nature portfolio

Peer Review File

mop1 affects maize recombination landscapes by modulating methylation of MITEs near genes in open chromatin

Corresponding Author: Professor Meixia Zhao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, Hasan et al. investigated the distribution of crossover (CO) events in the mop1 mutant of maize in comparison to the wild type. MOP1 is considered the ortholog of RDR2 in Arabidopsis and plays a key role in the RdDM pathway. As a result, mop1 mutants display significantly altered (mainly reduced) DNA methylation, particularly in the CHH context, and to a lesser extent in CG and CHG.
The authors generated and sequenced large BC1 populations for both wild-type and mop1 genotypes, enabling high-quality and comprehensive mapping of CO locations in both male and female meiosis. They then conducted a multi-layered characterization of crossover patterns across genotypes and sexes. Their findings indicate that mop1 mutants exhibit a reduction in COs during male, but not female meiosis, and that the distribution of COs differs substantially from that of the wild type in both sexes. Moreover, the authors report that COs in mop1 more frequently occur in regions with high SNP density and show stronger associations with MITE transposable elements. Interestingly, the observed CO redistribution overlaps only marginally with DMRs, suggesting that reduced DNA methylation alone is not sufficient to render these regions permissive for recombination.
This work offers valuable insights not only into the recombination landscape of mop1 mutants with impaired DNA methylation but also into crossover dynamics in wild-type maize, which have not previously been explored in such depth. I found the results regarding the relationship between crossovers, polymorphism density, and transposable element types particularly compelling. However, I also identified some conclusions that are not strongly supported by the presented data, and I have concerns regarding aspects of the methodology.

Major Points:
1. The conclusion regarding a shift in COs from pericentromeric regions to chromosome arms lacks sufficient support. In Figure 1, this effect is illustrated only for a single chromosome (panel 1e), and Supplementary Fig 4 does not show a statistically significant redistribution. Similarly, analyses in Supplementary Figs 5–7 remain ambiguous. I suggest the authors simply state that there are changes in teh local redistribution of crossovers relative to the wild type and not emphasize pericentromere to arm shift.
2. To better capture the observed shifts in CO distributions, the authors should consider presenting correlations between the chromosomal distribution of COs across genotypes and sexes. Such analysis would offer a more comprehensive view of CO landscape changes.
3. Line 217 onward: The comparison of sequence motif frequency across genotypes/sexes must rely on a shared motif dataset. Otherwise, it becomes a comparison of unrelated elements. I recommend merging all CO datasets from the four populations to identify a common set of motifs, and then quantifying the frequency of those motifs in each population separately.
4. Transposon analysis (Figure 4d): While the general association between COs and MITEs is convincing, some aspects of the analysis require improvement. For example, in line 277, the authors state: “in female WT, 33.8% of COs within a 2 kb interval overlapped with MITEs.” However, this percentage lacks context unless it is compared to a random expectation. The authors should determine what proportion of randomly selected 2 kb genomic windows would overlap with each TE type. A similar issue arises in the analysis of CO hotspots and TE association (line 306 onward).
5. Hotspot Analysis: Although the definition of hotspots is inherently somewhat arbitrary, the relatively low threshold used in this study limits the value of comparing hotspot conservation across genotypes and sexes, due to potential sampling effects. For example, if the authors were to randomly split the 110 WT female BC1 individuals into two groups and identify hotspots separately in each, how much overlap would they observe between the resulting sets, even if the threshold was halved?

Likely very little — hence, the authors' analysis of hotspot conservation is, in my view, uninformative. I propose a revised strategy similar to the one mentioned for motif analysis: pool all CO data across the four groups, define a common hotspot set, and then evaluate the frequency of hotspot usage in each population.

Minor Comments:
- Line 293: Should read: "...in female WT, female mop1, male WT, and male mop1, respectively..."
- Consider including a chromosomal distribution map of DMRs relative to COs. While the DMR data may originate from another publication, most readers will not be familiar with their distribution in mop1 versus WT.

Reviewer #2

(Remarks to the Author)
In this manuscript, Hasan et al. investigate the effect of the mop1 mutation on meiotic crossovers in the maize genome. They generated high-resolution maps of meiotic crossovers in male and female meiosis through NGS of backcrossed populations in wild-type and mop1 maize. Their results are consistent with previous reports on high-resolution maps of meiotic DSBs or crossovers in various plant species, showing that crossovers are enriched in euchromatin, euchromatin-associated TEs near genes, and low-nucleosome-density regions with active histone marks, while being suppressed in retrotransposon-enriched pericentromeric regions.

