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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-Ubⁿ) 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*^{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-Ubⁿ in mammals. As PCNA-Ub and PCNA-Ubⁿ both make use of K164, error-free PCNA-Ubⁿ-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-Ubⁿ is prohibited in *PCNA*^{K164R} MEFs, the formation of PCNA-Ubⁿ 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-Ubⁿ.

Keywords

PCNA; RAD5; HLTF; SHPRH; Somatic hypermutation; Translesion synthesis

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2010.12.008.

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 (PCNA^{K164}) 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-Ubⁿ in mammalian DNA damage tolerance [9,10].

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 anti-bodies 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 gapfilling 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^{K164R}) were generated. This point mutation prohibits PCNA^{K164} modifications without interfering with other pivotal functions of the protein. Analysis of the mutation spectra of mutated Ig genes in B cells from PCNA^{K164R} knock-in mice revealed a selective 90% reduction of A/T mutations [22,23]. In agreement, PCNA knock-out mice reconstituted with a PCNA^{K164R} 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-Ubⁿ 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-Ubⁿ on TLS-mediated mutagenesis, recent studies in *S. pombe* suggest that more complex TLS pathways may actually depend on PCNA-Ubⁿ to coordinate a serial activation of lesion bypass and extender TLS polymerases [29]. In addition to its catalytic function in generating PCNA-Ubⁿ, 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*.

2. Materials and methods

2.1. Generation and maintenance of mice

To inactivate *Shprh* in the mouse germ line, the embryonic stem cell clone RRJ453 was obtained from BayGenomics. Germ-line competent chimeric mice were generated and crossed onto a 129/Sv background. The following primers were used for PCR genotyping: *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'; *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 *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 genetrapp.

Generation of the *Hltf*-deficient and *Shprh*-deficient mice will be described else-where (HD, unpublished data) (JH, unpublished data). To determine HLTF mRNA expression in WT and *Hltf/Shprh* mutant B cells, total RNA was extracted using RNeasy (Qiagen) according to manufacturers protocol. Synthesis of cDNA with Superscript III reverse transcriptase (Invitrogen) was primed with oligo(dT). The following primers were used for detection of *Hltf*: 5'-GCC ATA CCC ATC TTC TTA CAA ACG-3' and 5'-TTG CAT GTG GCT GCT CAC TGT GA-3' and *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^{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

Naïve 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 μ M 2-mercapthoethanol, penicillin/streptomycin and 50 μ 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, 10^5 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

Germinal 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 min 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 min on ice, the samples were sonicated and further incubated for 10 min on ice before centrifugation to isolate 'chromatin bound fraction'. For immunoprecipitation, proteins were precleared 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.

3. Results and discussion

3.1. Generation of *Shprh/Hltf* double mutant mice

To study the role of PCNA-Ubⁿ 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, *Shprh*Δ mutant mice lack transcripts containing exon 17 (Fig. 2d). Furthermore, no transcripts containing the RING and helicase domain could be identified in *Shprh*Δ 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-Ubⁿ. 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 can not be excluded in this strain of mouse. To investigate this possibility, we also generated *Shprh*-deficient mice (JH, unpublished data and Supplementary Fig. 1). To generate *Shprh/Hltf* double mutant mice, the *Shprh*-mutant and -deficient strain of mice were intercrossed with *Hltf*-deficient mice, in which the 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 (Supplementary 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 polyubiquitination in *Shprh*- and *Hltf*-single deficient MEFs is not completely eliminated [10]. The residual PCNA-Ubⁿ 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* double deficient, and PCNA^{K164R} mice were UV-C irradiated and analyzed for the formation of PCNA-Ub and PCNA-Ubⁿ. As expected, no modification of PCNA could be detected in PCNA^{K164R} mutant MEFs, whereas PCNA-Ub and PCNA-Ubⁿ are detectable in wild type cells (Fig. 3). Interestingly, although reduced, the formation of PCNA-Ubⁿ is not completely abolished in *Shprh/Hltf* double deficient MEFs. Apparently, there is at least a third E3 ligase present in MEF, that can generate PCNA-Ubⁿ upon DNA damage.

