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MEIOTIC RECOMBINATION IS CONTROLLED BY PCNA K164 MODIFICATION

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ABSTRACT

Proliferating cell nuclear antigen (PCNA) is known to be modified by either ubiquitin(s) or small ubiquitin-like modifier(s) (SUMO) at the conserved lysine 164 residue (K164) in S. cerevisiae, while no SUMOylated PCNA has been detected in mammalian cells to date. Previous studies have shown that homozygous Pcna<sup>K164R/K164R</sup> knockin mice display severe defects in germ cell development. Here we have analysed the function of PCNA modification in spermatogenesis in more detail. We show that the essential role of PCNA modification for primordial germ cell survival and/or proliferation is independent of a functional HR6B/RAD18 complex, that functions upstream of PCNA ubiquitylation. In addition, mutation of Polymerase η or double knockout of Htf1/Shprh, that are enzymes known to function downstream of the PCNA ubiquitylation, do not affect primordial germ cell development. However, we cannot exclude partial redundancy between different enzymes involved in translesion synthesis.

In heterozygote Pcna<sup>WT/K164R</sup> knockin mice, we observed an increase of 20% in meiotic recombination frequency in comparison to wild type controls. We show that although HR6B also modifies meiotic recombination, PCNA modification and HR6B most likely act independently in this mechanism. HR6B regulates the number of meiotic DSBs, whereas PCNA modification regulates the choice between crossover and noncrossover formation. We propose that PCNA SUMOylation rather than ubiquitylation is required to recruit an anti-recombinogenic downstream helicase in meiotic prophase nuclei. Increased meiotic recombination is the only phenotypic defect that has been observed to date in heterozygote Pcna<sup>WT/K164R</sup> knockin mice. This points to a possible requirement for SUMOylation of all three subunits of the homotrimeric PCNA ring, whereas ubiquitylation of a single PCNA subunit may be sufficient for functionality.
INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is a processive clamp for DNA polymerases and an essential binding platform for numerous proteins involved in DNA replication, repair, and cell cycle regulation. PCNA forms a homotrimer that encircles double-stranded DNA, and operates as a sliding clamp to keep the DNA polymerase machinery firmly on the DNA during DNA replication (reviewed in [1]).

During DNA replication, the presence of unrepaired DNA lesions threatens to block progression of the replication machinery, which might result in the formation of DNA double-strand breaks (DSBs) and gross chromosomal rearrangements, or even to a permanent cell-cycle arrest and cell death. Replication damage bypass (RDB) is a special pathway that allows progression of DNA replication without actually removing the lesion (reviewed in [2]).

In *Saccharomyces cerevisiae*, the E2 ubiquitin-conjugating enzyme Rad6 is essential for this pathway. Depending on interactions with downstream components, error-free or error-prone sub-pathways can be activated. The first step in both pathways involves mono-ubiquitylation of PCNA at conserved lysine residue 164 (K164) by the Rad6-Rad18 complex, in which Rad18 acts as an ubiquitin ligase (E3 enzyme) [3]. Mono-ubiquitylation of PCNA by the Rad6-Rad18 complex recruits specific translesion synthesis (TLS) polymerases that can incorporate nucleotides in the strand opposite the site of the DNA lesions, and this process may usually be error-prone (reviewed in [4,5]). Alternatively, mono-ubiquitylation by the Rad6-Rad18 complex may be followed by Rad5-Mms2-Ubc13-mediated poly-ubiquitylation [3]. This polyubiquitylation involves the formation of K63-linked polyubiquitin chains. Ubc13 is the only known enzyme that can stimulate the formation of K63-linked polyubiquitin chains. K48-linked polyubiquitylation usually targets substrates for degradation by the proteasome, whereas K63-linkage does not. The E3 ubiquitin ligase Rad5 interacts with both the Rad6-Rad18 and the Mms2-Ubc13 complexes to stimulate poly-ubiquitylation of PCNA. Subsequently, polyubiquitylated PCNA enables template switching to the intact sister chromatid and as a consequence an error-free damage bypass pathway can be activated [6]. In *S. cerevisiae*, K164 residue of PCNA is known to be modified not only by ubiquitin but also by small ubiquitin like modifier (SUMO) [3]. This modification is mediated by the SUMO ligase Siz1 and causes the recruitment of a helicase Srs2 to replication forks during S phase in order to prevent unwanted crossover events through its ability to disrupt Rad51 nucleoprotein filaments [7-11]. Srs2 was originally identified as a suppressor of *rad6* and *rad18* mutants, and has been previously proposed to be a regulator of the Rad6-dependent pathways [12].

