A/T mutagenesis in hypermutated immunoglobulin genes strongly depends on PCNA^K164 modification

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High-affinity antibodies are generated by somatic hypermutation (SHM) within the variable regions of Ig heavy and light chain genes (1). SHM enables antigen-specific B cells of the germinal center to mutate their Ig genes at a rate of 10⁻³ base pairs per generation, compared with 10⁻⁹ for spontaneous mutations (2). The sequential action of the single B cell–specific DNA lesion inducer activation-induced cytidine deaminase (AID) as well as proteins involved in DNA repair and DNA damage tolerance increase the mutation rate locally by six orders of magnitude (3, 4). AID initiates this process by deamination of cytosine (C) to generate uracil (U) in the variable region of immunoglobulin genes (5). Removal of uracil by the uracil N-glycosylase 2 (UNG2) generates an abasic site. In hypermutating B cells, high fidelity replication over the uracil lesion favors transitions (C to T and G to A), whereas low fidelity replication over abasic sites favors transversions (C to G/A or G to C/T). Consistent with these predictions, mutations at C/G pairs are shifted toward transitions in mice lacking UNG2 (6). These data indicated that lesions, like C to U transitions and noninstructive abasic sites favor the generation of mutations at G/C base pairs (phase I or UNG2-dependent pathway of SHM).

Alternatively, the mismatch repair (MMR) complex human mutS homologue 2 (MSH2)–MSH6 can recognize U/G mismatches (7). Lack of either MSH2 or MSH6 results in a reduction of mutations at template A/T, normally accounting for 50% of all mutations generated, and simultaneously increases mutations at G/C (8–10). The increased mutation frequency at template G/C (G/C bias) observed in MSH2–deficient mice implicates that processing of U/G mismatches by MSH2–MSH6 likely triggers an alternative mutagenic repair pathway that is responsible for the establishment of most mutations at template A/T (phase II or MMR-dependent pathway of SHM). Although SHM is perturbed by a single deficiency in either UNG2, MSH2, or MSH6, combined UNG2/MSH2 or UNG2/MSH6 deficiency leads to a total ablation of SHM at A/T pairs, but, as expected, does not affect replication across the initiating U/G mispair, enabling G to A and C to T transitions (6, 8–12). Thus, two repair pathways, base excision repair and MMR, which are normally effective in restoring U/G lesions, provide alternative pathways in generating somatic mutations.

What allows base excision repair and MMR to become mutagenic, i.e., what prohibits the completion of these faithful repair pathways to enable programmed mutagenesis? The identification and
characterization of damage-tolerant, error-prone replication pathways in yeast provide a possible scenario. Extensive screenings of DNA damage-sensitive mutants led to the identification of the Rad6 epistasis group comprising the ubiquitin-conjugating/ligating complexes Rad6–Rad18 and Mms2–Ubc13–Rad5, the translesion DNA polymerases polη (Polη; Rad30), Rev1, and Polɛ (a heterodimer of Rev3 and Rev7), the high fidelity polymerase Polδ, the SRS2 helicase, and proliferating cell nuclear antigen (PCNA; references 13, 14; Fig. 1).

Although the RAD6 epistasis group was first described in the budding yeast, *Saccharomyces cerevisiae*, functional orthologues have been identified in higher eukaryotic organisms, implying that this pathway is of general importance (15–18). The Rad6 epistasis group provides two alternative pathways to allow stalled DNA replication to continue across damaged templates, such as noninstructive abasic sites (19). Lesions in the DNA template cause replication forks to arrest and trigger Rad6–Rad18–mediated monoubiquitination of PCNA at lysine residue K164 (reference 20; PCNA-Ub). PCNA-Ub serves as a molecular switch for the high fidelity, error-free DNA Polδ and the low-fidelity, error prone translesion DNA synthesis (TLS) polymerases Polη, Rev1, and Polɛ (21). TLS polymerases such as Polη and Rev1 can bypass DNA lesions, and Polɛ can extend replication from non–Watson Crick base pairs to rescue stalled replication forks, albeit often at the expense of accuracy (22). TLS across lesions or even intact templates can be highly error prone and therefore requires stringent control. The existence of PCNA-interacting peptide box (23) and the ubiquitin binding domains Ub-binding motif and Ub-binding zinc finger in TLS polymerases of the Y family of DNA polymerases provided molecular insights into how TLS polymerase are targeted to stalled replication forks to accomplish polymerase switching and TLS activation (24). PCNA-interacting peptide is likely to provide the PCNA specificity, and Ub-binding motifs increase the avidity of this binding. In fact, several TLS polymerases are shown to become more processive when binding to PCNA-Ub (25–27).

