CXXC finger protein 1-mediated histone H3 lysine-4 trimethylation is essential for proper meiotic crossover formation in mice

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MS TITLE: CxxC Finger Protein 1-mediated Histone H3 Lysine-4 Trimethylation is Essential for Proper Meiotic Crossover Formation in Mice

AUTHORS: Yu Jiang, Hui-Ying Zhang, Zhen Lin, Ye-Zhang Zhu, Chao Yu, Qian-Qian Sha, Ming-Han Tong, Li Shen, and Heng-Yu Fan

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Particularly, it will be critically important to clarify how phenotypic differences shown in this and previous study could be caused. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The author found that deletion of CFP1 caused delay in DSB repair and improper crossover formation in pachytene, indicating that CFP1 may be involved in the programmed meiotic DSB repair.

They found increased crossover in both CFP1 deficient spermatocytes and oocytes,in contrary, they also found more than half diplotene cells with precocious homologous chromosomes segregation in both male and female meiosis, which is quite new to me, maybe indicate some new findings/regulations.

Comments for the author

In this manuscript, Jiang et al. used Stra8-Cre to generate mice with specific deletion of CFP1 in germ celss before the initiation of meiosis. They showed that the mice werre completely sterile and attributed to spermatogenetic arrest at MII stage. They also found that deletion of CFP1 caused delay in DSB repair and improper crossover formation in pachytene and more than half diplotene cells with precocious homologous chromosomes segregation in both male and female meiosis. CFP1 deletion also led to a significant decrease of H3K4me3 enrichment at DMC1-binding sites, they suggested that which might compromise DSB generation. They also found increased crossover in both CFP1 deficient spermatocytes and oocytes. However, I think that their many results are contradictory, their conclusions and reasoning lack experimental evidence. Here I just give a few examples:
1.The authors show in CFP1 KO mice, increased univalents in both diplotene spermatocytes and oocytes and an increase in CO numbers. Actually, if MLH1 foci/CO number in a cell increased, one usually do not expected to see increased univalents in diplotene spermatocytes and oocytes, so these results are not consistent. If this is true, the authors should answer: How is chromosome missegregation caused with increased CO? This may be due to the loss of obligate CO, however, the author did not provide any evidence.
2.The authors found increased univalents in diplotene spermatocytes and oocytes. If these are true, one should expect to see meiosis arrest at MI stage but (most probably) not at MII stage, because of the activation of spindle checkpoint. However, the author reported the contrary results, they must explain why.
3. The authors found a reduction of H3K4me3 on DSB hot-spots in leptotene/zygotene spermatocytes from KO mice, and suggested that this may cause an alternation on DSB distribution as well as CO formation. However, they did not provide any evidence that the DSB distribution as well as CO position are altered. It has been reported that hot-spot related H3K4me3 sites are far more than DSB sites. Thus, the reduction in H3K4me3 level at hot spots does not necessarily mean changes on DSB distribution. Further experiments such as DMC1-CHIP or SPO11-CHIP should be done to see whether DSB distribution is really changed in KO mice.
4. The authors showed more dramatic change in H3K4me3 level in spermatocytes since pachytene stage. It has recently been reported that hot-spot related H3K4me3 are demethylated in pachytene, which means that the H3K4me3 after pachytene are related to transcription regulation or other events rather than DSB regulation. Thus, it is highly possible that the phenotype observed in CFP1 KO mice is related to disturbed gene expression caused by CFP1 depletion. The authors should provide evidence to show whether the phenotype they observed are caused by hot-spot specific or promoter-specific H3K4me3 loss.

Minors:
1. Immunofluorescence staining cannot be used for the quantification of protein level, especially when the cells are on two different slides. Thus, it is inappropriate to quantify H3K4me3 level from Fig1 C&E.
2. IF staining on testicular sections rather than on spermatocyte spreads would be better to show the changes in H3K4me3 level in WT and KO mice.
3. The purity of isolated spermatocytes used for Western bolt in Figure 1 should be shown in supplementary figures.

In conclusion, the phenotypes of the knockout mice are interesting, but more experiments are needed to explain missing links among different phenotypes, and among phenotypes and the possible mechanisms.

Reviewer 2

Advance summary and potential significance to field

This study reports the essential function of CFP1 in controlling H3K4me3 in meiotic prophase I, both in males and females. This is an important contribution to the field because a previous study reported that CFP1 is not required for meiotic prophase I (Tian et al. Plos Genet 2018); however, this new study reveals positive results using a different Cre line recently developed by the authors.

Comments for the author

Overall, the quality of data is high and the data are clearly and logically presented. I am highly enthusiastic about this study for publication. I have some technical concerns, major suggestions, and minor points be addressed to improve the quality of the study.

Technical concerns:

1. Based on the FACS data, arrest at the secondary spermatocytes stage was concluded. However, in general, secondary spermatocytes are transient populations, and it is hard to believe so many secondary spermatocytes can be obtained using FACS (at the level we can perform western blot). Relating to this point, what is the major population detected between "RS" and "MII" in the mutant FACS panel of Figure 3A? Also, for the sake of data interpretation, it would be informative to add the purity data of FACS populations for each stage (used in panel 1B). Leptotene/zygotene, pachytene, and diplotene stages can be examined using SYCP3 staining. However, there is no established marker for secondary spermatocytes. My best suggestion is to perform dual color DNA FISH using X and Y-linked probes. If this is indeed secondary spermatocytes, 50% of the cells show 2 dots of X-loci (no Y-foci), and another 50% of cells show 2 dots of Y loci (no X foci). This is because X and Y are already segregated, but they are still 2n.
2. Although the authors concluded the secondary spermatocyte arrest, as far as I can see in the pictures Fig. 3B, C, arrest occurs at metaphase I.
3. Evaluation of crossover sites is not appropriate. MLH1 is the marker of future crossover sites, but MLH1 foci do not necessarily indicate crossover formation. Indeed, they found increased MLH1 foci, but decreased crossover was shown in Fig. 3C. Therefore, this is not appropriate interpretation: "The Cxxc1fl/−;Stra8-Cre mice formed more crossovers in pachytene than WT". The best way to score crossover formation is to score the chiasmata counts (as performed in Fig. 4DE of Holloway et al. Plos Genet 2008: PMID 18787696). This can be done using the data shown in Figure 3C. Interestingly, the increase of MLH1 foci was also observed in this paper (Holloway et al. Plos Genet 2008). Is Class II crossover affected in the Cfp1 mutants?
4. I do not agree with the interpretation of the RNA seq data "Indeed, expression of these genes between WT and Cxxc1-null spermatocytes became similar at the MII stage (Fig. 6C, right panel)". Relating to question #1 above, are they really MII?