Functional orthologs of proteins involved in the RDB pathway have been identified in mammals, implying that this pathway is generally well conserved [13]. In mammalian cells, RAD18 complexes with the mammalian orthologs of yeast Rad6, HR6A (UBE2A) and HR6B (UBE2B) [14], and mediates PCNA mono-
ubiquitylation at K164 when the replication machinery is stalled by UV-induced DNA damage to recruit one of the TLS polymerase families, Polη [15]. Mammalian orthologs of yeast Rad5, HLTF and SHPRH, mediate PCNA polyubiquitylation with mammalian MMS2-UBC13, and maintain genomic stability probably via the conserved error-free pathway of RDB [16-19]. PCNA is highly conserved as is K164, suggesting that PCNA might be regulated by SUMOylation as well as ubiquitylation in mammals, in a manner similar to that in S. cerevisiae. However, no SUMOylated forms have so far been detected in mammalian PCNA.

Recently, mice carrying a lysine (K) to arginine (R) mutation at lysine residue 164 of PCNA (K164R) were generated, using two different approaches [20,21]. In the first approach, knockin mice carrying the K164R mutation in Pcna were generated [20], whereas in the second approach a transgene carrying the K164R mutation was expressed on a Pcna knockout background [21]. Both K164R mouse models showed reduced somatic hypermutation and male infertility, but the K164R knockin mice showed a much severer phenotype, in particular in the testis [20] than the K164R transgenic mice. The latter displayed meiotic arrest in early pachytene with elongated chromosome axes [21], whereas no germ cells were detected in testes from K164R Pcna knockin mice [20]. The difference in phenotypes might be caused by differences in expression level of mutant PCNA; if the transgene is overexpressed, this may somehow help to overcome the lack of PCNA modification, resulting is a less severe phenotype. The role of PCNA modification in gametogenesis is at present unknown. The mammalian Rad6 orthologs HR6A and HR6B have essential functions in female and male gametogenesis, respectively [22,23], but it is not known whether any of the reproductive functions of HR6A and HR6B depends on the ability of HR6A and HR6B to ubiquitylate PCNA. Elongated chromosomal axes in spermatocyte nuclei as described for the transgenic K164R mice [21] have also been observed in Hr6b knockout mice [24]. In Hr6b knockout mice, these elongated axes are associated with increased meiotic recombination frequency ([24], see also below). This fits with the general correlation between chromosomal axis length and meiotic recombination frequency that has been described [25]. Meiotic recombination is initiated with the induction of meiotic DSBs by the topoisomerase II-like enzyme SPO11. In mitotic cells, DSBs may be repaired either through the non-homologous end-joining pathway (NHEJ) or via homologous recombination (HR). In meiotic cells, the error-prone NHEJ process is repressed [26], leaving HR as the only available pathway for repair. HR, using one of the chromatids of the homologous chromosome as a template for repair, may lead to the formation of crossovers. In mouse and man only a small minority of the DSBs lead to the formation of crossovers, but each chromosome pair has at least one (obligate) crossover. Repair of most meiotic DSBs leads to so-called gene conversions or noncrossovers (reviewed in [27]). Repair of meiotic DSBs is accompanied by, and essential for, chromosome paring and synapsis of homologous chromosomes. Synapsis is achieved through the formation of the synaptonemal complex (SC) along the
chromosomal axes of the paired chromosomes. The SC consists of two lateral elements that follow the chromosomal axes of each chromosome, connected by a central element. Before synapsis, the lateral elements are called axial elements, and these start to form in the leptotene stage of meiotic prophase, when the DSBs have just formed. In zygotene, the axial elements are complete and synapsis is initiated. In pachytene, synapsis is complete. At this stage, the crossover sites can be visualized through immunocytochemical analysis of the mismatch repair enzyme MLH1. This protein forms a single focus at each crossover site on the SC. Subsequently, the SC is disassembled in diplotene, and in metaphase I, the actual crossovers are visible as chiasmata. The number of crossovers is tightly regulated. A mechanism termed crossover interference reduces the likelihood of two crossovers occurring in close proximity of one another. Furthermore, it is ensured that each chromosome pair contains at least a single crossover site. The most outspoken change in crossover frequency is observed in mice that are deficient for the ubiquitin conjugating enzyme HR6B [24]. Hr6b knockout male mice are infertile, associated with dysregulation of chromatin structure in meiotic and post-meiotic cells [23,24,28]. In late pachytene nuclei of these cells, the SCs are longer, and the number of MLH1 foci is 20% increased in comparison to controls [24], but crossover interference is not affected [29].