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-Ubⁿ 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 Fig. 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-Ubⁿ 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 Δ *Shprh/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-Ubⁿ, which demonstrated elevated levels of TLS-dependent mutagenesis, no difference in the frequency of point mutations in somatically mutated Ig genes from Δ *Shprh/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).

3.5. Point mutation spectra in wild type, PCNA^{K164R}, *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 residue 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 Supplementary 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- [1]. Friedberg EC. Suffering in silence: the tolerance of DNA damage. *Nat. Rev. Mol. Cell Biol.* 2005; 6:943–953. [PubMed: 16341080]
- [2]. Jansen JG, Fousteri MI, de WN. Send in the clamps: control of DNA translesion synthesis in eukaryotes. *Mol. Cell.* 2007; 28:522–529. [PubMed: 18042449]
- [3]. Prakash S, Johnson RE, Prakash L. Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu. Rev. Biochem.* 2005; 74:317–353. [PubMed: 15952890]
- [4]. Hoegge C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature.* 2002; 419:135–141. [PubMed: 12226657]

- [5]. Stelter P, Ulrich HD. Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature*. 2003; 425:188–191. [PubMed: 12968183]
- [6]. Kannouche PL, Wing J, Lehmann AR. Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol. Cell*. 2004; 14:491–500. [PubMed: 15149598]
- [7]. Watanabe K, Tateishi S, Kawasuji M, Tsurimoto T, Inoue H, Yamaizumi M. Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. *EMBO J*. 2004; 23:3886–3896. [PubMed: 15359278]
- [8]. Chiu RK, Brun J, Ramaekers C, Theys J, Weng L, Lambin P, Gray DA, Wouters BG. Lysine 63-polyubiquitination guards against translesion synthesis-induced mutations. *PLoS Genet*. 2006; 2:e116. [PubMed: 16789823]
- [9]. Motegi A, Sood R, Moinova H, Markowitz SD, Liu PP, Myung K. Human SHPRH suppresses genomic instability through proliferating cell nuclear antigen polyubiquitination. *J. Cell Biol*. 2006; 175:703–708. [PubMed: 17130289]
- [10]. Motegi A, Liaw HJ, Lee KY, Roest HP, Maas A, Wu X, Moinova H, Markowitz SD, Ding H, Hoeijmakers JH, Myung K. Polyubiquitination of proliferating cell nuclear antigen by HLTf and SHPRH prevents genomic instability from stalled replication forks. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:12411–12416. [PubMed: 18719106]
