nature portfolio

Peer Review File

FIGNL1-FIRRM is essential for meiotic recombination and prevents DNA damage-independent RAD51 and DMC1 loading

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The DNA binding activity of RecA family recombinases is modulated by a variety of accessory factors that act as positive or negative regulators of filament initiation or stability. In spite of a good deal of work on these proteins, there is little understanding regarding why regulation of recombinase filaments is so complex, and this is a central issue in the field of homologous recombination. Here the authors analyze the role of one of these regulatory proteins, the AAA-ATPase FIGNL1 and its partner protein FIRRM. These proteins have been characterized in mitotic mouse cells and meiotic plant cells previously. Here the function of these proteins is characterized in mouse spermatocytes. FIRRM and FIGNL1 limit the formation or life span of recombinases RAD51 and DMC1 foci at cites of ongoing recombination. This activity is associated with efficient progression to later stages of crossover recombination. The work also provides evidence that FIGNL1 and FIRRM counteract a tendency of the recombinases to form structures that are not associated with recombination intermediates. For the most part, the experiments are done appropriately and provide good support for the key conclusions of the paper. If the appropriate changes are made, I think this paper is appropriate for publication in Nature Communications as it makes a significant contribution to understanding of the mechanisms that regulate recombinase activity in the mouse.

I have two main criticisms of the paper; experiments need to be substantially improved or removed from the paper.

The epistasis analysis involving SWSAP1 is not carried out properly because it lacks the Swsap1-/- single mutant control. The authors wish to use historical data as the control, but this is not appropriate. The entire experiment needs to be redone with the two single mutant controls or the partial experiment eliminated from the paper. Given the time and expense required to redo the experiment, I think it best to simply eliminate it as it is not a critical component of the paper.

The biochemical data could reflect non-specific aggregation of RAD51 and RAD51-DNA complexes caused by addition of the sample containing accessory protein. The fact that no detectable dissociation of preformed complexes is observed is consistent with this possibility as are the EM data. If the authors decide to carry out further experiments to show that FIGNL1 or FF can dissociate pre-formed complexes, or to exclude the possibility that non-specific aggregation explains the current observations, they should be careful to add storage buffer in place of protein dissolved in storage buffer when carrying out controls, because solubility can be sensitive to buffer components.

Another issue that I think should be addressed is that mention was made of differences in average RAD51 or DMC1 focus staining intensity. Mutants defective in dissociation of filamentous proteins tend to display foci with increased length or staining intensity. It appears that the differences in staining patterns examined here are restricted to focus number, not focus length or intensity. I think average focus staining intensities should be reported with appropriate interpretation of the results.

Additional corrections/suggestions for relatively minor issues are as follows:

54. aberrant gametes formation. (singular)

59. breaks (DSBs) formation (singular)

74. …foci which colocalize extensively at DSB sites.

78. Reference 18 is not relevant.

86. positively or negatively (I am unaware of a protein that does both).

88. …that, in mammals, comprises

89. The Shu complex promotes…… It is also important for assembling…

96. negatively regulate

106. Saying the role “remains unknown” is a bit awkward since the paper goes on to make claims about what the role is. How about “the role of FIGNL1 and FIRRM during meiotic recombination in mammals was unknown. “

116. filaments (plural)

160. are indicative (the evidence is better than “suggestive” for the claims made).

200. the control.

214. Consider changing title to “RAD51 accumulates on chromatin during premeiotic replication in Firrm cKO and Fignl1 cKO spermatocytes”

226. …foci did not colocalize with….

235. throughout the meiotic

237…and to greater numbers…

242. Awkward run-on sentence. Rewrite.

266. partial synapsis (not synapses)

269. indicate a highly aberrant…

327-334 This section should be re-written for clarity.

394. What structures are observed in the absence of DNA? This finding could also reflect end-to-end association of DNA bound filaments.
.
430. I do not think the list of differences in genetic requirements among species “explains” why FIGNLl1-FIRRM is seems to be more important for meiotic recombination in mouse. The results here simply add to the list of differences, but those differences remain

unexplained. One could say the difference in the requirement for FGNL1-FIRRM might be functionally related to the other differences.

460. Entire paragraph. This somewhat speculative section relies on the uncontrolled experiment referred to above. The Shinohara group did the proper experiment. That paper could be cited here if the authors would like to retain the speculation that the the function of FIGNL1 regulation is to keep RAD51 away from 3' filament ends. I also think the paragraph could be shortened.

574. The antibodies used….

578. primary antibody. Primary antibody incubation was performed in….

1088. The title of this figure is an interpretation of the data not a description of the result. The result is that foci of late acting recombination proteins are reduced which suggests a block before later stages. Also, I don't think the measurement of a single double mutant testes is sufficient. This observation could be eliminated without significant impact.

1128. I think this experiment is looking at co-localization of RAD51 and DMC1, not RAD51 and RPA2.

1164. Color differences for forward and reverse strands are difficult to distinguish. Suggest they be modified to make it easier to read the result. How is the fact that the total area under the curve is less in the cKO than the control explained? Is this not the opposite result from what is predicted?

In general, the figure legends have a lot of redundant information regarding closely related cytological experiments. The authors might use a phrase like "methods and statistical analysis are the same as described in Figure X."

Reviewer #2 (Remarks to the Author):

Zainu et al reports the functions of FIGNL1 and its partner FIRRM in mouse meiosis. The orthologues of these two proteins are known to function in meiosis in Arabidopsis and rice. FIGNL1 is also known to negatively regulate RDA51 in human cells. In this study, germ cell-specific conditional mutants of Fignl1 and Firrm (with Stra8-Cre) were generated and extensively characterized. Double mutants with Spo11 mutant or Swsap1 mutant were also studied. Both mutants showed meiotic blocks and similar meiotic defects: reduction in the number of recombination intermediates and accumulation of RAD51/DMC1 foci on intact chromatin. The excess RAD51 and DMC1 foci in Firrm cKO spermatocytes is Spo11-independent. Biochemical experiments show that recombinant human FIGNL1 changes RAD51/DMC1 nucleofilaments and inhibits D-loop formation. This comprehensive study demonstrates the essential role of FIGNL1 and FIRRM in mouse meiosis.

Major concerns:

1) Localization of FIGNL1 and FIRRM in testis is unknown. It is important to perform immunofluorescent analysis of FIGNL1 and FIRRM in testis sections to determine in which

germ cells they are expressed and their subcellular localizations. In addition, it is important to perform IF on spread nuclei of spermatocytes to determine if they form foci. Such results will be very informative and help explain the knockout phenotypes.

2) Lines 281-284: Fig. 5b, c: The description “Overall, the pattern of RAD51 281 and DMC1 in Firrm cKO and Spo11YF/YF Firrm cKO were similar:…” is not accurate. The number of RAD51 loci is significantly lower in Spo11YF/YF Firrm cKO than in Firrm cKO in every type of spermatocytes shown. The number of DMC1 foci is also lower in Spo11YF/YF Firrm cKO than in Firrm cKO in three out of four types of spermatocytes. These decreases show that loss of Spo11 function does have an impact on the number of RAD51 and DMC1 foci in Firrm cKO. Some RAD51/DMC1 foci in Firrm cKO are meiotic DSB (SPO11)-dependent and the extra RAD51/DMC1 foci are Spo11-independent. This needs to be described and discussed in the result section.