Other major suggestions:
1. Since the authors report a contradicting result from Tian et al. Plos Genet 2018, it would be important to examine the efficiency of Cre-mediated recombination using the new Cre-line they used, which seems to be pretty high.
2. I understand that the initial data set is sufficient for publication. However, a major remaining question is how CFP1 regulates H3K4me3 in meiotic prophase I. It would be important to determine whether CFP1 works with PRDM9 methyltransferase to designate the sites of meiotic recombination or to determine if CFP1 works with SETD1 methyltransferase to regulate gene expression. To distinguish these possibilities, a straightforward analysis can compare the H3K4me3 distribution at in vivo binding sites of PRDM9 (Grey et al. Genome Res 2017: PMID: 28336543).
3. Also, this is just a suggestion for future experiments (not essential to this study): if CFP1 works with PRDM9 as predicted, it would be interesting to test whether CFP1 works with PRDM9 to designate the sites of meiotic recombination. This can be tested by mapping the sites of meiotic

recombination (as described by Khill PP et al. Genome Res 2012: PMID 2237190). If it is the case, we may predict a similar phenotype with the Prdm9 mutants (Brick et al. Nature 2012: PMID 22660327).

Minor points:
1. The data quantification in Figure 1D and F are based on very small numbers of cells. This should be increased using several independent mice. Regarding Fig. 1F pachytene oocytes, it is hard to believe that such significant p value can be obtained with this minor difference in H3K4me3 intensity from the small numbers of samples (n=5 vs n=4).
2. The H3K4me3 level at the DMC1 binding sites are characterized, but there is no description as to how they were identified. This information should be included.
3. In the discussion, GSE120994 was cited and another study is mentioned. If this information was derived from personal communication, it should be described with the permission of the authors.
4. At the end of the discussion, H2AK119ub1 was mentioned in relation to H3K4me3. However, this argument is not evidence based. This should be deleted.
5. Figure 8 legend: oocyte spreads are described as “spermatocyte spreads”.

Reviewer 3

Advance summary and potential significance to field

CXXC finger protein 1(Cfp1) is known to be a member of the PRDM9 complex in germ cells. Cfp1 has two types of zinc finger motifs, FYVE/PHD and CXXC. Cfp1 reportedly exhibits unique DNA binding specificity for unmethylated CpG dinucleotides. Cfp1 is also known to be a component of the euchromatic Setd1A complex. Previously, the complete gene deletion of Cfp1 was reported. The mice were lethal at the early developmental stage (Tian et al. PLoS Genet, 2018). During spermatogenesis, Cfp1 forms a complex together with PRDM9, which is crucial for proper H3K4me3 deposition, DSB formation, and homologous recombination. PRDM9 plays a significant role in the deposition of H3K4me3 and H3K36me3 at hotspots. In ES cells, CFP1 is crucial for proper genome targeting of the Setd1A kinase. However, functions of CFP1 in the germ cell lineage have not been unveiled yet. To address this question, Petkov's group generated CFP1 conditional knockout mice lacking exon 2 and 3 and reported that CFP1 is not essential for normal DSB formation and meiotic recombination in mice (Tian et al. PLoS Genet, 2018).
In this manuscript, Jiang et al. used the conditional KO mice lacking exon 6 and 7 and showed Cfp1 is essential for meiotic cross over the formation. They showed the arrest of the secondary spermatocyte differentiation accompanied by a number of defects: abnormal gene expression during premeiotic stages; impairment of DSB repair and cross over; abnormal deposition of H3K4me3; impairment of oogenesis due to similar phenomena.
Petkov's work showed quite similar sets of results and their experiments were seemingly adequately performed as this study. However, the results are contradictory. Leaving this question unsolved would lead to new problems being brought about in future studies involving CFP1.
Finally, the reviewer did not have access to the raw NGS files so that the reviewer was not able to judge their NGS data in detail.

Comments for the author

Major points
1. Although the authors cited Cao's paper (Cao et al. Nat Immunol, 2016), the mice Cao et al. established lacks exon 4 and 5. Did the authors use a different conditional line?
2. The mice may produce a truncated protein, which would make it possible to explain the difference in the phenotypes from different reports. This is also very important because such deletion, no matter whether it is exon 4/5 or exon 6/7, may cause infertility in humans. Thus, please use an antibody that recognizes the N-terminal region of CFP1(commercially available) but not the one that recognizes aa100-200.
3. Related to 2, has GWAS been done, or have any significant SNPs been published for the gene?
4. In the previous ES cell study, CFP1 deficiency caused the reduction of Dnmt1 and Setd1A. Please show the expression levels of Dnmt1, Setd1A, Setd1B, and PRDM9 in leptotene/zygotene and Pachytene stages.

4. Is PRDM9 binding generally decreased or is some specific binding affected in L/Z cells? A genome-wide PRDM9 analysis is necessary. This experiment is quite essential because CFP1 itself does not have a histone methyltransferase activity, and PRDM9 is a critical factor for the deposition of the H3K4me3 mark and the recruitment of Spo11 for the formation of DSB.
5. The function of CFP1 in ES cells is to suppress Setd1A activity. Do the authors think that a similar mechanism causes the reduction of H3K4me3 at the regulatory region in male germ cells? Please include more mechanistic interpretation in the discussion.
5. In Figure 3, the x-axis and y-axis indicate Indo-1 and DAPI, respectively. Indo-1 is a Ca-binding dye, and DAPI does not stain live cells, which is different from the method description.
6. There is no indication of mitotic cells (type A, In, and B spermatogonia) in the testes. Where are they?
7. I don't understand the interpretation of Figure 3E. In their Cfp1 conditional KO mice, division II of meiosis does not occur (Figure 3A); total H3K4me3 after the Pachytene stage is almost gone (Figure 1B); H3K4me3 levels at TSS are globally reduced and DSB does not form properly (Figure 6). However, I see proper PNA signals with a typical shape and intensity at acrosomes of spermatid-like cells in Figure 1E. The acrosome is a membranous organelle consisting of many proteins, such as enzymes, structural proteins, and vesicle trafficking proteins, suggesting acrosome-related pathways should proceed properly under the abnormal meiotic processes in this work. Please check the gene expression of acrosome-related proteins using the RNA-sequence data sets.
Minor points
1. In Figure 1, it seems that spermatogonia are highly positive for CFP1 (squamous cells). This expression pattern is also one of the contradictions seen between Tian's and this work. Please check CFP1 expression in spermatogonia and early meiotic cells using specific markers such as PLZF and Stra8, respectively.
2. Please show a snapshot of the RNA-seq data of Cfp1.

