DNA polymerase β is critical for mouse meiotic synapsis

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We have shown earlier that DNA polymerase β (Pol β) localizes to the synaptonemal complex (SC) during Prophase I of meiosis in mice. Pol β localizes to synapsed axes during zygonema and pachynema, and it associates with the ends of bivalents during late pachynema and diplonema. To test whether these localization patterns reflect a function for Pol β in recombination and/or synapsis, we used conditional gene targeting to delete the *PolB* gene from germ cells. We find that Pol β -deficient spermatocytes are defective in meiotic chromosome synapsis and undergo apoptosis during Prophase I. We also find that SPO11-dependent γ H2AX persists on meiotic chromatin, indicating that Pol β is critical for the repair of SPO11-induced double-strand breaks (DSBs). Pol β -deficient spermatocytes yielded reduced steady-state levels of the SPO11-oligonucleotide complexes that are formed when SPO11 is removed from the ends of DSBs, and cytological experiments revealed that chromosome-associated foci of replication protein A (RPA), RAD51 and DMC1 are less abundant in Pol β -deficient spermatocyte nuclei. Localization of Pol β to meiotic chromosomes requires the formation of SPO11-dependent DSBs. Taken together, these findings strongly indicate that Pol β is required at a very early step in the processing of meiotic DSBs, at or before the removal of SPO11 from DSB ends and the generation of the 3' single-stranded tails necessary for subsequent strand exchange. The chromosome synapsis defects and Prophase I apoptosis of Pol β -deficient spermatocytes are likely a direct consequence of these recombination defects.

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Introduction

Meiosis consists of a highly regulated and coordinated series of events in which diploid precursor cells first double their DNA content and then go through two rounds of cell division to give rise to haploid cells. An essential feature of this process is crossing over between homologous chromosomes, giving rise to genetic variation. During the leptotene substage of Prophase I of meiosis, SPO11-dependent double-strand breaks (DSBs) are introduced into the DNA (Roeder, 1997), then are repaired as cells progress into and through zygonema. The appearance of DSBs can be inferred by staining meiocytes with antiserum against γ H2AX, a phosphorylated form of the H2AX histone variant (Mahadevaiah *et al*, 2001). Genetic exchange in many organisms is facilitated by formation of the synaptonemal complex (SC) (Roeder, 1997). The axial elements of the SC are visualized by the appearance of short fragments that are immunoreactive with antiserum raised against SYCP3, a protein component of these elements (Heyting *et al*, 1987; Moens *et al*, 1987; Offenberg *et al*, 1991). Once DSBs are introduced and as the axial elements of the SC form, both DMC1 and RAD51 foci appear, marking the sites of ongoing recombination (Tarsounas *et al*, 1999). During zygonema, the homologs begin to synapse as evidenced by the appearance of SYCP1, a component of the transverse filaments of the SC (Dobson *et al*, 1994; Liu *et al*, 1996; Ashley, 2004). The homologs are fully synapsed by the time they reach pachynema, and significantly fewer RAD51 and DMC1 foci are observed in each nucleus (Plug *et al*, 1996; Moens *et al*, 1997, 2002). As the homologs desynapse during diplonema, chiasmata are observed, which are the cytological manifestations of crossovers (Cohen and Pollard, 2001). During the reductional meiotic division, the homologs move to opposite poles. In the second, equational meiotic division, the sister chromatids separate and segregate. Many other proteins localize to the axial elements and SC during Prophase I of meiosis and are likely to have a function in this process (Bannister and Schimenti, 2004).

Once DSBs are produced, several different DNA transactions including 5' end resection, a homology search, D-loop formation, branch migration and junction resolution are likely catalysed by proteins that localize to the chromosome axes and/or the SC (Ashley and Plug, 1998; Cohen and Pollard, 2001). Little is known about the identities of enzymes that catalyse DNA synthesis during meiotic recombination in mammals. Synapsis-associated and homologous recombination-associated DNA synthesis have been shown to take place along the SC in mice and appear to be coincident with the appearance of RAD51 (Ashley *et al*, 1995). Expression of DNA polymerase β (Pol β) is highest in the testes compared with other organs, and Pol β is expressed in zygotene and pachytene spermatocytes (Hirose *et al*, 1989; Alcivar *et al*, 1992; Plug *et al*, 1997a). Pol β is a 39-kDa enzyme that has both polymerase and deoxyribose phosphate (dRP) lyase activities

and is known to function in base excision repair (BER) (Sobol and Wilson, 2001). Meiotic DNA synthesis is sensitive to dideoxythymidine, which preferentially inhibits Pol β (Alcivar *et al*, 1992). Importantly, we have shown that Pol β localizes as discrete foci to the SCs of mouse meiotic chromosomes (Plug *et al*, 1997a; Jonason *et al*, 2001). Pol β is first observed on chromosomes in zygotene nuclei of both spermatocytes and oocytes (Plug *et al*, 1997a). The majority of Pol β foci localize to synapsed axes. At mid-pachynema, Pol β is arrayed along the SC at a density of ~ 5 –7 Pol β foci per bivalent. By late pachynema, few foci of Pol β are observed along the SC, but foci are detected at the ends of the chromosomes (Plug *et al*, 1997a). As the nuclei proceed into diplotene, the terminal Pol β signals persist. The localization of Pol β to the SC as discrete foci during the zygotene and pachytene substages of meiotic Prophase I indicate that this polymerase may function during Prophase I of meiosis.

To determine whether Pol β is critical for meiosis, we constructed mice in which Pol β is conditionally deleted in germ cells, using the Cre-loxP system of gene targeting. We observe defective synapsis of axial elements in spermatocytes of mice deleted of Pol β . Our results are consistent with the interpretation that Pol β facilitates one or more steps early in the processing of meiotic DSBs, possibly removal of the SPO11 complex from the 5' ends of DSBs.

Results

Construction of mice deficient for Pol β in germ cells

Deletion of the *PolB* gene in mice using targeted gene disruption results in lethality because of massive apoptosis of postmitotic neurons (Gu *et al*, 1994; Sugo *et al*, 2000). Thus, to determine whether Pol β is critical for meiosis, we deleted the *PolB* gene specifically from gonads, using Cre-loxP tissue-specific gene targeting (Bunting *et al*, 1999). Two components for this system are required: mice carrying loxP sites that flank the gene to be deleted, and mice that express Cre protein in the tissue in which the deletion is to occur. Cre is a site-specific recombinase that catalyses recombination between two loxP sites, resulting in excision of the fragment between the two sites and retention of one loxP site (Figure 1A). As detailed in Materials and methods, we used the tissue non-specific alkaline phosphatase (TNAP)-Cre and *PolB*^{flox} mice to produce mice that were deleted of the *PolB* gene in primordial germ cells (PCGs) and in the cells descended from them, including meiocytes.