The alternative damage tolerance pathway requires Mms2–Ubc13–Rad5–mediated, K63-linked polyubiquitination of monoubiquitinated PCNAK164. Polyubiquitinated PCNAK164 enables template switching to the intact sister chromatid and as a consequence an error-free damage bypass (19). Besides its role as a processivity factor for high and low fidelity polymerases, the PCNA trimer interacts with multiple damage control and repair factors, including the mismatch recognition proteins MSH6 and MSH3 (23).

![Figure 1. Role of the Rad6 epistasis group in DNA damage bypass.](image)

Figure 1. Role of the Rad6 epistasis group in DNA damage bypass. The ring-shaped PCNA homotrimer encircles DNA, and by tethering DNA Polδ to the template it serves as an important processivity factor for DNA replication. In the presence of DNA damage (star), PCNA becomes mono-ubiquitinated at K164 by the ubiquitin-conjugating/ligating complex Rad6–Rad18. PCNA-Ub can directly activate TLS polymerases (like Polη, Rev1, and Polɛ), enabling error-prone damage bypass. Alternatively, K63-linked polyubiquitination of PCNA-Ub by the Rad5–Mms2–Ubc13 complex enables template switching and thus an error-free damage bypass. Besides ubiquitination, PCNA can also be SUMOylated at K164, PCNA-SUMO recruits the anti-recombinogenic Srs2 helicase, which prohibits Rad51 filament formation and is thought to favor damage bypass indirectly. The figure was adapted from Hoege et al. (reference 20). Red circle, ubiquitin; black circle, SUMO.

![Figure 2. Wild-type and mutant PCNA are expressed at equal levels.](image)

Figure 2. Wild-type and mutant PCNA are expressed at equal levels. (A) RT-MLPA (reference 54) on RNA isolated from B and T cells derived from the spleen of wild-type, heterozygous, and homozygous PCNAK164R mice. The relative mRNA level of wild-type and mutant PCNA mRNA determined by MLPA is shown. Heterozygous mutant mice express the wild-type and mutant allele at equal levels. Actin and heat shock protein 90 (HSP90) serve as controls. The error bars represent the standard deviation from six independent experiments. (B) PCNA trimer formation in PCNA mutants. Whereas in wild-type and homozygous mice, only monomorph homotrimers of a 3K or 3R composition are formed, respectively, the mixed pool of PCNA molecules in heterozygous mice allows four distinct compositions: 3K, 2K1R, 1K2R, or 3R at a ratio of 1:3:3:1.
ES cells to transmit the mutation into the mouse germline was tested by intercrossing male chimeras to wild-type mice. Considering the homotrimeric nature of the PCNA sliding clamp and its biallelic expression (Fig. 2A), monomorph PCNA trimers with a 3K and a 0K composition will assemble in the wild-type and homozygous setting, respectively. In the heterozygous setting two equal pools of wild-type PCNA and mutant PCNA K164R coexist (Fig. 2B), enabling the formation of trimers with a 0K, 1K, 2K, and 3K composition at a relative frequency of 1:3:3:1, respectively. If a single wild-type PCNA suffices in mediating damage tolerance, seven out of eight replication complexes (1K, 2K, and 3K complexes) are expected to be functional, and, consequently, the phenotype of heterozygous PCNA K164R mutants should be similar to that of wild type. If a 3K composition is required, replication across damaged templates is effective in just one out of eight cases and heterozygous mutants should be similar to homozygous PCNA K164R mutants. Interestingly, chimeras do transmit the PCNA K164R mutation, giving rise to normally developed heterozygous offspring. To test whether a homozygous PCNA K164R mutation is compatible with mammalian life, the offspring (n = 397) from 70 intercrosses between