- [11]. Unk I, Hajdu I, Fatyol K, Szakal B, Blastyak A, Bermudez V, Hurwitz J, Prakash L, Prakash S, Haracska L. Human SHPRH is a ubiquitin ligase for Mms2-Ubc13-dependent polyubiquitylation of proliferating cell nuclear antigen. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103:18107–18112. [PubMed: 17108083]
- [12]. Unk I, Hajdu I, Fatyol K, Hurwitz J, Yoon JH, Prakash L, Prakash S, Haracska L. Human HLTf functions as a ubiquitin ligase for proliferating cell nuclear antigen polyubiquitination. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:3768–3773. [PubMed: 18316726]
- [13]. Di Noia JM, Neuberger MS. Molecular mechanisms of antibody somatic hypermutation. *Annu. Rev. Biochem.* 2007; 76:1–22. [PubMed: 17328676]
- [14]. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*. 2000; 102:553–563. [PubMed: 11007474]
- [15]. Jansen JG, Langerak P, Tsaalbi-Shtylik A, van den BP, Jacobs H, de WN. Strand-biased defect in C/G transversions in hypermutating immunoglobulin genes in Rev1-deficient mice. *J. Exp. Med.* 2006; 203:319–323. [PubMed: 16476771]
- [16]. Ross AL, Sale JE. The catalytic activity of REV1 is employed during immunoglobulin gene diversification in DT40. *Mol. Immunol.* 2006; 43:1587–1594. [PubMed: 16263170]
- [17]. Zeng X, Winter DB, Kasmer C, Kraemer KH, Lehmann AR, Gearhart PJ. DNA polymerase eta is an A→T mutator in somatic hypermutation of immunoglobulin variable genes. *Nat. Immunol.* 2001; 2:537–541. [PubMed: 11376341]
- [18]. Delbos F, De SA, Faili A, Aoufouchi S, Weill JC, Reynaud CA. Contribution of DNA polymerase eta to immunoglobulin gene hypermutation in the mouse. *J. Exp. Med.* 2005; 201:1191–1196. [PubMed: 15824086]
- [19]. Martomo SA, Yang WW, Wersto RP, Ohkumo T, Kondo Y, Yokoi M, Masutani C, Hanaoka F, Gearhart PJ. Different mutation signatures in DNA polymerase eta- and MSH6-deficient mice suggest separate roles in antibody diversification. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:8656–8661. [PubMed: 15939880]
- [20]. Schenten D, Gerlach VL, Guo C, Velasco-Miguel S, Hladik CL, White CL, Friedberg EC, Rajewsky K, Esposito G. DNA polymerase kappa deficiency does not affect somatic hypermutation in mice. *Eur. J. Immunol.* 2002; 32:3152–3160. [PubMed: 12555660]
- [21]. Faili A, Sary A, Delbos F, Weller S, Aoufouchi S, Sarasin A, Weill JC, Reynaud CA. A backup role of DNA polymerase kappa in Ig gene hypermutation only takes place in the complete absence of DNA polymerase eta. *J. Immunol.* 2009; 182:6353–6359. [PubMed: 19414788]
- [22]. Langerak P, Nygren AO, Krijger PH, van den Berk PC, Jacobs H. A/T mutagenesis in hypermutated immunoglobulin genes strongly depends on PCNAK164 modification. *J. Exp. Med.* 2007; 204:1989–1998. [PubMed: 17664295]