PolB^{flox/ Δ} mice with or without the Cre transgene are identified by polymerase chain reaction (PCR) of genomic DNA isolated from tails, as shown in Figure 1B (Gu *et al*, 1994). Mice that have the Cre⁺ *PolB*^{flox/ Δ} genotype in tail DNA have the potential to have the Cre⁺ *PolB* ^{Δ / Δ} genotype in the gonad, because excision of the *flox* allele should have taken

Figure 1 Molecular characterization of *PolB* mice. (A) Schematic representation of the *PolB* gene locus before and after exposure to Cre. The WT, *flox* and *delete* alleles are pictured. The loxP sites in the floxed allele are denoted by filled arrowheads. When crossed with mice expressing Cre protein from the TNAP promoter, there is site-specific recombination between the loxP sites, leading to deletion of part of the promoter region (denoted by a bent arrow), and exon 1. This leaves one loxP site in the deleted allele. The *flox* allele is detected by amplification with the β metF1 and β metF2 primers, shown above the floxed allele in the cartoon. The deleted allele is detected by amplification with the β F1 and β R1 primers shown above and below, respectively, the deleted allele in the cartoon. (B) The *PolB*^{flox} and *PolB*^{delete} alleles can be detected by PCR. Genomic DNA was isolated from tail clippings and used as templates in a PCR reaction with the primers as described in Materials and methods. An aliquot of the PCR reaction was resolved on an agarose gel, and the image was captured using a Multimage Light Camera (Alpha Innotech). M, marker (base pairs); lane 1, tail DNA with the *flox* allele alone; lane 2, DNA with WT allele alone; lane 3, DNA with both WT and *flox* alleles; lane 4, DNA with the *delete* allele; lane 5, DNA with the Cre⁺ transgene. (C) The *PolB* deletion index can be estimated by Southern blotting. Genomic DNA was isolated from testes, digested with BamHI, resolved on an agarose gel, and analysed by Southern blotting as described in Materials and methods. Lane 1, mouse DNA carrying only the WT allele; lane 2, mouse DNA carrying only the *PolB*^{flox} allele; lane 3, Cre⁻ mouse carrying both *PolB*^{flox} and *PolB*^{delete} alleles; lane 4, Cre⁺ mouse carrying both the *PolB*^{flox} and *PolB*^{delete} alleles, present at 10 and 90% of total, respectively, as determined by image analysis. The presence of the Cre⁺ transgene was determined by PCR as in (B).

place in the PCGs during embryogenesis. To determine whether the *PolB* gene is deleted in the testis, we isolated genomic DNA from testes and used quantitative Southern blotting to obtain a deletion index, as described in Materials and methods. In Figure 1C, we show a representative Southern blot with DNA from the testis of a 17-day-old mouse along with the appropriate controls in the first three lanes, demonstrating that we can distinguish the wild-type (WT; 10 kb), floxed (4.5 kb) and deleted (3 kb) alleles using this approach. Lane 4 contains genomic DNA isolated from the testis of a *Cre⁺PolB^{flox/Δ}* male, which does indeed carry both the floxed and deleted alleles in the testis, but with the deleted allele predominating. Bands were quantified and used to calculate the deletion index, which is an estimate of the percent of cells that have deleted the *flox* allele. We estimate the testis deletion index of the mouse characterized in Figure 1C to be 90%. We obtained an average testis deletion index of 86% (number of mice (*n*) = 27; range is 77–97%) in *Cre⁺PolB^{flox/Δ}* mice that were between 14 and 23 days of age. This result suggests that on average Cre reduces the original floxed allele from 50% of total DNA to 14%, implying in turn that ~72% of the cells of the testes are homozygous for the deleted allele. Importantly, the deletion index applies to the whole testes, which contain somatic cells that will contribute to the non-deleted fraction. Thus, the majority of cells derived from PGCs of *Cre⁺PolB^{flox/Δ}* mice are deleted of the

PolB gene. In studies below, we characterized meiosis in mice that had a testis deletion index of at least 86%.

Pol β -deficient seminiferous tubules have few germ cells

To characterize the seminiferous tubules of Pol β -deficient mice, testes were excised from euthanized mice at 14, 17 and 23 days of age from WT (*n* = 21, 20 and 9, respectively) and *Cre⁺PolB^{flox/Δ}* (*n* = 19, 21 and 9, respectively) mice, and histological analysis was performed. The average testis weight of *Cre⁺PolB^{flox/Δ}* mice was significantly less than for WT controls (*P* < 0.001) at 14 (8.4 mg $\pm$ 1.9 (WT); 4.5 mg $\pm$ 0.86 (*Cre⁺*)), 17 (13.4 mg $\pm$ 4 (WT); 8 $\pm$ 2.7 (*Cre⁺*)) and 23 (29 mg $\pm$ 7 (WT); 14 mg $\pm$ 4 (*Cre⁺*)) days of age (Figure 2A and B).

In male mice, spermatogonial stem cells are located at the periphery of the seminiferous tubules. These cells undergo several mitotic divisions before giving rise to spermatocytes, the cells in which meiosis takes place. The first wave of spermatocytes entering meiosis occurs in juvenile mice during the second and third weeks after birth. Representative examples of the tubules from 17-day-old *Cre⁺PolB^{flox/Δ}* and WT mice are shown in Figure 2C and D. At this age, when many of the spermatocytes are expected to have reached or passed the pachytene stage, the tubules from the WT mouse looked normal, in that both abundant numbers of spermatogonial stem cells and spermatocytes were present. In

Figure 2 Pol β -deficient seminiferous tubules have few germ cells. (A) Testes of 14-day-old *Cre⁺PolB^{flox/Δ}* mice (right) are smaller than those of WT (left). (B) Average testis weight (in mg) versus age (■: WT mice (red); ▲: *Cre⁺PolB^{flox/Δ}* mice (green)). (C) Tubule histology of 17 WT pups. (D) Histology of tubules of 17-day-old *Cre⁺PolB^{flox/Δ}* pups. Image was taken at 400 $\times$ magnification (scale bar = 10 μ m). Note that the pups with the *Cre⁺* transgene exhibit aberrant testis morphology. Black and red symbols designated by (*) denote tubules with few and moderate numbers of cells, respectively. (E, F) TUNEL assay of two seminiferous tubules (tubules marked 1 and 2) from 17-day-old WT and *Cre⁺PolB^{flox/Δ}* mice. Image taken at 200 $\times$ magnification (scale bar = 5 μ m). (G) Quantification of TUNEL assay. (H) Activation of caspase-3 in *Cre⁺PolB^{flox/Δ}* spermatocytes. Western blot analysis was performed using whole testis extracts from 17-day-old mice and immunoblotted with anti-caspase-3 antibody. 1, WT; 2, *Cre⁺PolB^{flox/Δ}* mice.

contrast, many of the tubules from the Cre^+ mouse had spermatogonia but few spermatocytes. We analysed Bouins fixed tubules from the WT and Cre^+ mice (four littermate pairs) at various days postpartum (dpp) (data not shown). At 9 dpp, the WT mice exhibit advanced spermatogonial cell types including A and B spermatogonia and also preleptotene spermatocytes. At this age, the Cre^+ mice have A spermatogonia that are clearly proliferating. At 12 dpp, the majority of tubules from WT mice have late spermatogonia and/or spermatocytes up to pachytene, with some germ cell apoptosis present. The Cre^+ mice also have advanced spermatogonial cell types, and some germ cell apoptosis is observed. At 15 dpp, virtually all tubules from the WT mice have spermatocytes, some with two generations. Occasional apoptosis is observed. The Cre^+ mice have both zygotene and pachytene spermatocytes. Apoptosis is observed but cannot be pinpointed to a specific stage. At 17 dpp, more tubules with two generations of spermatocytes are observed in tubules from WT mice, but no meiotic divisions or spermatids are observed. There are few apoptotic cells. In the Cre^+ mice, spermatocytes are observed in more tubules than at 15 dpp. Apoptosis is observed that occasionally looks like Stage IV arrest, but some apoptosis also looks like 'normal developmental apoptosis' of germ cells. There is no accumulation of abnormal looking leptotene spermatocytes. At 17 weeks of age, the tubules from WT cells look normal and the tubules from Cre^+ mice exhibit normal spermatogenesis throughout the tubules except that some tubules have poor spermatogenesis because of missing generations of cells. Thus, it appears that tubules from Cre^+ mice may exhibit developmental delay. Nonetheless, they do have meiotic cells, and some apoptosis is observed that looks like Stage IV arrest.

