DNA Polymerase Beta is Critical for Mouse Meiotic Synapsis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

05 June 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has now been evaluated by three referees whose comments are enclosed below. As you will see from their reports the referees express potential interest in role of Polbeta in meiosis, however, it is clear that further experimental analysis is required to support the proposed role and to make it suitable for publication in the EMBO Journal.

The referees raise a number of important concerns, among these referee #1 (and to some extent referee #2) is concerned about the meiotic staging of some of the experiments and would like to see this clarified by the use of different markers and some additional histology. Both referee #2 and #3 express interest in the potential role of Polbeta in removal of Spo11, however, they both find that the same phenotype could also be a result of decreased or delayed DNA double strand break formation, this issue also needs to be addressed. Should you be able to address the concerns of the referees we would be willing to reconsidered a revised version of your manuscript. I would also like to point out that in the pdf file that was submitted online the figures were not of the highest quality and some thing that was commented on by one of the referees, I am sure that this occurred during the conversion to pdf format but needs to be resolved in revised version.

I would like to remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your revisions included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.
Yours sincerely,

Editor
The EMBO Journal

REFeree COMMENTS

Referee #1 (Remarks to the Author):

It has previously been shown that DNA polymerase beta (Polβ) localises to the synapsed chromosome axes in meiotic prophase (unfortunately I was unable to access this earlier paper in the course of refereeing the current MS). Mice lacking Polβ function die soon after birth, so in the present study a conditional targeting strategy to remove Polβ function specifically from the germ line (using a germ line specific Cre expressed in primordial germ cells). Unfortunately, as fully appreciated by the authors, the Cre was not fully efficient in removing Polβ function, and this complicated the interpretation of the results, and the reduced the impact of the conclusions. There is much that is good in this paper but there are issues that need to be addressed in relation to the interpretation and analysis of the phenotype, which I have outlined below.

1. First of all, as the authors make clear, there are important age effects on the phenotype, so the authors should make it clear for each set of data, what age mice were used to provide material.
2. From Fig 2c and the accompanying text the impression is given that there is little progression to meiosis and the germ cells were predominantly spermatogonia. The tubule cross section shown in fact has no meiotic cells and very few spermatogonia; given that the Cre is active in PGCs this raises in my mind the possibility that there is spermatogonial deficiency.
3. Convincing evidence is presented that there is a severe disruption to synapsis. Since full synapsis is a defining feature of pachynema, this raises problems as to staging. Another marker of entry into pachynema is the formation of a sex body, but recently Mahadevaiah et al (JCB paper) presented cogent evidence that pachytene cells with moderate levels of asynapsis do not form a sex body (leading to MSCI failure which is of itself sufficient to cause apoptosis at tubule stage IV). It is apparent that these authors had expended great effort in wrestling with the problems of classifying cells as being in pachynema when there is extensive asynapsis.
4. In the present paper, the proportion of spermatocytes in pachynema or diplonema at 17dpp was said to be reduced from 73% to 25% - all these cells were Polβ positive so had not deleted the locus. However, had the authors excluded cells with asynapsis/no sex body as being zygotene cells? When analysing cells with long axial cores 87% were said to have extensive asynapsis - this statement implies that these cells were considered to be late zygonema or pachynema. It is very hard to interpret these data as to what stage of meiosis the lack of Polβ causes a problem without additional markers of meiotic stage that are independent of the phenotypic consequences of Polβ deficiency.
5. There is extensive evidence that the predominant apoptotic loss in meiotic mutants is at tubule stage IV, which equates with early/mid pachynema in normal male meiosis. It would therefore have been useful to know at what stage the apoptotic losses are occurring in the Polβ deficient mice.
6. In females from an analysis at 17 and 19 days gestation, meiosis is said to block in leptonema. To have an earlier block in females than in males is I think a unique finding; however, it could be that in males and females Polβ deficiency delays meiotic progression, and that the females had been analysed too early. So I would like to see female data from females processed just after birth. Also, it would be good to assess whether things are progressing on a normal schedule in males - good Bouin’s-fixed histology with an expert assessor such as De Rooij (or indeed Terry Ashley) might be sufficient to establish if there is delayed progression from leptonema.
7. P 8, first para: the data are presented as if the females studied were from the conditional targeting (i.e. only in the germ line). However, in the discussion (bottom P15) the females were said to be 'completely (and not conditionally) deleted for Polβ’. If this was indeed the case, other non germ line specific consequences of Polβ deficiency may have played a role in the failure to proceed beyond leptonema - was there overall developmental retardation?