Herein, we analyzed the mice carrying a point mutation at lysine K164 (K164R) in Pcna to study the role of K164 modification of PCNA during spermatogenesis. In addition, we analysed the meiotic phenotype of several knockout mouse models that carry mutations in genes that function downstream or upstream of PCNA ubiquitylation in the RDB pathway.

MATERIALS AND METHODS

Mice
Generation of PCNA K164R knockin mice [20] and Polh knockout mice [30] has been described. HLTF/SHPRH double knockout mice were kindly provided by Kyungjae Myung (National Institute of Health, USA).

Antibodies
For primary antibodies, we used the mouse monoclonal antibodies anti-MLH1 (Becton and Dickinson), the rabbit polyclonal antibodies anti- RAD51 [31], and anti-SYCP3 (gift from C. Heyting). For secondary antibodies, we used a goat anti-rabbit IgG alexa 488/564 or goat anti-mouse alexa IgG 488/564 (Molecular Probes).

Meiotic spread nuclei preparations and immunocytochemistry.
Testis tissues were processed to obtain spread nuclei for immunocytochemistry as described by Peters et al [32]. Spread nuclei of spermatocytes were stained with antibodies mentioned above. Before incubation with antibodies, slides
were washed in PBS (3x10 min), and non-specific sites were blocked with 0.5% w/v BSA and 0.5% w/v milk powder in PBS. Primary antibodies were diluted in 10% w/v BSA in PBS, and incubations were overnight at room temperature in a humid chamber. Subsequently, slides were washed (3x10 min) in PBS, blocked in 10% v/v normal goat serum (Sigma) in blocking buffer (supernatant of 5% w/v milk powder in PBS centrifuged at 14,000 rpm for 10 min), and incubated with secondary antibodies in 10% normal goat serum in blocking buffer at room temperature for 2 hours. Finally, slides were washed (3x10 min) in PBS (in the dark) and embedded in Prolong Gold with DAPI (invitrogen).

**Histological analysis and TUNEL assay**

Testes were isolated from control, *Hlf/Hlf* double knockout, *Po1* knockout, heterozygous PCNA WT/K164R, and homozygous PCNA K164R/K164R mice that were 2 and 4 weeks old. Testes were fixed in 4% paraformaldehyde (TUNeL) or Bouins’ fixative for morphological analysis, and embedded in paraffin according to standard procedures. For histological analysis, mounted sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. For TUNeL assay, sections were mounted on aminoalkylsilane-coated glass slides, dewaxed, and pretreated with protease K (Sigma) and peroxidase as described elsewhere [33]. Slides were subsequently washed in terminal deoxynucleotidyl transferase (TdT) buffer for 5 min [34] and then incubated for at least 30 min in TdT buffer containing 0.01 mM Biotin-16-dUTP (Roche) and 0.4 U of TdT enzyme (Promega). The enzymatic reaction was stopped by incubation in TB buffer, and the sections were washed [33]. Slides were then incubated with StreptABComplex-horseradish peroxidase conjugate (Dako) for 30 min and washed in PBS. dUTP-biotin labelled cells were visualized with 3,3-diaminobenzidine tetrahydrochloride-metal concentrate (Pierce, Rockford). Tissue sections were counterstained with nuclear fast red–5% (wt/vol) (Al₂(SO₄)₃). For each animal, the number of TUNeL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling)-positive cells was counted in at least 200 tubule sections, and the average number of positive cells per 100 cross sections was calculated. Data were analyzed by using the Mann-Whitney U test.