All of the cells in tubules from the WT mice stain with Pol β antibody. However, only some of the cells in tubules from $Cre^+ PolB^{flox/\Delta}$ stain with Pol β antibody; this suggests that many of the cells, including spermatocytes, in these tubules are deficient for Pol β , as shown in Supplementary Figure 1.

To determine whether the absence of germ cells results from apoptosis, we characterized the seminiferous tubules of 17-day-old mice using the TUNEL assay. We observed little apoptosis in the tubules of WT mice (Figure 2E and G), but the apoptosis index of nuclei from $Cre^+ PolB^{flox/\Delta}$ mice was much greater (Figure 2F and G). We also analysed the activation of pro-caspase-3 in whole-cell testis extracts from 17-day-old mice by immunoblotting. Induction of apoptosis is accompanied by cleavage of pro-caspase-3 (35 kDa) into subunits that are 17–19 and 10–12 kDa. Pro-caspase-3 was evident in WT and $Cre^+ PolB^{flox/\Delta}$ testes extracts. The 17–19-kDa activated subunit was barely detectable in testis extracts isolated from WT mice and was greatly increased in the testis extracts from $Cre^+ PolB^{flox/\Delta}$ (Figure 2H). This suggests that spermatocytes induce apoptosis as a consequence of the absence of Pol β .

Pol β -deficient spermatocytes do not progress through meiosis

As we observed few spermatocytes in the seminiferous tubules of $Cre^+ PolB^{flox/\Delta}$ mice at an age when Prophase I-stage cells should have been abundant, we reasoned that spermatocytes from these mice might not progress through meiosis. To address this possibility, we stained WT and $Cre^+ PolB^{flox/\Delta}$ spread spermatocyte nuclei from 17-day-old

Figure 3 Pol β -deficient germ cells do not progress through meiosis. (A) Example of a spermatocyte nucleus in the pachytene sub-stage of meiotic Prophase I from a 17-day-old WT mouse. The bivalents are stained with SYCP3 (red) and Pol β (green). (B) Tabulation of cells exhibiting extensive synapsis in each of two genetic backgrounds. Genotype obtained by PCR analysis of tail DNA. Staging of randomly chosen nuclei was assessed by the staining pattern of SYCP3 antiserum and the DAPI image. At least three different 17-day-old mice of each genotype were assessed for progression to pachynema.

littermates with antisera raised against SYCP3, which is a component of axial cores chromosomes (Jonason *et al*, 2001), and Pol β , and counted the number of nuclei that exhibited extensive synapsis of chromosomes. An example of a WT pachytene nucleus with completely synapsed bivalents is shown in Figure 3A. We found that 73% of spermatocytes isolated from WT mice exhibited complete synapsis (Figure 3B). In contrast, only 25% of the total number of nuclei isolated from $Cre^+ PolB^{flox/\Delta}$ mice appeared to exhibit fully or near fully synapsed axes ($P < 0.0001$ when compared with WT mice). Importantly, all of the spermatocytes in $Cre^+ PolB^{flox/\Delta}$ mice that exhibited extensive synapsis also stained positive for Pol β (Figure 3B). The staining was similar to what we observed in WT mice (data not shown) (Plug *et al*, 1997a), suggesting that the pachytene spermatocytes that stain with Pol β antiserum originated from PCGs in which the *PolB* gene had not undergone Cre-mediated excision. These results suggest that Pol β -deficient nuclei are arrested before they reach pachynema. Note that spermatocytes isolated from $Cre^- PolB^{flox/\Delta}$ mice undergo synapsis, as shown in Supplementary Figure 2. On examination of 210 spermatocyte nuclei obtained from $Cre^- PolB^{flox/\Delta}$ mice, 158 (75%) exhibited extensive synapsis. This is not significantly different from WT (73%) and suggests that the presence of one allele of Pol β is sufficient for this process.

Pol β is critical for synapsis in males and females

To characterize synapsis in spermatocyte nuclei, we stained them with antisera raised against SYCP3 and SYCP1. In mouse spermatocytes, as axial elements form they also begin to synapse. Complete formation of axial cores without appreciable synapsis is rare in WT male mice (Scherthan *et al*, 1996). Therefore, we characterized nuclei that exhibited significant axial element formation for their ability to synapse, as judged by staining with anti-SYCP1. Figure 4C

Figure 4 Pol β is critical for synapsis. (A–C) Example of spermatocyte nuclei from a 17-day-old WT pup. (A) SYCP3 (red); (B) SYCP1 (green); (C) merge of panels (A) and (B). (D–I) Examples of spermatocyte nuclei from a 17-day-old *Cre⁺PolB^{flox/Δ}* pup. (D, G) SYCP3 (red); (E, H) SYCP1 (green); (F, I) merge of panels (D) and (E) and (G) and (H), respectively. (J–L) Example of centromere localization (CREST) in WT spermatocyte nuclei. (J) Zygotene substage stained with CREST ($n=40$); (K) early pachynema ($n=21$); (L) pachynema. (M–O) Example of CREST localization from *Cre⁺PolB^{flox/Δ}* spermatocyte nuclei. (M) Zygonema, ($n=40$); (N) late zygonema ($n=40$); (O) pachynema like ($n=29$). SYCP3 (red), CREST (green). (P–Q) Examples of 17-day-old nuclei labelled with CREST (green), SYCP3 (white) and Pol β (red). (P) WT nucleus. (Q, P) Nucleus from *Cre⁺PolB^{flox/Δ}* pup that appears to have progressed to pachynema. White arrows point to Pol β foci.

provides an example of a WT spermatocyte showing nearly complete synapsis when long axial cores are present (Figure 4C). Note from the unmerged images of SYCP3- and SYCP1-stained nuclei that the SYCP1 (green) and SYCP3 (red) signals overlapped almost completely but that there were a few asynapsed axes present at this substage (i.e. late zygonema). In WT mice, 104 of 106 spread nuclei that exhibited extensive axial element formation also exhibited nearly complete synapsis. In contrast, although we observed extensive axial element formation in spermatocytes from *Cre⁺PolB^{fllox/Δ}* mice, 87% (79/91) of the nuclei exhibited extensive asynapsis (Figure 4D–I; $P < 0.0001$).

To characterize synapsis in Pol β -deficient oocytes, we crossed *Cre⁺PolB^{fllox/Δ}* mice to each other and harvested the ovaries of female *Cre⁺PolB^{Δ/Δ}* embryos at 17 and 19 days of gestation and just after birth (see below regarding fertility of the *Cre⁺PolB^{fllox/Δ}* mice). We isolated the oocytes, prepared spread nuclei and stained them with antisera raised against SYCP3 and SYCP1. We could not identify any oocytes ($n = 40$) from *Cre⁺PolB^{Δ/Δ}* ovaries that had progressed further than leptotene in mice at 1 dpp (Supplementary Figure 3). Oocytes isolated from the WT and *Cre⁺PolB^{fllox/Δ}* female embryos were able to reach zygonema and pachynema and stained with antibodies to SYCP1 and SYCP3 (Supplementary Figures 3 and 4). Thus, Prophase I was blocked at the leptotene substage in the Pol β -deficient oocytes. In combination, our results indicate that Pol β is critical for synapsis in spermatocytes and oocytes and likely for specific DNA transactions required for these processes.