3) DMC1 ChIP (SSDS) was done in control and Firrm cKO testes but not in Spo11YF/YF Firrm cKO double mutant testes. While DMC1 ssDNA ChIP in Firrm cKO testes did provide some insights into DMC1 distribution at meiotic DSB hotspots, the outcome is expected because of wild type SPO11 function. I think that DMC1 ChIP in Spo11YF/YF Firrm cKO double mutant testes would be more informative. It would address whether the excess DMC1 foci correspond to ssDNA, or dsDNA, or meiotic DSB hotspots. From the genetic studies and spread analysis, it was assumed that RAD51/DMC1 in the absence of FIGNL1 or FIRRM bind to intact chromatin (dsDNA) (the excess foci). DMC1 ChIP or RAD51 ChIP might provide such evidence. At the minimum, this possibility needs to be considered and discussed.

4) What is FIGNL1deltaN? Is it a truncated version? Please define it.

5) Is localization of HORMAD1/HORMAD2 normal in Fignl1 and Firrm cKO spermatocytes?

Minor points:
Role of FIGNL1 and FIRRM in female meiosis? The Stra8-Cre transgene used in this study is not active in female germ cells. This can be mentioned.

Fig. 4d, e: the inset boxes needed to be labeled with numbers or letters. It is hard to follow which is which.

Line 140: change “is suggested play a role in…” to “is suggested to play a role in..”

Line 410: change “FIGL1” to “FIGNL1”.

Reviewer #3 (Remarks to the Author):

In this study, Zainu et al present an impressive piece of work related to the role of Fignl1 and Firrm during mouse meiosis. Understanding the dynamics of RAD51 filament is a major question in field of homologous recombination and DNA repair and the meiosis offer unique opportunities to unveil the role of key players. Here, the authors propose that the FIGNL1-FIRRM complex is required to dismantled the RAD51/DMC1 nucleofilament at DSBs preventing the further processing of recombination intermediates and thus DNA repair,

synapsis and crossover formation. They also describe a DSB-independent function preventing the excessive deposition of recombinases on ‘intact’ DNA during pre-meiotic S phase. An accompanying manuscript from Shinohara’s team and a very recently published article from Yu’s team (NAR) also reach similar conclusions with minor discrepancies. Such does not withdraw any merit to the present work as the role of FIGNL1 in plants presents quite substantial differences, thus having several teams from different horizons gaining independent demonstrations is most welcome. The present study (Zainu et al) is well conducted, robust and provides a fine description of the phenotype of Firrm and Fignl1 mutants.

Major comments:
1- Are the animals fertile -and if so do they transmit the flox or the excised allele-? The question may seem trivial regarding the testicular sections presented but it would be a nice way to demonstrate that ‘escapees’ are indeed due the Stra8-cre inefficiency.
2- The authors based this study on the assumption that Firrm-/- cells do not present any defect prior meiosis due to the use of the Stra8-cre. However, Stra8 is also present in some spermatogonia. In this line, it would be important to clarify whether any defect (spindle or RAD51 accumulation) exists in mitotic germ cells prior the time of meiotic initiation.
3- Showing the presence of the complex (in WT meiosis) at the time the recombinase filament is organized or dismantled would be an additional point to position a role FIGNL1-FIRRM at this stage. Immunostaining on spreads with DMC1 and TEX11 might answer the question. I am actually surprised it is not reported here and if a technical obstacle prevented it, it should be mentioned.

Minor comments:
1- It remains unclear whether the accumulation of RAD51 on ‘intact’ chromatin is due to meiotic specificities. Similarly do meiotic specificities on the ssDNA (apart from DMC1 presence) may explain the recombinase abundancy in the Swsap1KO; Fignl1cKO. These points should be clarified.
2- Introduction line 89-91 is unclear.
3- Results, line127. The message regarding the dKO should be tempered with n=1. Actually, it may even seem the lowest weight recorded. Can it be back-up by histological examinations?
4- line 129, not all tubules seem to contain both spermatogonia and spermatocytes.
5- Fig1c, Protein amount should be quantified. Is there any change in cytoplasmic RAD51?
6-Fig1e, from this figure, meiotic progression appears to slightly differ between Firrm and Fignl1 mutant mice. Could a similar age be provided (18dpp versus 8weeks)?
7- line 149, ‘normal meiotic entry’. This point warrants attention due to the observed effect during pre-meiotic S phase. Is the timing of meiotic entry indeed normal? A mere quantification of the percentage of meiotic cells (meiotic vs total germ cells) at 12 dpp might offer a first hint.
8- Please specify as much as possible the age in the legend of figures (especially for Fig1 & 2).
9- Fig2a-d; The ‘leptotene’ is confusing having already an ‘early leptotene’ category. Mid-late leptotene?
10- line 175, Fig 2d. This is not a trend it is significant (*** and ****). Thus it might be an oversimplification to say that first step of recombination were not affected. Please temper. Still regarding Fig2d, RPA2 behaves differently in Firrm and Fignl1cKO in mid zygotene. This should be noted/commented. In the mid-zygotene illustration chosen in Fig2c, the RPA2 foci appear quite abundant in the Fignl1cKO. Last, why no late zygonema are observed in Fignl1 cKO ?

11- line231, Fig3 h-i. The colocalization of RPA and RAD51 during pre-meiotic S phase is a very interesting observation, though I do not follow the rationale that prompt the author to propose that DNA is ‘intact’. This interrogation goes with the following assumption that the later accumulation of RAD51 and DMC1 is DNA damage-independent (including in Spo11 mutant). See also fig 5b-c below.
12- typo line 243 (RD51)
13- The nice observation from Fig4f warrants additional comments. How such might be related to the possibly altered distribution of RAD51 and DMC1 on the filament.
14- Fig5b-c, line 282. How do the authors explain the differences between Firrm cKO and the double mutant (Firrm Spo11). The difference between both is highly significative. Doesn’t this suggest that RAD51 and DMC1 accumulation is partly dependent of DSB ? Please clarify or comment.
15- Similarly, though I globally agree with authors’ conclusion, I am not sure whether one can claim that the formation of RAD51 and DMC1 foci is (fully) independent from SWSAP1 in Fignl1 mutant? I see statistically significant differences (Fig 7 and Ext fig 8). Partly independent ?
16- Line 378 For readers, please explain the use of DeltaN-FIGNL1.
17- Discussion, I do not follow the type of role considered before strand invasion (line 444) and whether a relationship is considered between the two proposed roles of the FIGNL1-FIRRM complex (pre-meiotic replication, meiotic breaks).
18- Ext data Fig1. A draw of FIGNL1 protein might be useful here and may help positioning the N-ter for later biochemistry (Fig8).
19- A single study had suggested a role for Fignl1 during male mouse meiosis -prior these- (L’Hote et al PLoS One. 2011;6(11):e27582. doi: 10.1371). It might be worth an additional reference.
20- line 615-616, How does ‘simulating the random localization’ is performed? Specific soft for randomizing numbers/XY coordinates?
21- line 629, typo ‘ntot’ instead of ‘nT’

We want first to thank the reviewers for their comments, which allowed us to improve significantly the manuscript. We have addressed comments in our point-by-point response below. All changes are highlighted in blue within the manuscript.