RESULTS

Generation of mice carrying a homozygous PCNA K164R mutation
To block PCNA-dependent damage tolerance, a lysine (K) to arginine (R) mutation at residue 164 of PCNA (PCNA K164R) was introduced by targeting an A to G transition in exon 4 of the mouse PCNA locus. The generation of the PCNA K164R targeting construct, targeting of embryonic stem (ES) cells, and development of a high throughput screening system for the simultaneous identification of homologous and nonhomologous recombinants have been published elsewhere (36). ES cells heterozygous for the PCNA K164R mutation were used to derive chimeric mice (37). The capacity of targeted ES cells to transmit the mutation into the mouse germline was tested by intercrossing male chimeras to wild-type mice. Considering the homotrimeric nature of the PCNA sliding clamp and its biallelic expression (Fig. 2A), monomorph PCNA trimers with a 3K and a 0K composition will assemble in the wild-type and homozygous setting, respectively. In the heterozygous setting two equal pools of wild-type PCNA and mutant PCNA K164R coexist (Fig. 2B), enabling the formation of trimers with a 0K, 1K, 2K, and 3K composition at a relative frequency of 1:3:3:1, respectively. If a single wild-type PCNA suffices in mediating damage tolerance, seven out of eight replication complexes (1K, 2K, and 3K complexes) are expected to be functional, and, consequently, the phenotype of heterozygous PCNA K164R mutants should be similar to that of wild type. If a 3K composition is required, replication across damaged templates is effective in just one out of eight cases and heterozygous mutants should be similar to homozygous PCNA K164R mutants. Interestingly, chimeras do transmit the PCNA K164R mutation, giving rise to normally developed heterozygous offspring. To test whether a homozygous PCNA K164R mutation is compatible with mammalian life, the offspring (n = 397) from 70 intercrosses between

Figure 3. Homozygous PCNA K164R mice are born at sub-Mendelian frequency and are infertile. (A) 397 offspring from 70 intercrosses between heterozygous PCNA K164R mutants were genotyped. In contrast to the 25% expected homozygous mutants, only 5% were observed. Wild-type and heterozygous mice were born at a frequency of 34 and 61%, respectively. (B) Embryos from E14.5 intercrosses between heterozygous PCNA K164R mutants with the respective Applied Biosystems sequencing profile of their PCNA alleles are shown. (C) Failure of germ cell development in homozygous PCNA K164R mice. Histological sections of testis and ovary of 3-mo-old mice. Bars: (small) 500 μm; (large) 200 μm. (top four panels) Control or PCNA K164R testes. The control testis shows all stages of normal spermatogenesis whereas PCNA K164R testes show atrophy of spermatogenesis: only Sertoli cells are found, no sperm is detected. The PCNA K164R testes show strong hyperplasia of Leydig cells. (bottom four panels) The normal ovary contains numerous follicles in all stages of development. The PCNA K164R ovary consists predominantly of interstitial cells.
heterozygous mice was genotyped (Fig. 3 A). To our surprise, homozygous mutants are born, albeit at a sub-Mendelian frequency (Fig. 3 B). Overall, only 5% of the progeny carried the PCNA^{K164R} mutation on both alleles compared with the expected 25%. To address whether the PCNA^{K164R} mutation provides a selective disadvantage to homozygous mutant embryos, we genotyped 71 embryonic day (E) 14.5 embryos derived from intercrosses between heterozygous carriers. At day 14.5 of embryonic development 4% homozygous PCNA^{K164R} embryos were found, which is consistent with the 5% observed for the viable offspring. Apparently the selection against homozygous embryos occurs before day E14.5.

**Homozygous PCNA^{K164R} mice are infertile**

Despite the fact that homozygous PCNA^{K164R} mutants are born rather infrequent, survivors develop and grow normally, indicating that the fitness of somatic cells carrying a homozygous PCNA^{K164R} is not drastically altered. In contrast, the finding that homozygous PCNA^{K164R} female and male mice are infertile, in conjunction with a severe hypotrophy of the gonads, suggested a selective defect in germ cell development. A histopathological examination of ovaries and testes revealed a virtual complete absence of germ cells (Fig. 3 C). The selective failure of germ, but not somatic, cell development suggests the existence of a specific PCNA modification essential for germ cells.