To further validate the synaptic failure in *Cre⁺PolB^{fllox/Δ}* spermatocytes, we analysed synapsis at centromeric regions by staining with CREST antiserum (Brenner *et al*, 1981). In WT leptotene spermatocytes, the maximum number of CREST foci is 40 because sister centromeres are tightly paired. As chromosomal pairing and synapsis progresses during zygonema and pachynema, the number of CREST foci decreases to 21 because the centromeres of homologous pairs of autosomes become closely juxtaposed, whereas the centromeres of the X and Y chromosomes remain separated (Figure 4J–L). We analysed the number of CREST foci in *Cre⁺PolB^{fllox/Δ}* spermatocytes in which long axial elements were assembled (Figure 4M–O). These mutant spermatocytes exhibited less juxtaposition of centromeres during zygonema (Figure 4M and N) than spermatocytes from WT mice (Figure 4K) and had on average 29 ± 6.7 foci ($n = 25$ nuclei analysed) during a pachytene-like substage (Figure 4O). This result suggests that some of the homologous chromosomes failed to synapse, at least in the centromeric regions. Note that *Cre⁺PolB^{fllox/Δ}* spermatocytes that progress to pachynema stain with Pol β antibody (Figure 4Q), just as WT cells (Figure 4P). We did not observe cells with >40 CREST foci, suggesting that sister chromatid cohesion at the centromeric regions is not defective in *Cre⁺PolB^{fllox/Δ}* spermatocytes.

DSBs persist in Pol β -deficient spermatocytes

The disappearance of γ H2AX staining from meiotic chromatin is coincident with synapsis (Mahadevaiah *et al*, 2001), so the synapsis defect in Pol β -deficient meiocytes led us to test whether these cells are defective in the repair of these breaks. In WT cells, we observed abundant localization of γ H2AX in 100% of nuclei ($n = 32$) during leptotene (Figure 5A), as shown earlier (Mahadevaiah *et al*, 2001). The abundance and

intensity of the γ H2AX dropped precipitously by late zygonema ($n = 36$); and by pachynema, γ H2AX domains were observed around the sex body and few foci were visible on the autosomes (Figure 5B). In spermatocytes isolated from *Cre⁺PolB^{fllox/Δ}* mice, localization of γ H2AX during leptotene ($n = 43$) was similar to that observed in WT, indicating that Pol β activity is not required for DSB formation (Figure 5C). However, staining of γ H2AX was profuse in the *Cre⁺PolB^{fllox/Δ}* cells during the zygonema-like substage when compared with WT (Figure 5B and D). In this regard, the phenotype of the Pol β -deficient mice is similar to that of mice that are deleted of the *Hop2*, *Dmc1*, *Msh4* and *Msh5* genes, which encode factors known or suspected to be directly involved in DSB repair (Pittman *et al*, 1998; Edelmann *et al*, 1999; Kneitz *et al*, 2000; Petukhova *et al*, 2003). These results thus indicate that Pol β is important for the repair of DSBs during meiosis.

To determine whether the persistent γ H2AX seen in Pol β -defective spermatocytes is a result of failure to repair SPO11-induced breaks, we generated *Cre⁺PolB^{fllox/Δ} Spo11^{-/-}* mice and characterized the localization of γ H2AX. Spermatocyte nuclei from *Spo11^{-/-}* mice are deficient in chromosome synapsis (Baudat *et al*, 2000; Romanienko and Camerini-Otero, 2000) and form localized accumulations of γ H2AX that resemble a sex body, although these rarely include the sex chromosomes (Barchi *et al*, 2005; Bellani *et al*, 2005). Importantly, spermatocyte nuclei from *Cre⁺PolB^{fllox/Δ} Spo11^{-/-}* mice showed no additional γ H2AX staining aside from that of the pseudo sex body (Figure 6B), consistent with an absence of DSBs in Pol β -deficient *Spo11^{-/-}* spermatocytes. Thus, the DSBs that occur in Pol β -deficient cells are induced by the SPO11 complex and do not result as a consequence of a Pol β deficiency. Importantly, we observed no localization of Pol β antiserum to the chromosomes of SPO11-deficient mice (Figure 6D), as compared with WT (Figure 6C), suggesting that Pol β associates with chromosomes that have been acted on by SPO11. In contrast, Pol β was localized to the chromosomes in DMC1-deficient mice (Figure 6E), suggesting that Pol β may be required for meiotic DNA transactions subsequent to the formation of DSBs.

Decreased levels of SPO11-oligo complexes released from DSB ends in Pol β -deficient spermatocytes

Meiotic recombination is initiated by the formation of DSBs made by the SPO11 protein complex. The SPO11 dimer coordinately breaks both strands of a DNA molecule, creating a DSB with covalent linkages between the newly created 5' DNA strand ends and the catalytic tyrosine residue in each SPO11 monomer (Keeney, 2001). In order for the DSB to be repaired, SPO11 protein is removed from DSB ends by an endonucleolytic mechanism that releases SPO11 covalently attached to a short oligonucleotide (Neale *et al*, 2005). To gain further insight into the persistence of DSBs in the spermatocytes of *Cre⁺PolB^{fllox/Δ}* mice, we examined whether SPO11 release from the chromosomes occurs in the absence of Pol β . We characterized the release of a SPO11-oligonucleotide complex from the DNA of mouse spermatocytes by immunoprecipitation followed by labelling with terminal transferase (TdT) and 32 P-labelled nucleotide. Importantly, we found that on average three-fold (3.4 ± 1.2 , from three experiments) more SPO11-oligonucleotide complex was recovered from testis extracts from WT mice as compared with testis extracts from *Cre⁺PolB^{fllox/Δ}* mice (Figure 7A). (Note that, as discussed above, Cre-mediated

Figure 5 Meiotic DSBs are generated but not repaired in Pol β -deficient spermatocytes. Shown are surface spreads of spermatocyte nuclei from WT (A, B) and $Cre^+ PolB^{flox/\Delta}$ (C, D) 17-day-old mice. Each of the nuclei were stained with antisera raised against γ H2AX (green) and SYCP3 (red). Note that this signal is much more intense and persists in the nuclei isolated from $Cre^+ PolB^{flox/\Delta}$ pups, whereas it only stains the sex body in WT pups and those without the *Cre* transgene.

Figure 6 Pol β functions in the repair of SPO11-induced double-strand breaks. The $PolB^{flox/\Delta}$ mice with or without the *Cre* transgene were crossed into a SPO11 background. (A) Example of a spermatocyte nucleus from 17-day-old $SPO11^{-/-}$ mice. The axial elements are stained with SYCP3 (red) and γ H2AX (green). (B) Example of a spermatocyte nucleus from $Cre^+ PolB^{flox/\Delta} SPO11^{-/-}$ mice. Note the presence of a pseudo sex body and other staining by γ H2AX on the autosomes. (C–E) Examples of localization of Pol β on spermatocyte spreads obtained from (C) WT; (D) $SPO11^{-/-}$; (E) $DMC1^{-/-}$ (SYCP3 (red), Pol β (green)). Note that there is more than one nucleus in each panel.

excision of the floxed allele is incomplete, so this assay likely underestimates the magnitude of the defect in spermatocytes that lack Pol β .) Testis extracts from both WT and Cre^+ mice

contain similar levels of Spo11 β , but a 1.4-fold reduction of Spo11 α was observed in Cre^+ mice (Figure 7B). Furthermore, we also found that the transcript levels of Spo11 β are similar

in both types of mice, but that Spo11 α transcript is decreased 1.8-fold in the Cre⁺ mice (Supplementary Figure 5). These findings are consistent with other studies, which have estab-

lished that Spo11 β is the predominant form expressed in leptotene and zygotene spermatocytes, and that Spo11 α is specifically depleted in mutants in which spermatocytes are eliminated during pachynema (Romanienko and Camerini-Otero, 2000; Neale *et al*, 2005). As the levels of SPO11-dependent γ H2AX in leptotene spermatocytes are comparable in Cre⁺ and WT (see above), it is unlikely that reduced numbers of SPO11-oligo complexes are a consequence of reduced numbers of DSBs. Thus, the decrease in steady-state levels of SPO11-oligonucleotide complexes could reflect either a defect in SPO11 release or more rapid turnover of SPO11-oligonucleotide complexes. Results in the next section strongly suggest that Pol β is critical for the timely and efficient removal of SPO11 from DSB ends and/or for DSB-processing events that occur soon after SPO11 release.