In addition to modifications made in response to reviewer's comments, a few major additional changes are listed below.

- Raphaël Mercier was included among the authors, because the project was initiated on his initiative, relied on unpublished information obtained from him (he identified Firrm as the mouse ortholog of the plant FLIP gene), and beneficiated from his input at all stages.
- Statistics: Mann-Whitney tests for comparing focus counts have been by Dunn's multiple comparison tests to consider multiple comparisons. This led to less significant differences, as discussed below for specific cases pointed by reviewers.
- The antibody against FIRRM used in Western Blot was replaced by another reference (supplementary Table 2 has been updated).
- Given the extended time taken for the revision, several additional references were included, and are highlighted in blue in the bibliography.
- For clarity and avoiding redundancy, the paragraph describing RAD51-DMC1 colocalization has been moved after the description of Spo11YF Firrm cKO double mutant. Thus, RAD51-DMC1 colocalization in single (Firrm cKO) and double mutants are described together, because an important conclusion is that RAD51-DMC1 high level of colocalization is maintained in Firrm cKO and is DSB-independent (lines 296-311).

REVIEWER COMMENTS

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The DNA binding activity of RecA family recombinases is modulated by a variety of accessory factors that act as positive or negative regulators of filament initiation or stability. In spite of a good deal of work on these proteins, there is little understanding regarding why regulation of recombinase filaments is so complex, and this is a central issue in the field of homologous recombination. Here the authors analyze the role of one of these regulatory proteins, the AAA-ATPase FIGNL1 and its partner protein FIRRM. These proteins have been characterized in mitotic mouse cells and meiotic plant cells previously. Here the function of these proteins is characterized in mouse spermatocytes. FIRRM and FIGNL1 limit the formation or life span of recombinases RAD51 and DMC1 foci at cites of ongoing recombination. This activity is associated with efficient progression to later stages of crossover recombination. The work also provides evidence that FIGNL1 and FIRRM counteract a tendency of the recombinases to form structures that are not associated with recombination intermediates. For the most part, the experiments are done appropriately and provide good support for the key conclusions of the paper. If the appropriate changes are made, I think this paper is appropriate for publication in Nature Communications as it makes a significant contribution to understanding of the mechanisms that regulate recombinase activity in the mouse.

I have two main criticisms of the paper; experiments need to be substantially improved or removed from the paper.

The epistasis analysis involving SWSAP1 is not carried out properly because it lacks the Swsap1-/- single mutant control. The authors wish to use historical data as the control, but this is not

appropriate. The entire experiment needs to be redone with the two single mutant controls or the partial experiment eliminated from the paper. Given the time and expense required to redo the experiment, I think it best to simply eliminate it as it is not a critical component of the paper.
The experiment was redone with Swsap1-/- single mutant control, and samples include now both Swsap1-/- Firrm cKO and Swsap1-/- Fignl1 cKO (lines 372-403).
We confirm that the number of DMC1 foci is lower in Swsap1-/- single mutant than in control, and similar between double mutants and single Fignl1cKO or FirrmcKO. However, because most DMC1 foci in FirrmcKO are Spo11-independent, this information alone did provide any clue on the recruitment of DMC1 at SPO11-dependent DSBs.
Thus, to assess more specifically DMC1 recruitment at DSB sites, we counted DMC1-RPA co-foci: whereas the number of RPA foci colocalized with DMC1 was reduced in Swsap1-/- (as expected from the overall reduced number of DMC1 foci), this number was similar in double mutants and in single Fignl1cKO or FirrmcKO mutants, similar or higher than in control (Fig. 7d and Extended Data Fig. 8c-e). This suggests that SWSAP1 is not necessary for forming normal numbers of detectable DMC1 foci at meiotic DSBs when FIGNL1-FIRRM is absent. We believe that this observation supports an early role for FIGNL1-FIRRM in assembling/stabilizing the nucleo-filament, as discussed in the Discussion section (paragraph reformulated for clarity, lines 520-533).

The biochemical data could reflect non-specific aggregation of RAD51 and RAD51-DNA complexes caused by addition of the sample containing accessory protein. The fact that no detectable dissociation of preformed complexes is observed is consistent with this possibility as are the EM data. If the authors decide to carry out further experiments to show that FIGNL1 or FF can dissociate pre-formed complexes, or to exclude the possibility that non-specific aggregation explains the current observations, they should be careful to add storage buffer in place of protein dissolved in storage buffer when carrying out controls, because solubility can be sensitive to buffer components.
The control experiments were always done by adding the same volume of storage buffer without protein, and this is now mentioned explicitly in Methods section (EMSA and TEM experiments). We did not observe any particular effect of the storage buffer of FIGLN1DN on RAD51 filaments.
Regarding the properties of the observed aggregation, we have increased the ionic strength (up to 150mM NaCl, 5mM MgCl2) to release non-specific electrostatic interactions: it did not affect the effect of FIGLN1DN on RAD51 filaments.
In addition, we really checked the homogeneity of each of proteins by EM and did not identify any contaminant nor heterogeneity in the preparations.
In conclusion, here we showed that FIGLN1DN protein preparation was homogeneous, and that the inhibitory effect of FIGLN1DN on RAD51-mediated D-loop formation was not influenced by the FIGLN1 storage buffer but increased with the concentration of FIGLN1DN, so we conclude to the specificity of this effect.

Another issue that I think should be addressed is that mention was made of differences in average RAD51 or DMC1 focus staining intensity. Mutants defective in dissociation of filamentous proteins tend to display foci with increased length or staining intensity. It appears that the differences in staining patterns examined here are restricted to focus number, not focus length or intensity. I think average focus staining intensities should be reported with appropriate interpretation of the results.
We measured RAD51 and DMC1 focus intensity in RAD51-DMC1 co-labeled control and Firrm cKO spermatocytes with RAD51 and DMC1 co-labeling: RAD51 focus intensity was increased while DMC1 focus intensity was similar (replicate 1) or lower (replicate 2) than in control (Extended Data Fig. 4a-b). A sentence has been added to mention this observation: “Consistently with this observation, the

intensity of RAD51 foci was increased compared to that of DMC1 foci in *Firrm* cKO spermatocytes (Extended Data Fig. 4a-b)."(lines 260-262)

Additional corrections/suggestions for relatively minor issues are as follows:

54. aberrant gametes formation. (singular) fixed

59. breaks (DSBs) formation (singular) fixed

74. …foci which colocalize extensively at DSB sites. fixed

78. Reference 18 is not relevant. Citation removed.

86. positively or negatively (I am unaware of a protein that does both). Fixed.

88. …that, in mammals, comprises fixed.

89. The Shu complex promotes…… It is also important for assembling… fixed

96. negatively regulate fixed.

106. Saying the role "remains unknown" is a bit awkward since the paper goes on to make claims about what the role is. How about "the role of FIGNL1 and FIRRM during meiotic recombination in mammals was unknown. " fixed.

116. filaments (plural) fixed.

160. are indicative (the evidence is better than "suggestive" for the claims made). Fixed.

200. the control. Fixed.