Some other minor points:
(a) Abstract: The statement that Polβ deficient spermatocytes 'are unable to complete meiosis' is overstating the strength of the findings.
(b) P5 three lines from bottom: I didn't understand how the ~72% was arrived at.
(c) P6, 2nd para, line 9: I suggest 'and the germ cells that were present.'
(d) P6, last line: I suggest removing 'direct'. In the Mahadevaiah et al JCB paper asynapsis was shown to be linked to MSCI failure, which of itself will cause apoptosis. Thus the apoptosis associated with Polβ deficiency could also be a downstream consequence of the disrupted synapsis leading to MSCI failure.
(e) P10, line 3: I am concerned that the reduced amount of Spo11-oligo complex found might simply be a consequence of the much reduced numbers of the relevant spermatocyte stage in the Polβ deficient mice. This is perhaps unlikely given the equivalent Spo11 levels in figure 7B, but it would have been nice to have some kind of loading controls.
(f) P10, 2nd para, line 8: As Terry Ashley has shown in a subsequent study, RPA is actually detectable on the axes prior to synapsis as would be expected as it should be present on the ssDNA tails that drive synapsis. With immunostaining it is wise to be cautious in concluding when a protein first appears.
(g) Page 11, 2nd para. I would draw attention to the fact that when a testis has only a few spermatogonia that are meiosis proficient, and many spermatogonia that are destined for meiotic failure, there will be selection in favour of the former over time. That is one of the reasons why the age of the mice when material is gathered is of crucial importance when interpreting the findings.
(h) Fig 7A legend: This talks of using testis extracts and then goes on to talk of WT spermatocytes - I presume these experiments used whole testis extracts?

Referee #2 (Remarks to the Author):

DNA polymerase beta is critical for mouse meiotic synapsis by Kidane et al.

In order to analyze the function of Polbeta in meiosis, essential for mouse development, the authors take advantage of a floxed Polbeta gene and induced the deletion of exon1 by expressing Cre from the TNAP promoter, driving expression in PGC cells. This strategy has the advantage of allowing the analysis of meiotic cells, it has the disadvantage of leading to a mixed populations of cells, some with a functional Polbeta, some with a deleted Polbeta gene, due to the incomplete recombination between Lox sites. The authors evaluate the efficiency of Polbeta deletion by southern hybridization. The mice obtained have several striking phenotypes indicating the importance of Polbeta for meiosis: reduced testis weight, deficiency in meiotic prophase progression in spermatocytes (spreads and sections), in oocytes (spreads), induction of apoptosis, synapsis defect in spermatocytes, defect in Spo11 removal and defect in DSB repair (gH2AX accumulation, decreased Rad51 and RPA foci). The mice are fertile, probably due to cells escaping Cre mediated deletion of Polbeta.

This work is very interesting as it provides a direct functional support for the initial observation made by Plug et al. in 1997 about the localization of Polbeta in spermatocytes. This work provides important and unexpected finding about the role of this polymerase in meiosis even though the precise role of Polbeta is not known. The paper is clearly presented and most of the interpretations are convincing. A few points need to be revised however. This referee thinks that some interpretations although interesting are not well supported by the data.

General comments:
1) One important issue is to validate the lower amount of Spo11-oligos
2) The interpretation about RPA, Rad51 and Dmc1 needs to be clarified and it cannot be concluded as written in the abstract that the formation of these foci is delayed.
3) It is not clear why the authors conclude that Polbeta has more than one role during meiosis. There seems to be some confusion about the relationship between DSB repair and synapsis. I personally consider it is not the best choice for a title to refer to the synapsis defect.
4) The impact on oocyte meiosis needs to be further validated.