**Confocal microscopy**

Images of cells were obtained using a Zeiss LSM510NLO microscope (Carl Zeiss) with a 63 × /1.40 NA oil immersion lens. Proteins stained with alexa 488 were detected by exciting the probes with a 488 nm Argon gas laser and monitoring the emission through a 500-550 band-pass filter. Proteins stained with alexa 564 were detected by exciting the probes with a 543 nm helium neon laser and monitoring the emission through a long-pass 560 filter. To minimize the effect of photo-bleaching, images were taken with 10 μW for a 488 nm laser, and with 20 μW for a 543 nm laser. For quantification of immunofluorescent signal, slides were analyzed on the same day. Fluorescent images were taken under identical
conditions for all slides, and images were analysed using the ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA [http://rsb.info.nih.gov/ij/]). Nuclei were selected and the mean signal ($\mu$) and standard deviation ($\sigma$) was calculated by Image J software, and threshold was determined [35]. To count the number of RAD51 foci, threshold was set at; $\mu + 2.5*\sigma$, and the number of foci was calculated using Image J. To prevent counting of non-specific background as RAD51 foci, areas less than 2 pixels were excluded from counting.

**RESULTS**

**Lack of primordial germ cells in homozygous PCNA$^{K164R/K164R}$ mice, and increased apoptotic cells in heterozygous PCNA$^{WT/K164R}$ mice**

We previously found that mutation of lysine 164 of PCNA (K164R) leads to infertility in both female and male mice, in association with complete absence of germ cells [20]. All wild-type, heterozygous, and homozygous mice showed similar body weight, while the testis of PCNA$^{K164R/K164R}$ mice weighed only 20% of testis of wild type and PCNA$^{WT/K164R}$ mice (Figure 1A). Next, we analyzed young mice to study whether some germ cells, including cells in meiotic prophase, might still be present in immature testes. However, similar to our previous observations [20], already in 4-week-old mice, the seminiferous tubules were devoid of germ cells. To investigate this further, we decided to analyse testes before birth. Primordial germ cells arise outside the gonads and migrate into the gonads around day 11 of mouse embryonic development (E11). During this period, the primordial germ cells increase in number through mitotic proliferation. Around E13, the germ cells enter a mitotic arrest, which is maintained until the process of spermatogenesis is initiated shortly after birth. In E18, neither seminiferous tubules formation nor primordial germ cells were found in PCNA$^{K164R/K164R}$ mice, suggesting a developmental retardation of gonads as well as primordial germ cells (Figure 1B). The lack of germ cells in homozygous PCNA$^{K164R/K164R}$ mice indicates that modification of PCNA is essential for the proliferation or survival of primordial germ cells. Heterozygous PCNA$^{WT/K164R}$ mice did not show any phenotype with respect to the number of germ cells compared with wild-type (Figure 1B and C). However, we observed an approximately 1.5-fold increase in the number of apoptotic spermatocytes in heterozygous PCNA$^{WT/K164R}$ mice by TUNEL at age of 4 weeks (Figure 1D). Downstream of PCNA monoubiquitylation by HR6A/B-RAD18, Pol $\eta$ can be recruited to mediate error-prone RDB. Alternatively, RAD5 orthologs may mediate PCNA polyubiquitylation together with UBC13, to allow error free RDB. To analyse whether impairment of any of these downstream events may cause the loss of germ cells in the homozygous PCNA knockin mice, and the increased apoptosis of the heterozygote, we analysed testes from mice that
carried mutations in both mouse RAD5 orthologs, Hltf/Shprh double knockout mice (Figure 2A) and from Polh knockout mice (Figure 2B). The total number of germ cells as well as the number of apoptotic spermatocytes was not different from controls in testis sections from these mice (Figure 2C and D).