214. Consider changing title to "RAD51 accumulates on chromatin during premeiotic replication in Firrm cKO and Fignl1 cKO spermatocytes" The title has been modified accordingly (line 230).

226. …foci did not colocalize with…. fixed.

235. throughout the meiotic identical to original text.

237…and to greater numbers… fixed.

242. Awkward run-on sentence. Rewrite. The sentence has been rewritten.

266. partial synapsis (not synapses) fixed.

269. indicate a highly aberrant… fixed.

327-334 This section should be re-written for clarity.

This section has been re-written to explain more clearly the differences in DMC1-SSDS profiles at hotspots between control and Firrm cKO, and their interpretations. (lines 353-370)

394. What structures are observed in the absence of DNA? This finding could also reflect end-to-end association of DNA bound filaments.
We agree with the reviewer, as it is difficult to prove that there was no contaminating DNA at all (although their formation in absence of added DNA substrate was resistant to incubation with benzonase). Since we observed the formation of similar long filaments in presence of short DNA molecules (400nt) or in the absence of DNA added in the reaction, we're inclined to think that the polymerization is DNA-free. But we can't prove that these long filaments formed in the reaction with DNA do not contain DNA, and their architecture does not appear to be different. However, it would be interesting in another study to resolve their structure at high resolution using cryo-EM, in order to find out whether FIGLN1DN is involved in their formation, or whether their structure is different from that of filaments assembled on DNA.
The sentence was reworded to mitigate the conclusion (removal of “confirming”, replaced with “consistent with this hypothesis”): “Their length was not compatible with the length of the used DNA substrate, suggesting a DNA-independent polymerization in the presence of FIGNL1ΔN. Consistent with this hypothesis, similar structures were detected by incubating RAD51 with FIGNL1ΔN without adding any DNA (Extended Data Fig. 10b-d).” (lines 423-427)

430. I do not think the list of differences in genetic requirements among species “explains” why FIGNLl1-FIRRM is seems to be more important for meiotic recombination in mouse. The results here simply add to the list of differences, but those differences remain unexplained. One could say the difference in the requirement for FGNL1-FIRRM might be functionally related to the other differences.
This sentence has been reworded according to reviewer comment.(lines 465-467)

460. Entire paragraph. This somewhat speculative section relies on the uncontrolled experiment referred to above. The Shinohara group did the proper experiment. That paper could be cited here if the authors would like to retain the speculation that the the function of FIGNL1 regulation is to keep RAD51 away from 3’ filament ends. I also think the paragraph could be shortened.
The Swsap1-/- experiment has been redone with appropriate control (Swsap1-/- single mutant), with analysis of RPA2 and DMC1 foci. An important conclusion is that SWSAP1 is not required for forming normal numbers of DMC1-RPA2 co-foci in Swsap1-/- Firrm/Fignl1 cKO spermatocytes, which is consistent with SWSAP1 being required to protect the RAD51/DMC1 filaments from FIGNL1-FIRRM-dependent destabilization. The whole paragraph has been reformulated extensively for clarity. (lines 495-533)

574. The antibodies used…. Fixed.

578. primary antibody. Primary antibody incubation was performed in…. fixed.

1088. The title of this figure is an interpretation of the data not a description of the result. The result is that foci of late acting recombination proteins are reduced which suggests a block before later stages.
This title has been modified accordingly: “Meiotic DSBs are formed and processed in Firrm cKO and Fignl1 cKO spermatocytes”. (line 1164)

Also, I don't think the measurement of a single double mutant testes is sufficient. This observation could be eliminated without significant impact.
We have now 4 double mutant testes, 2 adults and 2 juv (Fig 1a and Extended Data Fig2a). Double mutants are similar to single cKO for testis weight and histology (Extended Data Fig2b).

1128. I think this experiment is looking at co-localization of RAD51 and DMC1, not RAD51 and RPA2.
Yes indeed, this has been fixed. (Fig. 5, line1194)

1164. Color differences for forward and reverse strands are difficult to distinguish. Suggest they be modified to make it easier to read the result. How is the fact that the total area under the curve is less in the cKO than the control explained? Is this not the opposite result from what is predicted?
The colors on Fig. 6f were changed to more contrasted ones for clarity.

Smaller area under cKO curve on Fig. 6e. The DMC1-SSDS data were normalized only for the number of reads (RPM)/fragments (FPM) in each library. Therefore, the profiles can be compared between samples, but the enrichments values cannot. For example, the Extended Data Fig. 7b-c illustrates that the average enrichment at hotspots was lower in Firrm cKO than in control in replicate 1, whereas it was quite the same in both genotypes in replicate 2. On Fig. 6e, where reads from replicates 1 and 2 were pooled, the resulting profile is closer to replicate 1, because more reads were obtained in replicate 1 than in replicate 2. However, because the experimental setup lacks normalizing enrichments to a reference, we cannot conclude whether or not this difference reflects a moderate decrease in DMC1 recruitment on ssDNA at DSB hotspots, or not. Given the observed enrichments, and the detection of ~7,000 peaks in FirrmcKO versus ~10,000 peaks in control, we can however conclude that, if the recruitment of DMC1 on ssDNA at hotspots is lowered, the decrease is not dramatic.

On normalized Fig. 6f, the area under cKO curve looks also smaller in the "shoulder" region (Fig. 6f). However, the total areas within the -2.5kb-+2.5kb were normalized such that the total area is the same: higher values in FirrmcKO in side intervals compensate fully for lower values in the central "shoulder" intervals. We thus interpret the difference as suggesting a change in average distribution of DMC1-SSDS signal at DSB hotspots.

Would one predict higher or lower DMC1-SSDS enrichment at Spo11-dependent DSB hotspots? Assuming alternative hypotheses, different predictions might be done:
(1) Higher enrichment is predicted, if DMC1 is recruited efficiently (possibly on longer ssDNA fragments?) and persists longer (repair defect) on ssDNA at DSB hotspots in FirrmcKO. However, it is not clear whether a moderate overall increase would be detectable in our experiments. For example, DMC1-SSDS signal to DSB (SPO11-oligo) is higher on chromosome X (likely because of delayed repair) than on autosome hotspots (e.g. Lange et al, 2020; this manuscript, Extended Data Fig. 7d). However, detecting this effect relies on direct comparison between chromosome X and autosomes within a single library. It seems unlikely to detect this range of variation between samples, if it occurs genome-wide (see below).
(2) Conversely, a strongly reduced or abolished DMC1-SSDS enrichment at DSB hotspots in Firrm cKO:
    (a) if all available DMC1 is titrated away, e.g. to Spo11-independent sites (on dsDNA or ssDNA);
    (b) or, even if DMC1 amount is not limiting, if Spo11-independent DMC1 foci visualize DMC1 recruitment on persistent ssDNA throughout the genome, by diluting the signal: if these ssDNA fragments are long enough to be retrieved in DMC1-SSDS experiments, resulting in a

strong reduction of the relative enrichment in DSB hotspot fragments within the library. Even if Spo11-independent DMC1 recruitment on ssDNA is widely spread throughout the genome, no new peaks are expected, but a strong decrease in hotspot peak signal is still expected.
Our detection of a number of a number of PRDM9- and SPO11-dependent peaks similar to control indicates that the recruitment of DMC1 on ssDNA at hotspots is not impaired dramatically, and suggests that the abundant Spo11-independent DMC1 foci do not visualize DMC1 bound on persistent ssDNA, retrievable by the DMC1-SSDS method.
The main text section related to this figure has been rewritten for clarity, as mentioned above (lines 353-370).