Pol β -deficient spermatocytes are defective for formation of chromosome-associated protein complexes diagnostic of single-stranded DNA

To further define the recombination defect in Pol β -deficient spermatocytes, we examined localization of replication protein A (RPA) and the strand exchange proteins RAD51 and DMC1. RPA is a stable complex consisting of the RPA70, RPA34 and RPA14 subunits that preferentially associates with single-stranded DNA (for a review see Fanning *et al* (2006)). RPA cytological foci appear during leptotene and associate with the SC at the time of synapsis (Plug *et al*, 1997b; Moens *et al*, 2002). Strikingly, fewer RPA foci were associated with the spermatocytes isolated from the Cre⁺ PolB^{fllox/ Δ} mice versus WT mice (Figure 8A–D). Quantitative analysis of leptotene cells showed a slight reduction in the average number of RPA foci per cell in Pol β -deficient spermatocytes, but also suggested the presence of a subpopulation of leptotene cells with no or very few foci (Figure 8E). Such a subpopulation was not observed in WT cells of equivalent stage. In zygonema, Pol β -deficient cells showed on average a >2-fold reduction in the number of RPA foci ($P < 0.001$) (Figure 8E), although RPA foci that do form in the mutant are axis-associated as in WT (Figure 8A). Taken together, these findings are consistent with the interpretation that

Figure 7 SPO11 is not completely released from the SCs of Pol β -deficient spermatocytes. (A) Whole testis extracts, isolated from 17-day-old mice, were incubated with SPO11 antiserum for 2 h, and then with agarose beads overnight. After centrifugation and washing, as described in Materials and methods, TdT and γ -³²P were added to label the SPO11-DNA complex. Lane 1, extracts from WT spermatocytes; lane 2, extracts from Cre⁺ PolB^{fllox/ Δ} ; lane 3, mock control (background). In this case, PBS was added to the extracts prepared from WT spermatocytes instead of antiserum against SPO11. Arrow points to SPO11 DNA complex. See Materials and methods for details. (B) Immunoblotting of the blot in (A) with monoclonal antibody raised against SPO11. The secondary antibody in this case with mouse HRP-protein A to reduce background. The bands corresponding to SPO11 α and SPO11 β are denoted with arrows and labels.

Figure 8 Fewer RPA foci associate with the SC in Pol β -deficient spermatocytes. Spread spermatocyte nuclei of 17-day-old WT (top two panels; A, B) and Cre⁺ PolB^{fllox/ Δ} mice (bottom two panels; C, D) during leptotene (left) and zygonema (right). The SC is visualized with antiserum raised against SYCP3 (red) and RPA is visualized with antiserum raised against the 34-kDa subunit of RPA (green). Note that RPA appears to be associated with the synapsed axes during zygonema. (E) Quantification of the number of foci per nucleus of RPA in leptotene and pachynema in the WT and Pol β -deficient spermatocytes.

Figure 9 Fewer RAD51 and DMC1 foci are observed on the SCs of Pol β -deficient spermatocytes. (A, B) Examples of RAD51 localization in 17-day-old mice; (A) WT; (B) $Cre^+ PolB^{flox/\Delta}$; (C) Scatter plot of leptotene/zygotene spermatocyte nuclei that display RAD51 foci versus the number of foci per nucleus. Red squares indicate focus counts in WT cells; green triangles indicate counts in mutant cells. RAD51 focus counts in leptotene cells (mean $\pm$ s.e.m.) were 242 ± 13 in WT and 60 ± 14 in spermatocytes from $Cre^+ PolB^{flox/\Delta}$ mice ($P < 0.0001$). Counts in zygotene cells were 180 ± 13 and 132 ± 13 for WT and mutant, respectively ($P < 0.01$). (D–F) Examples of DMC1 localization; (D) WT; (E) $Cre^+ PolB^{flox/\Delta}$; (F), Scatter plot of leptotene/zygotene spermatocyte nuclei that display DMC1 foci versus the number of foci per nucleus. DMC1 focus counts in leptotene cells were 169 ± 15 and 58 ± 14 in WT and mutant, respectively ($P < 0.0001$). Counts in zygotene cells were 159 ± 15 and 99 ± 21 , respectively ($P < 0.01$). Note that P -value was calculated by using Mann–Whitney test.

formation of single-stranded DNA is delayed and/or reduced in cells lacking Pol β .

RAD51 and DMC1 bind to 3'-single-stranded DSB tails and displace RPA (Keeney, 2001; Fanning *et al*, 2006). We stained spermatocyte nuclei from WT and $Cre^+ PolB^{flox/\Delta}$ mice with antisera raised against RAD51 ($n = 95$ and $n = 123$ for WT and mutant, respectively) and against DMC1 ($n = 48$ and 54 , respectively). A significantly lower average number of both RAD51 and DMC1 foci were observed in spermatocytes from $Cre^+ PolB^{flox/\Delta}$ versus WT controls, both at leptotema and zygonema (Figure 9). Note that leptotene cells from $Cre^+ PolB^{flox/\Delta}$ mice appeared to fall into two categories: most cells displayed few or no foci of either RAD51 or DMC1, whereas a smaller subset displayed focus counts that were closer to the WT range (Figure 9C and F). Importantly, the cells with appreciable numbers of RAD51 foci were also Pol β positive (Supplementary Figure 6). Thus, the subset of cells that exhibited similar numbers of RAD51 and DMC1 foci as WT were likely those in which Cre-mediated excision of the floxed allele had not occurred, as shown by double staining of RAD51 and Pol β . These findings reveal that Pol β -deficient spermatocytes are extremely defective for forming recombination-associated cytological foci of RAD51 and DMC1. These results further support the conclusion that formation of single-stranded DNA at meiotic DSBs is delayed and/or reduced in Pol β -deficient spermatocytes.

$Cre^+ PolB^{flox/\Delta}$ mice are fertile

To determine whether the $Cre^+ PolB^{flox/\Delta}$ mice were fertile, we mated 8–14-week-old male mice and their Cre^- littermates to WT females at 8, 10 and 12 weeks of age. No significant differences were observed in the litter sizes of these mice. The $Cre^- PolB^{flox/\Delta}$ fathers sired a total of 198 pups among 28 litters, and their Cre^+ littermates sired 224 offspring among

33 litters. We genotyped the offspring and found that the deleted and floxed alleles were passed on in the expected Mendelian ratios to offspring of the $Cre^+ PolB^{flox/\Delta}$ fathers. Surprisingly, 211 of 224 offspring of the $Cre^+ PolB^{flox/\Delta}$ fathers inherited a $PolB^\Delta$ allele. One possible explanation for this result is that the Pol β -deficient spermatocytes progress through Prophase I of meiosis and differentiate into sperm. This scenario seems highly unlikely, however, given the profound defects in synapsis and recombination foci formation as well as the substantial spermatocyte apoptosis observed in spermatocytes from the Pol β -deficient mice. A more likely possibility is that spermatocytes containing a floxed allele that was not deleted by Cre protein in PCGs progressed through meiosis, whereupon a later Cre-mediated excision event took place. This interpretation would be consistent with the demonstration that the *TNAP* gene is expressed in secondary spermatocytes or spermatids as well as in PCGs (Shima *et al*, 2004).

