EMBO reports

M1AP interacts with the mammalian ZZS complex and promotes male meiotic recombination

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Transaction Report:

(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Prof. Shi,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out that the data (especially the coIP data) must be strengthened before the study can be considered for publication here. I think all points raised are valid and need to be addressed. Points 1 and 2 by referee 3 can be addressed in the manuscript text.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (24th Nov 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public

database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready.

Please let me know if you have any comments or questions regarding the revisions.

Yours sincerely,

Esther Schnapp, PhD

Senior Editor
EMBO reports

Referee #1:

This article describes the identification of a splicing mutation in the M1AP gene as responsible for oligozoospermia in a family of human patients. By generating antibodies against the mouse protein and by modelling the mutation in the mouse model, they find that M1AP, previously shown for being important for male fertility, is a novel actor of meiotic recombination, during which is interacts with a pro-crossover complex, the ZZS, and localizes to recombination intermediates together with the RPA protein. In the point mutant, or another more extensive mutant, male fertility is reduced, recombination intermediates (labelled by the MSH4 protein) are reduced, and final crossover intermediates, marked by the MLH1-MLH3 complex, are slightly reduced. Interestingly, binding of M1AP to chromosomes depends on the ZZS SPO16 protein, but not TEX11. Rather, M1AP is required to maintain TEX11 at the recombination intermediates.
This provides an important conceptual advance in the formation of meiotic crossovers, with implications for human fertility, that deserves to be published, although the molecular details of the interactions and the cooperation between the proteins are not fully elucidated.
In general, the experiments are well carried out and controlled.
However, there are a number of issues that need to be addressed, detailed below.

- My major comment is that the authors should perform co-localization of their protein, M1AP, with at least one of the ZZS proteins, SHOC1, TEX11 (both for which antibodies are available) or SPO16. Indeed, the authors show some faint co-IP in testes between M1AP and the ZZS, and confirm this interaction using tagged proteins in human cells. However, it is essential for the paper to assess colocalization on meiotic chromosome spreads, especially since they find that M1AP shows discrete foci along chromosome axes, that are dependent on SPO11.
- Another major point is the lack of experimental detail and raw data for the mass spectrometry experiments. The authors should indicate how many times the experiment was done, if a negative control was done, and provide a link for the raw data deposited to a public database.

The following other comments also need to be addressed, in order of appearance in the manuscript:
- Introduction, line 39: it occurs preferentially between nonsister chromatids.
- Line 44: for the TOPOVIBL, the paper of Vrielynck et al (2016) Science should also be cited.
- Line 51: also cite the remaining "ZMM" proteins, in addition to MSH4/5, involved in stabilizing the recombination intermediates (reviewed recently in Pyatnitskaya et al 2019)
- Line 62: cite De Muyt et al 2018 as well.
- Line 103 : please precise how low 0.8 {plus minus} 0.5 millions/ml is low compared to wild type.
- Line 166 and Figure EV2B: it is not clear why in the homozygous M1ap KI/KI testes, there are 2 bands, including one predicted to be the canonical splicing product. Please explain.
- Line 248: replace "indicate" by "suggest", since you have no evidence that there is any strand invasion here.
- Line 249: remove "following strand invasion" (same reason as above comment)
- Line 286: remove "directly", since this is an assay in human cells, where the interaction between the tagged proteins may be mediated by many of the human cells proteins. For direct interaction assay, one would need purified proteins.
- Line 289: add De Muyt et al 2018 citation for the ZZS localization at meiotic recombination intermediates.
- Line 299: "suggesting" rather than "indicating". Indeed, M1AP may be recruited prior to strand invasion.
- Line 327: rather than "stabilizes recombination intermediates", suggest "stabilizes Tex11 at recombination intermediates", which reflects better the experiments actually done here.
- Line 328: promoting optimal crossover formation.
- Line 343: please use Sertoli cells only instead of "SCO".
- Line 342-343: this sentence is not clear. Suggest something like: 'We believe that disruption of M1AP not resulting in Stertoli cell only microtubules is consistent with M1AP being first detected..."
- Line 362: was it shown that Dmc1-/- repair some meiotic DSB using the sister chromatid? While this is the case in plants like Arabidopsis, I am not sure this has been shown in mammals. If it has, please ad a reference.
- Figure 5, panel B: could the authors comment why the TEX11 band in the IP migrates higher than in the input? And why the SHOC1 band in the IP is fuzzier than in the input?
- Figure EV2, panel A: please use a font (like Courier) that allows alignment between letters of the two strands.
- Legend of Fig. EV4, panel B: Please specify what the PCR products of the lower panel are. Control locus?

Referee #2:

In current study, Li et al investigated the functions of M1AP both in human patients and mutant/knockout mouse models. Mechanically, the authors demonstrated that M1AP localizes to recombination intermediates and promotes homologous

recombination by interacting with the ZZS complex. These results add M1AP to the "ZZS" model and provide new insights into meiotic recombination. In general, the experiments are well designed and performed at high qualities, and most results are supported by direct datasets. I would suggest the authors to revise this manuscript, considering my following concerns.

Major concerns:
1. If in male spermatocytes, M1AP is required for the recruitment of TEX11 to the sites of SHOC1-SPO16; why this interaction is dispensable for female meiosis in embryonic ovary? Mutation of SPO16, SHOC1 or TEX11 lead to meiotic recombination defects in both males and females.
2. I would like to encourage the authors to discuss the differences (phenotypically and mechanically) among mutations in M1AP, SHOC1, SPO16 and TEX11.
3. The reviewer is not fully convinced by the explanation of the splicing variant. Because the mutation is in the beginning of intron 7-8, the splicing between exon 6 and exon 7 should not be affected by this mutation. Instead, intron 7-8 retention is predicted due to this mutation. The conclusion is drawn by transfection of the sequence between intron 6-7 and intron 8-9, however without intact exon 6.
4. The quality of co-IP results could be improved. For example, endogenous IP could be performed with M1AP knockout testes or SPO16 knockout testes on hand.