**Normal SYCP3 length in pachytene spermatocytes of PCNA<sup>WT/K164R</sup> mice**

Although we could not identify meiotic prophase cells in Pcna<sup>K164R/K164R</sup> testes, the increased apoptosis of spermatocytes in the heterozygotes suggested a meiotic phenotype in these mice. Since elongated chromosomal axes have been

![Figure 1. Characterization of wild-type, heterozygous, and homozygous PCNA<sup>WT/K164R</sup> mice.](image)

(A) Body and testis weight from 4-week-old wild type, heterozygous Pcna<sup>WT/K164R</sup> and homozygous Pcna<sup>K164R/K164R</sup> mice. Error bars indicate SEM values from 7 wild type, 8 heterozygous, and 4 homozygous mice. (B, C) Histological sections of embryonic day 18 (B) and 4-week-old (C) wild type, heterozygous Pcna<sup>WT/K164R</sup> and homozygous Pcna<sup>K164R/K164R</sup> mouse testes were stained with GCNA. (D) The number of apoptotic cells per 100 tubuli from 4-week-old wild type and heterozygous Pcna<sup>WT/K164R</sup> mice. Error bars indicate SEM values from 2 wild type and 2 heterozygous mice.
reported for spermatocytes from transgenic $Pcna^{+/tgK164R}$ mice [21], we analysed this parameter in our heterozygous $PCNA^{WT/K164R}$ mice. The length of SYCP3 was measured in MLH1-positive mid-pachytene nuclei. In contrast to transgenic PCNA K164R mice, we did not find elongation of SYCP3 length in $PCNA^{WT/K164R}$ mice (Figure 3A).

**PCNA modification regulates meiotic recombination frequency**

In *S. cerevisiae*, PCNA SUMOylation is mediated by the SUMO ligase Siz1 and this modification causes the recruitment of the helicase Srs2 to replication forks
during S phase. At these sites, Srs2 prevents unscheduled crossover events through its ability to disrupt Rad51 nucleoprotein filaments [7-11,36]. To investigate whether PCNA modification may control meiotic crossover frequency...
in mice, we examined the nuclear localization of RAD51 and MLH1 in meiotic spread preparations of wild type and PCNA WT/K164R spermatocytes. RAD51 functions as a single-stranded DNA-binding protein that has DNA-dependent ATPase activity and stimulates strand exchange. This protein accumulates in foci in leptotene and zygotene spermatocytes. These foci depend on the presence of the DSB-inducing enzyme SPO11, and are therefore thought to represent meiotic DSB repair sites. We observed no abnormalities in the overall pattern of RAD51 localization in PCNA WT/K164R spermatocytes (data not shown). In addition, the number of RAD51 foci in leptotene nuclei was not different between wild type and PCNA WT/K164R spermatocytes (Figure 3B). Subsequently, MLH1 foci were counted in mid-to-late pachytene spermatocytes to determine the crossover number. Intriguingly, we detected an increased number of MLH1 foci in leptotene PCNA WT/K164R spermatocytes (27.0 +/- 0.1) compared to wild type pachytene spermatocytes (22.3 +/- 0.2) (Figure 3C and D). The same analyses were performed for testes from Hltf/Shprh (Figure 4A), and Polη (Figure 4B) deficient mice, but no differences in the number of MLH1 foci between the knockouts and control mice were observed. Previously, we have shown that spermatocytes from Hr6b knockout mice also show a significantly increased number of MLH1 foci [24]. To analyse whether these effects on meiotic recombination could occur through the same pathway, we also analysed the number of RAD51 foci in Hr6b knockout mice. In contrast to the normal number of RAD51 foci in leptotene PCNA WT/K164R spermatocytes, an increased number of RAD51 foci was found in leptotene and zygotene Hr6b knockout spermatocytes compared with wild type spermatocyte (Figure 3E). This indicates that in Hr6b knockout mice, an increased number of meiotic DSBs can explain the increased number of MLH1 foci. In addition, we found that the increased number of RAD51 foci in Hr6b knockout mouse spermatocytes was not damage-induced but SPO11 dependent (Figure 3E). Taken together, these results suggest that the increased number of crossover sites observed in PCNA WT/K164R and Hr6b knockout mice are regulated by distinct mechanisms.
DISCUSSION