First revision

Author response to reviewers' comments

We thank reviewers for providing insightful comments and constructive suggestions to solidify the presented data. During the revision, we payed careful attention to reviewers' comments and did our best to address the questions raised by reviewers. We hope that the reviewers find our responses satisfactory.

Editor: Particularly, it will be critically important to clarify how phenotypic differences shown in this and previous study could be caused.
Response: We verified the efficiency of our *Stra8-Cre*-mediated recombination and have included the data in the revised manuscript (Fig. S1C). Moreover, we crossed our *Cxxc1* floxed mice with the transgenic *Stra8* mice (Sadate-Ngatchou et al., 2008), and observed the same phenotype with sterile and abnormal epididymis (Fig. R1), demonstrating that our *Stra8*-Cre-mediated recombination knockout worked fine.
We venture a guess that the most likely cause of the phenotypic differences between previous and our work is the construction strategy of the *Cxxc1* floxed mice. In our *Cxxc1* floxed mice, exons 4 and 5 of the *Cxxc1* allele are flanked by loxP sites, which will lead to the deletion of CXXC domain as well as the frameshift mutation upon Cre-mediated recombination. And the *Cxxc1* floxed mice that were used had been crossed with different cre mice and all these crossings resulted in different phenotypes in the offspring, for example, Lck-cre, Zp3-cre, and Gdf9-cre (Cao et al., 2016; Sha et al., 2018; Yu et al., 2017). However, despite the construction strategy of *Cxxc1* floxed mice in previous work, with exons 2 and 3 flanked by loxP sites, will also lead to frameshift mutation upon Cre-mediated recombination, the CXXC domain coding sequence is intact. With no evidence, we just bring forward a propose that a truncated CXXC1 protein containing CXXC domain with alternative translation initiation sites might be produced under stress. Given that CXXC domain is indispensable for histone methyltransferase genomic targeting (Lee et al., 2001; Tate et al., 2010), we believe that our construction strategy is more radical in the inactivation of CFP1 functions.

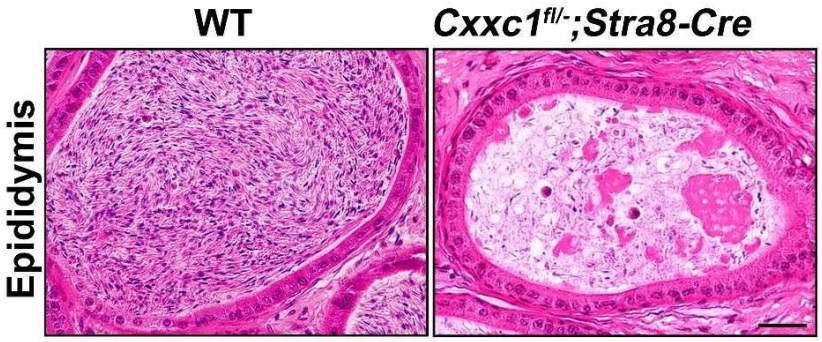

Fig. R1

Reviewer 1

1. The authors show in CFP1 KO mice, increased univalents in both diplotene spermatocytes and oocytes and an increase in CO numbers. Actually, if MLH1 foci/CO number in a cell increased, one usually do not expected to see increased univalents in diplotene spermatocytes and oocytes, so these results are not consistent. If this is true, the authors should answer: How is chromosome missegregation caused with increased CO? This may be due to the loss of obligate CO, however, the author did not provide any evidence.

Response: We thank the reviewer for this important suggestion. To study the CO obligation, we scored the number of MLH1 foci on individual chromosomes. However, we did not observe a significant loss of obligate CO in the *Cxxc1*-cKO pachynema compared to that in the WT (Fig. S1H). After further comparation of the distribution of MLH1 signals from *Cxxc1*-cKO and WT pachynema, we found that the COs in the *Cxxc1*-cKO showed a distribution preference in regions adjacent to the telomere, which are less able to enhance the segregation fidelity (Fig. 5E and Fig. S1I) (Mezard et al., 2015; Sherman et al., 1994). Thus, despite the increased CO numbers, the two terminals COs are less effective in enhancing the disjunction.

2. The authors found increased univalents in diplotene spermatocytes and oocytes. If these are true, one should expect to see meiosis arrest at MI stage but (most probably) not at MII stage, because of the activation of spindle checkpoint. However, the author reported the contrary results, they must explain why.

Response: We apologize for our inappropriate description of the phenotype. Actually, we could see a lot of apoptotic meiotic cells at the epithelial stage XII, indicating a partial arrest at MI stage (Fig. 3B), which is consistent with our observation that the diplotene spermatocytes in the *Cxxc1*-cKO mice showed an increase in the univalents. However, there were a significant number of spermatocytes that could escape from the spindle checkpoint and these could develop into the secondary spermatocytes but finallybe arrested at the MII stage.

3. The authors found a reduction of H3K4me3 on DSB hot-spots in leptotene/zygotene spermatocytes from KO mice, and suggested that this may cause an alternation on DSB distribution as well as CO formation. However, they did not provide any evidence that the DSB distribution as well as CO position are altered. It has been reported that hot-spot related H3K4me3 sites are far more than DSB sites. Thus, the reduction in H3K4me3 level at hot spots does not necessarily mean changes on DSB distribution. Further experiments such as DMC1-CHIP or SPO11-CHIP should be done to see whether DSB distribution is really changed in KO mice.

Response: We appreciate the good suggestion made by the reviewer regarding the direct testing of DSB distribution using DMC1-CHIP or SPO11-CHIP. However, we were unable to perform this experiment because of the unavailability of an effective antibody. We hope that this experiment would be accomplished in future studies by other researchers. In the present study, the H3K4me3 levels in the leptotene/zygotene spermatocytes were not changed significantly at the global level (Fig. 1B) but showed a sharp decrease at the DMC1-binding sites (Fig. 7D). Given the hallmark role of DMC1 in DSB distribution (Brick et al., 2012; Smagulova et al., 2011), we inferred that the lack of CFP1 influenced the global DSB distribution.

