap.

Generation of the \textit{Hltf}-deficient and \textit{Shprh}-deficient mice will be described elsewhere (HD, unpublished data) (JH, unpublished data). To determine HLT f mRNA expression in WT and Hltf/Shprh mutant B cells, total RNA was extracted using RNeasy (Qiagen) according to manufacturer’s protocol. Synthesis of cDNA with Superscript III reverse transcriptase (Invitrogen) was primed with oligo(dT). The following primers were used for detection of \textit{Hltf}: 5’-GCC ATA CCC ATC TTC TTA CAA ACG-3’ and 5’-TTG CAT GTG GCT GCT CAC TGT GA-3’ and \textit{Hprt}: 5’-CTG GTG AAA AGG ACC TCT CG-3’ and 5’-TGA AGT ACT CAT TAT AGT CAA GGG CA-3’. Analyses were carried out using Fast SYBR Green PCR master mix (Applied Biosystems) and the Roche Light-Cycler 480. Results were normalized with respect to HPRT expression. mRNA levels were quantified according to the \( \Delta \Delta \) cycle threshold (Ct) method.

The generation of PCNA\textsuperscript{K164R};Flpe knock-in mice have been described elsewhere [22, 23, 33]. Mice were maintained at the animal facility of the Netherlands Cancer Institute (Amsterdam, Netherlands) and the National Institute of Health (Bethesda, MD). All experiments were approved by an independent animal ethics committee of the Netherlands Cancer Institute and NIH and executed according to national guidelines.

2.2 Class Switch recombination

Naive splenic B cells from three mice per genotype were obtained by CD43 depletion using biotinylated anti-CD43 antibody (Clone S7, BD Biosciences) and the IMag system (BD Biosciences), as described by the manufacturer. Purified B cells were cultured at 10\(^5\) cells/ml in 24 well plates in IMDM, 8% FCS, 50 \( \mu \)M 2-mercaptoethanol, penicillin/streptomycin and 50\( \mu \)g/ml E.Coli LPS (055:B5,
Sigma) either in the presence or absence of IL-4-containing supernatants generated from X63-m-IL-4 cell cultures. Flow cytometric analysis of surface Ig expression was performed on day 4 of culture using goat anti mouse IgM-APC, IgG1-PE and IgG3-PE (Southern Biotech). Data were analyzed using FlowJo 7.6 software.

2.3 Survival
Naïve splenic B cells were obtained and cultured as described above. For UV-C irradiation, 10^6 B cells were irradiated in 0.5 ml medium (254 nm, UV Stratalinker® 2400, Stratagene). Following irradiation, cells were cultured in 1 ml complete medium and LPS. For the survival upon Cisplatin and MMS induced DNA damage, 10^5 B cells were grown in 1 ml complete medium and LPS in the continuous presence of different doses of Cisplatin or MMS. For determining the survival, B cells were harvested after four days of culture and live (propidium iodine negative) B cells were counted by FACS. Data were analyzed using FlowJo 7.6 software.

2.4 Isolation of germinal center B cells and mutation analysis
Germinai center (CD19+, PNA high, CD95+) B cells were sorted from Peyer’s patches. DNA was extracted using proteinase K treatment and ethanol precipitation. The JH4 3′flanking intronic sequence of endogenous rearrangements of VHJ558 family members were amplified during 40 cycles of PCR using PFU Ultra polymerase (Stratagene) [34]. PCR products were purified using the QIAquick Gel Extraction kit (Qiagen) and cloned into the TOPO II blunt vector (Invitrogen Life Technologies) and sequenced on a 3730 DNA analyzer (Applied Biosystems). Sequence alignment was performed using Seqman software (DNAStar). Calculations exclude non-mutated sequences, insertions and deletions. Clonally related sequences were counted only once.