Specific comments:
1) The authors evaluate meiotic progression by analysis of testis sections (Fig.2C). It is not sufficient to present one section and to refer to it as a representative example. A quantification is needed (% of sections with different stages of progression for instance). There should be a fraction of cells with functional polbeta and thus progressing in meiosis until end pachytene with a normal sexbody and detectable in these sections. What is the signal of the polbeta antibody in sections? Could it be used to actually correlate meiotic progression with the genotype?
2) Similar question is raised for the analysis by spreads: is polbeta present or absent in a nucleus like on Fig4O?

3) The result about oocytes is very surprising and unlike other DSB repair mutants to my knowledge. This needs to be better documented. What is the number of oocytes at birth? When are they eliminated? Oocytes can be easily lost when doing spread preparations, so the absence of stages later than leptotene could an experimental artefact.

4) Rephrase, p9, L17, "Thus, the only...", this sentence is not clear.

5) The release of Spo11 from DSB. This observation is very interesting, however not fully convincing. The question is about the normalization control (the Spo11 western blot). First, western blot are poorly quantitative. Although it is not an assay at the protein level, if Spo11 protein amounts are identical, this could be shown by qPCR on Spo11. Second I do not understand how, with the cell composition shown on Fig2C (as representative), one could have similar levels of Spo11 protein in wt vs polbeta. I therefore still consider possible that the lower amount of Spo11-oligos reflects a lower amount of cells making DSB in the testis population from polbeta floxed (+cre). This is a key issue to be answered.

6) RPA, DMC1 and Rad51 foci interpretations. The observed effects could be a delay, a reduced formation or a lower stability. These various possibilities should be presented. P11, lane 11. It is proposed that cells with high level of RAD51 or DMC1 foci express polbeta: could this be validated with the use of the polbeta antibody?

7) P13: I do not understand why it is surprising to see a deficiency in synapsis in polbeta mutant and to see polbeta at synapsed region in wt. Whatever the precise action of polbeta, the defect of synapsis could be explained by one role in DSB repair which leads to impair synapsis (this is actually written on p15).

8) Are the polbeta females fertile?

9) Homogenize annotations on figures.

10) Qualities of Fig5 and 6 are poor.

Referee #3 (Remarks to the Author):

Kidane and coworkers examined the role of DNA polymerase-beta (pol-beta) in meiosis by making a germline-specific null mutant by Cre/Lox technology. Although a background of pol-beta positive cells limits some interpretation, an overall picture emerges of an important role for pol-beta in the earliest stages of meiotic recombination. In particular, indirect evidence is provided that cells lacking pol-beta show a defect/delay in removal of Spo11 from double-strand break ends. This work in this paper is on the whole well-executed, and the implications are important and of broad general interest.

I think that the following issues ought to be addressed in a revised manuscript:

1. The flox allele used is not a meiosis-specific knockout, but rather appears to be being excised from the time of establishment of germ cell primordia onwards. Therefore, there is a remote possibility that defects seen in meiosis I prophase reflect an absence of pol-beta activity during pre-meiotic S phase or earlier, rather than a direct activity of pol-beta during prophase itself. This issue should be addressed in the discussion.

2. Similarly, it would be interesting to know if the flox allele is removed just before meiosis, or if it is absent in much of the germ line. The latter is likely, because of the loss of late-meiosis cells that are lacking pol-beta. However, this should also be addressed, either in the discussion or preferably by staining whole-sections for pol-beta.
3. The conclusion of inefficient Spo11 removal is based on a reduced recovery of Spo11-linked oligonucleotides, as well as an apparent delay in the appearance of break-associated ssDNA (assayed by RPA and Rad51/Dmc1 foci). However, this phenotype would also occur if double-strand break formation were delayed/less synchronous and inefficient. It therefore is important to the argument to show that break formation occurs on time and about the same level as in wild-type. This is not easily done, but a start could be made by more carefully quantifying the stage and extent of gamma-H2AX formation. The suspicion, from reading the paper, is that this was not done as rigorously as was the RPA and Rad51/Dmc1 focus quantification.

Minor points:

4. There is an inconsistency between text and figures in terms of SYCP3 and SYCP1 protein nomenclature.

5. Figures would be much more readable if meaningful labels were provided—for example, in matrices of micrographs, labeling horizontal rows as wild-type, mutant, etc and vertical rows as leptotene, zygotene, etc. would make it much easier for the reader to comprehend. Similarly, labeling gel lanes with their contents, in addition to numbering them, would help. In graphs, adding a red "wildtype" and green "Cre+polBfloxdel" would help. Etc. Etc.