In general, the figure legends have a lot of redundant information regarding closely related cytological experiments. The authors might use a phrase like "methods and statistical analysis are the same as described in Figure X."
We added "Dunn's multiple comparison tests for comparisons of focus numbers unless stated otherwise." At first occurrence (Fig. 2b legend), and removed that and some additional redundant information throughout legends.

Reviewer #2 (Remarks to the Author):

Zainu et al reports the functions of FIGNL1 and its partner FIRRM in mouse meiosis. The orthologues of these two proteins are known to function in meiosis in Arabidopsis and rice. FIGNL1 is also known to negatively regulate RDA51 in human cells. In this study, germ cell-specific conditional mutants of Fignl1 and Firrm (with Stra8-Cre) were generated and extensively characterized. Double mutants with Spo11 mutant or Swsap1 mutant were also studied. Both mutants showed meiotic blocks and similar meiotic defects: reduction in the number of recombination intermediates and accumulation of RAD51/DMC1 foci on intact chromatin. The excess RAD51 and DMC1 foci in Firrm cKO spermatocytes is Spo11-independent. Biochemical experiments show that recombinant human FIGNL1 changes RAD51/DMC1 nucleofilaments and inhibits D-loop formation. This comprehensive study demonstrates the essential role of FIGNL1 and FIRRM in mouse meiosis.

Major concerns:

1) Localization of FIGNL1 and FIRRM in testis is unknown. It is important to perform immunofluorescent analysis of FIGNL1 and FIRRM in testis sections to determine in which germ cells they are expressed and their subcellular localizations. In addition, it is important to perform IF on spread nuclei of spermatocytes to determine if they form foci. Such results will be very informative and help explain the knockout phenotypes.
We agree with the reviewer, but unfortunately, despite several attempts with several antibodies, we did not succeed in detecting FIGNL1 or FIRRM on sections, or spermatocyte squashes or spreads.

2) Lines 281-284: Fig. 5b, c: The description "Overall, the pattern of RAD51 281 and DMC1 in Firrm cKO and Spo11YF/YF Firrm cKO were similar:…" is not accurate. The number of RAD51 loci is significantly lower in Spo11YF/YF Firrm cKO than in Firrm cKO in every type of spermatocytes shown. The number of DMC1 foci is also lower in Spo11YF/YF Firrm cKO than in Firrm cKO in three out of four types of spermatocytes. These decreases show that loss of Spo11 function does have an impact on the number of RAD51 and DMC1 foci in Firrm cKO. Some RAD51/DMC1 foci in Firrm cKO are meiotic DSB (SPO11)-dependent and the extra RAD51/DMC1 foci are Spo11-independent. This needs to be described and discussed in the result section.

When multiple comparisons are considered (non-parametric Dunn's multiple comparison test, which is conservative), the differences between Firrm cKO and double Spo11YF Firrm cKO in individual side-by-side comparisons are significant only for axis-associated RAD51 foci in early leptotene and mid-zygotene (Fig. 5b-c, Extended Data Fig 5a-b), although we acknowledge that the trend remains consistent in early leptotene and zygotene for both RAD51 and DMC1.
As suggested by reviewers #2 and #3, the higher counts in zygotene may be interpreted as visualizing the formation of DSB-dependent RAD51/DMC1 foci in Firrm cKO. This is consistent with the DMC1-SSDS experiment where DMC1 is detected at DSB hotspots in Firrm cKO.
In early leptotene however, we have no clear explanation for a difference between single and double mutants. gH2AX and RPA2 staining suggests that the number of processed DSB is still very low at this stage in Firrm cKO, not different from control (Fig. 2).
Because this experiment (with all genotypes) has only been done in duplicate, with a limited number of nuclei, we chose not to emphasize these differences and to focus our conclusions rather on the major result that most RAD51 and DMC1 foci are SPO11 DSB-independent. Nevertheless, we added a short description and discussion of this difference at the end of this section of Results. (lines 288-294).

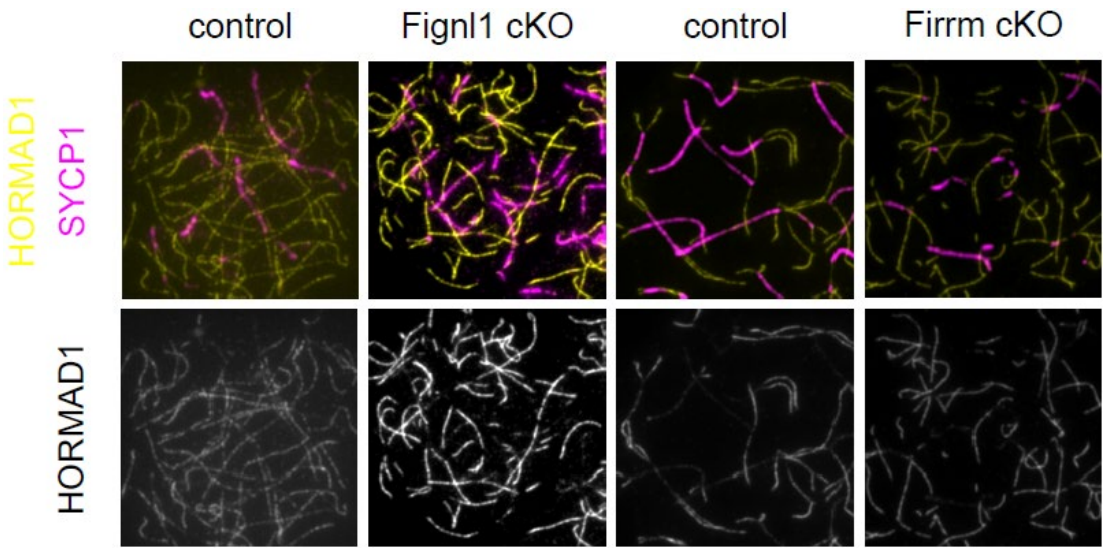
3) DMC1 ChIP (SSDS) was done in control and Firrm cKO testes but not in Spo11YF/YF Firrm cKO double mutant testes. While DMC1 ssDNA ChIP in Firrm cKO testes did provide some insights into DMC1 distribution at meiotic DSB hotspots, the outcome is expected because of wild type SPO11 function. I think that DMC1 ChIP in Spo11YF/YF Firrm cKO double mutant testes would be more informative. It would address whether the excess DMC1 foci correspond to ssDNA, or dsDNA, or meiotic DSB hotspots. From the genetic studies and spread analysis, it was assumed that RAD51/DMC1 in the absence of FIGNL1 or FIRRM bind to intact chromatin (dsDNA) (the excess foci). DMC1 ChIP or RAD51 ChIP might provide such evidence. At the minimum, this possibility needs to be considered and discussed.
We agree with the reviewer about the interest of performing DMC1 ChIP-seq in Spo11 mutant. However, it was beyond the scope of this project.