2.5 Detection of PCNA Ubiquitination
The chromatin bound fraction was isolated as previously described [6], with slight modifications. In brief, the harvested cells were resuspended in buffer A (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPes (pH 6.8), 1 mM EGTA, 0.2% Triton X-100, 100 μM NaVO4, 50 mM NaF, and protease inhibitors (Roche)) and incubated for 5 minutes on ice with gentle inverting and centrifugation. The pellet was resuspended in buffer B (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 100 μM NaVO4, 50 mM NaF, and protease inhibitors). After 10 minutes on ice, the samples were sonicated and further incubated for 10 minutes on ice before centrifugation to isolate ‘chromatin bound fraction’. For immunoprecipitation, proteins were pre-cleared by pre-incubation with protein G Sepharose beads (GE healthcare) and incubated with anti-PCNA antibody (PC10, Santa-Cruz Biotechnology). Immune complexes were precipitated with protein G Sepharose beads. Proteins were separated on NuPAGE® Novex 4-12% Bis-Tris Gel (Invitrogen) and transferred to PVDF membranes (Bio-Rad) for immunoblotting following manufacturer’s recommendations.
RESULTS AND DISCUSSION

3.1 Generation of Shprh/Hltf double mutant mice

To study the role of PCNA-Ub in mammalian DDT, we inactivated Shprh and Hltf, the two known mammalian orthologs of S. cerevisiae Rad5, in the mouse germline. To inactivate Shprh we initially took advantage of an exon-trapped ES cell clone, in which a lacZ-Neo exon was inserted into intron 16 of the mouse Shprh locus (Fig. 2a). This insertion results in a fusion protein in which both the ring and the helicase domains are replaced by βgal-neo (Fig. 2b), generating a higher molecular weight product (Fig. 2c). Consistent with the insertion site of the exon trap, ShprhΔ mutant mice lack transcripts containing exon 17 (Fig. 2d). Furthermore, no transcripts containing the ring and helicase domain could be identified in ShprhΔ mutant mice (Fig. 2d). As the ring domain is pivotal for the E3 activity of SHPRH [9], the mutant SHPRH/βgal-neo fusion protein is defective in generating PCNA-Ub. However, given the potential non-catalytic role of Rad5 in controlling TLS in S. cerevisiae [30, 31], a structural role of the remaining SHPRH portion in regulating TLS polymerases independent of PCNA modifications cannot be excluded in this strain of mouse. To investigate this possibility, we also generated Shprh-deficient mice (JH, unpublished data and sup. fig. 1). To generate Shprh/Hltf double mutant mice, the ShprhΔ-mutant and -deficient strain of mice were intercrossed with Hltf-deficient mice, in which the first five exons were replaced by insertion of a selection cassette (HD, unpublished data). Further details on the generation of the Hltf-deficient and Shprh-deficient mice will be described elsewhere. Importantly, no SHPRH protein could be detected in Shprh-deficient mice (Sup. Fig 1). Furthermore, as antibodies specific for mouse Hltf are not available, quantitative RT-PCR reactions covering the ring domain of Hltf were performed. While readily detectable in wild type B cells, Hltf mutant B cells express very low amounts of ring-domain transcripts at one percent of wild type levels (Fig. 2e). To determine the 5’ extension of these residual ring-domain transcripts, we performed RT-PCR reactions with primer sets covering the 5’UTR and the ring-domain of Hltf. These reactions revealed very short Hltf specific ring-domain containing transcripts (data not shown), indicating that the Hltf knock-in renders Hltf non-functional.

3.2 PCNA ubiquitination is significantly reduced, but not abolished in the Shprh/Hltf-deficient cells

As previously reported, PCNA polyubiquination in Shprh- and Hltf- single deficient MEFs is not completely eliminated [10]. The residual PCNA-Ub was explained by a partial redundancy of the two E3 ligases. The derivation of Shprh/Hltf double-deficient MEFs enabled us for the first time to address this issue. MEFs from wild type, Shprh/Hltf, and PCNAK164R mice were UV-C irradiated and analyzed for the formation of PCNA-Ub and PCNA-Ub. As expected, no modification of PCNA could be detected in PCNAK164R mutant MEFs, whereas PCNA-Ub and PCNA-Ub
are detectable in wild type cells (Fig. 3). Interestingly, although reduced, the formation of PCNA-Ub$^\text{n}$ is not completely abolished in \textit{Shprh/Hltf double} deficient MEFs. Apparently, there is at least a third E3 ligase present in MEF, that can generate PCNA-Ub$^\text{n}$ upon DNA damage.