Notably, the authors found that mop1 led to a reduction in crossover frequency compared to the wild type. Moreover, mop1 female meiosis reshaped the crossover landscape, leading to a preferential occurrence of crossovers in MITEs overlapping hypoDMRs in open chromatin regions. This study reinforces the importance of DNA methylation and the genetic and epigenetic state in shaping genome-wide meiotic crossover patterns.

However, the novelty of their findings appears somewhat limited, apart from the observed moderate reshaping of meiotic crossovers in mop1. Additionally, I have several critical concerns and comments that the authors need to address to improve the manuscript.

Comments:
Lines 164–169, Fig. 1d: Please provide a histogram of the high-resolution crossover mapping sites (i.e., the physical distance between two SNPs per crossover) and a histogram plot showing the number of crossovers per BCF1 individual.

Line 171: It does not appear that the authors aligned crossover sites to the complete maize genome (from telomere to telomere). Therefore, the phrase "with no COs observed in the centromeric regions" should be revised to "with no COs observed in the pericentromeric regions close to the centromeric regions."

Fig. 1c: The circular plot is not appropriate for comparing crossover landscapes between samples. Please provide XY plots for each chromosome to better visualize and compare crossover distributions.

Lines 176-177 and Fig 1: In this manuscript, the authors did not explain why crossover rates are significantly reduced in mop1 males but remain unchanged in females (Fig. 1d), even though this observation represents a major effect of mop1 on meiotic crossovers. Given that mop1 leads to a decrease in CHH methylation and may potentially affect gene expression via hypo-CHH methylation, the authors should examine the transcript levels of meiotic genes in wild-type and mop1 meiocytes. In particular, the transcript level of HEI10, a dosage-dependent pro-crossover factor, should be investigated in wild-type and mop1 mutants, as the levels of HEI10 mRNAs and protein abundance correlate with crossover number in Arabidopsis and mice.

Line 198-201: The authors argued that they observed unique crossover peaks along Chr1, indicating variations. However, this claim is not supported by statistical analysis. Please provide statistical analysis and highlight the peaks or regions showing significant differences. The crossover frequency data along chromosomes should be carefully analyzed and compared between genotypes, as the number of individuals and crossovers is relatively low.

Lines 226-232: These sentences and Fig 3a regarding CO intervals are unclear. The crossover site is precisely determined by two SNPs between B73 and Mo17 via reciprocal exchange, indicating the positions of these SNPs and their physical distance on the genome. What is definition of CO intervals here? Have the authors analyzed 1 kb upstream and downstream of the SNPs from the crossover midpoint? Did the authors use all crossover sites or only high-resolution crossover sites here? Why not show a histogram of the physical distances (mean distance) between the two SNPs at crossover sites? Additionally, the authors could present a plot showing SNP density (SNPs per 1 kb) within 1 kb, 2 kb, 3 kb, or 10 kb flanking the crossover midpoint.

Lines 257-286: These observations are consistent with previous reports in Arabidopsis. The TEs associated with crossovers are preferentially located near gene start and end sites in Arabidopsis. The authors should present a plot showing the location and distribution of MITEs associated with crossovers around gene start and end sites. Additionally, why not plot all crossovers from the genotypes in this study around gene start and end sites within a 2 kb window?

Line 362-375 (Fig. 6c): These data and descriptions are related to the previously suggested plot showing crossovers and MITEs around gene start and end sites within a 2 kb window. DMRs could also be included in the same plot. The current proportion plot is unclear and difficult to interpret intuitively, along with the corresponding description in the text.

Line 398, 403 and 406: There is no statistically significant difference in interference. These sentences containing "weaker" and "greater" should be revised and clarified; otherwise, they might lead to misinterpretation.

Reviewer #3

(Remarks to the Author)
This is a very exciting follow up study on the effects of mop1 RdDM mutant on crossovers in maize. The authors use high-resolution crossover mapping and extensive bioinformatic analysis to show: 1) different effects of mop1 on total crossover numbers in male and female meiosis; 2) excitingly, the authors find de novo crossovers in female mop1 mutants. This study is original, novel and would be of interest to the meiosis and epigenetics research community. The methodology is sound, the work meets the expected standard in the field.