4. The authors showed more dramatic change in H3K4me3 level in spermatocytes since pachytene stage. It has recently been reported that hot-spot related H3K4me3 are demethylated in pachytene,

which means that the H3K4me3 after pachytene are related to transcription regulation or other events rather than DSB regulation. Thus, it is highly possible that the phenotype observed in CFP1 KO mice is related to disturbed gene expression caused by CFP1 depletion. The authors should provide evidence to show whether the phenotype they observed are caused by hot-spot specific or promoter-specific H3K4me3 loss.
Response: In the *Cxxc1*-cKO mice, meiosis was partially defective at the MI stage and was finally arrested at the MII stage (please refer to our response to your concern mentioned at point no. 3 above). We believe that the effect of *Cxxc1*-cKO on meiosis has two aspects. First, the loss of *Cxxc1* leads to the ineffective deposition of H3K4me3 on DSB hotspots at the leptotene/zygotene stage, which causes a delay in DSB repair and a disproportionate CO distribution, and finally results in a partial defect at the MI stage. Second, the loss of *Cxxc1* blocked the re-establishment of H3K4me3 in the diplotene stage. The H3K4me3 at the diplotene stage is related to transcription rather than to meiotic recombination. Therefore, transcription of key genes required for meiosis II is largely silenced because of the deficiency of *Cxxc1*, which results in the arrest at the MII stage. In accordance with our supposition, the RNA-seq results showed that the number of down-regulated genes at the MII stage was much higher than at the pachytene or diplotene stages. The GO analysis of down-regulated genes at the MII stage showed an enrichment of the cell division and M-phase related genes.

Minors:
1. Immunofluorescence staining cannot be used for the quantification of protein level, especially when the cells are on two different slides. Thus, it is inappropriate to quantify H3K4me3 level from Fig1 C&E.
Response: To make the data more convincing, we quantified the H3K4me3 level using the bands obtained in the western blot analysis with the Image J software (Fig. 1B).

2. IF staining on testicular sections rather than on spermatocyte spreads would be better to show the changes in H3K4me3 level in WT and KO mice.
Response: We did the IF staining on testicular sections as suggested by the reviewer (Fig. S1B), and found that the pattern was consistent with the results obtained for spermatocyte spreads.

3. The purity of isolated spermatocytes used for Western bolt in Figure 1 should be shown in supplementary figures.
Response: As recommended by the reviewer, we have added the purity data for the isolated spermatocytes in Supplementary Figure S1F-G.

Reviewer 2
Technical concerns:
1. Based on the FACS data, arrest at the secondary spermatocytes stage was concluded. However, in general, secondary spermatocytes are transient populations, and it is hard to believe so many secondary spermatocytes can be obtained using FACS (at the level we can perform western blot). Relating to this point, what is the major population detected between "RS" and "MII" in the mutant FACS panel of Figure 3A? Also, for the sake of data interpretation, it would be informative to add the purity data of FACS populations for each stage (used in panel 1B). Leptotene/zygotene, pachytene, and diplotene stages can be examined using SYCP3 staining. However, there is no established marker for secondary spermatocytes. My best suggestion is to perform dual color DNA FISH using X and Y-linked probes. If this is indeed secondary spermatocytes, 50% of the cells show 2 dots of X-loci (no Y-foci), and another 50% of cells show 2 dots of Y loci (no X foci). This is because X and Y are already segregated, but they are still 2n.
Response: As recommended by the reviewer, we have added the purity data for the isolated spermatocytes in Supplementary Figure S1F-G.
We appreciate the reviewer's specific comment on the experimental design for the verification of MII cell population by DNA FISH; however, we are sorry that we could not perform the experiment because of the following reasons:
a) The method for the isolation of spermatocytes using flow cytometry had been optimized earlier and the MII population gating method had been reported (Gaysinskaya et al., 2014). As shown in the cited paper, the gating population density of MII cells was comparable to that of L/Z cells. In the present study, we used the same isolation method to separate the cells in different stages.
b) The MII cells isolated from *Cxxc1*$^{fl/-}$;*stra8*-cre using FACS were positive for the PNA signal (Fig. 3F). Except for those arrested abnormal MII cells, other diploid cells including spermatogonia and

sertoli cells ought not to display PNA signals. Therefore, we believe that the gating cell population was composed of MII cells.

2. Although the authors concluded the secondary spermatocyte arrest, as far as I can see in the pictures Fig. 3B, C, arrest occurs at metaphase I.
Response: We answered a similar question in response to comment #2 of reviewer 1. The cells with lagging chromosomes in Fig. 3B were in stage XII of the spermatogenic cycle, in which the center of lumen ought to be metaphase cells and elongating spermatids. In stages I–XI, the center of lumen ought to be round spermatids and/or elongating spermatids. However, in the *Cxxc1*$^{fl/-}$;*stra8*-cre mice, lumens were filled with abnormal cells that were bigger than the round spermatids (Fig. 2E), and it was observed that these cells were not in chromosome condensing state as MI.

3. Evaluation of crossover sites is not appropriate. MLH1 is the marker of future crossover sites, but MLH1 foci do not necessarily indicate crossover formation. Indeed, they found increased MLH1 foci, but decreased crossover was shown in Fig. 3C. Therefore, this is not appropriate interpretation: "The Cxxc1fl/−;Stra8-Cre mice formed more crossovers in pachytene than WT". The best way to score crossover formation is to score the chiasmata counts (as performed in Fig. 4DE of Holloway et al. Plos Genet 2008: PMID 18787696). This can be done using the data shown in Figure 3C. Interestingly, the increase of MLH1 foci was also observed in this paper (Holloway et al. Plos Genet 2008). Is Class II crossover affected in the Cfp1 mutants?
Response: We appreciate the reviewer for pointing out our inappropriate description of the crossover; we have corrected it in the revised manuscript. Besides, we measured the chiasmata counts and did not find any significant difference between the WT and *Cxxc1*$^{fl/}$;*stra8*-cre mice (WT, n = 21.25; *Cxxc1*$^{fl/}$;*stra8*-cre, n = 22). The reason for the increased number of univalents in Fig. 3C is that the MLH1 signal in Cxxc1-cKO mice showed a distribution preference in regions adjacent to the telomere, which are less able to enhance the segregation fidelity. (Please also refer to our response to comment #1 of Reviewer 1). As suggested by the reviewer, the affected crossovers might also include Class I and Class II crossovers; we have appended the discussion in this regard in the revised manuscript.