Minor concerns:
1. Fig 1C: it would be better to provide an H3S10p-positive control, showing aligned chromosomes at MI.
2. Fig 3: the authors may use arrows to indicate unaligned chromosomes.
3. Fig 6A-B: needs quantification.

Referee #3:

Li and coworkers have examined the effect of mutations in the M1AP protein on meiosis in mouse spermatocytes, starting with a splice-site mutant at the exon 6 boundary that recapitulates one found in a human family, but also examining a deletion/frameshift in exon 4 with similar phenotypes. While the human mutants display complete oligozoospermia and a high frequency of univalent at meiosis I, the mouse mutants have a much more hypomorphic phenotype, and are partially fertile, with about 25% of spermatocytes showing fully-paired bivalents at meiosis I. Cytological studies show that, while markers of double-strand breaks are unaffected, crossover-associated MLH1/MLH3 foci are reduced to about 80% of wild-type. M1AP co-immunoprecipitates with the SPO16-TEX11-SHOC1 complex, although the recovery of TEX11 and SHOC1 from anti-M1AP immunoprecipitates is substantially less that stoichiometric. The authors conclude that M1AP interacts with and stabilizes the mammalian "ZZS" complex that is important for crossover formation.

Unfortunately, there are several issues that complicate interpretation of the current data. These include:

1. The hypomorphic phenotype, which contrasts to the complete lack of sperm in human mutants, as well as the absence of a phenotype in females. It is difficult (although not impossible) to imagine how a part of such an essential complex would have sex-specific functions. This hypomorphic phenotype stands in contrast to the more severe phenotype of mutants lacking proteins of the mammalian ZZS complex.
2. The antiserum used, which is made from a later part of the protein. The antiserum is not of great quality (there are many other bands in Western blots), and, because all mutants examined do not affect exons 1-3, leaves open the possibility that the remaining N-terminal part of the protein has at least partial function.
3. The poor quality of the in vivo immunoprecipitation experiments, which show very poor recovery of SPO16 and TEX11 relative to M1AP, calling into question the validity of the interaction. At a minimum, at control experiment with lysate from "-/-" mutant mice should be performed, to show that it is anti-M1AP, and not a cross-reacting activity, that is responsible for the pull down. Because of these issues, it is not possible to determine whether or not M1AP has an important role in meiotic recombination, or if the phenotypes observed are indirect effects of a defect in another process. Therefore, insight into what M1AP is actually doing during meiosis is minimal.

Other comments:
4. Authors tend to ignore primary references (which often are in yeast and Arabidopsis literature) and cite either review articles or articles on mammalian systems, especially in introduction and discussion. This should be corrected in revision or version sent elsewhere.
5. References in some subsection headings are made to stabilizing recombination intermediates where none are scored-foci are scored, and their stability is never assayed.
6. The section on the human mutants is redundant with previous literature and should be shortened considerably.

Point-by-point responses to the Reviewers

General response: We would like to thank you for taking the time to review our manuscript. We sincerely appreciate all your insightful comments and suggestions, which have greatly helped us to improve the quality of the manuscript. New data and discussion are now added to the revised manuscript. Please see below for the detailed Point-by-Point Responses.

Referee #1:

This article describes the identification of a splicing mutation in the M1AP gene as responsible for oligozoospermia in a family of human patients. By generating antibodies against the mouse protein and by modelling the mutation in the mouse model, they find that M1AP, previously shown for being important for male fertility, is a novel actor of meiotic recombination, during which is interacts with a pro-crossover complex, the ZZS, and localizes to recombination intermediates together with the RPA protein. In the point mutant, or another more extensive mutant, male fertility is reduced, recombination intermediates (labelled by the MSH4 protein) are reduced, and final crossover intermediates, marked by the MLH1-MLH3 complex, are slightly reduced. Interestingly, binding of M1AP to chromosomes depends on the ZZS SPO16 protein, but not TEX11. Rather, M1AP is required to maintain TEX11 at the recombination intermediates.

This provides an important conceptual advance in the formation of meiotic crossovers, with implications for human fertility, that deserves to be published, although the molecular details of the interactions and the cooperation between the proteins are not fully elucidated.

In general, the experiments are well carried out and controlled.

However, there are a number of issues that need to be addressed, detailed below.- My major comment is that the authors should perform co-localization of their protein, M1AP, with at least one of the ZZS proteins, SHOC1, TEX11 (both for which antibodies are available) or SPO16. Indeed, the authors show some faint co-IP in testes between M1AP and the ZZS, and confirm this interaction using tagged proteins in human cells. However, it is essential for the paper to assess colocalization on meiotic chromosome spreads, especially since they find that M1AP shows discrete foci along chromosome axes, that are dependent on SPO11.

Response: Thanks for your constructive suggestion. Our M1AP antibody was raised in rabbits. The anti-SHOC1 antibody we have, gifted by Prof. Chao Yu (Zhejiang University, China), was also raised in rabbits, and could not be used for co-immunofluorescence staining with M1AP. Fortunately, we obtained a goat anti-TEX11 antibody, gifted by Prof. Chao Yu (Zhejiang University, China, Zhang et al., 2019, Sci Adv), for co-immunofluorescence staining to assess the co-localization of M1AP with TEX11 on meiotic chromosome spreads. As shown in Fig 7A and B, M1AP showed a high degree of colocalization with TEX11. In late zygonema, the average fraction of M1AP foci co-localizing with TEX11 is 95.90 ± 0.53%, and the average fraction of TEX11 co-localizing with M1AP is 96.35 ± 0.71%. We have added this information in our revised manuscript (Fig 7A and B and lines 305-309).