To determine whether a Cre-mediated deletion occurred before the cells differentiated into spermatids, populations of testicular cells from 10–12-week-old mice were separated using fluorescence-activated cell sorting (FACS) based on DNA content similar to what has been described earlier (Kovtun *et al*, 2004) (Figure 10A and B). After sorting, cellular populations were visualized by microscopy, examples of which are shown in Figure 10C–E. We rarely observed contamination of purified populations of cells with any other cell type. Spermatids, predominantly of the elongating type, were collected in the P1 fraction (1C DNA content). Spermatogonia and spermatocytes were collected in the 2C and 4C fractions, respectively. We then isolated DNA from the spermatocytes and spermatids and used PCR to determine the genotype of each cell population. In each PCR, we also amplified part of Intron 10 of Pol β as an internal control.

Figure 10 *PolB* undergoes excision before cells reach the spermatid stage. (A, B) Display FACS analysis of testicular cells from 10-week-old *Cre⁺PolB^{flox/Δ}* mice. FSC is forward scatter and PI-A is propidium iodide staining. Each population was examined using light microscopy as shown in panels (C–E), which contain spermatogonia (arrows), spermatocytes (arrows) and spermatids (mostly elongating and arrow points to a round spermatid), respectively. (F) Shows an example of PCR amplification of DNA isolated from various testicular cell fractions from *Cre⁺PolB^{flox/Δ}* mice. M is the marker. Lane 1, spermatocyte DNA with primers for Intron 10 and the *flox* allele; lane 2, spermatocyte DNA with primers for Intron 10 and the *delete* allele. The band that resolves just under the Intron 10 band in this and other lanes is unknown, but consistently appears when primers for Intron 10 and the *delete* allele are used in the PCR reaction; lane 3, spermatid DNA with primers for Intron 10 and the *flox* allele; lane 4, spermatid DNA with primers for Intron 10 and the *delete* allele.

DNA isolated from the *Cre⁺PolB^{flox/Δ}* spermatocytes contains both the *PolB* *flox* and *delete* alleles in equal proportion, as expected (data not shown). Although both the *flox* and *delete* alleles are present in DNA isolated from the spermatocytes of *Cre⁺* mice, only the deleted allele is detected in spermatids of these mice, as shown in Figure 10F. These data are consistent with the possibility of a Cre-mediated deletion event taking place before spermatocytes differentiated into spermatids.

Discussion

We showed earlier that Pol β is arrayed as discrete foci along the synapsed axes of the SC during zygonema and pachynema of Prophase I of meiosis (Plug *et al*, 1997a).

Towards the end of pachynema, Pol β was observed exclusively at the ends of the bivalents. These initial findings suggested that Pol β could have a critical function in meiosis. In this study, we found that conditional deletion of the *PolB* gene in PGCs leads to a defect in synapsis of meiotic chromosomes, reduced steady-state levels of SPO11-oligonucleotide complexes, and reduced and/or delayed formation of RPA, RAD51 and DMC1 foci. These findings reveal that Pol β is important for early steps in the meiotic recombination pathway. As it is known that homologous pairing and chromosome synapsis in mouse meiosis require the formation and proper repair of DSBs through homologous recombination, it is likely that the synapsis defect in Pol β -deficient spermatocytes is an indirect consequence of a defect in processing meiotic DSBs.

Pol β may have more than one function during Prophase I of meiosis

One key finding that emerges from these studies is that spermatocytes that are deficient in Pol β exhibit very little interhomolog synapsis. This observation was surprising considering that our earlier study showed that Pol β cytological foci only associate with synapsed bivalents (Plug *et al*, 1997a). The simplest interpretation of our results is that Pol β has more than one function during Prophase I of meiosis. The first function (whether direct or indirect) is early, at or soon after the removal of SPO11 from the ends of DSBs. A later, undefined function would then be during or after SC formation. Because of the synapsis defect in Pol β -deficient spermatocytes, the conditional knockout approach used here does not permit us to examine the function of Pol β after synapsis has occurred. An alternative explanation, which we do not favour, is that Pol β cytological foci on synapsed axes represent the accumulation of Pol β protein that has already facilitated SPO11 removal, and that Pol β is not required at later steps during meiotic Prophase I. As Cre-mediated excision of the *POLB* gene occurs in PCGs, the absence of Pol β activity during premeiotic S-phase or earlier could lead to the defects we observe during meiosis.

Pol β is important for the repair of SPO11-induced breaks

As Pol β is essential for both long and short patch BER (Sobol and Wilson, 2001), one possible way to interpret our results is that Pol β also functions in this process during meiosis. The pattern of γ H2AX localization indicates that SPO11-induced breaks are not repaired in Pol β -deficient spermatocyte nuclei, similar to what was observed in *Hop2*-deficient cells (Petukhova *et al*, 2003) and other meiotic recombination-defective mutants. The wide-spread appearance of γ H2AX across the genome was not observed in spermatocyte nuclei isolated from *Cre⁺PolB^{fllox/Δ}Spo11^{-/-}* mice. Instead, these mice demonstrated a restricted domain of γ H2AX staining similar to the pseudo-sex body previously demonstrated in *PolB^{+/+}Spo11^{-/-}* mice. These results indicate that unrepaired DSBs in Pol β -deficient spermatocytes represent bona fide SPO11-dependent breaks as opposed to DSBs that arise because of the absence of Pol β *per se*. Our results also show that SPO11-induced breaks are necessary for Pol β to localize to meiotic chromosomes. Thus, it is likely that SC-associated Pol β complexes function in the repair of SPO11-induced breaks and not breaks that result from oxidative base damage. Our inability to detect localization of APE I and XRCC1 to the SC (data not shown) supports this interpretation.

Importantly, we found that equivalent amounts of γ H2AX appeared to be formed in WT and Pol β -deficient leptotene spermatocytes, suggesting that DSBs are formed in the mutant with normal timing and in roughly normal numbers. In contrast, less SPO11-oligonucleotide complex is recovered from testis extracts of Pol β deficient versus proficient spermatocytes. Moreover, very few RPA, RAD51 and DMC1 foci are formed in leptotene spermatocytes from *Cre⁺PolB^{fllox/Δ}* mice, indicating that generation of ssDNA at DSB ends is highly defective and/or substantially delayed in the absence of Pol β . One interpretation of these findings is that Pol β is critical for efficient and/or timely removal of the SPO11 complex from DSB ends. If so, it is important to note that the three-fold reduction in steady-state levels of these

complexes is likely to be a significant underestimate of the magnitude of the defect in Pol β -deficient cells because Cre-mediated deletion of *PolB* from the germ cells of *Cre⁺PolB^{fllox/Δ}* mice is incomplete. An alternative possibility is that Pol β is required for 5'-3' exonucleolytic resection after endonucleolytic removal of SPO11 from DSB ends. Interestingly, most of the leptotene spermatocytes that lack Pol β had very few or no foci of RPA, RAD51 or DMC1, despite abundant γ H2AX, suggesting that SPO11-oligonucleotide release and/or DSB resection are very strongly dependent on the presence of Pol β . It is also noteworthy that a subset of mutant cells still had few or no DMC1 foci at zygonema (Figure 9F), whereas significant numbers of RPA and RAD51 foci were observed in equivalent stage cells (but still reduced compared with WT controls). This finding may indicate that at least some SPO11-oligonucleotide release and/or resection can occur in Pol β -deficient cells, or, alternatively, that ssDNA can be generated by partial unwinding of DSBs that still have SPO11 covalently attached to the ends. A final possibility is that SPO11-oligo release and resection still occur in Pol β -deficient cells, but that there is a delay in assembly of RPA, RAD51 and DMC1 complexes. Given the high affinity with which RPA binds ssDNA, however, we consider it unlikely that substantial amounts of ssDNA are generated in leptotene cells that lack Pol β .