6. page 6. "apoptosis index" implies a numerical measurement. Was one made? "greater" rather than "larger".

7. page 10, 11. The discussion of RPA and Rad51/Dmc1 is somewhat problematic, as Moens and coworkers (2002, J Cell Sci) report that RPA focus formation occurs after Rad51/Dmc1, and suggest that this reflects the presence of strand invasion intermediates. In my opinion, the priority placed on RPA is...misplaced, especially given the relatively low number of RPA foci relative to Rad51 and Dmc1 foci seen at similar stages. In addition, the absence of an overlap between wt and mutant RPA distributions at zygotene seems problematic. Certainly the Rad51 and Dmc1 data are more compelling.

8. page 11. The interpretation that cells with no or few Rad51/Dmc1 foci are pol-beta negative should be directly tested. This could readily be done, since the antisera against Sycp3, Rad51 and pol-beta were raised in goats, rabbits and mice, respectively.

9. page 23 (Fig 4 legend). What is meant by (n=XX) for each of the stages?

10. page 32 (Fig 2). A very minor point, but clarity would be increased if wild-type was always on the left, mutant always on the right, or vice versa.

11. page 38 (Fig 7a). The claim of reduced Spo11-linked oligo in pol-beta minus gonads needs to be supported with some sort of loading control, especially since the overall mass of pol-beta minus testes is considerably less that in wild-type. Also, this experiment seems to provide an opportunity to detect Spo11 that is still linked to chromosomal DNA--this should be detected as a high-molecular-weight signal labeled by TdT. Was any seen?

12. page 38 (Fig 7b). A spo11- load to confirm that the minor band is, indeed, Spo11 would be useful.

13. page 41 (Fig 10). A schematic showing the logic of the PCR assay would be useful (perhaps simply including primers in Figure 1?). Panel F is not labeled.
2. Spermatogonial deficiency may be observed in the Polβ deleted spermatocytes as shown in the tubule in Figure 2C. We have included a new version of Figure 2C that is most representative of our observations. It shows that, in general, tubules from the Cre+ PolB flox/∆ mice have spermatogonia and do not appear to have spermatogonial deficiency. Spermatocytes are observed in some of the tubules from these mice.

3. Classification of cells in pachynema. Because Mahadeviah and colleagues showed that cells that exhibit some asynapsis do not form a sex body, we did classify cells with extensive synapsis as being in pachynema; we felt that we could not use the sex body as a conclusive marker of this stage.

4. The issue of classification of 17dpp cells as being in pachynema or diplonema and exclusion of cells from these categories if they exhibited asynapsis/no sex body. Cells in which most of the chromosomes were synapsed were classified as being in pachynema even if they did not have a sex body. We realize this is not ideal, but it is the best we were able to do, given the phenotype. The heading in the table in Figure 3 has been changed to make this clear. We have also stated our rationale in the legend to Figure 3.

5. Stage of apoptotic losses. Dr. Dirk DeRooij examined Bouins fixed tubules from the Cre+ and WT mice from 9-17 dpp and at 17 weeks of age. At 9 dpp the WT mice exhibit advanced spermatogonial cells types including In and B spermatogonia and also preleptotene spermatocytes. At this age, the Cre+ mice have A spermatogonia that are clearly proliferating. At 12 dpp, the great 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+ cells 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. At 17 weeks of age some tubules from WT mice and tubules from Cre+ mice exhibit normal spermatogenesis throughout the tubules except that some tubules have poor spermatogenesis due to missing generations of cells. Thus, it appears that tubules from Cre+ mice may exhibit developmental delay, but that they do have meiotic cells and that some apoptosis is observed that looks like Stage IV arrest.

6. Processing of females just after birth instead of 17-19 day of gestation. We have performed this experiment and the results are in Supplementary Figure 2. We never detected cells that had progressed past leptonema. This is a unique finding as the Reviewer points out. The analysis of Bouins fixed tubules by Dr. De Rooij is discussed in point 5, above.