**PCNA^{K164R} prohibits damage–induced ubiquitination**

To test whether the PCNA^{K164R} mutation actually prohibits damage-induced PCNA ubiquitination, primary mouse embryonic fibroblasts (MEFs) were derived and genotyped by multiplex ligation-dependent probe amplification (MLPA) to identify mutant and wild-type alleles (reference 36; Fig. 4 A). The chromatin-associated PCNA fractions from nontreated and UV-irradiated primary wild-type and homozygous mutant MEFs were isolated and analyzed for the presence of ubiquitin–conjugated PCNA (Fig. 4 B). Although in nontreated wild-type MEFs the subfraction of PCNA-Ub is clearly detectable and readily increased upon UV irradiation, monoubiquitination of PCNA is lacking in homozygous PCNA^{K164R} mutants and cannot be detected even after prolonged exposure of the x-ray film. These data confirm that the PCNA^{K164R} mutation prohibits damage-induced mono-ubiquitination of PCNA and exclude the existence of alternative damage-induced ubiquitin conjugation sites within mouse PCNA. Because PCNA–Ub is the substrate for Mms2–Ubc13–Rad5–mediated, K63-linked polyubiquitination, homozygous PCNA^{K164R} mutants are expected to be completely defective in PCNA-dependent damage tolerance.

**Survival and proliferation capacity of LPS–activated B cell blasts**

The proliferative capacity and survival of PCNA^{K164R} mutant cells were determined ex vivo for LPS–activated B cell blasts. B cells isolated from the spleen of wild-type, heterozygous, and homozygous mutant mice were loaded intracellularly with the fluorescent cell tracker CFSE and stimulated polyclonally with LPS for 3 d. Comparing the overlay histograms of the CFSE dilution profiles, no substantial differences in the percentage of B cells triggered to divide upon LPS activation (responder frequency), the mean of divisions among those cells that divided at least once (burst size), and the relative frequency of cells with n divisions (proliferative capacity) were found (Fig. 5 A). The survival of LPS–activated B cell blasts was measured by staining phosphatidyl serine in the outer leaflet of the plasma membrane with fluorochemistry-conjugated Annexin V (AnV) and the uptake of the fluorescent, DNA binding molecule propidium iodine (PI) was used to measure the permeabilization of the plasma membrane for small molecules. The frequency of live (AnV\(^{-}\), PI\(^{-}\)), apoptotic (AnV\(^{+}\), PI\(^{-}\)), and dead (AnV\(^{+}\), PI\(^{+}\)) cells at defined time points after polyclonal LPS activation remained indistinguishable (Fig. 5 B). In line with the growth capacity of homozygous PCNA^{K164R} mice, these data suggest that PCNA-dependent damage tolerance plays no major role in determining the proliferative capacity and survival of PCNA^{K164R},\^{K164R} cells in the presence of spontaneous damage.

**Class switch recombination is unaltered in PCNA^{K164R} mutant mice**

Besides SHM, AID is critical in initiating class switch recombination by the deamination of cytosines in Ig switch regions.
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PCNA modification controls A/T mutagenesis

To explore the role of PCNA-dependent damage tolerance in programmed mutagenesis, i.e., the intentional introduction of AID lesions into Ig variable regions and the subsequent recruitment of TLS polymerases to subserve mutator functions (4, 38), the JH4 intronic regions of memory B cells isolated from wild-type, heterozygous, and homozygous PCNAK164R mutants were amplified, sequenced, and analyzed for mutations. Interestingly, the frequency of point mutations in the JH4 intron of memory B cells from these mice (n = 5/genotype) remains quite similar: 1.06% (PCNA+/+), 1.10% (PCNA+/K164R), and 0.79% (PCNAK164R/K164R). The decrease in the mutation frequency is linked to a decrease in the mean number of point mutations per sequence, which is 4.5 in homozygous, 5.6 in heterozygous, and 5.8 in wild-type B cells. The frequency of mutated sequences with n mutations is shown in Fig. 6 A. The distributions of point mutations along the JH4 intron were comparable for all genotypes (Fig. 6 B). Striking alterations were observed when comparing the base exchange pattern of wild-type, heterozygous, and homozygous PCNAK164R mutants. The failure to modify PCNAK164R in homozygous mutant B cells resulted in a 10-fold reduction of transitions and transversions at template A/T—normally accounting for 50% of all mutations generated. The selective failure to mutate template A/T is compensated by an increase in mutations at template G/C, with a noted exception of C to G transversions (Fig. 6, C and D). The decrease of mutations at template A/T and simultaneous increase at template G/C further indicate the existence of two alternative pathways, a major PCNAK164R-dependent A/T mutator pathway and a major PCNAK164R-independent G/C mutator pathway. Interestingly, no significant differences were observed in the base exchange pattern when comparing heterozygous PCNAK164R and wild-type B cells (p-values are provided in Table S1, available at http://www.jem.org/cgi/content/full/jem.20070902/DC1).