4. I do not agree with the interpretation of the RNA seq data "Indeed, expression of these genes between WT and Cxxc1-null spermatocytes became similar at the MII stage (Fig. 6C, right panel)". Relating to question #1 above, are they really MII? Response: We are sorry for the inaccurate description. We meant that the expression levels of these genes in the Cxxc1-null P/D spermatocytes became comparable to those in the MII stage cells in the WT mice. We have made the correction in the revised manuscript.

Other major suggestions:
1. Since the authors report a contradicting result from Tian et al. Plos Genet 2018, it would be important to examine the efficiency of Cre-mediated recombination using the new Cre-line they used, which seems to be pretty high.
Response: We have appended the results in Supplementary Figure S1C.

2. I understand that the initial data set is sufficient for publication. However, a major remaining question is how CFP1 regulates H3K4me3 in meiotic prophase I. It would be important to determine whether CFP1 works with PRDM9 methyltransferase to designate the sites of meiotic recombination or to determine if CFP1 works with SETD1 methyltransferase to regulate gene expression. To distinguish these possibilities, a straightforward analysis can compare the H3K4me3 distribution at in vivo binding sites of PRDM9 (Grey et al. Genome Res 2017: PMID: 28336543).
Response: We appreciate the reviewer's suggestion and we have appended the results in Figure 7E in the revised manuscript.

3. Also, this is just a suggestion for future experiments (not essential to this study): if CFP1 works with PRDM9 as predicted, it would be interesting to test whether CFP1 works with PRDM9 to designate the sites of meiotic recombination. This can be tested by mapping the sites of meiotic recombination (as described by Khill PP et al. Genome Res 2012: PMID 2237190). If it is the case, we may predict a similar phenotype with the Prdm9 mutants (Brick et al. Nature 2012: PMID 22660327).
Response: We appreciate this specific suggestion by the reviewer and would consider it in our future studies.

Minor points:

1. The data quantification in Figure 1D and F are based on very small numbers of cells. This should be increased using several independent mice. Regarding Fig. 1F pachytene oocytes, it is hard to believe that such significant p value can be obtained with this minor difference in H3K4me3 intensity from the small numbers of samples (n=5 vs n=4).
Response: We appreciate the reviewer's suggestion and have updated the results for experiments with a greater number of biological replicates in the revised manuscript.

2. The H3K4me3 level at the DMC1 binding sites are characterized, but there is no description as to how they were identified. This information should be included.
Response: We have corrected this in the revised manuscript and would like to thank the reviewer for pointing it out.

3. In the discussion, GSE120994 was cited and another study is mentioned. If this information was derived from personal communication, it should be described with the permission of the authors.
Response: We are thankful to the reviewer for bringing this to our notice. We have corrected the discrepancy in the revised manuscript.

4. At the end of the discussion, H2AK119ub1 was mentioned in relation to H3K4me3. However, this argument is not evidence based. This should be deleted.
Response: We have discussed this issue in the revised manuscript.

5. Figure 8 legend: oocyte spreads are described as "spermatocyte spreads". Response: We are sorry for this error and have corrected it in the revised manuscript.

Reviewer 3
Major points
1. Although the authors cited Cao's paper (Cao et al. Nat Immunol, 2016), the mice Cao et al. established lacks exon 4 and 5. Did the authors use a different conditional line?
Response: We apologize for this mistake; it should have been exon 4 and 5; we have corrected the error in the revised manuscript. We would like to thank the reviewer for pointing this out.

2. The mice may produce a truncated protein, which would make it possible to explain the difference in the phenotypes from different reports. This is also very important because such deletion, no matter whether it is exon 4/5 or exon 6/7, may cause infertility in humans. Thus, please use an antibody that recognizes the N-terminal region of CFP1 (commercially available) but not the one that recognizes aa100-200. Response: As recommended by the reviewer, we performed western blot with FACS-isolated spermatocytes to verify the knockout efficiency. And no truncated form of CFP1 was detected (Fig. R2).

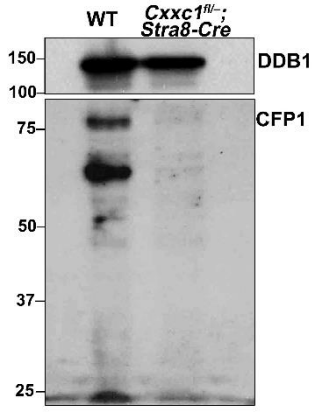

Fig. R2

3. Related to 2, has GWAS been done, or have any significant SNPs been published for the gene?
Response: In the published data, a synonymous variant of *cxxc1* at exon 10 is involved in the body height in GWAS (GCST007841) (Kichaev et al., 2019). No other identified SNP has been reported in

genome-wide studies.

4. In the previous ES cell study, CFP1 deficiency caused the reduction of Dnmt1 and Setd1A. Please show the expression levels of Dnmt1, Setd1A, Setd1B, and PRDM9 in leptotene/zygotene and Pachytene stages.
Response: We have tried our best to address the reviewer's concerns. We performed western blot analysis using the available DNMT1 antibody and did not detect any signal with 5×10^4 cells per sample. To confirm that the antibody worked normally, we added a sample of 150 mouse GV oocytes and detected a normal DNMT1 band (Fig. R3). Therefore, we believe that the expression level of DNMT1 in spermatocytes is too low to be detected by western blot analysis, which is different from that in the ES cells.
In our RNA-seq data, the levels of Setd1a and Prdm9 were not influenced in the leptotene/zygotene and pachytene stages. The level of Setd1b is too low to be detected.
Moreover, in ES cells and spermatocytes CFP1 might play different roles, and therefore, its absence influenced different pathways.