- We performed one DMC1 ChIP-seq experiment designated to detect binding on dsDNA, in parallel to one DMC1-SSDS replicate (on same chromatin sample). We found no detectable difference between control and Firrm cKO. The absence of detectable peaks might be due either to the absence of DMC1 binding on dsDNA, to the spreading of dsDNA binding on long/dispersed/random regions throughout the genome, or to some technical failure. Because there is no significant signal in control (maybe because DMC1 is essentially bound to ssDNA in WT), we could not conclude about DMC1 binding on dsDNA from this assay.
- The efficient detection of DMC1-SSDS peaks at SPO11-dependent DSB hotspots suggests that this signal was not outcompeted by an excess of retrievable DMC1 bound on additional long, persistent stretches of ssDNA expected to give rise to additional peaks, which were not detected, or no peaks if the SPO11-independent signal is spread throughout the genome). Although we agree that the experiment should be done to give a definitive answer (and tell whether there is DSB-independent binding of DMC1 on hotspots in Spo11YF/YF Firrm cKO), our result suggests that the SPO11-independent DMC1 foci do not result massively from binding to long stretches of ssDNA.
- Since the submission of our manuscript, RAD51-SSDS experiment was performed by the group of Akira Shinohara, showing no enrichment of RAD51 on ssDNA specific to Spo11-/-Fignl1cKO compared with Spo11-/- (Ito et al, 2023, ncomms). This is the more direct evidence for the absence of RAD51 and DMC1 binding to specific, detectable ssDNA regions in the absence of FIGNL1/FIRRM in mouse spermatocytes.

We added a short discussion and citation of Ito et al work in the text (lines 344-347).

4) What is FIGNL1deltaN? Is it a truncated version? Please define it.
The organization of FIGNL1 and the truncation FIGNL1DeltaN are now depicted on Extended Data figure 1b, and the role of this truncation (to allow producing soluble protein) is mentioned in Methods, with citation of the original publication (Matsuzaki et al, ncomms, 2019).(line 752)

5) Is localization of HORMAD1/HORMAD2 normal in Fignl1 and Firrm cKO spermatocytes?
We did not confirm reproducible difference in localization or intensity of HORMAD1 on unsynapsed and synapsed regions (representative zygotene/Z-like images below; we did not assay HORMAD2). There was significant nucleus-to-nucleus variation in the detected level of HORMAD1 remaining on synapsed regions (SYCP1), both in cKO and in control. Determining with certainty whether or not an excess of "residual" HORMAD1 correlates with the cKO genotypes, as recently reported by Zhang and col. (Nuc Acids Res), would require more extensive analysis. As it is, we chose not to draw any conclusion within this manuscript.



Minor points:
Role of FIGNL1 and FIRRM in female meiosis? The Stra8-Cre transgene used in this study is not active in female germ cells. This can be mentioned.
We added this mention in Methods section: "This transgene expresses Cre specifically in male germ cells, from undifferentiated spermatogonia to preleptotene spermatocytes (Sadate-Ngatchou et al, 2008).".(lines 584-585)

Fig. 4d, e: the inset boxes needed to be labeled with numbers or letters. It is hard to follow which is which.
We followed the reviewer's suggestion and labeled insets with Roman numerals.

Line 140: change "is suggested play a role in…" to "is suggested to play a role in.." fixed.

Line 410: change "FIGL1" to "FIGNL1". Here, FIGL1 refers to the plant ortholog of FIGNL1. This is now made explicit in the text. (line 444)

Reviewer #3 (Remarks to the Author):

In this study, Zainu et al present an impressive piece of work related to the role of Fignl1 and Firrm during mouse meiosis. Understanding the dynamics of RAD51 filament is a major question in field of homologous recombination and DNA repair and the meiosis offer unique opportunities to unveil the role of key players. Here, the authors propose that the FIGNL1-FIRRM complex is required to dismantled the RAD51/DMC1 nucleofilament at DSBs preventing the further processing of recombination intermediates and thus DNA repair, synapsis and crossover formation. They also describe a DSB-independent function preventing the excessive deposition of recombinases on 'intact' DNA during pre-meiotic S phase. An accompanying manuscript from Shinohara's team and a very recently published article from Yu's team (NAR) also reach similar conclusions with minor discrepancies. Such does not withdraw any merit to the present work as the role of FIGNL1 in plants presents quite substantial differences, thus having several teams from different horizons gaining independent demonstrations is most welcome. The present study (Zainu et al) is well conducted, robust and provides a fine description of the phenotype of Firrm and Fignl1 mutants.

Major comments:

1- Are the animals fertile -and if so do they transmit the flox or the excised allele-? The question may seem trivial regarding the testicular sections presented but it would be a nice way to demonstrate that 'escapees' are indeed due the Stra8-cre inefficiency.

We performed sperm count in epididymis, and found no sperm at all in epididymis from 4-month-old Firrm cKO males (Extended Data Fig. 2d).(line 143)

We assessed the efficiency of the transmission of the excised allele to the progeny in phenotypically WT animals, by mating wild-type females with Firrm flox/+ Stra8-Cre Tg males: among 97 pups, 52 were Firrm+/+ and 45 were Firrm+/-. The Firrm flox allele was not detected in any, indicating a 100% (45/45) transmission of the excised allele to the progeny. (lines 144-145)

The residual WT-looking spermatocytes and spermatids might thus represent either a small proportion of FIGNL1/FIRRM-depleted germ cells with no (observed) phenotype, or a small proportion of cells in which the excision of the exon, and/or the depletion of the protein, is delayed. We did not succeed in detecting FIGNL1 or FIRRM in individual cells on sections or spreads, which would have provided a direct evidence for the presence of the proteins in these cells. Nevertheless, we favor the second hypothesis (delay in protein depletion), in line with our previous experience with same Stra8-Cre transgene on floxed Hells (Imai et al., eLife, 2020), and with the observations reported by Ito et al (ncomms, 2023) on Fignl1 cKO.

These results are now reported with interpretations in the first section of Results.(lines 142-145)

2- The authors based this study on the assumption that Firrm-/- cells do not present any defect prior meiosis due to the use of the Stra8-cre. However, Stra8 is also present in some spermatogonia. In this line, it would be important to clarify whether any defect (spindle or RAD51 accumulation) exists in mitotic germ cells prior the time of meiotic initiation.

We agree that the exact timing of Cre-driven gene excision and the depletion of the proteins FIGNL1 or FIRRM might occur before the time of meiotic initiation in some cells.

Our Stra8-Cre transgene was reported to lead to detectable excision-dependent expression of a GFP reporter in "only a small proportion of spermatogonia (…) at P4 and P8", while "at P14, all meiotic cells displayed strong (GFP) signal (Bao et al, genesis, 2013, DOI: 10.1002/dvg.22389). Thus, pre-meiotic depletion of FIRRM would be predicted to affect only a minority of cells, at least during first meiotic wave, whereas RAD51 accumulation in pre-leptotene cells was observed in a majority of cells

(Fig. 3b). Moreover, we did not observe RAD51 accumulation in non-meiotic cells (SYCP3-negative cells) suggesting that RAD51 accumulation before entry in meiotic initiation is rare if there is any in juveniles. Thus, pre-leptotene RAD51 accumulation is unlikely to result from FIGNL1/FIRRM depletion in spermatogonia prior entry into the meiotic cycle.