- [23]. Krijger PH, Langerak P, van den Berk PC, Jacobs H. Dependence of nucleotide substitutions on Ung2, Msh2, and PCNA-Ub during somatic hypermutation. *J. Exp. Med.* 2009; 206:2603–2611. [PubMed: 19901081]
- [24]. Roa S, Avdievich E, Peled JU, MacCarthy T, Werling U, Kuang FL, Kan R, Zhao C, Bergman A, Cohen PE, Edelman W, Scharff MD. Ubiquitylated PCNA plays a role in somatic hypermutation and class-switch recombination and is required for meiotic progression. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:16248–16253. [PubMed: 18854411]
- [25]. Broomfield S, Chow BL, Xiao W. MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. *Proc. Natl. Acad. Sci. U.S.A.* 1998; 95:5678–5683. [PubMed: 9576943]
- [26]. Brusky J, Zhu Y, Xiao W. UBC13, a DNA-damage-inducible gene, is a member of the error-free postreplication repair pathway in *Saccharomyces cerevisiae*. *Curr. Genet.* 2000; 37:168–174. [PubMed: 10794173]
- [27]. Johnson RE, Henderson ST, Petes TD, Prakash S, Bankmann M, Prakash L. *Saccharomyces cerevisiae* RAD5-encoded DNA repair protein contains DNA helicase and zinc-binding sequence motifs and affects the stability of simple repetitive sequences in the genome. *Mol. Cell Biol.* 1992; 12:3807–3818. [PubMed: 1324406]
- [28]. Li Z, Xiao W, McCormick JJ, Maher VM. Identification of a protein essential for a major pathway used by human cells to avoid UV-induced DNA damage. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99:4459–4464. [PubMed: 11917106]
- [29]. Coulon S, Ramasubramanian S, Alies C, Philippin G, Lehmann A, Fuchs RP. Rad8(Rad5)/Mms2-Ubc13 ubiquitin ligase complex controls translesion synthesis in fission yeast. *EMBO J.* 2010
- [30]. Gangavarapu V, Haracska L, Unk I, Johnson RE, Prakash S, Prakash L. Mms2-Ubc13-dependent and -independent roles of Rad5 ubiquitin ligase in postreplication repair and translesion DNA synthesis in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 2006; 26:7783–7790. [PubMed: 16908531]
- [31]. Pages V, Bresson A, Acharya N, Prakash S, Fuchs RP, Prakash L. Requirement of Rad5 for DNA polymerase zeta-dependent translesion synthesis in *Saccharomyces cerevisiae*. *Genetics.* 2008; 180:73–82. [PubMed: 18757916]
- [32]. Sood R, Makalowska I, Galdzicki M, Hu P, Eddings E, Robbins CM, Moses T, Namkoong J, Chen S, Trent JM. Cloning and characterization of a novel gene SHPRH, encoding a conserved putative protein with SNF2/helicase and PHD-finger domains from the 6q24 region. *Genomics.* 2003; 82:153–161. [PubMed: 12837266]
- [33]. Langerak P, Nygren AO, Schouten JP, Jacobs H. Rapid and quantitative detection of homologous and non-homologous recombination events using three oligonucleotide MLPA. *Nucleic Acids Res.* 2005; 33:e188. [PubMed: 16340005]
- [34]. Jolly CJ, Klix N, Neuberger MS. Rapid methods for the analysis of immunoglobulin gene hypermutation: application to transgenic and gene targeted mice. *Nucleic Acids Res.* 1997; 25:1913–1919. [PubMed: 9115357]
- [35]. Sood R, Makalowska I, Galdzicki M, Hu P, Eddings E, Robbins CM, Moses T, Namkoong J, Chen S, Trent JM. Cloning and characterization of a novel gene, SHPRH encoding a conserved putative protein with SNF2/helicase and PHD-finger domains from the 6q24 region. *Genomics.* 2003; 82:153–161. [PubMed: 12837266]