**DISCUSSION**

Here we report on the generation of PCNAK614R mutant mice. This mutation prohibits site-specific modifications of PCNAK164R required for PCNA-dependent DNA damage tolerance (20). Surprisingly, homozygous PCNAK614R mice are born, albeit at a sub-Mendelian frequency. Only 5% of the offspring from heterozygous parents were homozygous for the PCNAK164R allele, suggesting a counter selection of homozygous mutants during embryonic development. The counter selection occurs before E14.5, as the same frequency is found at this stage of mouse development. Despite the initial counter selection, our data indicate that mammals can develop in the absence of PCNA-dependent DNA damage tolerance. We are currently addressing the possible activation of compensatory DNA repair or damage tolerance pathways enabling the survival of some homozygous mutants in the presence of spontaneous DNA damage. With the exception of germ cells, somatic cells appear to develop normally in surviving homozygous mice. The infertility, caused by the lack of germ cells, suggests the existence of a PCNAK164 modification that is essential for a germ cell–specific process, possibly linked to meiosis. Besides ubiquitination, the alternative conjugation of the small ubiquitin-like modifier (SUMO) to PCNAK164R has to be considered as an essential PCNA modification for germ cells.
indicating that PCNA K164R mutant B cells switch normally in response to LPS, LPS/IL-4, and LPS/IFN-γ stimulation. To determine the impact of the PCNA K164R mutation on SHM, nonselected mutations from the 5′ region of the JH4 intron were analyzed. PCNA K164R B cells are able to mutate their Ig genes, but at a reduced mutation frequency of 0.79% compared with 1.06 and 1.10% for wild-type and heterozygous mutants. This finding contrasts the phenotype observed in a PCNA K164R chicken DT40 clone (31). Although the overall mutation frequency in the DT40 clone is reduced sevenfold to 15% of wild-type levels, homozygous PCNA K164R mutant B cells maintain the mutation frequency at 75%.

Considerable changes in the base exchange pattern were found in homozygous but not heterozygous PCNA K164R B cells. We observed a 90% decrease in mutations at template A/T, which is a phenotype lacking in a gene conversion-defective

Monoubiquitination of PCNA K164 has been proposed to regulate the recruitment and activity of TLS polymerases, enabling direct replication across damaged as well as undamaged templates (39–41). As TLS polymerases were found to play a role in SHM (29–35) and some TLS polymerases are activated by PCNA-Ub (25–27), we determined the requirement for PCNA-Ub in establishing somatic mutations. Because the mutation load is highest in memory B cells, we chose to analyze SHM in small, class switched CD19+ IgM−, and IgGhigh B cells of the spleen. As these B cells are isolated from nonimmunized mice they are unlikely to be recently activated and therefore are referred to as memory B cells. The normal frequency of these memory B cells in homozygous mutants (unpublished data) already suggested that the PCNA mutation does not affect the ability of B cells to switch their Ig isotype. This is further supported by in vitro observations indicating that PCNA K164R mutant B cells switch normally in response to LPS, LPS/IL-4, and LPS/IFN-γ stimulation. To determine the impact of the PCNA K164R mutation on SHM, nonselected mutations from the 5′ region of the JH4 intron were analyzed. PCNA K164R B cells are able to mutate their Ig genes, but at a reduced mutation frequency of 0.79% compared with 1.06 and 1.10% for wild-type and heterozygous mutants. This finding contrasts the phenotype observed in a PCNA K164R chicken DT40 clone (31). Although the overall mutation frequency in the DT40 clone is reduced sevenfold to 15% of wild-type levels, homozygous PCNA K164R mutant B cells maintain the mutation frequency at 75%.

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chicken DT40 cell line with a PCNA<sup>K164R</sup> alteration (31). The lack of an A/T mutator activity in chicken DT40 cell lines is consistent with other observations indicating that this activity is low in hypermutation-proficient cell lines (42–45). Our in vivo system clearly reveals the existence of a major PCNA-dependent A/T mutator pathway.