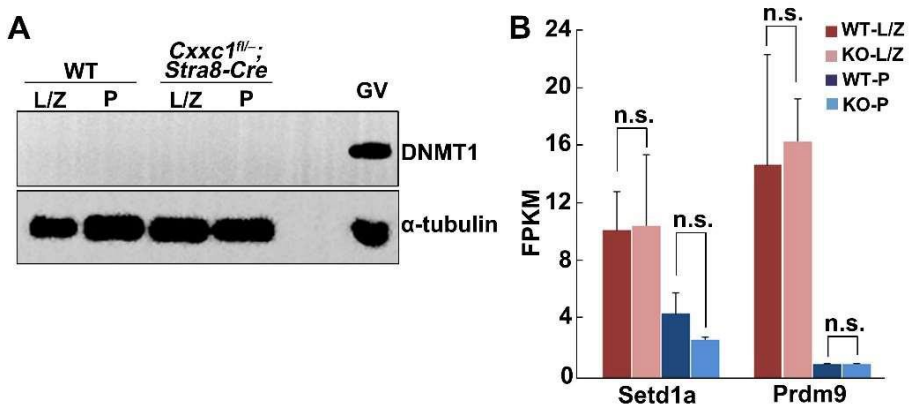

Fig. R3

5. Is PRDM9 binding generally decreased or is some specific binding affected in L/Z cells? A genome-wide PRDM9 analysis is necessary. This experiment is quite essential because CFP1 itself does not have a histone methyltransferase activity, and PRDM9 is a critical factor for the deposition of the H3K4me3 mark and the recruitment of Spo11 for the formation of DSB.
Response: We appreciate the reviewer's suggestion and have appended the results in Figure 7E in the revised manuscript.

6. The function of CFP1 in ES cells is to suppress Setd1A activity. Do the authors think that a similar mechanism causes the reduction of H3K4me3 at the regulatory region in male germ cells? Please include more mechanistic interpretation in the discussion.
Response: As mentioned in the response to comment #4 above, in ES cells and spermatocytes CFP1 might play different roles; we have discussed this in the revised manuscript.

7. In Figure 3, the x-axis and y-axis indicate Indo-1 and DAPI, respectively. Indo-1 is a Ca-binding dye, and DAPI does not stain live cells, which is different from the method description.
Response: Indo-1 (blue) and DAPI are the default channel names in the BD Aria2 cell sorter. Hoechst 33342 was excited using a 355 nm UV laser, and the dye's wide emission spectrum was detected in two distinct channels: the "Hoechst Blue" (DAPI, 450/50 nm band-pass filter) and the "Hoechst Red" (Indo-1 (Blue), 530/30 band-pass filter). To make this clearer, we have added the description in the *Methods* section.

8. There is no indication of mitotic cells (type A, In, and B spermatogonia) in the testes. Where are they?
Response: This question is a little confusing and we can make two interpretations. If the reviewer is confused about the expression levels of CFP1 in spermatogonia, we have answered a similar question in response to the minor comment #1 listed below. However, if the reviewer is wondering about the effect of CFP1 knockout on spermatogonia, we would like to inform that we performed IHC for stra8, which is a marker for differentiated spermatogonia, on cross sections of testes from the WT and *Cxxc1*$^{fl/-}$;*stra8*-cre mice and did not find any influence on spermatogonia.

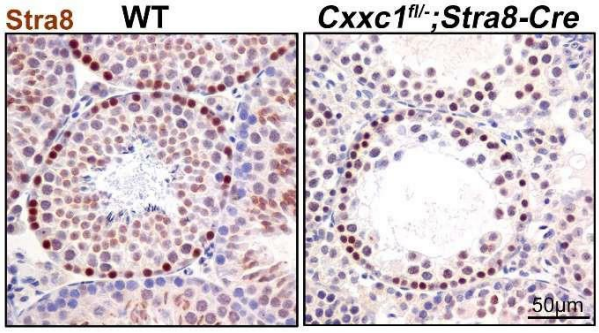

Fig. R4

9. I don't understand the interpretation of Figure 3E. In their Cfp1 conditional KO mice, division II of meiosis does not occur (Figure 3A); total H3K4me3 after the Pachytene stage is almost gone (Figure 1B); H3K4me3 levels at TSS are globally reduced and DSB does not form properly (Figure 6). However, I see proper PNA signals with a typical shape and intensity at acrosomes of spermatid-like cells in Figure 1E. The acrosome is a membranous organelle consisting of many proteins, such as enzymes, structural proteins, and vesicle trafficking proteins, suggesting acrosome-related pathways should proceed properly under the abnormal meiotic processes in this work. Please check the gene expression of acrosome-related proteins using the RNA-sequence data sets.
Response: We appreciate the reviewer's suggestion. We have provided the results asked for the reviewer in Supplementary Figure 2 F-G in the revised manuscript.

Minor points
1. In Figure 1, it seems that spermatogonia are highly positive for CFP1 (squamous cells). This expression pattern is also one of the contradictions seen between Tian's and this work. Please check CFP1 expression in spermatogonia and early meiotic cells using specific markers such as PLZF and Stra8, respectively.
Response: The co-staining of CFP1 and LIN28A confirmed that CFP1 is highly expressed in undifferentiated spermatogonia (Fig. S1B). However, we did not have any antibodies against the marker for differentiating spermatogonia for co-staining with CFP1. We have tried our best to address the reviewer's concerns. We checked the expression level of CFP1 in the published single-cell RNA-seq dataset (Chen et al., 2018) and found that the expression levels of CFP1 are high from spermatogonia to meiotic cells (Fig. S1A).

2. Please show a snapshot of the RNA-seq data of Cfp1.
Response: As asked for by the reviewer, we provide the snapshot of our RNA-seq data for Cfp1 below:

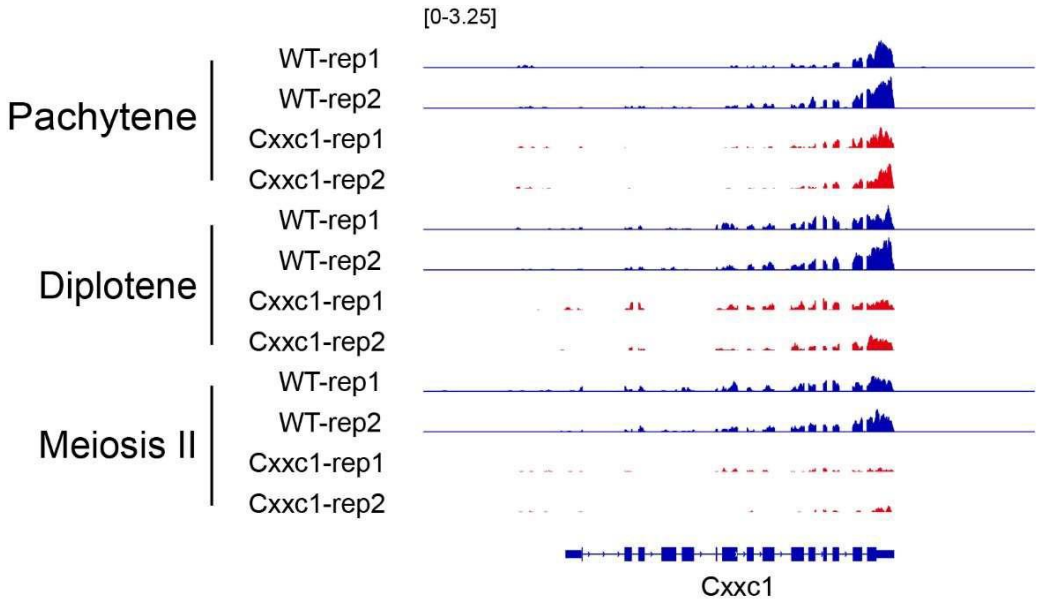

Fig. R5

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Second decision letter

MS ID#: DEVELOP/2019/183764

MS TITLE: CxxC Finger Protein 1-mediated Histone H3 Lysine-4 Trimethylation is Essential for Proper Meiotic Crossover Formation in Mice