**Figure 2. Generation of Shprh-mutant mice.** (A) Shprh was inactivated in the mouse germ line by making use of an exon-trapped ES cell clone. As indicated schematically, this clone carries a lacZ/Neo$^\text{R}$ exon in intron 16 of mouse Shprh. The location of the three different primer sets (P1-3) used in figure 2D, are indicated. (B) The Shprh mutation generates a ΔShprh-βgal-neo fusion in which the C-terminal portion of SHPRH harboring the ring and helicase domain of SHPRH are replaced by βgal-Neo. The location of the three different primer sets (P1-3) used in figure 2D, are indicated. (C) As revealed by Western blotting, the exon trap strategy only allows the generation of a single mutant protein species, which is consistent with the location of the exon trap and compared to the wild type SHPRH protein has lower mobility. (D) Expression of exon 17 (P1) and the ring and helicase domain (P3) of shprh gene, and the lacZNeo$^\text{R}$ exon (P2) revealed by RT-PCR with Actin transcript level as a loading control. (E) mRNA Levels of the ring domain of Hltf were determined by quantitative real-time PCR in stimulated B cells and related to mRNA levels of Hprt. Data are means of 2 independent reactions.
3.3 Survival and class switch recombination of *Shprh/Hltf*-deficient B cells

It has been previously described that clones with a stable shRNA knockdown of *Shprh* or *Hltf* in HCT116 cells displayed a mild sensitivity to MMS [9, 10]. To explore these findings further, we here determined the DNA damage sensitivity of *Shprh/Hltf* double deficient cells. Primary-activated B cells from wild type and *Shprh/Hltf* double deficient mice were exposed to increasing doses of DNA-damaging agents. Strikingly, wild type and *Shprh/Hltf* double deficient cells are equally sensitive to UV-C, DNA crosslinks, and DNA alkylation, i.e replication blocking lesions (fig. 4). These data suggests that *Shprh/Hltf*-mediated PCNA-Ub is not essential for survival of replicating B cells.

In addition, we determined the capacity of wild type and *Shprh/Hltf* double deficient B cells to undergo class switch recombination. As indicated in figure 4d, no significant differences were observed between wild type and *Shprh/Hltf* double deficient cells.

3.4 Mutation frequencies in wild type, Δ*Shprh/Hltf* and *Shprh/Hltf* double deficient B cells

To determine the contribution of *Shprh/Hltf*-dependent PCNA-Ub in the regulation of SHM, we analyzed SHM in the JH4 intronic region of germinal center B cells using two sets of mice. The first set comprised three Δ*Shprh/Hltf* double mutant and three wild type controls. By sequencing clonally unrelated mutated introns, 1452 mutations were found in 194 sequences from wild type
B cells, and 872 mutations in 124 sequences from ΔShprh/Hltf mutant B cells. The second set comprised three Shprh/Hltf double deficient mice and three wild type controls. 235 mutations were found in 56 sequences from wild type B cells, and 211 mutations in 57 sequences from Shprh/Hltf double deficient B cells. In contrast to previous observations made in S. cerevisiae and human cells defective in generating PCNA-Ub, which demonstrated elevated levels of TLS-dependent mutagenesis, no difference in the frequency of point mutations in somatically mutated Ig genes from ΔShprh/Hltf double mutant B cells and their WT controls or the Shprh/Hltf double deficient B cells and their WT control was found (Table 1).