In yeast, K164 of PCNA is known to be modified by ubiquitin, and also by SUMO, depending on the context [3]. PCNA SUMOylation is a key regulator to suppress unwanted homologous recombination and to facilitate error-free RDB during replication in yeast somatic cells [8,9]. Here we have analysed the role of PCNA K164 modification during spermatogenesis in mice.

**PCNA modification is crucial for proliferation of primordial germ cells**

Our results show that disruption of the modification of PCNA at K164 residue results in the complete absence of primordial germ cells. The number of primordial germ cells was not reduced in the heterozygote, in Polh–deficient testes nor in testis of a double mutant for the known mouse Rad5 homologs. This raises the question whether this defect is caused by defective SUMOylation or ubiquitylation. PCNA ubiquitylation is thought to depend on the HR6A/B-RAD18 complex. Mice deficient for HR6A or HR6B have normal numbers of primordial germ cells (our own unpublished observations), and double-mutant mice are not viable. Rad18 knockout mice show no overt defects in spermatogenesis in young adults, indicating that the number of spermatogonial stem cells is normal [37]. We have analyzed Rad18 knockdown mice, and also observed that the number of spermatogonial stem cells was normal (Inagaki et al., manuscript in preparation). These data indicate that primordial germ cell proliferation/survival might be independent of PCNA ubiquitylation. Alternatively, another E2/E3 complex may mediate basal PCNA ubiquitylation required for primordial germ cell proliferation/survival. In this context it is of interest to note that a severe reduction of stem cells has also been observed in Rev1-deficient mice (our own unpublished observations). These mice display transient growth retardation, and defects in C/G transversions in hypermutating immunoglobulin genes [38]. REV1 is a Y polymerase that can be recruited to a damaged replication template, and like mono-ubiquitylated PCNA, regulates TLS polymerases. [38-40]. Another DNA repair enzyme that is required for primordial germ cell survival and proliferation is FANCC [41]. This protein is part of an E3 ubiquitin ligase complex that ubiquitylates FANCD2/FANCI complex in the Fanconi anaemia (FA) pathway of DNA damage signalling. Apparently, primordial germ cells have special requirements for the RDB and FA pathways.

Roa et al. showed that in Pcna+/tg<sup>K164R</sup> mice, germ cells are able to reach the early pachytene stage [21]. Although it is possible that the number of stem cells is also reduced in these mice, the phenotype is obviously milder. This difference may be explained by possible differences in genetic background (FVB versus C57BL/6J) of the two strains, or the two- to eight-fold overexpression of the mutant PCNA transgene in the Pcna+/tg<sup>K164R</sup> mice may somehow rescue part of the defect [21].
HR6B modifies meiotic recombination frequency through regulation of the number of meiotic DSBs