AUTHORS: Yu Jiang, Hui-Ying Zhang, Zhen Lin, Ye-Zhang Zhu, Chao Yu, Qian-Qian Sha, Ming-Han Tong, Li Shen, and Heng-Yu Fan

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees found the revised manuscript improved based on their comments, but also have found some significant concerns remained and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. Please consider that this is the last opportunity for revision of the manuscript.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

I have provided this info before.

Comments for the author

In this revised manuscript, the authors made some changes based on my and other reviewers' comments, and added some new data accordingly. However, there are still a few points left to be answered:
1. To answer: "How is chromosome missegregation caused with increased CO?", the authors checked the formation of obligate crossover, and found no significant loss in obligate crossover in Cxxc1-cKO spermatocytes. Thus, they then studied the distribution of COs and "found that the COs in the Cxxc1-cKO showed a distribution preference in regions adjacent to the telomere, which are less able to enhance the segregation fidelity". However, given the increased number of MLH1 foci in Cxxc1-cKO spermatocytes (about 1 more in each spermatocyte as shown in Fig5D), it is hard to imagine that only 2 more MLH1 foci (in average) distributed at telomeres (as shown in Fig S1I) would result in the appearance of lagging chromosome in more than 30% MI spermatocytes (Fig3D). Thus, the authors should show the number of MLH1 in regions "adjacent to the telomere" rather than "MLH1 foci distributed at telomeres".
2. The response to the second major comment is acceptable.
3. The third major comment, the authors should show direct evidence for an altered distribution of DSBs by DMC1-CHIP or SPO11-CHIP, but the authors "were unable to perform this experiment because of the unavailability of an effective antibody". However, as I know the antibody for DMC1-CHIP is a commercially available antibody (anti-DMC1, Santa Cruz, sc-8973) [1] which is also widely used for DMC1 staining.
4. To answer the fourth major comment, the authors suggest that the effect of Cxxc1-cKO on meiosis has two aspects:
1) The loss of Cxxc1 leads to the ineffective deposition of H3K4me3 on DSB hotspots at the leptotene/zygotene stage, which causes a delay in DSB repair and a disproportionate CO distribution, and finally results in a partial defect at the MI stage.
2) Second, the loss of Cxxc1 blocked the re-establishment of H3K4me3 in the diplotene stage. The H3K4me3 at the diplotene stage is related to transcription rather than to meiotic recombination. Therefore, transcription of key genes required for meiosis II is largely silenced because of the deficiency of Cxxc1 which results in the arrest at the MII stage.
However, the problems are:
1) From Fig7B, we can see that H3K4me3 at promoter region is sharply decreased at leptotene/zygotene stage in Cxxc1-cKO spermatocytes, suggesting the gene expression pattern in Cxxc1-cKO spermatocytes could be changed even since leptotene/zygotene stage (or even earlier). This may also affect the expression of genes related to DSB repair and lead to the delay in DSB repair and a disproportionate CO distribution.

2) The altered gene expression in pachytene and diplotene stage could also affect distribution of MLH1 and the formation of crossover products after the loading of MLH1, which may explain the failure of chiasma formation despite of increased MLH1 foci.
It could be very hard for the authors to check these possibilities and find the mechanism behind the phenotype in Cxxc1-cKO spermatocytes due to the limitation of animal models, but authors should at least discuss these possibilities objectively in their discussion.

Taken together, I think that the manuscript still need to be revised.

1. Khil PP, Smagulova F, Brick KM, Camerini-Otero RD, Petukhova GV (2012) Sensitive mapping of recombination hotspots using sequencing-based detection of ssDNA. Genome Res 22: 957-965.

Reviewer 2

Advance summary and potential significance to field

The authors addressed many of my previous suggestions. However, I am not fully convinced of the validity of MII cells and secondary spermatocytes they detected. In this revision, the authors did not perform validation experiments that I suggested previously. I still think these cells can be MI. Having said that, this is a relatively minor point in the main story. Overall, this is an important study that should be published soon.

Comments for the author

In their response, the authors present two reasons for this argument: 1) the similarity with Gaysinskaya et al., 2014, and 2) the PNA signals in mutant secondary spermatocytes (Figure 3F). Reason 1) sounds indirect to me. This reference does not prove validity. Reason 2) could be a result of a phenotype. Did the authors detect PNA signals in wild-type secondary spermatocytes isolated by FACS?

Without any direct evidence, I believe the definitive description of MII cells and secondary spermatocytes is misleading. These cells should be described as “MII-like” cells and “secondary spermatocyte-like” cells, and the stage should be described as “Meiosis II-like” stage, both for controls and mutants, throughout the manuscript (including the abstract and figure panels). In the text, this phrasing should be explained like “These cells appear to be MII cells (termed MII-like cells)”. This could be the most feasible revision at this point.

This is an important point for publication. If this paper is published in the current form, one may cite this study as evidence of these cells, similarly to how the authors are citing Gaysinskaya et al., 2014.

Reviewer 3

Advance summary and potential significance to field

The authors have significantly revised the original submission and have adequately addressed most of the concerns and suggestions from this reviewer.

Comments for the author

However, I have a couple of minor suggestions to make clear what the authors’ observations are accurate because the conflicting results are previously published. Firstly, it is better to include Fig. R2 into the supplemental information, as well as the antibody information such as recognition sites used in the Fig.2R. Secondly, would it possible to show the deletion of exon 5 and 6 as the snapshots of the RNA-seq?. That is the main reason to show the snapshots of the RNA-seq.

In addition, in Fig.3, the authors can change the channel name to Hoechst Blue and Hoechst Red. The authors used Hoechst dye but not DAPI and Iodo-1. Finally, the initials of the first name of the authors in the text should be deleted.