Because most A/T mutations (naturally accounting for half of all mutations) are lacking in homozygous PCNA<sup>K164R</sup> B cells, the mutation frequency is expected to be reduced by 50%. The finding that the overall mutation frequency is reduced by only 25% indicates a partial compensation by G/C mutator activities, with the noted exception of C to G transversions. As reported previously, the deoxycytidyl-transferase Rev1 is involved in the generation of both C to G and G to C transversions in mutated Ig genes (29, 30). Remarkably, both TLS polymerases Polη and Rev1 were shown to depend on binding to PCNA-Ub for effective damage bypass (26, 46). The relative reduction in C to G transversions observed in PCNA mutant B cells may therefore be explained by an impaired Rev1 activity. The striking observation that G/C mutations are not impaired in the absence of PCNA<sup>K164</sup> modification strongly implicates the existence of an alternative pathway that allows other TLS polymerases (mainly G/C mutators) to become activated. In fact, the heterotrimeric Rad9–Rad1–Hus1 complex (also known as the 9–1–1 complex), which is structurally similar to PCNA (47), was shown in yeast to interact with TLS polymerases (48, 49) and may thereby provide a platform for PCNA-independent TLS.

Considering the trimeric nature of the PCNA sliding clamp, it is interesting that the mutation frequency, mutation load, and base exchange pattern in heterozygous PCNA<sup>K614R</sup> mice do not differ considerably from wild type. These observations have great implications regarding the dependence of Polη activity on the ubiquitination status of the PCNA trimer. Analysis of PCNA messenger RNA (mRNA) of heterozygous B and T cell blasts reveals that both PCNA alleles are transcribed at equal levels. Therefore, in this setting, only one out of eight PCNA trimers will have a 3K composition and become ubiquitinated at all three K164 residues. Although normally all monomers within the PCNA trimer can become ubiquitinated (28, 39), the unpaired generation of A/T mutations in heterozygous PCNA<sup>K164R</sup> B cells favors the idea that a single PCNA-Ub within the PCNA trimer suffices in activating Polη.

Our data clearly indicate that mutations at template A/T, which resembles 50% of all mutations in hypermutated Ig genes of mammals, strongly depend on PCNA<sup>K164</sup> modification. Interestingly, a very similar phenotype was observed in B cells from Polη<sup>−/−</sup> and MMR-deficient MSH2<sup>−/−</sup> or MSH6<sup>−/−</sup> mice (8–10, 34, 35). By normalizing that published data on MSH2<sup>−/−</sup>, MSH6<sup>−/−</sup>, and Polη-deficient mice, we have compared the hypermutation phenotype between PCNA mutant, Polη-deficient, and MMR-deficient mice for substantial differences in the frequency of A/T mutations as well as base-exchange pattern. Except for Polη-deficient mice, which have a slightly higher remaining frequency of A/T mutations, the aforementioned parameters remain very similar between the different mutants. This suggests that mismatch recognition, PCNA-Ub, and Polη act in concert to establish A/T mutations during SHM in mammalian B cells. The somewhat higher frequency of A/T mutations in the Polη-deficient B cells is likely to be attributed to remaining A to C and T to G transversions. This suggests the existence of a TLS polymerase other than Polη that depends on MMR and PCNA ubiquitination to generate these transversions.

PCNA is highly expressed during S phase and becomes ubiquitinated upon DNA damage to allow replication across DNA lesions (20, 50). These observations implicate that the introduction of mutations at template A/T occurs during replication, which is consistent with the notion that Rad6-dependent damage tolerance is linked to replication (51). This assumption is further supported by the observations that G to A and C to T transitions are increased not just in homozygous PCNA<sup>K164R</sup> mutant B cells but also in Polη- and MMR-deficient B cells (8, 34, 35). The failure to modify PCNA<sup>K164</sup> prohibits Polη and Rev1 activation and thereby favors replicative bypass of uracils by the high fidelity Polδ. Although Polδ can easily explain the increased frequency of G to A and C to T transitions, transversions at G and C likely relate to an Ung2-dependent G/C mutator activity. We speculate that the 25% reduction in the mutation frequency observed in homozygous PCNA<sup>K164R</sup> mice might therefore be attributed to the differential processing of AID-induced lesions, i.e., allowing relatively more A/T mutations to be introduced during long patch MMR-dependent resynthesis compared with G/C mutations in Ung2-dependent short patch resynthesis or Ung2-independent replicative bypass (52).