Crossovers are crucial for faithful meiotic chromosome segregation, and their formation requires the formation of programmed DSBs by SPO11 [42], followed by HR repair using one of the chromatids of the homologous chromosome as a template for repair. When DSB formation is reduced in S. cerevisiae spo11 mutants, normal meiotic crossover frequencies are maintained. This phenomenon is referred to as crossover homeostasis [43]. Overexpression of Spo11 in S. pombe does not increase meiotic frequency above wild type levels [44], and in C. elegans there are indications that crossover homeostasis also quenches the effect of extra (radiation)-induced DSBs on crossover frequencies. In mice, the induction of approximately 100 extra DSBs through irradiation leads to only 1 extra crossover site on average, indicating that these breaks are preferentially repaired via noncrossover pathways. Previously, we had shown that Hr6b-deficient spermatocytes show an approximately 20% increase in meiotic recombination frequency. Here we show that this increase can be explained by a 15% increase in SPO11-induced DSB formation. Lynn et al. have shown that there appears to be a positive correlation between normal length of the SC and the number of meiotic recombination sites, indicating that the length of the SC reflects genetic rather than physical distance [25]. In Hr6b knockout mice, in accordance with the increased recombination rate, we also measured an increase in SC length in comparison to wild type, but this effect was only seen in late pachytene nuclei. This suggests that the increased SC length may be a manifestation of aberrant chromatin structure caused by Hr6b deficiency [24,28]. Still, aberrant chromatin structure, leading to a more open chromatin configuration, may allow the formation of more DSBs in leptotene, and subsequent extra crossover sites. In C. elegans, chromosome axis length is determined by condensin [45], which in turn controls the number of meiotic DSBs. Introducing extra DSBs with ionizing radiation or eliminating meiotic DSB formation altogether using a Spo11 mutation had no effect on axis length in either the wild type or in the condensin I complex mutants [45], indicating that axis length is not influenced by DSBs, but axis structure regulates DSB frequency. If this mechanism is conserved from worm to mouse, it might be suggested that HR6B may functionally interact with condensin, regulating chromosome structure and hence, the number of DSBs, SC length, and crossover frequency.

PCNA modification at K164 regulates crossover frequency

In contrast to the positive correlation between the number of DSBs, SC axis length and crossover frequency in Hr6b knockout spermatocytes, we observed a 19% increase in the number of MLH1 foci in Pcna WT/K164R mice compared to controls, but no effect on the number of DSBs or SYCP3 length. Thus, although this indicates that both PCNA K164 modification and HR6B activity regulate meiotic crossover
frequency to the same extent, they may operate via distinct and independent mechanisms. In support of this conclusion, we have not found an effect of RAD18 depletion in testes on meiotic recombination frequency (Inagaki et al., manuscript in preparation), indicating that HR6B regulates crossover frequency independent of the RDB pathway, possibly involving functional interaction with condensins, as described above. For PCNA, the regulation of crossover frequency through its modification at K164, most likely involves modification of the choice between crossover and noncrossover pathways, since the number of meiotic DSBs in leptotene was not different from controls. PCNA has been implicated in the suppression of recombination events during replication [8,9]. This function of PCNA requires its SUMOylation at K164. This modification recruits the helicase Srs2 [8,9]. Srs2 functions as an anti-recombinase through different mechanisms during separate stages of HR. At an early stage, Srs2 dismantles the RAD51 presynaptic filament. During the next stage of HR, when RAD51 coated ssDNA has invaded the homologous template, Srs2 disrupts the D-loop intermediate [7,46]. It has not yet been established whether this mechanism is also operative in mammals, but several possible functional homologs of Srs2 have been identified. Three different proteins, named RecQL5, FBH1 and BLM have been shown to be able to displace RAD51 from ssDNA and to suppress recombination [47-49]. BLM and an additional possible functional Srs2 homolog named RTEL1 have been shown to dissociate D-loop recombination intermediates [47,50]. In C. elegans, mutation of Rtel1 leads to elevated numbers of crossover [51]. Taking these data into account it may be suggested that the effect of K164 mutation on recombination frequency that we observed is caused by a lack of PCNA SUMOylation, leading to decreased function of downstream anti-recombinogenic helicases at sites of meiotic DSB repair. An attractive candidate helicase that may function downstream of PCNA SUMOylation in meiosis is BLM. BLM localizes as foci in leptotene and zygotene nuclei. Using a conditional knockout approach, it was recently shown that deletion of Blm leads to a dramatic increase in meiotic recombination frequency in affected cells [52]. To obtain more insight in the possible anti-recombinogenic effect of the K164R mutation we analyzed RAD51 and RPA foci in zygotene and pachytene spermatocytes of wild type and Pcna<sup>WT/K164R</sup> mice, but no overt change in the patterns of RAD51 and RPA foci was observed (data not shown). However, it must be noted that the foci pattern were highly dynamic, making it impossible to detect subtle shifts in the appearance and disappearance of RAD51 and RPA foci. Further investigations are required to determine which, if any, helicase is recruited to regulate meiotic crossover frequency via SUMOylated PCNA. In addition, it will be important to establish whether the distribution of crossovers is similar in spermatocytes of wild type and heterozygote mice.
PCNA ubiquitylation and SUMOylation