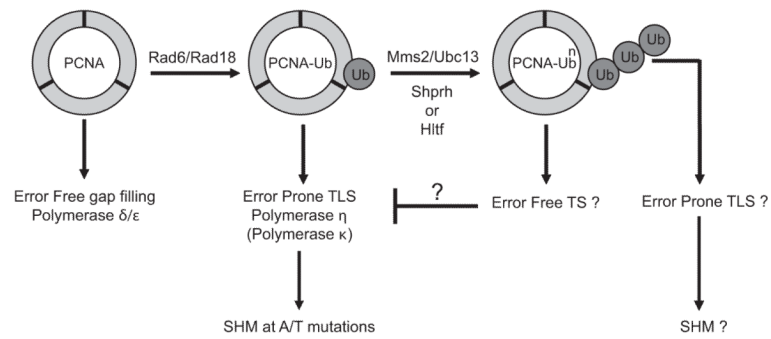


Fig. 1.

Role of the Rad6 epistasis group in SHM. The ring-shaped PCNA homotrimer 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 η 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-Ubⁿ may stimulate TLS polymerases and affect SHM.

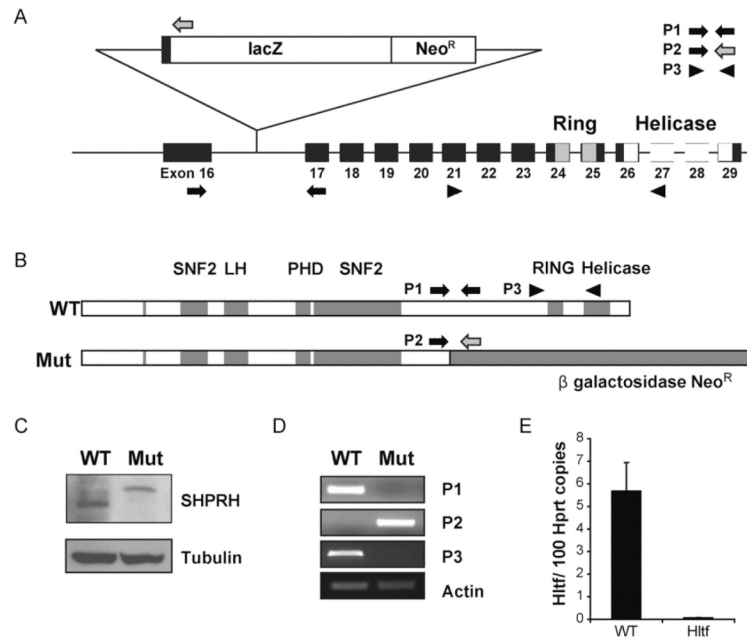


Fig. 2. Generation of Shprh-mutant mice. (A) Shprh was inactivated in the mouse germ line by making use of an exon-trapped ES cell clone. As indicated schematically, this clone carries a lacZ/Neo^R exon in intron 16 of mouse Shprh. The location of the three different primer sets (P1–3) used in (D), are 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 (D), 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 lacZ/Neo^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.

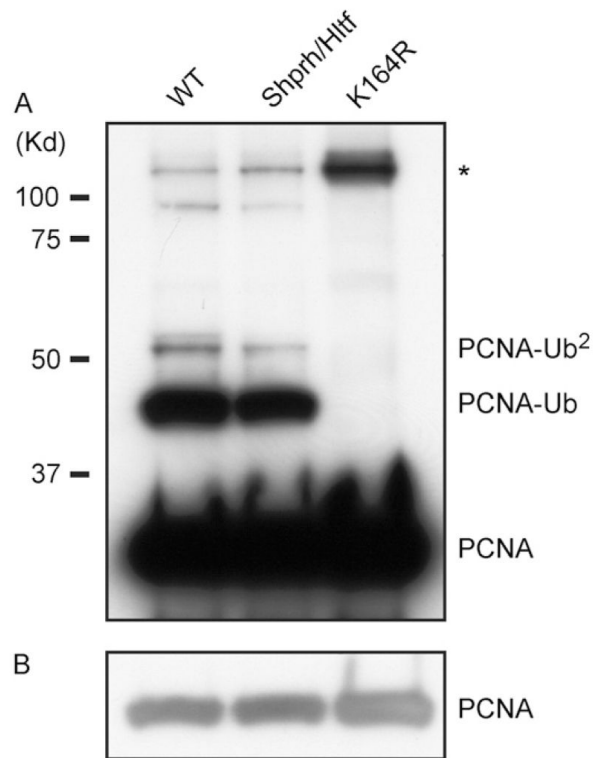


Fig. 3. PCNA Polyubiquitination. (A) MEFs from wild type, *Shprh/Hltf*-deficient, and $PCNA^{K164R}$ mice were irradiated with 60 J/m^2 UV-C and incubated 12 h at 37°C . After isolating chromatin bound fraction from the cells, immunoprecipitations were carried out using an anti-PCNA antibody. The immunoprecipitated proteins were analyzed by immunoblotting with the anti-PCNA antibody to detect the presence of the PCNA ubiquitination. *A protein species of unknown identity. (B) Short exposure of the unmodified PCNA band.