References:

Qianting Zhang, Shu-Yan Ji, Kiran Busayavalasa, Chao Yu. SPO16 binds SHOC1 to promote homologous recombination and crossing-over in meiotic prophase I. Sci Adv. 2019 Jan 23;5(1):eaau9780. doi: 10.1126/sciadv.aau9780. eCollection 2019 Jan.

- Another major point is the lack of experimental detail and raw data for the mass spectrometry experiments. The authors should indicate how many times the experiment was done, if a negative control was done, and provide a link for the raw data deposited to a public database.

Response: Thank you for the comment and suggestion. We have added a brief description of how the experiment was done, including how many times the experiment was done and what negative control was used, and the experimental details (lines 562-611). The raw data for the mass spectrometry is also provided in the revised manuscript (Dataset EV1).

The following other comments also need to be addressed, in order of appearance in the manuscript:

- Introduction, line 39: it occurs preferentially between nonsister chromatids.

Response: Thanks for the suggestion. We have corrected this accordingly.

- Line 44: for the TOPOVIBL, the paper of Vrielynck et al (2016) Science should also be cited.

Response: Thanks for the suggestion. We have added the citation of Vrielynck et al (2016) Science in the revised manuscript.

- Line 51: also cite the remaining "ZMM" proteins, in addition to MSH4/5, involved in stabilizing the recombination intermediates (reviewed recently in Pyatnitskaya et al 2019)

Response: Thank you for the suggestion and comment. We have reworded this sentence and related parts in the revised manuscript (please see lines 52-58).

- Line 62: cite De Muyt et al 2018 as well.

Response: Thanks for the suggestion. We have added the citation of De Muyt et al 2018 in the revised manuscript.

- Line 103 : please precise how low 0.8 {plus minus} 0.5 millions/ml is low compared to wild type.

Response: Thanks for your suggestion and comment. According to the "WHO laboratory manual for the examination and processing of human semen (the fifth edition)" published by World Health Organization (WHO), the lower reference limit for sperm concentration is 15×10^6 spermatozoa per ml (5th centile, 95% CI $12\text{-}16 \times 10^6$). In the revised manuscript, we have indicated this reference range when describing that the sperm concentrations in the patients were low (please see lines 105-109).

- Line 166 and Figure EV2B: it is not clear why in the homozygous $M1ap^{KI/KI}$ testes, there are 2 bands, including one predicted to be the canonical splicing product. Please explain.

Response: Thank you very much for the question. As reported in previous studies on splicing mutations, the effect of a specific splicing mutation could cause more than one type of splicing alterations and generate several different mRNA isoforms, including the canonical mRNA (examples: *IKBKAP* c.2204+6T>C; *CFTR*, 3849+10 kb C>T; *SMN2*, c.830C>T) (Slaugenhaupt et al. AJHG, 2001; Nissim-Rafinia et al, EMBO Rep, 2004; Cartegni and Krainer, Nat Genet, 2002). The *M1AP* (c.1074+2T>C) mutation affects the +2 residual at the 5' donor splice site of intron 7-8 and leads to the generation of a canonical splicing product and a product with exon 7 skipping, both of which are much lesser than the canonical splicing product in the WT mice. We infer that, in the presence of the c.1074+2T>C mutation, the binding affinity of the splicing machinery to the 5' donor splice site of intron 7-8 may be weakened, rather than abolished, the splicing site at the intron 6-7 is preferentially recognized and exon 7 is skipped under some circumstances. It is worthy to investigate the molecular basis of the splicing alterations, which may provide some clues for the treatment of patients harboring the mutation. Nonetheless, given the priority of the present study, we would like to explore this further in our following research.

References:

Luca Cartegni, Adrian R Krainer. Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. Nat Genet. 2002 Apr;30(4):377-84. doi: 10.1038/ng854. Epub 2002 Mar 4.

Malka Nissim-Rafinia, Micha Aviram, Scott H Randell, Liat Shushi, Efrat Ozeri, Ornit Chiba-Falek, Ofer Eidelman, Harvey B Pollard, James R Yankaskas, Batsheva Kerem. Restoration of the cystic fibrosis transmembrane conductance regulator function by splicing modulation. EMBO Rep. 2004 Nov;5(11):1071-7. doi: 10.1038/sj.embor.7400273.

S A Slaugenhaupt 1, A Blumenfeld, S P Gill, … J F Gusella. Tissue-specific expression of a splicing mutation in the IKBKAP gene causes familial dysautonomia. Am J Hum Genet. 2001 Mar;68(3):598-605. doi: 10.1086/318810. Epub 2001 Jan 22.

- Line 248: replace "indicate" by "suggest", since you have no evidence that there is any strand invasion here.

Response: Thanks for the suggestion. We have corrected this accordingly.

- Line 249: remove "following strand invasion" (same reason as above comment)

Response: Thank you for the suggestion. We have corrected this accordingly.

- Line 286: remove "directly", since this is an assay in human cells, where the interaction between the tagged proteins may be mediated by many of the human cells proteins. For direct interaction assay, one would need purified proteins.

Response: Thank you for the suggestion. We have corrected this accordingly.

- Line 289: add De Muyt et al 2018 citation for the ZZS localization at meiotic recombination intermediates.

Response: Thank you for the suggestion. We have added this citation in the revised manuscript.