Due to the virtually complete absence of germ cells from $Pcna^{K164R/K164R}$ mouse testes, we were unable to analyse the effect of complete absence of K164 modification on recombination frequency. Considering the homotrimeric structure of PCNA, wild type PCNA from control mice and mutated PCNA from homozygous $Pcna^{K164R/K164R}$ mice will form monomorph PCNA trimers that can be normally modified in the wild type, and for which all K164 modification is prevented in the homozygous mutant mice. In $Pcna^{WT/K164R}$ heterozygotes, four distinct compositions of the trimeric PCNA ring are possible from the combination of 3WT PCNA molecules, 2WT+1K164R PCNA molecule, 1WT+2K164R PCNA molecules, and 3K164R PCNA molecules at a ratio of 1:3:3:1. Langerak et al. described that the presence of only a single wild type $Pcna$ allele might be enough for a normal damage tolerance function of ubiquitylated PCNA rings, since seven out of eight PCNA trimer complexes can be ubiquitylated at one site at least in heterozygous mice [20]. From this perspective, it is very surprising that we observed a relatively strong increase in meiotic recombination frequency in the heterozygote mice. The detrimental effect of the presence of 50% of K164R mutated and 50% wild type PCNA indicates that perhaps for this function of PCNA, all three subunits of the PCNA trimer need to be modified in order to function properly. Since this is expected to happen for only 1/8th of the trimeric PCNA rings, this may explain why the heterozygote displays such a dramatic effect on recombination frequency. Taken together with the above described data that point to a function of PCNA SUMOylation instead of PCNA ubiquitylation in the regulation of meiotic recombination, it might be suggested that SUMOylation is only effective when all three PCNA subunits are modified, whereas the ubiquitylation of a single PCNA in the ring is sufficient to stimulate the RDB pathway. SUMOylation of PCNA has not been reported for mammalian cells. So far, we have also been unable to establish that PCNA is modified by SUMO in wild type testes. This may be caused by active deSUMOylation during protein isolation, lack of proper antibody recognition, or actual lack of PCNA SUMOylation.

In summary, we have shown that PCNA modification at K164 is essential for the survival and/or proliferation of primordial germ cells. Since RAD18-deficient mouse testis appear to contain normal numbers of primordial germ cells, the observed lack of primordial germ cells in the $Pcna$ mutant mice could be caused by defective SUMOylation instead of ubiquitylation. Alternatively, another E3 may mediate basal PCNA ubiquitylation required for primordial germ cell proliferation/survival. Heterozygote $Pcna^{WT/K164R}$ mouse testes have normal numbers of spermatogonial stem cells, indicating that a single wild type allele is sufficient to rescue the primordial germ cells, similar to the wild type properties of $Pcna^{WT/K164R}$ mouse cells with respect to DNA repair. In contrast, meiotic recombination frequency is 20% higher in heterozygote K164R mutant mouse spermatocytes.
compared to wild types. We speculate that this phenotype is caused by defective PCNA SUMOylation, resulting in reduced recruitment of a downstream antirecombinogenic helicase, possibly BLM protein. We propose that this function requires SUMOylation of all three subunits of the homotrimeric PCNA ring.

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