7. The female mice were completely and not conditionally deleted of Polβ because we did not need the mice to live until 14-21 dpp. POLB deleted mice die within a day of birth. The mice are small and known to exhibit apoptosis in postmitotic neurons but other studies have not suggested that there is any type of developmental retardation.

Minor points

a. We have deleted that statement, "unable to complete meiosis" from the Abstract.
b. The deletion index is ~86% which means that each testis on average is likely to have the floxed allele in approximately 14% of the cells. 14 x 2 =28% (two alleles) and 100-28=72% of the cells are homozygous for the deleted allele.

c. The word "germ" was added.
d. The word "direct" was deleted.

e. Concern that the reduced amount of Spo11-oligo complex found might be a consequence of the much reduced numbers of the relevant spermatocyte stage. The Referee suggested loading controls. As shown in Figure 7 of the revised version, we probed the first blot (the one in part A with the Spo11-oligo complex) with mouse antibody raised against Spo11. To reduce background, the secondary antibody we used was mouse HRP-Protein A. As can be seen in Figure 7B, similar amounts of Spo11ß, and slightly lower amounts (1.4-fold reduction) of Spol1α, were observed in cells from the WT and Delete mice, respectively. These findings are consistent with other studies, which have established that Spo11ß is the predominant form expressed in leptotene and zygotene spermatocytes, and that Spol1α is specifically depleted in mutants in which spermatocytes are eliminated during pachynema (Romanienko and Camerini-Otero, 2000; Neale et al., 2005). We have also shown that SPO11ß transcript levels are similar in both the WT and Delete mice (Supplementary Figure 2). Because the levels of SPO11-dependent γH2AX in leptotene spermatocytes are comparable in the WT and Delete mice, it is unlikely that the reduced amounts of the SPO11-oligo complex in the Pol ß-deficient mice are a consequence of reduced numbers of DSBs. We feel that the best "loading control" in the case of the SPO11-oligo blot would be SPO11 itself because this is in fact an immunoprecipitation and we would have no reason to suspect that the proteins normally used as loading controls for western blots, ie, tubulin, should be in complex with SPO11.

f. We state that cytological foci of RPA appear during leptonema, which seems to be consistent with the Reviewer's statement.

g. We have added this to the Discussion in the paragraph with the Heading, "Pol ß-deficient mice are fertile".

h. Whole testis extracts were used. This has been changed in the legend to Figure 7.

Referee 2

General Comments:

1. Validation of the lower amount of Spo11-oligos. We did this. Please see comment e above, for Referee 1.

2. Delay of focus formation for RPA, DMC1, and RAD51. We have removed delayed from the Abstract and state that they are less abundant.

3. Pol ß having greater than one role during meiosis. We suggest this to be the case in the Discussion, because we do not observe Pol ß to be localized to the axes until AFTER synapsis, yet in the study presented here we observe a defect in synapsis. Because we observe a defect in synapsis, we have included this in the title.

4. The impact on oocyte meiosis has been evaluated further. See point 6 for Referee 1, above.

Specific comments:

1. Representative tubule picture and progression. We have presented a section in Figure 2C that shows that tubules from 14 dpp Cre+ mice have spermatogonia and some spermatocytes. Dr. Dirk De Rooij evaluated tubules of various dpp and that discussion is also presented in the manuscript and above in point 5 for Referee 1. We also stained the tubules with monoclonal antibody raised against Pol ß and found that there is a fraction of cells in the tubule with Pol ß and a fraction that does not stain with Pol ß. We do not think that the staining of the tubules with Pol ß antibody can be used to correlate meiotic progression with genotype because we are not comfortable basing our results on negative staining. We have shown in our meiotic spread preparations that there are Pol ß proficient cells that progress through meiosis.

2. In a nucleus like the former Figure 4O, Pol ß is present, as shown in Figure 4P and Q of the revised version. This suggests that if a majority of axes have synapsed, Pol ß is present in the cells.
3. Observation of only leptotene oocytes in female spread preparations. We consulted with two experts in ovarian histology who harvested and examined oocyte preparations at birth. These experts both characterized the follicles observed in the WT and deleted mice as primordial follicles and observed that there were somewhat fewer of these in the deleted females. As stated in point 6, above, for Referee 1, in spread preparations from the deleted mice we only detected oocytes in leptotene and not beyond. Because we have repeatedly prepared (six times) spreads from both WT and Pol β-deleted mice at the same time, and found pachytene oocytes in cells from WT mice, we think it unlikely that this pattern is caused by losing material in the preparation.