- Line 299: "suggesting" rather than "indicating". Indeed, M1AP may be recruited prior to strand invasion.

Response: Thank you for the suggestion. We have corrected this accordingly.

- Line 327: rather than "stabilizes recombination intermediates", suggest "stabilizes Tex11 at recombination intermediates", which reflects better the experiments actually done here.

Response: Thank you for the suggestion. We agree with you and have corrected this accordingly.

- Line 328: promoting optimal crossover formation.

Response: Thank you for the suggestion. We have corrected this accordingly.

- Line 343: please use Sertoli cells only instead of "SCO".

Response: Thank you for the suggestion. We have corrected this accordingly.

- Line 342-343: this sentence is not clear. Suggest something like: 'We believe that disruption of M1AP not resulting in Stertoli cell only microtubules is consistent with M1AP being first detected..."

Response: Thank you for the suggestion. We have corrected this accordingly.

- Line 362: was it shown that Dmc1$^{-/-}$ repair some meiotic DSB using the sister chromatid? While this is the case in plants like Arabidopsis, I am not sure this has been shown in mammals. If it has, please add a reference.

Response: Thank you for the comment. We do not find any study that provides direct evidence regarding whether some of the DSBs in *Dmc1*$^{-/-}$ mice were repaired using the sister chromatid in mice.

- Figure 5, panel B: could the authors comment why the TEX11 band in the IP migrates higher than in the input? And why the SHOC1 band in the IP is fuzzier than in the input?

Response: Thank you for the question. We think that these might be due to unoptimized Western blot processing. We have repeated the Co-IP and WB experiments and the results are now better. The new results are now shown in Fig 6B.

- Figure EV2, panel A: please use a font (like Courier) that allows alignment between letters of the two strands.

Response: Thanks for the suggestion. We have used the font (Courier) and make sure that the letters of the two strands are aligned.

- Legend of Fig. EV4, panel B: Please specify what the PCR products of the lower panel are. Control locus?

Response: Thank you very much for the critical reading of our manuscript. We are very sorry for our negligence for not labeling the PCR products. The lower panel of the PCR products are the *Actb* cDNA, which served as the loading control. We have added the information in our revised manuscript (now shown in Fig EV3).

Referee #2:

In current study, Li et al investigated the functions of M1AP both in human patients and mutant/knockout mouse models. Mechanically, the authors demonstrated that M1AP localizes to recombination intermediates and promotes homologous recombination by interacting with the ZZS complex. These results add M1AP to the "ZZS" model and provide new insights into meiotic recombination. In general, the experiments are well designed and performed at high qualities, and most results are supported by direct datasets. I would suggest the authors to revise this manuscript, considering my following concerns.

Major concerns:

1. If in male spermatocytes, M1AP is required for the recruitment of TEX11 to the sites of

SHOC1-SPO16; why this interaction is dispensable for female meiosis in embryonic ovary? Mutation of SPO16, SHOC1 or TEX11 lead to meiotic recombination defects in both males and females.

Response: Thank you very much for the question. Yes, it is confusing why M1AP does not localize onto chromosome axes if it is indeed expressed in oocytes, given that ZZS proteins are expressed and form discrete foci on the chromosome axes in oocytes. We first examined the level and localization of M1AP protein in fetal ovaries. As shown in Fig 5A, western blotting showed that M1AP protein was detected in fetal ovaries at 16.5 days postcoitum (dpc), at a comparable level as in the testes of 14-dpp-old mice, indicating that the absence of M1AP foci on the chromosome axes of oocytes was not likely a result of low protein expression (as observed in our *M1ap*$^{KI/KI}$ male mice). Intriguingly, confocal laser scanning microscopy analyses of spermatocyte or oocyte smears showed that, M1AP signals were detected in the nucleus of zygotene/pachytene spermatocytes, forming discrete foci; in contrast, dispersed M1AP signals were detected only in the cytoplasm, but not detected in the nucleus, of oocytes (Fig 5C). These results indicate that M1AP is expressed but cannot be translocated into nuclei, and not likely interacts with the ZZS proteins in oocytes.

As you and reviewer #3 pointed out: in male spermatocytes, M1AP is required for the recruitment of TEX11 to the sites of SHOC1-SPO16; why this interaction is dispensable for female meiosis in embryonic ovary. We infer that this could suggest the molecular differences in meiotic recombination between females and males. Several meiotic recombination–related proteins exhibit sexually dimorphic functions in mice, with a specific meiotic phenotype in spermatocytes but not in oocytes when knocked out (as reviewed in Hua & Liu et al, 2021, Front Cell Dev Biol). For example, *Tex15*$^{-/-}$ male mice were infertile owing to complete losses of RAD51 and DMC1 foci on chromosome axes, but the mutant females were fertile (Yang et al., 2008, J Cell Biol). RAD51AP2 foci were detected in both spermatocytes and oocytes, but this protein is required for crossover formation only on XY chromosomes but not on XX and autosomes in mice (Ma et al., 2022, Sci Adv). In addition, knockout of *Meilb2* or *Zcwpw1* also results in a much more severe meiotic DSB repair defects in males than in females (Zhang et al., Nat Comm, 2019; Li et al., Sci Adv, 2019). These findings, along with observations in *M1ap* mutants in the present study, indicate that the meiotic recombination in males may require additional factors, likely owing to the longer meiotic DSB repair duration in males (7-8 days) than in females (4-5 days) (Baudat et al, 2013, Nat Rev Genet) and specific mechanism ensuring XY recombination which is restricted to a tiny homologous region.

We have added this in the discussion of the revised manuscript (lines 417-436).