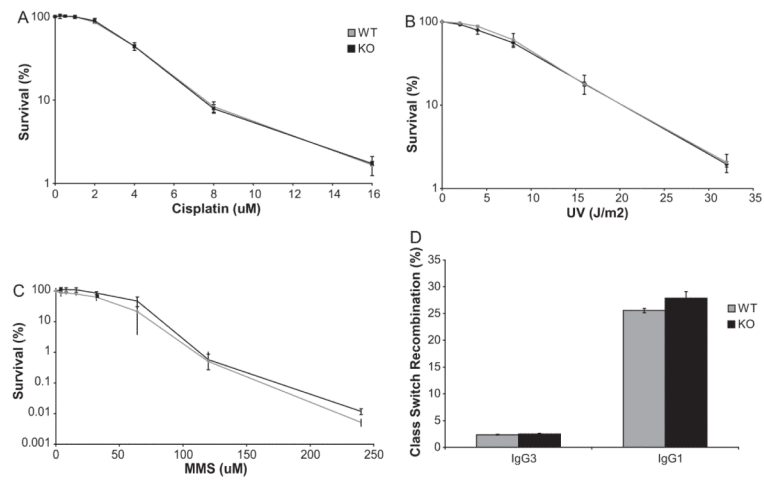


Fig. 4. Survival and Class Switch Recombination. *Shprh/Hltf*-deficient (black) and wild-type (grey) B cells were stimulated with LPS and exposed to increasing doses of either cisplatin (a), UV-C (b), or MMS (c). The percentage of survival after four days of culture is shown. Comparison of wild-type (grey) and *Shprh/Hltf* double-deficient B cells (black bars) switched to IgG3 upon activation by LPS, or switched to IgG1 upon activation by LPS and IL-4 (d).

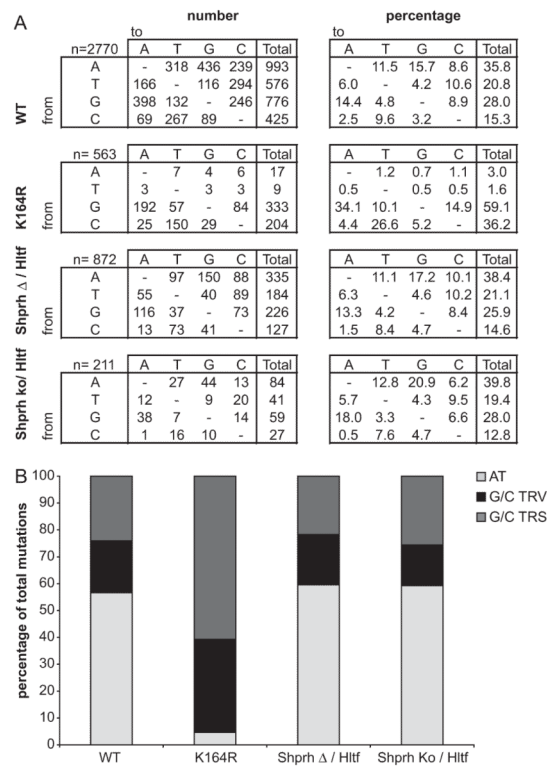


Fig. 5. Mutations in rearranged J_{H4} intronic sequences from wild type, PCNA^{K164R}, Δ Shprh/Hltf mutant and Shprh/Hltf-deficient B cells. (A) Pattern of nucleotide substitution. Values are expressed as the total number of mutations and percentage of total mutations. (B) Relative contribution of A/T mutations, G/C transitions (TRS) and G/C transversions (TRV). Values are expressed as the percentage of total mutations.

Table 1

Mutations in the JH4 intron in Peyer's patch B cells from Δ *Shprh/Hltf* mutant, *Shprh/Hltf* deficient mice and their wild type (WT) controls.

| | WT | Δ <i>Shprh/Hltf</i> | WT | <i>Shprh ko/Hltf</i> |
|--------------------------|--------|----------------------------|--------|----------------------|
| Number of mice | 3 | 3 | 3 | 3 |
| Number of mut. sequences | 194 | 124 | 56 | 57 |
| Total mutations | 1452 | 872 | 235 | 211 |
| Total bp sequenced | 94,602 | 60,567 | 27,948 | 28,479 |
| Mutations/bp (%) | 1.5 | 1.4 | 0.8 | 0.7 |