4. We rephrased line 17 on page 9. We wanted to point out that DSBs are only being induced by Spo11 in both the Pol β deleted and WT cells.

5. Release of Spo11 from DSB. We have performed qRT-PCR as requested and observed that SPO11ß transcript levels are similar in both the WT and Cre+ mice (Supplementary Figure 2), whereas SPO11α levels are slightly lower in the Cre+ mice. This was confirmed by reprobing the first blot (the one in Figure 7, part A, with the Spo11-oligo complex) with mouse antibody raised against Spo11. To reduce background, the secondary antibody we used was mouse HRP-Protein A. As can be seen in Figure 7B, similar amounts of Spo11ß, and slightly lower amounts (1.4-fold reduction) of Spo11α, were observed in cells from the WT and Delete mice, respectively. These findings are consistent with other studies, which have established that Spo11ß is the predominant form expressed in leptotene and zygotene spermatocytes, and that Spo11α is specifically depleted in mutants in which spermatocytes are eliminated during pachynema (Romanienko and Camerini-Otero, 2000; Neale et al., 2005). Because the levels of Spo11ß were similar in testes from Cre+ and WT it suggests that they contain similar numbers of leptotene and perhaps early zygotene spermatocytes. We have also shown by γH2AX staining there are similar levels of SPO11ß-induced DSBs in leptotene cells from both WT and Cre+ mice, but that the DSBs do not disappear in cells from deleted mice. Our data when taken together indicate that there is sufficient SPO11 in the testes of Cre+ mice to induce DSBs in similar amounts to what is observed in WT cells.

6. The various possibilities are now presented in the revised version.

P11 L11: In Supplemental Figure 2, we show that cells that exhibit large numbers of Rad51 foci also have Polß foci and that cells with fewer Rad51 foci do not stain with Pol β. This suggests that Pol β-deficient spermatocytes are extremely deficient in forming recombination-associated cytological foci.

7. Pol β has one role. As stated by the Referee, we have presented this possibility in the Discussion.

8. The Pol β females we are working with are not conditionally deleted but completely deleted of Pol β and are dead one day 1 after birth so we do not know if they are fertile.

9 and 10. We have attempted to improve the quality and clarity of the Figures.

Referee 3

1. Deletion of Pol β in primordial germ cells. We have addressed this issue in the Discussion.

2. Removal of the Pol β allele just before meiosis. We have stained tubules with the Pol β antibody and show that spermatogonia appear to be deleted of Pol fl. See Supplemental Figure 1.

3. Quantification of γH2AX. We attempted to quantify γH2AX foci but in the process we could not mainly because the "foci" are not really focal at all, especially after leptotena. After leptotena, the γH2AX formation appears to cover large chromosomal domains, making it difficult to precisely define a focus. Thus, we do not feel that counting "blobs" covering large chromosomal domains is a faithful representation of the number of DSBs. It is important to point out that γH2AX is very abundant on leptotene chromosomes in both the WT and mutant, strongly suggesting that there is not a DSB defect in the Cre+ PolB flox/∆ mutant.

Minor Points.
4. The labeling of the Figures is now consistent.

5. We have tried to provide meaningful labels to the Figures.

6. Apoptosis index. We have calculated the apoptosis index. To obtain the apoptosis index from WT (n=85) and Cre’ PolB flox/∆ (n=124), TUNEL-positive cells were counted and divided by the number of tubules for each cross-section.

7. RPA, RAD51, and DMC1 discussion is somewhat problematic/priority placed on RPA is... misplaced, given the relatively low number of RPA foci relative to DMC1/RAD51 foci. First, the numbers of RPA foci range from 0–200 and the numbers of RAD51 and DMC1 foci range from 0–325 per cell. Thus, we are studying similar numbers of foci per cell. For each protein, the distribution of foci is different in the WT versus the Pol β deleted cells. There appears to be a subpopulation of Pol β deficient leptotene cells that have zero to very few foci of any of these proteins and subpopulations in zygotene that also have fewer foci per cell for each of these proteins compared with WT. Because each of these proteins is likely involved in synopsis, the low levels of foci in these subpopulations of cells supports our finding that deletion of Pol β results in defective synopsis. In each case, there appear to be cells in both leptotene and zygotene that have similar amounts of RPA, RAD51 and DMC1 foci in both WT and the Pol β-deleted cells. Our demonstration, shown in Supplemental Figure 2, that cells that have appreciable numbers of RAD51 foci are also Pol β proficient, suggests that there are in fact two populations of cells. We feel that inclusion of all three proteins in our study is important because they all appear to participate in synopsis.