In conclusion, to explore the role of PCNA monoubiquitination in SHM, we have generated PCNA<sup>K164R</sup> mutant mice. Homozygous mice show a marked reduction in mutations at template A/T and a compensatory increase of mutations at template G/C. The normal base exchange pattern in heterozygous PCNA<sup>K164R</sup> mice implies that a single PCNA-Ub suffices to activate the A/T mutator Polη. The striking similarities regarding the alterations in the base exchange patterns derived from Polη<sup>−/−</sup>, MSH2<sup>−/−</sup>, MSH6<sup>−/−</sup>, and PCNA<sup>K164R</sup> mice strongly indicate that mismatch recognition, PCNA-Ub, and Polη cooperate during replication to establish mutations at template A/T within mammalian Ig genes. This favors a model for phase II mutagenesis in which the MMR pathway is intersected by PCNA-dependent damage tolerance, enabling the recruitment and activation of specific PCNA-Ub–dependent TLS polymerases (preferentially Polη) to generate somatic mutations during replication.

**MATERIALS AND METHODS**

**Generation of PCNA<sup>K164R</sup> mice.** The generation of the targeting construct and the targeting of the ES cells have been described previously (36). The pFlexible construct was provided by A. Bradley (Wellcome Trust Sanger Institute, Cambridge, UK). ES cells were introduced in C57BL/6J blastocysts according to standard procedures (53). Male PCNA<sup>K164R</sup> chimeras were bred to C57BL/6J females to generate heterozygous PCNA<sup>K164R</sup> offspring (F1). To determine germline transmission, tail DNA was analyzed.
for the presence of PCNA\textsuperscript{K164R} by MLPA, as well as sequence analysis. The sequences of the MLPA probes have been published elsewhere (36). PCR primers used for sequence analysis are wild-type PCNA\textsuperscript{K164R} forward (5' GCTGAGGCTTCCCCCTTTCTGACT 3') and reverse (5' GCCGGGCTCCATCCGTCTCCA 3'). The PCR program used was as follows: 3 min at 95°C, 5 min at 75°C, and 1.5 min at 72°C, followed by 30 cycles of 1 min at 94°C, 1 min at 63°C, and 1.5 min at 72°C. The final extension was 10 min at 72°C. The resulting PCR fragments were cloned into a TOPO TA cloning kit (Invitrogen) and sequenced. F1 as well as F2 heterozygous PCNA\textsuperscript{K164R} mice were intercrossed to generate homozygous PCNA\textsuperscript{K164R} offspring. Wild-type and heterozygous littermates of homozygous PCNA\textsuperscript{K164R} mice served as controls for all analyses. All experiments were performed according to national ethical guidelines and all required permissions were obtained.

Histological analysis. Mice were killed and isolated organs were fixed in 4% paraformaldehyde in PBS, pH 7.8, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. These organs include testes, ovary, uterus, mammary glands, prostate, skin, kidney, liver, intestines, thymus, heart, lungs, salivary gland, eye, brain, and pituitary gland.

Characterization of PCNA\textsuperscript{K164R} expression. To compare the expression of wild-type PCNA and mutant PCNA\textsuperscript{K164R}, mRNA in wild-type, heterozygous, and homozygous mice, total RNA was extracted from Con A and LPS blasts using an RNeasy kit (QIAGEN). The RNA was used for RT-MLPA analysis. Synthetic MLPA probes consisting of either two (for the LPS blasts using an RNeasy kit (QIAGEN). The RNA was used for RT-PCNA K164R offspring. Wild-type and heterozygous littermates of homozygous PCNA\textsuperscript{K164R} mice served as controls for all analyses. All experiments were performed according to national ethical guidelines and all required permissions were obtained.

Class switch recombination assay. The assay was performed as described previously (36). In short, T cell–depleted splenocytes were grown per 100,000 cells in a 24-well plate in RPMI medium containing 8% FBS, non-essential amino acids, sodiumpyruvate, 50 μM 2-mercaptoethanol, penicillin/streptomycin, and 25 μg/ml LPS alone (Salmonella typhimurium DIFCO) or in combination with 50 U/ml of recombinant mouse IL–4 (PeproTech) or 100 ng/ml IFN-γ (R&D Systems) for 3 or 4 d. Switching was determined by FACS analysis.

Mutation analysis and statistics. Clonally related sequences (based on their identical third complementarity determining region rearrangements) and duplicated sequences were excluded from the analysis. Statistical analysis of the base exchange pattern was performed using the χ² test.

Online supplemental material. Fig. S1 shows class switch recombination in the absence and presence of PCNA\textsuperscript{K164} modification. Table S1 shows the p-values as determined by the χ² test for the base exchanges shown in Fig. 6. Table S2 shows the oligonucleotide sequences, probe lengths, and competitor to oligonucleotide ratios as used for the RT-MLPA reactions. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070902/DC1.

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