In summary, a straightforward interpretation of our findings is that a defect in one or more very early steps in DSB processing in the absence of Pol β , leading to inefficient assembly of RAD51 and DMC1 strand exchange complexes, in turn leads to defective homologous synapsis because homology searching and strand invasion do not occur or are substantially delayed.

Pol β catalyses DNA synthesis and dRP removal using its dRP lyase activity. In our conditional gene-targeting approach, Pol β protein is not produced from the deleted allele (Gu *et al*, 1994), so it is not known whether the polymerase, dRP lyase, or both activities of this protein are critical for SPO11 removal. SPO11 has homology to the type II topoisomerases of archaeobacteria (Bergerat *et al*, 1997), and DNA cleavage by SPO11 results in two-base 5' overhangs (Keeney, 2001). Pol β is known to fill small gaps during BER, so it could create blunt ends using its polymerase activity after the SPO11 cleavage reaction. Creation of these blunt ends could be a prerequisite for the subsequent cleavage reaction that removes SPO11 from the DSB ends, for example, by creating a DNA substrate that would be of the proper conformation to associate with the endonuclease(s) or other proteins that are directly involved in the removal of SPO11. The dRP lyase activity of Pol β removes a sugar-phosphate moiety but has not been shown to facilitate removal of protein that is covalently bound to DNA, so the possible contribution of this activity to SPO11 removal is not clear. Finally, it is also possible that Pol β could function non-catalytically, for example, as a 'landing platform' for another protein that itself participates in the SPO11 removal and/or resection.

Pol β -deficient mice are fertile

We used conditional gene targeting to delete Pol β from the germ line of mice. On the basis of our deletion index analysis, immunolocalization studies and PCR-genotyping of fractionated testicular cells, it seems that *PolB* was not deleted in a

significant subset of the germ cells. It is likely that when few spermatogonia are present that are meiosis proficient, and many spermatogonia are destined for meiotic failure, there will be a selection for the ones that are proficient. Moreover, our results suggest that the spermatocyte population is enriched for Pol β -deficient cells in pups but not in adults. We have also shown that spermatogonia in both juvenile and adult males are enriched for the *PolB* *flox* allele relative to germ cells at later stages in spermatogenesis. Thus, we suggest that after the first wave of meiosis in pups, the spermatogonia likely give rise to a spermatocyte population that contains roughly equivalent numbers of Pol β -proficient and -deficient cells. The Pol β -deficient spermatocytes are eliminated by apoptosis during meiotic Prophase I, but the Pol β -proficient spermatocytes are able to progress through meiosis and differentiate into secondary spermatocytes and spermatids. In these cells, the Cre protein is once again produced (Shima *et al*, 2004), resulting in deletion of Pol β from the majority of spermatids. These spermatids differentiate into sperm, which produce mice carrying the *PolB* ^{Δ} allele. Of note, the complete absence of oocytes in substages of Prophase I beyond leptotema in females completely (and not conditionally) deleted for Pol β is consistent with a function for Pol β in processes that occur after leptotema, such as synapsis.

In summary, we show that Pol β is required for meiotic recombination and chromosome synapsis. Our data support a model in which Pol β facilitates removal of the SPO11 complex from the DNA ends, permitting 5' resection of the ends of the breaks that results in the generation of single-stranded DNA with which RAD51 and DMC1 associate.

Materials and methods

Conditional gene targeting

Mice carrying the *PolB* *flox* allele were a gift from Dr Samuel Wilson (National Institutes of Environmental Health Sciences) and were constructed by Dr Klaus Rajewsky (Gu *et al*, 1994). In these mice, *loxP* sites were inserted within the promoter region and intron 1 of the Pol β gene. Thus, on treatment with the Cre protein, some of the promoter and the entire sequence of exon 1 are deleted (Gu *et al*, 1994). Mice carrying the Cre gene fused to the TNAP (TNAP-Cre mice) promoter were a gift from Dr Andras Nagy (University of Toronto) (Lomeli *et al*, 2000). The TNAP promoter is active in PCGs (MacGregor *et al*, 1995), and Cre expression is turned on at this time in the TNAP-Cre mice. Mice were mated to produce offspring with the tail genotype of *Cre*⁺ or *Cre*⁻*PolB*^{*flox*/ Δ} . Tail genotyping was performed on DNA isolated from small tail clippings using a DNA isolation kit (Viagen). To determine the *Cre* and *PolB* genotypes, DNA was amplified using the PCR with allele-specific primers. Amplification of the *Cre* transgene was performed with the creF1 and creR1 primers which have the sequences 5'-TGATGGACAT GTTCAGGGATCGC and 5'-CAGCCACCAGCTTGATGATCTC, respectively. Amplification of the *PolB* *flox* allele was performed with the primers β metF1 and β met R1, which have the sequences 5'-CCC TCTTCTCTTCTATTATATCTCC and 5'-CCTCAACTCCACCACAACAC, respectively. Amplification of the deleted (Δ) allele was performed with the β F1 and β R1 primers, which have the sequences 5'-AAGG ACGGAAGGTGGAGGGAGAGCTAATGC and 5'-CGGCTTCCATCCCT GCTAAGTGACAGC, respectively. Approximately 1 μ l of DNA was mixed with 10 $\times$ Taq buffer (Promega), 1.5 mM MgCl₂, 0.25 mM dNTPs, 0.4 μ M each primer, and 1.25 units of Taq polymerase (Promega). The amplification conditions were 95°C, 1 min; then 35 cycles of: 95°C, 30 s; 55°C, 30 s; 72°C, 1 min. The DNA was then extended at 72°C for 10 min and held at 4°C. Approximately 10 μ l of products were resolved on a 3% agarose gel. As shown in Figure 1A, we were able to detect the Pol β *flox*, WT, and *delete* (Δ) alleles and the *Cre*⁺ transgene. All experiments on live

vertebrates were performed in accordance with institutional and national guidelines and regulations and were approved by the Yale University Animal Care and Use Committee.

Deletion index

Mice that have the tail genotype *Cre*⁺*PolB*^{*flox*/ Δ} could have undergone a Cre-mediated deletion event in PCGs, making them *Cre*⁺*PolB* ^{Δ / Δ} in the testis or ovary. To determine whether a Cre-mediated deletion had occurred in these organs, we obtained a deletion index using Southern blotting. DNA was isolated from testis or ovary using a Qiagen Kit. Approximately 5 μ g of DNA was digested with BamHI (New England BioLabs) and resolved on a 1% agarose gel. After denaturation, the DNA was transferred to a nylon membrane using standard Southern blotting procedures (Sambrook *et al*, 1989). Hybridization was performed in Perfect Hyb Plus buffer (Sigma). The probe and washing conditions were as described (Gu *et al*, 1994). Bands were quantified using the Imagequant program on a Storm phosphorimager (Molecular Dynamics). After subtraction of background, the deletion index, which is an estimate of the percentage of cells that carry the deleted allele, was calculated as (the number of pixels of a specific band (4.5 kb for *flox* or 3.5 kb for Δ) divided by the total number of pixels in all bands) times 100.