References:

Rong Hua, Mingxi Liu. Sexual Dimorphism in Mouse Meiosis. Front Cell Dev Biol. 2021 May 10;9:670599. doi: 10.3389/fcell.2021.670599. eCollection 2021.

Miao Li, Tao Huang, Meng-Jing Li, … Kui Liu. The histone modification reader ZCWPW1 is required for meiosis prophase I in male but not in female mice. Sci Adv. 2019 Aug 14;5(8):eaax1101. doi: 10.1126/sciadv.aax1101. eCollection 2019 Aug.

Hui Ma, Tao Li, Xuefeng Xie, … Qinghua Shi. RAD51AP2 is required for efficient meiotic recombination between X and Y chromosomes. Sci Adv. 2022 Jan 14;8(2):eabk1789. doi: 10.1126/sciadv.abk1789. Epub 2022 Jan 12.

Fang Yang, Sigrid Eckardt, N Adrian Leu, K John McLaughlin, Peijing Jeremy Wang. Mouse TEX15 is essential for DNA double-strand break repair and chromosomal synapsis during male meiosis. J Cell Biol. 2008 Feb 25;180(4):673-9. doi: 10.1083/jcb.200709057. Epub 2008 Feb 18.

Jingjing Zhang, Yasuhiro Fujiwara, Shohei Yamamoto, Hiroki Shibuya. A meiosis-specific BRCA2 binding protein recruits recombinases to DNA double-strand breaks to ensure homologous recombination. Nat Commun. 2019 Feb 13;10(1):722. doi: 10.1038/s41467-019-08676-2.

2. I would like to encourage the authors to discuss the differences (phenotypically and mechanically) among mutations in M1AP, SHOC1, SPO16 and TEX11.

Response: Thank you very much for the constructive suggestion. In the revised manuscript, we have discussed the differences among *M1ap*, *Shoc1*, *Spo16* and *Tex11* mutant mice (for details, please see lines 370-389).

3. The reviewer is not fully convinced by the explanation of the splicing variant. Because the mutation is in the beginning of intron 7-8, the splicing between exon 6 and exon 7 should not be affected by this mutation. Instead, intron 7-8 retention is predicted due to this mutation. The conclusion is drawn by transfection of the sequence between intron 6-7 and intron 8-9, however without intact exon 6.

Response: Thank you very much for the suggestion. We have re-constructed the minigene vectors, which now contains the genomic sequence spanning intron 5-6 and intron 8-9 of the M1AP gene (a schematic is shown below, **Fig A**). We transfected the newly-constructed minigene vectors into HEK-293T cells, followed by reverse transcription-PCR (RT–PCR). Similarly, a DNA product with a smaller size was obtained from cells transfected with the mutant minigene vector than the product obtained from cells transfected with a wild-type minigene vector. Sanger sequencing further confirmed that exon 7 was skipped in DNA products obtained from cells transfected with the mutant minigene vector. The new results are shown in Fig 1E and F in the revised manuscript.

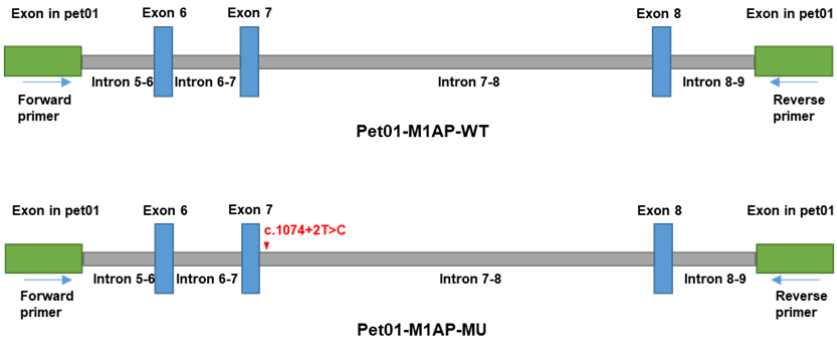

Fig A. Schematic illustrating the re-construction of the minigene vectors.

4. The quality of co-IP results could be improved. For example, endogenous IP could be performed with M1AP knockout testes or SPO16 knockout testes on hand.

Response: Thank you very much for the suggestion. As you and reviewer #3 suggested, we have repeated the co-IP with *M1ap*$^{-/-}$ testes on hand. As shown in Fig 6B, M1AP, SHOC1 and TEX11 proteins were detected in the input and M1AP-IPed lysate of the WT mice. M1AP was not detected in the *M1ap*$^{-/-}$ input or M1AP-IPed lysates obtained from *M1ap*$^{-/-}$ testes. Both SHOC1 and TEX11 were detected in the *M1ap*$^{-/-}$ input but not in M1AP-IPed lysates obtained from *M1ap*$^{-/-}$ testes. We believe that these new findings could provide sufficient evidence to support that M1AP interacts with SHOC1 and TEX11. The new results are now added in our revised manuscript (Fig 6B).

Minor concerns:

1. Fig 1C: it would be better to provide an H3S10p-positive control, showing aligned chromosomes at MI.

Response: Thank you for the suggestion. We have replaced the image.

2. Fig 3: the authors may use arrows to indicate unaligned chromosomes.

Response: Thanks for the suggestion. We have used arrows to indicate the unaligned chromosomes in our revised manuscript.

3. Fig 6A-B: needs quantification.

Response: Thank you for the suggestion. We have quantified the co-localization of M1AP-TEX11, M1AP-DMC1 and M1AP-RPA2 in our revised manuscript (Fig 7 B, D and F).