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Figure 5. Mutations in rearranged J₄ intraclonic sequences from wild type, PCNA<sup>K164R</sup>, ΔShprh/Hltf mutant and Shprh/Hltf-deficient B cells. (A) Pattern of nucleotide substitution. Values are expressed as the total number of mutations and percentage of total mutations. (B) Relative contribution of A/T mutations, G/C transitions (TRS) and G/C transversions (TRV). Values are expressed as the percentage of total mutations.
3.5 Point mutation spectra in wild type, PCNA$^{K164R}$, $\Delta$Shprh/Hltf and Shprh/Hltf double deficient B cells

We previously reported on the analysis of SHM in the JH4 intronic region of germinal center B cells from homozygous PCNA$^{K164R}$ mice, in which all K164-specific PCNA modification are prohibited by the introduction of a lysine to arginine modification at lysine 164 [23]. Hypermutated Ig genes in B cells of these mice revealed a selective reduction of mutations at template A/T, suggesting that PCNA modifications are required in regulating the mutagenic outcome of SHM. To determine the relative contribution of PCNA-Ub and PCNA-Ubn in regulating TLS polymerases during SHM, we here compared SHM between $\Delta$Shprh/Hltf, Shprh/Hltf double deficient, PCNA$^{K164R}$ and wild type mice (Fig. 5). Considering previous findings in yeast ([25-27]), where PCNA-Ubn prohibits TLS, one may expect that SHPRH/HLTF-mediated PCNA-Ubn may interfere with the generation at A/T mutations during SHM (Fig. 1). As the mutation spectra of the wild type controls were very similar, the data sets were combined. Despite the strong inhibition of A/T mutations in K164R mutant B cells, the mutation spectra between wild type, $\Delta$Shprh/Hltf mutant and Shprh/Hltf-deficient B cells remained unaltered (Fig. 5a and Sup Fig 2.). To visualize this more clearly, the relative contribution of G/C transitions generated by replication across U, G/C transversions generated by replication across Ung2 generated AP sites, and PCNA$^{K164}$-dependent A/T mutations were compared (Fig. 5b). While A/T mutations are strongly reduced in the absence of PCNA modification (K164R), the pattern of SHM remained normal in the absence of SHPRH and HLTF. These data exclude a non-catalytic role for SHPRH and HLTF in regulating the outcome of SHM, however the existence of an alternative E3 ligase does not exclude a role for PCNA-Ubn in controlling SHM.

3.6 Concluding remarks

This report indicates for the first time, that inactivation of both SHPRH and HLTF are dispensable for DNA damage survival of primary B cells. This observation likely relates to residual formation of PCNA-Ubn in Shprh/Hltf double deficient cells. These data suggest that in mammals damage-inducible polyubiquitination of PCNA at lysine 164 can be accomplished by at least three alternative E3 ligases. Identification of this alternative E3 ligase will finally help to delineate the contribution of PCNA-Ub and PCNA-Ubn in mammalian DDT and SHM.

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REFERENCE LIST


Sup Figure 1. Generation Shprh-deficient mice. A) Structure of the wild-type genomic SHPRH locus (top) and targeted locus (bottom). Exons 5-13 are indicated by numbered squares. 5' and 3' indicate the position and size of the probes used to screen ES clones for targeted disruption. Most upper line represents the EcoR1 fragment detected in non-targeted alleles, the two bottom lines represent the EcoR1 fragments after targeted disruption of the allele. B) Southern analysis of EcoR1 digested DNA of four individual G418-resistant ES clones. The position of the wild-type allele (12.4 kb) and the targeted allele, detected with the 5' and 3' probe (7.1 and 3.2 kb, respectively) are indicated. C) Immunoblot of protein extracts from wild-type (+/+) and knockout (-/-) mouse embryonic fibroblasts at early (p3) and late (p37) passages using α-SHPRH antibodies[35]. The arrow points to the protein of approximately 193 kDa that is lacking in the knockout (-/-) protein extracts.
Sup Figure 2. Mutations in rearranged J_{4} intronic sequences from wild type, PCNA^{K164R}, ΔShprh/Hltf mutant and Shprh/Hltf-deficient B cells. Pattern of nucleotide substitution. Values are expressed as the total number of mutations and percentage of total mutations.

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