Second revision

Author response to reviewers' comments

Reviewer 1
1.To answer: “How is chromosome missegregation caused with increased CO?”, the authors checked the formation of obligate crossover, and found no significant loss in obligate crossover in Cxxc1-cKO spermatocytes. Thus, they then studied the distribution of COs and “found that the COs in the Cxxc1-cKO showed a distribution preference in regions adjacent to the telomere, which are less able to enhance the segregation fidelity”. However, given the increased number of MLH1 foci in Cxxc1-cKO spermatocytes (about 1 more in each spermatocyte as shown in Fig5D), it is hard to imagine that only 2 more MLH1 foci (in average) distributed at telomeres (as shown in Fig S1I) would result in the appearance of lagging chromosome in more than 30% MI spermatocytes (Fig3D). Thus, the authors should show the number of MLH1 in regions “adjacent to the telomere” rather than “MLH1 foci distributed at telomeres”.
Response: We greatly appreciate the reviewer for this suggestion. As the distribution of MLH1 in adjacent to centromere and telomere are different, we immunostained the spermatocyte spreads by CREST to further make a distinction between centromere and telomere. By dividing each SC into 10 equal length intervals, we studied the distribution of MLH1 along the SCs. As shown in revised Fig.5G, only a little proportion of MLH1 foci located near the centromere in wild-type spermatocytes, whereas a significant increase proportion of MLH1 foci was observed in Cxxc1-cKO spermatocytes.

2.The response to the second major comment is acceptable.

3.The third major comment, the authors should show direct evidence for an altered distribution of DSBs by DMC1-CHIP or SPO11-CHIP, but the authors “were unable to perform this experiment because of the unavailability of an effective antibody”. However, as I know the antibody for DMC1-CHIP is a commercially available antibody (anti-DMC1, Santa Cruz, sc-8973) [1] which is also widely used for DMC1 staining.
Response: We appreciate the reviewer’s suggestion. We had also paid attention to this antibody before, and this antibody was widely used in DMC1-related research, but it had already been commercially discontinued and thus it was unavailable for us. For details, please check: https://www.scbt.com/p/dmc1-antibody-c-20?productCanUrl=dmc1-antibody-c-20&_requestid=441328
The replaced antibody recommended by the manufacture is a mouse monoclonal antibody which is not recommended for ChIP. It is known that mouse antibodies usually do not work well on mouse tissue. Besides, our laboratory does not have experiences in ChIP-sequencing experiments using antibody other than histone antibodies. It will take a very long time for us to optimize the conditions, and significantly delay the project. We hope the reviewer can kindly understand the situation.

4.To answer the fourth major comment, the authors suggest that the effect of Cxxc1-cKO on meiosis has two aspects:
1)The loss of Cxxc1 leads to the ineffective deposition of H3K4me3 on DSB hotspots at the leptotene/zygotene stage, which causes a delay in DSB repair and a disproportionate CO distribution, and finally results in a partial defect at the MI stage.
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However, the problems are:

1)From Fig7B, we can see that H3K4me3 at promoter region is sharply decreased at leptotene/zygotene stage in Cxxc1-cKO spermatocytes, suggesting the gene expression pattern in Cxxc1-cKO spermatocytes could be changed even since leptotene/zygotene stage (or even earlier). This may also affect the expression of genes related to DSB repair and lead to the delay in DSB repair and a disproportionate CO distribution.
2)The altered gene expression in pachytene and diplotene stage could also affect distribution of MLH1 and the formation of crossover products after the loading of MLH1, which may explain the failure of chiasma formation despite of increased MLH1 foci.
It could be very hard for the authors to check these possibilities and find the mechanism behind the phenotype in Cxxc1-cKO spermatocytes due to the limitation of animal models, but authors should at least discuss these possibilities objectively in their discussion.
Response: We appreciate the reviewer's insightful suggestions. We have discussed this issue in the revised manuscript.

Reviewer 2
In their response, the authors present two reasons for this argument: 1) the similarity with Gaysinskaya et al., 2014, and 2) the PNA signals in mutant secondary spermatocytes (Figure 3F). Reason 1) sounds indirect to me. This reference does not prove validity. Reason 2) could be a result of a phenotype. Did the authors detect PNA signals in wild-type secondary spermatocytes isolated by FACS?
Without any direct evidence, I believe the definitive description of MII cells and secondary spermatocytes is misleading. These cells should be described as "MII-like" cells and "secondary spermatocyte-like" cells, and the stage should be described as "Meiosis II-like" stage, both for controls and mutants, throughout the manuscript (including the abstract and figure panels). In the text, this phrasing should be explained like "These cells appear to be MII cells (termed MII-like cells)". This could be the most feasible revision at this point.
This is an important point for publication. If this paper is published in the current form, one may cite this study as evidence of these cells, similarly to how the authors are citing Gaysinskaya et al., 2014.
Response: We have performed the DNA FISH experiment on the FACS-isolated secondary spermatocytes and we have appended this result in supplementary figure S1E.

Reviewer 3
However, I have a couple of minor suggestions to make clear what the authors' observations are accurate because the conflicting results are previously published. Firstly, it is better to include Fig. R2 into the supplemental information, as well as the antibody information such as recognition sites used in the Fig.2R.
Response: We appreciate the reviewer's suggestion and we added the result in revised manuscript Fig. S1G. We also provided the antibody information in the Supplementary Table S1.

Secondly, would it possible to show the deletion of exon 5 and 6 as the snapshots of the RNA-seq?. That is the main reason to show the snapshots of the RNA-seq.
Response: The snapshot of Cxxc1 deleted exons are displayed at Fig. S2H.

In addition, in Fig.3, the authors can change the channel name to Hoechst Blue and Hoechst Red. The authors used Hoechst dye but not DAPI and Iodo-1. Finally, the initials of the first name of the authors in the text should be deleted.
Response: We appreciate the reviewer's suggestion and we have corrected it.

Third decision letter

MS ID#: DEVELOP/2019/183764

MS TITLE: CxxC Finger Protein 1-mediated Histone H3 Lysine-4 Trimethylation is Essential for Proper Meiotic Crossover Formation in Mice

AUTHORS: Yu Jiang, Hui-Ying Zhang, Zhen Lin, Ye-Zhang Zhu, Chao Yu, Qian-Qian Sha, Ming-Han Tong, Li Shen, and Heng-Yu Fan
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.