Referee #3:

Li and coworkers have examined the effect of mutations in the M1AP protein on meiosis in mouse spermatocytes, starting with a splice-site mutant at the exon 6 boundary that recapitulates one found in a human family, but also examining a deletion/frameshift in exon 4 with similar phenotypes. While the human mutants display complete oligozoospermia and a high frequency of univalent at meiosis I, the mouse mutants have a much more hypomorphic phenotype, and are partially fertile, with about 25% of spermatocytes showing fully-paired bivalents at meiosis I. Cytological studies show that, while markers of double-strand breaks are unaffected, crossover-associated MLH1/MLH3 foci are reduced to about 80% of wild-type. M1AP co-immunoprecipitates with the SPO16-TEX11-SHOC1 complex, although the recovery of TEX11 and SHOC1 from anti-M1AP immunoprecipitates is substantially less that stoichiometric. The authors conclude that M1AP interacts with and stabilizes the mammalian "ZZS" complex that is important for crossover formation.

Unfortunately, there are several issues that complicate interpretation of the current data. These include:

1. The hypomorphic phenotype, which contrasts to the complete lack of sperm in human mutants, as well as the absence of a phenotype in females. It is difficult (although not impossible) to imagine how a part of such an essential complex would have sex-specific functions. This hypomorphic phenotype stands in contrast to the more severe phenotype of mutants lacking proteins of the mammalian ZZS complex.

Response: Thank you very much for the question. We have now shown that M1AP protein is localized in cytoplasm (Fig 5 A and C), and thus not likely to interact with the ZZS proteins in oocytes.

Yes, it is confusing as why M1AP is dispensable for female meiosis in embryonic ovaries. We infer that this could suggest the molecular differences in meiotic recombination between females and males. Several recombination–related proteins exhibit sexually dimorphic functions in mice, with a specific meiotic phenotype in spermatocytes but not in oocytes when knocked out (as reviewed in Hua & Liu et al, 2021, Front Cell Dev Biol). For example, *Tex15*$^{-/-}$ male mice were infertile owing to complete losses of RAD51 and DMC1 foci on chromosome axes, but the mutant females were fertile (Yang et al., 2008, J Cell Biol). RAD51AP2 foci were detected in both spermatocytes and oocytes, but this protein is required for crossover formation only on XY chromosomes but not on XX chromosomes and autosomes in mice (Ma et al., 2022, Sci Adv). In addition, knockout of *Meilb2* and *Zcwpw1* also results in a much more severe meiotic DSB repair defects in males than in females (Zhang et al., Nat Comm, 2019; Li et al., Sci Adv, 2019). These findings, along with observations in *M1ap* mutants in the present study, indicate that the meiotic recombination in males may require additional factors, likely owing to the longer meiotic DSB repair duration in males (7-8 days) than in females (4-5 days) (Baudat et al, 2013, Nat Rev Genet) and specific mechanism ensuring XY recombination which is restricted to a tiny homologous region.

We have added this in the discussion of the revised manuscript (lines 417-436).

References:

Rong Hua, Mingxi Liu. Sexual Dimorphism in Mouse Meiosis. Front Cell Dev Biol. 2021 May 10;9:670599. doi: 10.3389/fcell.2021.670599. eCollection 2021.

Miao Li, Tao Huang, Meng-Jing Li, … Kui Liu. The histone modification reader ZCWPW1 is required for meiosis prophase I in male but not in female mice. Sci Adv. 2019 Aug 14;5(8):eaax1101. doi: 10.1126/sciadv.aax1101. eCollection 2019 Aug.

Hui Ma, Tao Li, Xuefeng Xie, … Qinghua Shi. RAD51AP2 is required for efficient meiotic recombination between X and Y chromosomes. Sci Adv. 2022 Jan 14;8(2):eabk1789. doi: 10.1126/sciadv.abk1789. Epub 2022 Jan 12.

Fang Yang, Sigrid Eckardt, N Adrian Leu, K John McLaughlin, Peijing Jeremy Wang. Mouse TEX15 is essential for DNA double-strand break repair and chromosomal synapsis during male meiosis. J Cell Biol. 2008 Feb 25;180(4):673-9. doi: 10.1083/jcb.200709057. Epub 2008 Feb 18.

Jingjing Zhang, Yasuhiro Fujiwara, Shohei Yamamoto, Hiroki Shibuya. A meiosis-specific BRCA2 binding protein recruits recombinases to DNA double-strand breaks to ensure homologous recombination. Nat Commun. 2019 Feb 13;10(1):722. doi: 10.1038/s41467-019-08676-2.

2. The antiserum used, which is made from a later part of the protein. The antiserum is not of great quality (there are many other bands in Western blots), and, because all mutants examined do not affect exons 1-3, leaves open the possibility that the remaining N-terminal part of the protein has at least partial function.

Response: Thank you for the comment. We agree that the predicted truncated proteins in *M1ap*$^{KI/KI}$ mice and *M1ap*$^{-/-}$ mice (approximately two-thirds and one-third of the wild-type protein length, respectively) may be produced and retain some function of M1AP. However, due to the lack of antibodies recognizing the predicted truncated proteins, we could not test this possibility. We have discussed this issue in discussion (lines 365-371).

3. The poor quality of the in vivo immunoprecipitation experiments, which show very poor recovery of SPO16 and TEX11 relative to M1AP, calling into question the validity of the interaction. At a minimum, at control experiment with lysate from "-/-" mutant mice should be performed, to show that it is anti-M1AP, and not a cross-reacting activity, that is responsible for the pull down.

Response: Thank you very much for your comment and suggestion. In the *in vivo* immunoprecipitation experiment, because we do not have antibody against SPO16, we were unable to test the interaction between SPO16 and M1AP, and only the interactions between SHOC1/TEX11 and M1AP were tested.