8. See point 6, above, for Referee 2.

9. N=XX refers to the numbers of cells analyzed.

10. Wild-type is always on the left and the mutant is always on the right.

11. See comment e above, for Referee 1 and comment 5 for Referee 2. It is important to point out that we already know that covalent complexes of Spo11 bound to high molecular weight DNA will not resolve on SDS PAGE gels (see Neale et al. 2005).

12. Primers are now in Figure 1 and Panel F is labeled.

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2nd Editorial Decision 14 October 2009

I apologise for the delay in getting back to you with a decision on your revised manuscript but the referees were unavailable for some time after you resubmitted your study. Your manuscript has been reviewed by one of the original referees who is positive but would like to see an additional control experiment done.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):
This is a revised version of a MS that I refereed previously. The authors have done a good job in answering the referees’ criticisms.
Comments on the current MS:
1. p6, last line. Correct to "The Cre+ mice ...."
2. Top of page 9. For the study of the effect of PolB deficiency in female meiosis the authors opted to look at Cre+PolBΔ/Δ ovaries generated by mating Cre+PolBflox/Δ females with Cre+PolBflox/Δ males. At this point in the MS the reader will be puzzled that the Cre+PolBflox/Δ males are fertile - this surprising finding is explained much later in this long and complex MS. It is not clear why Cre is included in this cross, except perhaps as a control for non-specific effects of the Cre. The authors have followed my suggestion of looking at 1 dpp to make sure that female meiosis is arrested at leptotene rather than simply delayed, and this confirmed the arrest.
3. The finding with female meiosis is now I think unique in that females are apparently affected earlier in meiosis than males. This leads me to raise an additional point that I did not raise in my original report. |I apologise for this, as I know how irritating this can be.| The result for female meiosis is based on homozygous delete females, whereas the males studied have one obligatorily deleted allele and a second floxed allele that is subject to Cre mediated deletion which is not fully penetrant (as beautifully documented in the MS). This raised in my mind the possibility that the earlier block in the female might be a consequence of the differing genotypes. So what is the evidence that homozygous delete cells progress beyond leptotene in the male? The authors provide evidence that in males all the cells with extensive synopsis are PolB positive and thus are Cre+PolBflox/Δ. They nevertheless provide some evidence from Cre staining for disrupted synopsis in zygotene and a pachytene-like substage. However, it might be that these cells are also Cre+PolBflox/Δ and that the synaptic problems are a consequence of having only one functional (floxed) PolB allele. It would therefore be prudent to look at Cre-PolBflox/Δ mice to see if these also have synaptic problems.

Responses to the Referrees:
1. This has been corrected.
2. Top of page 9 is a point in the manuscript where the readers will be puzzled over the fertility of the Cre+PolBflox/Δ mice. We have added: (see below regarding fertility of the Cre+PolBflox/Δ mice). We do not want to present the fertility data first because we feel it will detract from the major focus of the manuscript.
3. The Referee raises the point that synaptic problems could arise from mice having only one functional allele of PolB and suggest that we analyze synopsis in mice that are Cre+PolBflox/Δ. This study has been performed using both male and female mice. In Supplementary Figure 5, we show representative examples of oocyte nuclei in leptonema, zygonema, and pachynema isolated from female embryos at day 17 of gestation. In Supplementary Figure 6, we show representative examples of spermatocyte nuclei isolated from male mice at 17 dpp. Upon examination of 210 spermatocyte nuclei obtained from Cre+PolBflox/Δ male mice, 158 (75%) exhibit extensive synopsis (compared to 73% in WT spermatocytes; see Figure 3). This has been added to the results section. Thus, synopsis takes place in the presence of one allele of Pol B.