Histology of mouse testis

Testes from mice of various ages were fixed in neutral buffered formalin and paraffin embedded by standard procedures. Sections of the seminiferous tubules that were ~5–6 μ m thick were deparaffinized in xylenes and rehydrated through a graded series of alcohols. The sections were stained with hematoxylin and eosin on a fee-for-service basis in the Yale Histology Laboratories, using standard procedures. Sections were viewed using light microscopy, and images were captured with a CCD camera and analysed using Adobe Photoshop.

TUNEL assay

Testes from 17-day-old mice were fixed in 4% paraformaldehyde for 3 h at 4°C, dehydrated in 30% sucrose overnight, paraffin embedded, and sectioned. TUNEL assays were performed with the ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (catalog no. S7110, Serologicals Corporation/Chemicon). To obtain the apoptosis index from WT ($n=85$) and *Cre*⁺*PolB*^{*flox*/ Δ} ($n=124$), TUNEL-positive cells were counted and divided by number of tubules for each cross-section.

Immunofluorescence studies of meiotic spread preparations

Surface spread spermatocytes were prepared as we have described earlier from 14- to 17-day-old male mice (Plug *et al*, 1997a). Antiserum raised in goat against SYCP3 was prepared as described using a clone that was a generous gift of Dr Christa Heyting (Agricultural University, Wageningen, The Netherlands). The polyclonal antiserum was affinity-purified using an Immunopure kit and according to the manufacturer's directions (Pierce). This antiserum was generally used at a dilution of 1:200. SYCP3 was detected using a rhodamine-labelled anti-goat antibody (Jackson ImmunoResearch). Antisera raised in rabbits against SYCP1 (Novus), γ H2AX (Upstate), RAD51 (Calbiochem) and SYCP3 (Novus) were used at dilutions of 1:400, 1:800, 1:200 and 1:500, respectively. These were detected using a FITC-labelled anti-rabbit antibody (Jackson ImmunoResearch), with the exception of SYCP3, which was detected with anti-rabbit rhodamine-labelled antibody (Jackson ImmunoResearch). Mouse monoclonal antibody raised against Pol β (Abcam) was used at a dilution of 1:20 and detected with anti-mouse FITC-labelled antibody. The affinity preparation and use of polyclonal antiserum raised against Pol β was described earlier (Plug *et al*, 1997a). Detection was with anti-rabbit FITC-labelled antibody (Jackson ImmunoResearch). All preparations were counterstained with 4',6-diamino-2-phenylindole (DAPI, Sigma) and mounted in diazabicyclo[2.2.2]octane (DABCO, Sigma). Antiserum raised against RPA was purchased from Calbiochem and detected as described for RAD51, above. Images were viewed on a Zeiss Axioscope microscope with narrow bandpass filters. Images were captured using a CCD camera as described (Plug *et al*, 1997a; Walpita *et al*, 1999) and merged using Adobe Photoshop, and further image analysis was carried out using the Image J Program. Normal serum was used as a control; and for studies with anti-rabbit Pol β serum, preimmune serum was used as a control. In all

cases, no signal was detected. We also used serum that had been mixed with a homogeneous preparation of Pol β protein before applying to slides and did not detect a signal (data not shown), confirming the specificity of the antiserum as we described earlier (Plug *et al*, 1997a).

Release of the SPO11-oligonucleotide complex

Eight testes at 17 days of age from *Cre⁺polB^{flax/Δ}* and WT mice were used for each experiment. Testes were decapsulated and lysed in 500 μ l lysis buffer (25 mM Hepes-NaOH pH 7.4, 5 mM EDTA, 1% Triton-100, 2 mM DTT; 1 mM phenylmethylsulphonyl fluoride, leupeptin, pepstatin, chymostatin and aprotinin, each at 10 μ g/ml). The lysates were centrifuged at 60 000 r.p.m. for 20 min in a TY65 rotor (Beckman). Supernatants were diluted with one volume of lysis buffer and then incubated with mouse-anti-SPO11 antibody (S Keeney) at 4°C for 2 h. Agarose beads were then added and incubated overnight at 4°C. The reaction was centrifuged at 3000 r.p.m., the supernatant was removed, the pellet was washed three times with lysis buffer, two times with 1xNEB4, and labelled with TdT (GE Healthcare) and α -³²P dCTP (6000 ci/mmol, 1 mCi) as described earlier (Neale *et al*, 2005).

Fluorescence-activated cell sorting

Isolation of testicular cells was based on earlier published procedures (Malkov *et al*, 1998; Kovtun and McMurray, 2001). Testes were dissected in a Petri dish containing ice-cold separation medium (4 mM L-glutamine, 1.5 mM sodium pyruvate, 10% fetal calf serum, 75 μ g/ml Ampicillin). Testes were decapsulated, and contents were released into a 15-ml Falcon tube containing 5 ml ice-cold separation medium. After adding 0.25 ml of 2 mg/ml collagenase that was prepared in separation medium, the solution was incubated at 35–37°C for 5 min with vigorous shaking. The mixture was incubated on ice until the seminiferous cords sedimented to the bottom. Seminiferous cords were washed twice in 10 ml of separation medium and resuspended in 12 ml of the same but containing 2.5 mg/ml trypsin and 1 Unit/ml DNase I. After incubation for 2 min at 37°C, the mixture was transferred to ice, and the seminiferous cords were disintegrated using a Pasteur pipet. After filtration through an 80 μ m mesh filter, the filtrates were centrifuged at 250 rcf for 5 min and washed twice with separation medium. Cells were then resuspended in a total of 2 ml separation medium and counted. Cells were diluted into separation medium at concentrations ranging from 5 to 10 $\times$ 10⁶ cells/ml. Cells were then

diluted 1:1 with a fresh propidium iodide solution (10 mM Tris pH 8.0, 1 ml NaCl, 0.1% nonidet p-40, 0.7 mg/ml RNase A, 0.05 mg/ml propidium iodide). Cells were sorted on a BD FACS Aria on a fee-for-service basis at the Yale Cancer Center, and data were analysed using FACS diva software. For microscopic analysis, cells were centrifuged at 2500 r.p.m. for 5–10 min and washed three times with PBS. After the last wash, ~100 μ l of solution was left in the tube and used to resuspend the cells. Cells were dropped onto a coated slide, air dried, fixed with 4% paraformaldehyde and washed three times with PBS. Cells were then stained with hematoxylin for 2 min. For DNA isolation, cells were centrifuged and resuspended 200 μ l of TENS (100 mM NaCl, 1% SDS, 10 mM Tris-Cl pH 8.0, 1 mM EDTA) containing 25 μ l of 20 μ g/ml proteinase K. After incubation at 50°C overnight, 100 μ l of 3 M NaAc, pH 5.2 was added; the solution was mixed, incubated at 55°C for 10 min and microcentrifuged at 7500 r.p.m. for 20 min. Approximately 2 μ l of a 20 mg/ml glycogen solution and 2 volumes of 100% EtOH were added to the supernatant, and the mixture was incubated overnight at –20°C. Samples were microcentrifuged, pellets were washed with 70% EtOH, centrifuged and air dried. Pellets were resuspended in 30–50 μ l TE buffer, and 1–3 μ l of DNA was used in each PCR reaction. Cycling conditions were as described above, and Intron 10 of Pol β was amplified in each reaction as an internal control. The sequences of the primers used to amplify Intron 10 are 5'-TAG GAA GGT GGG CAG TAG AG and 5'-TGG CTC AGT GGT TAA GAA.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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