As you and reviewer #2 suggested, we have repeated the co-IP with *M1ap*$^{-/-}$ testes on hand. As

shown in Fig 6B, M1AP, SHOC1 and TEX11 proteins were detected in the input and M1AP-IPed lysate of the WT mice. M1AP was not detected in the *M1ap*$^{-/-}$ input or M1AP-IPed lysates obtained from *M1ap*$^{-/-}$ testes. Both SHOC1 and TEX11 were detected in the *M1ap*$^{-/-}$ input but not in M1AP-IPed lysates obtained from *M1ap*$^{-/-}$ testes. We believe that these new findings could provide sufficient evidence to support that M1AP interacts with SHOC1 and TEX11. The new results are now added in our revised manuscript (Fig 6B).

Because of these issues, it is not possible to determine whether or not M1AP has an important role in meiotic recombination, or if the phenotypes observed are indirect effects of a defect in another process. Therefore, insight into what M1AP is actually doing during meiosis is minimal.

Response: Thank you very much for the comment. Though the meiotic defects of *M1ap*$^{-/-}$ spermatocytes are milder than ZZS mutants, we do not think *M1ap* is completely dispensable for male meiosis, as more than 70% of *M1ap*$^{-/-}$ MMI cells display univalents, indicating that crossover formation is deficient. Moreover, men harboring *M1AP* null mutations are infertile due to NOA or severe oligospermia (our study, as well as in Wyrwoll et al, 2020, AJHG; Tu et al, 2020, Clin Genet), and *M1ap*$^{-/-}$ mice display reduced fertility, demonstrating its importance in male fertility.

Though M1AP was previously shown essential for male fertility in men and mice, the localization and molecular role of this protein remain uncharacterized till date. In the present study, we have extensively investigated the localization patterns of M1AP in meiocytes and the interaction between M1AP and ZZS proteins, as well as a detailed analysis of meiotic defects of M1ap mutants, which allowed us to conclude that M1AP acts as a co-partner of the ZZS complex, and promotes TEX11 recruitment at recombination intermediates.

In addition, the specific requirement of M1AP in male meiotic recombination shed new insights to differences in meiotic recombination between females and males. Noticeably, the different localization patterns of M1AP in oocytes and spermatocytes imply that some sexual-dependent factors are implicated in the translocation of M1AP into the nuclei in spermatocytes, adding a new layer of sexual dimorphic regulation of meiotic recombination.

Altogether, we believe that our study would deepen the understanding on molecular role of M1AP and update the meiotic defects associates with M1AP ablation in mice.

References:

Margot J Wyrwoll, Şehime G Temel, Liina Nagirnaja, … Frank Tüttelmann. Bi-allelic Mutations in M1AP Are a Frequent Cause of Meiotic Arrest and Severely Impaired Spermatogenesis Leading to Male Infertility. Am J Hum Genet. 2020 Aug 6;107(2):342-351. doi: 10.1016/j.ajhg.2020.06.010. Epub 2020 Jul 15.

Chaofeng Tu, Ying Wang, Hongchuan Nie, … Juan Du. An M1AP homozygous splice-site mutation associated with severe oligozoospermia in a consanguineous family. Clin Genet. 2020 May;97(5):741-746. doi: 10.1111/cge.13712. Epub 2020 Feb 10.

Other comments:

4. Authors tend to ignore primary references (which often are in yeast and Arabidopsis literature) and cite either review articles or articles on mammalian systems, especially in introduction and discussion. This should be corrected in revision or version sent elsewhere.

Response: Thank you for the suggestion. We have added the original references (including in yeast and Arabidopsis) in the revised manuscript.

5. References in some subsection headings are made to stabilizing recombination intermediates where none are scored-foci are scored, and their stability is never assayed.

Response: Thank you for the suggestion. We agree with you and have toned down throughout the manuscript.

6. The section on the human mutants is redundant with previous literature and should be shortened considerably.

Response: Thanks for the suggestion and we have shortened the section in our revised manuscript.

Again, we are very grateful to all of you for taking the time to review our manuscript. We sincerely hope that this revised manuscript has now addressed most of your concerns and meet with approval.

Qinghua

Dear Prof. Shi,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees.

Referees 1 and 2 still have some minor suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript. Referee 3 is more critical but the other 2 referees do not agree with her/his concerns and this referee can therefore be overruled. Please double-check that no overstatements regarding your data and the conclusion that M1AP is required for TEX11 association with the SHOC1/SPO16 complex are made.

A few editorial requests also need to be addressed:

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- Please add the name and the legend of Dataset EV1 to the first tab of the excel file.

- The Appendix file is missing a table of content with page numbers. Appendix Fig S3 A and B panels are not labelled, please add.

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I would like to suggest a few minor changes to the abstract. Please let me know whether you agree with the following:

Following meiotic recombination, each pair of homologous chromosomes acquires at least one crossover, which ensures accurate chromosome segregation and allows reciprocal exchange of genetic information. Recombination failure often leads to meiotic arrest, impairing fertility, but the molecular basis of recombination remains elusive. Here, we report a homozygous M1AP splicing mutation (c.1074+2T>C) in patients with severe oligozoospermia owing to meiotic metaphase I arrest. The mutation abolishes M1AP foci on the chromosome axes, resulting in decreased recombination intermediates and crossovers in male mouse models. M1AP interacts with the mammalian ZZS (an acronym for yeast proteins Zip2-Zip4-Spo16) complex, SHOC1, TEX11, and SPO16. M1AP localizes to chromosomal axes in a SPO16-dependent manner and co-localizes with TEX11. Ablation of M1AP does not alter SHOC1 localization but reduces the recruitment of TEX11 to recombination intermediates. M1AP shows cytoplasmic localization in oocytes, and is dispensable for fertility and crossover formation in female mice. Our study provides the first evidence that M1AP acts as a co-partner of the ZZS complex to promote crossover formation and meiotic progression in males.