The likely depletion of spermatogonia in adults, with little expected effect on our analyses on spermatocytes in juvenile, is now discussed in the first paragraph of results.(lines 131-139)

3- Showing the presence of the complex (in WT meiosis) at the time the recombinase filament is organized or dismantled would be an additional point to position a role FIGNL1-FIRRM at this stage. Immunostaining on spreads with DMC1 and TEX11 might answer the question. I am actually surprised it is not reported here and if a technical obstacle prevented it, it should be mentioned.
We agree with the reviewer that the immunolocalization of FIGNL1 and/or FIRRM would be important to determine in which cells these proteins are present (cell type and stage in control, "escapees" in cKO), and their possible localization on meiotic chromosomes. Unfortunately, despite several attempts with several antibodies, we did not succeed in detecting them.

Minor comments:
1- It remains unclear whether the accumulation of RAD51 on 'intact' chromatin is due to meiotic specificities. Similarly do meiotic specificities on the ssDNA (apart from DMC1 presence) may explain the recombinase abundancy in the Swsap1KO; Fignl1cKO. These points should be clarified.
We did not observe RAD51 before preleptotene (in SYCP3-negative nuclei). However, we do not know whether the accumulation of RAD51 during premeiotic replication results from meiotic specificities, or just from the timing of FIRRM or FIGNL1 depletion in most cells (response to major comment 2, above).
Accumulation of RAD51 foci was reported in non-meiotic Fignl1/Firrm-deficient cells (not exposed to a replication challenging agent) in several studies (e.g., Stock et al, 2023, Cell Rep; Tischler et al, 2024, ncomms; Matsuzaki et al, 2024, NAR). Thus, the same mechanism likely takes place during premeiotic replication. We do not know whether the larger number of foci observed in preleptotene cells is a property of premeiotic replication, or the technical result of the spreading protocol.

The abundance of the recombinase in FirrmcKO and Fignl1cKO does not depend on SPO11-catalyzed DSBs. In Swsap1KO where SPO11-dependent DSBs form, we show that depleting FIGNL1-FIRRM restores the number of RPA-DMC1 co-foci to a WT level, but most foci might be DSB-independent with no evidence that these are on ssDNA. The number of RPA-DMC1 co-foci was similar in control, Fignl1/Firrm cKO and Swsap1KO Fignl1/Firrm cKO, suggesting that the abundance of recombinase foci on ssDNA generated at DSBs might not be different from WT. The section on Swsap1KO Firrm/Fignl1cKO has been rewritten to explain this with new results including the Swsap1KO control.(liness73-403)

2- Introduction line 89-91 is unclear.
This section has been reworded for clarity.(lines 87-91)

3- Results, line127. The message regarding the dKO should be tempered with n=1. Actually, it may even seem the lowest weight recorded. Can it be back-up by histological examinations?
We have now two 16-dpp juveniles and 2 adults: their testis weight/body weight ratios are in line with single cKO of same ages (kinetics shown on Extended Data Fig. 2a). A representative section is shown on Extended Fig. 2b, with qualitatively similar defect (now mentioned in the text).

4- line 129, not all tubules seem to contain both spermatogonia and spermatocytes.
As noticed by the reviewer, the density of germ cells in tubules tends to decrease with age in Fignl1cKO and FirrmcKO, suggesting that the pool of spermatogonia is affected in adults. We added images of sections from 4-week-old animals (now Fig. 1b), where the density of germ cells appears normal, and from 8-week-old and 10-11-week-old mice, where the depletion of germ cells is apparent (new Extended Data Fig. 2b-c). By comparing tubules from Spo11YF/YF (strictly meiotic) and Firrm cKO 8-week-old testes, the depletion is apparent in the second (Extended Data Fig. 2c). Thus, FIGNL1/FIRRM depletion in spermatogonia likely affects their proliferation/renewal during adult life. Nevertheless, this should not have significant consequences on our analysis during first meiotic waves in juveniles (see above, our response to major comment 2). (lines 131-139)

5- Fig1c, Protein amount should be quantified. Is there any change in cytoplasmic RAD51?
Quantifications of protein amounts are now displayed in Extended Data Fig. 2e. The experiment was done in duplicate for each genotype, and measurement in Fignl1cKO and FirrmcKO experiments were pooled for generating confidence intervals. The depletion of cytoplasmic and nuclear FIGNL1 and FIRRM, and the increase of nuclear RAD51 (but not DMC1) were detected. Despite a trend toward lower values, the change in cytoplasmic RAD51 was not significant. Given the ~10-fold larger volume of cytoplasmic versus nuclear extract and the small number of replicate experiments, a depletion in the cytoplasmic equivalent to the increase detected in nuclear fraction might remain below detection threshold however. (lines 151-155)

6-Fig1e, from this figure, meiotic progression appears to slightly differ between Firrm and Fignl1 mutant mice. Could a similar age be provided (18dpp versus 8weeks)?
Staging of an age-matched adult Firrm cKO (with control) is now provided for direct comparison on Fig. 1e. The distribution of meiotic prophase substages is more similar to adult Fignl1 cKO, indicating that the difference resulted from the age rather than from the genotype.

7- line 149, 'normal meiotic entry'. This point warrants attention due to the observed effect during pre-meiotic S phase. Is the timing of meiotic entry indeed normal? A mere quantification of the percentage of meiotic cells (meiotic vs total germ cells) at 12 dpp might offer a first hint.
The proportion of meiotic prophase cells (leptotene to diplotene) among all SYCP3-positive cells (i.e., meiotic prophase plus preleptotene) was assessed on spreads: they represented 72% vs 58% at 12-dpp, 80% vs 82% at 17-dpp, 78% vs 81% at 18-dpp in FirrmcKO and control, respectively. Preleptotene was chosen instead of all germ cells because the efficiency of spreading of non-meiotic cells and the identification of non-meiotic germ cells are not extremely reliable with our spreading protocol. Also, the question here is that of entry into meiotic prophase, the chosen ratio is independent on possible earlier defect before preleptotene. We see no evidence for a strong defect in meiotic entry and will therefore leave the sentence "suggesting a normal meiotic prophase entry".(line 162)

8- Please specify as much as possible the age in the legend of figures (especially for Fig1 & 2).
The age has been specified on figure legends.

9- Fig2a-d; The 'leptotene' is confusing having already an 'early leptotene' category. Mid-late leptotene?

”leptotene” is replaced with “mid-late leptotene” in all figures.