I look forward to seeing a final version of your manuscript as soon as possible.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have greatly addressed all my concerns, in particular my two major points, regarding nice colocalization between the ZZS protein, TEX11, and M1AP, and the details and raw data for mass spect analysis.

This is a great paper that deserves to be published.
I still have one very minor request that needs to be addressed before publication: lines 64-66: the Zip2, Zip4 and Spo16 proteins forming a complex, and the name for this complex, ZZS, are from the De Muyt et al 2018 paper. This paper should be cited here, in addition to the other references.

Referee #2:

The quality of this manuscript has been improved through revision, and the reviewer has been convinced by new results and explanations, except for several minor points.
1. In Fig 1C, the authors has changed the control image during the revision. However, it is still wired because spermatogenesis is synchronized in one cross section and should have more H3S10p-positive cells.
2. Poor quality of Western Blottings in Fig 5A, especially the M1AP band in testis do not match the one in Fig 2B.

Referee #3:

Authors have done an important control for the pulldowns (using the M1AP mutant) and have otherwise improved the manuscript, including showing that M1AP colocalizes with TEX11 and thus likely with the SHOC1/SPO16/TEX11 complex. However, the fact remains that the M1AP splicing mutant studied here has only a modest impact on male meiosis; crossovers and bivalents are only modestly reduced, and a substantial number of TEX11 foci (about 50% of wild type) still form. Moreover, TEX11 foci are present in the pseudoautosomal region of the X-Y bivalent at wild-type levels, even though this bivalent is more severely affect than autosome bivalents at the MutL gamma and bivalent level. This, coupled with the strong phenotypic disparity between spermatocytes and oocytes, and between mice and humans, calls into question the authors mechanistic conclusions that M1AP is required for TEX11 association with SHOC1/SPO16 complex. This article is more suited for a specialized journal of record.

Point-by-point responses to the Reviewers

General response: We would like to thank you again for taking the time to review our manuscript. We sincerely appreciate all your insightful comments, which have greatly helped us to improve the quality of the manuscript. Please see below for the detailed Point-by-Point Responses.

Referee #1:

The authors have greatly addressed all my concerns, in particular my two major points, regarding nice colocalization between the ZZS protein, TEX11, and M1AP, and the details and raw data for mass spect analysis.

This is a great paper that deserves to be published.

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Response: Thank you for the comment and suggestion. We have added the citation accordingly.

Referee #2:

The quality of this manuscript has been improved through revision, and the reviewer has been convinced by new results and explanations, except for several minor points.

1. In Fig 1C, the authors has changed the control image during the revision. However, it is still wired because spermatogenesis is synchronized in one cross section and should have more H3S10p-positive cells.

Response: Thank you for the comment. Actually, in the testis section from the control adult man who was diagnosed with obstructive azoospermia, the H3S10p-postive spermatocytes with aligned chromosomes were scarcely seen. We think it might be explained by that the meiotic metaphase stage is relatively rapid compared to the other substages of spermatogenesis and thus the ratio of tubules containing meiotic metaphase cells and the number of metaphase cells in these tubules are very low in the testicular cross sections of the adult control.

2. Poor quality of Western Blottings in Fig 5A, especially the M1AP band in testis do not match the one in Fig 2B.

Response: Thank you for the comment. In Figure 5A, we examined the expression of M1AP in fetal oocytes and compared the level of M1AP protein to that in spermatocytes. To make sure that the meiocytes were comparable between males and females, we chose fetal female embryo at 16.5 dpc and male mice at 14 dpp, in both of which meiosis proceeds to the zygotene stage. Because of the small size of the fetal mice ovaries (16.5 dpc), the concentrations of the ovarian protein lysates were low. Thus, to make sure that the levels of meiotic proteins were comparable between the testicular and fetal mice ovary samples (TEX11 serves as the loading control), we have to dilute the testicular lysates by approximately 15 times. Therefore, the band of M1AP in Figure 5A is much weaker than that in Figure 2B.

Referee #3:

Authors have done an important control for the pulldowns (using the M1AP mutant) and have otherwise improved the manuscript, including showing that M1AP colocalizes with TEX11 and thus likely with the SHOC1/SPO16/TEX11 complex. However, the fact remains that the M1AP splicing mutant studied here has only a modest impact on male meiosis; crossovers and bivalents are only modestly reduced, and a substantial number of TEX11 foci (about 50% of wild type) still form. Moreover, TEX11 foci are present in the pseudoautosomal region of the X-Y bivalent at wild-type levels, even though this bivalent is more severely affect than autosome bivalents at the MutL gamma and bivalent level. This, coupled with the strong phenotypic disparity between spermatocytes and oocytes, and between mice and humans, calls into question the authors mechanistic conclusions that M1AP is required for TEX11 association with SHOC1/SPO16 complex. This article is more suited for a specialized journal of record.

Again, we are sincerely grateful to all of you for taking the time to review our manuscript. We sincerely hope that this revised manuscript has now meet with approval.

Qinghua

Prof. Qinghua Shi
University of Science and Technology of China
Division of Reproduction and Genetics
Hefei
China

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- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
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- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - are tests one-sided or two-sided?
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  - definition of 'center values' as median or average;
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If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Materials and Methods, Table EV1

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes	Materials and Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Yes	Materials and Methods

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Yes	Acknowledgments

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	