10- line 175, Fig 2d. This is not a trend it is significant (*** and ****). Thus it might be an oversimplification to say that first step of recombination were not affected. Please temper. Still regarding Fig2d, RPA2 behaves differently in Firrm and Fignl1cKO in mid zygotene. This should be noted/commented. In the mid-zygotene illustration chosen in Fig2c, the RPA2 foci appear quite abundant in the Fignl1cKO. Last, why no late zygonema are observed in Fignl1 cKO ?
The Fignl1 cKO mid-zygotene illustration in Fig 2c contained images of 2 nuclei, explaining the abundance of foci. It has been replaced by a more representative image of a single nucleus.
Fignl1 cKO late zygotene RPA2 counts have been added (Fig. 2d and S2d). With more nuclei counted from additional mice, and taking into account multiple comparisons, RPA2 behaves quite similarly in Firrm cKO and Fignl1 cKO.
The higher number of RPA2 foci in early and mid-zygotene cKO spermatocytes is now discussed in the main text: for example, a staging artifact due to differences in synapsis progression, a different kinetics of DSB formation or repair, or the presence of different HR intermediates with different RPA2 detectability.(lines 187-191)

11- line231, Fig3 h-i. The colocalization of RPA and RAD51 during pre-meiotic S phase is a very interesting observation, though I do not follow the rationale that prompt the author to propose that DNA is ‘intact’. This interrogation goes with the following assumption that the later accumulation of RAD51 and DMC1 is DNA damage-independent (including in Spo11 mutant). See also fig 5b-c below.
We have no direct evidence demonstrating that DNA is “intact”, therefore the assertion was mitigated by replacing “likely on intact DNA” with “possibly”. There are some indications that there is no massive accumulation of DNA damage however.
First, RPA and gH2AX were not increased in early stages (preleptotene, early leptotene) in Firrm cKO compared to control, nor in Spo11YF/YF Firrm cKO compared to Spo11YF/YF.
Second, DMC1-SSDS experiments in Firrm cKO spermatocytes led to efficient detection of DSB hotspot but no additional peaks, suggesting that there is not an excess of DMC1-bound ssDNA outside hotspots (predicted to decrease the detection of hotspots, by diluting the signal at hotspots with additional ssDNA regions spread throughout the genome). Although we did not perform the experiment, RAD51-SSDS done by Shinohara’s group in Spo11-/- Fignl1 cKO failed to identify peaks, supporting the idea that there is probably no RAD51 binding on ssDNA in specific regions.

12- typo line 243 (RD51) Fixed

13- The nice observation from Fig4f warrants additional comments. How such might be related to the possibly altered distribution of RAD51 and DMC1 on the filament.
We do not have a clear interpretation for this observation, this is why we chose not to comment it in the Results section. One whole paragraph of Discussion deals with this observation however, in which we propose that transient interactions of RAD51/DMC1 with structural components of chromosome axis might help recruiting HR repair complex. FIGNL1-FIRRM would then be involved in preventing such RAD51/DMC1-axis protein interactions to be stabilized when there is no DNA damage. (lines 548-561)

14- Fig5b-c, line 282. How do the authors explain the differences between Firrm cKO and the double mutant (Firrm Spo11). The difference between both is highly significative. Doesn’t this suggest that RAD51 and DMC1 accumulation is partly dependent of DSB ? Please clarify or comment.

We agree with this comment, although this difference is less significant by taking into account multiple comparisons. This is explained above, in response to the similar comment from reviewer #2 (major concern 2). We added a short description with interpretation in the Result section. (lines 288-294)

15- Similarly, though I globally agree with authors' conclusion, I am not sure whether one can claim that the formation of RAD51 and DMC1 foci is (fully) independent from SWSAP1 in Fignl1 mutant? I see statistically significant differences (Fig 7 and Ext fig 8). Partly independent ?
More data on Swsap1-/- Fignl1 and Swsap1-/- Firrm cKO were added (including the Swsap1-/- single mutant control).
When multiple comparisons are considered, DMC1 counts do not differ significantly between Fignl1cKO single and Swsap1-/- Fignl1cKO double mutant, hence the first conclusion: the formation of a large number of DMC1 foci does not depend on SWSAP1 in the absence of FIGNL1-FIRRM.

Since a majority of DMC1 foci in Firrm cKO (and in Fignl1 cKO, see Shinohara's group study) are SPO11 DSB-independent, this observation is not sufficient to conclude whether or not SWSAP1 is required for forming normal numbers of DMC1 foci at meiotic DSBs. Thus, we show that the fraction of RPA2 foci that colocalize with DMC1 foci did not differ significantly between Fignl1 cKO single and Swsap1-/- Fignl1 cKO double (and Swsap1-/- Firrm cKO double) (Fig 7d, Extended Data Fig. 8d). This led to conclude that the formation of detectable DSB-dependent DMC1 foci (the subset that colocalizes with RPA2) is most likely fully independent from SWSAP1 in Fignl1/Firrm cKO.

The data are shown on Fig. 7 and Extended Data Fig. 8, and the description of Swsap1KO FirrmcKO/Fignl1cKO has been rewritten in the corresponding section of Results.(lines 373-403)

16- Line 378 For readers, please explain the use of DeltaN-FIGNL1.
The structure of FIGNL1deltaN is now depicted on Extended Data Fig. 1b. The deletion is now described there (N-ter 384aa deletion), and its role (it was required for producing soluble protein) is explained in the Methods section. The original reference of this truncation (Matsuzaki et al, 2019, ncomms) is cited at both places.(line 410, line 752)

17- Discussion, I do not follow the type of role considered before strand invasion (line 444) and whether a relationship is considered between the two proposed roles of the FIGNL1-FIRRM complex (pre-meiotic replication, meiotic breaks).
The paragraph discussing the putative role before strand invasion is now introduced explicitly: "the average DMC1-SSDS signal profile at meiotic DSB hotspots was altered in a way that suggests that FIRRM may be involved in regulating the length of DMC1-ssDNA filaments before strand invasion." (lines 495-497) This paragraph has been reformulated extensively for clarity.(lines 495-533)

18- Ext data Fig1. A draw of FIGNL1 protein might be useful here and may help positioning the N-ter for later biochemistry (Fig8).
The major FIGNL1 domains and the N-ter truncation are now depicted in Ext. Data Fig. 1b.

19- A single study had suggested a role for Fignl1 during male mouse meiosis -prior these- (L'Hote et al PLoS One. 2011;6(11):e27582. doi: 10.1371). It might be worth an additional reference.
This reference is now cited in first paragraph of Discussion. (line 447)

20- line 615-616, How does ‘simulating the random localization’ is performed? Specific soft for randomizing numbers/XY coordinates?

The images were analyzed with a custom ImageJ macro, which is described in a method article (now cited) in Methods in Molecular Biology (Cau et al., Methods Mol Biol, 2024, 2770:263-285. DOI: 10.1007/978-1-0716-3698-5_17). It is publicly available with documentation (https://github.com/MontpellierRessourcesImagerie/meiosis_bar).

Specifically, random X and Y coordinates are determined for n foci in channel A (“foci A”. n= number of experimentally detected “foci A”), localized within the selected ROI (either the whole nucleus, the axes, or outside the axes) and not with each other colocalizing (i.e. not within the defined custom distance of colocalization from each other). The number of experimentally detected “foci B” colocalized with the random “foci A” is determined. The whole process (randomization of “foci A” and count of “foci B” colocalized with random “foci A”) is repeated for a selected number of times (e.g., 100 x), resulting in a distribution and a mean number of “foci B colocalized with random foci A”. The same process is run with random “foci B” and experimental “foci A”. (lines 652-674)

21- line 629, typo ‘ntot’ instead of ‘nT’ Fixed.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The revisions to the paper are satisfactory and I recommend acceptance.

Reviewer #2 (Remarks to the Author):

The response to my concerns is satisfactory. I support its publication.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed all my comments in the revised version. Therefore I have no further issues with the manuscript.