My main comments/suggestions are:
1. I would like to see data and a discussion on gene expression in mop1 mutants. How does loss of CHH in MITEs near genes in mop1 affect proximal gene expression? Are any of these genes known meiotic factors (and, maybe, implicated in heterochiasmy)?
2. It would be great if authors added to the Results how many de novo crossovers per meiosis have been observed in female mop1 on average (compared to the wild type total crossovers per meiosis that the authors observed in this study).
3. This follows from my point above. What drives crossover redistribution to distal chromosome regions in mop1? If there are only very few (?) de novo crossovers in mop1 in females – would this be enough to cause chromosome-wide crossover redistribution to the distal regions? And what would the mechanism be in males? I could not find (apologies if I missed it) in the previous paper by the authors or this manuscript the total percentage loss of CG and CHG methylation over the pericentromeric region (or genome/chromosome-wide) in mop1. Whilst I agree with the authors that CHH loss could attract active chromatin marks that could drive crossover redistribution (an exciting new possibility that needs to be explored beyond this study), I would like the authors to discuss whether an overall reduction of CG methylation over maize pericentromeres in mop1 could drive crossover remodelling (similarly to Arabidopsis). In this case, CHH hypomethylation in MITEs would enable 'some new crossover sites' but would not necessarily drive the chromosome-wide crossover redistribution. It would also be good to report the overall percentage loss of CHG methylation, although if CHG methylation is reduced, one would expect increased pericentromeric crossovers. In any case, I would like to see these reported and discussed.
4. I would like to see a longer discussion of the implications of this finding for crop improvement – in maize and in crops in general

Other comments:
Abstract:
1. Lines 33-34 – please rephrase 'poorly understood' to 'no completely understood' or something like this. There is a lot known about the regulation of chromosome segregation.
Introduction:
2. Lines 71-73 – NCO can be formed from intermediates preceding dHJ – please correct
3. Lines 90-92 – instead of promoters, best to say transcription start and termination sites
4. Lines 96-98 – please rephrase for clarity – 'male meiosis exhibits higher recombination frequencies at low temperatures'. Is this compared to high temperatures or females?
5. Lines 100-102 'CO numbers and distributions are similar'. Are the authors comparing males and females?
6. Line 120 and throughout the text – gene names should be in italic.
Results
7. Lines 159-161 – could the authors please confirm that they used SNPs shared between 4 populations to map crossovers. If this was not the case, what was the reasoning behind it? What are the implications of using non-overlapping SNPs on crossover maps?
8. Could the authors please add details of statistical tests they used throughout the study, either to the text and/or figure legends next to p-values. For example, line 176 says 'COs per male meiosis were significantly reduced…' – please add stats text and p-values in brackets here and in similar cases throughout the text.
9. Lines 182-193 – Figures 1e, Supplemental Fig 4-7 are good, but I suggest plotting crossovers on the (y -axis) against all maize combined chromosomes oriented from telomere to centromere (x-axis), for example, as in https://doi.org/10.15252/embj.2020104858 Figure 1c. This will allow the authors get their message across quicker. It will be also easier for the reader to interpret.
10. Conclusion: 'mop1 introduces new CO sites by removing DNA methylation from MITEs within 2 kb flanking regions of genes'. This is a very exciting finding. Could the authors please add a close up (e.g. a genome browser screenshot with nucleosome density, gene/TEs, polymorphisms, DNA methylation in three contexts, and key histone marks, e.g listed in lines 380-382) with a 'representative' new CO associated with MITEs in mop1 (and how the same chromosome region looked in the wild type).
11. A figure of a fine-mapped de novo crossover would be nice to see too.
12. I find top panel of 4b confusing. Is there a way to replace with a clearer plot?
13. I find Figure 6a hard to interpret.
14. Is there a correlation between the loss of CHH in a MITE, changes in the proximal gene expression and probability of a de novo crossover at this MITE?
Discussion
15. I suggest rephrasing lines 443-444 stylistically to say that methylation is removed in mop1 mutant allowing deposition of

active histone marks (rather than 'mop1 removes methylation and deposits active histone marks').
16. Lines 454-456 – the authors propose that 'rather than altering the overall chromatin structure of the chromosomes, mop1 specifically modifies the chromatin state of particular regions, such as MITEs near genes'. Given the extensive bioinformatic analysis, can this information now be extracted from the DNA methylation/histone ChIP data and presented in the paper?
17. Lines 465-473 – a similar threshold has been found in other plants (e.g. https://doi.org/10.15252/embj.2020104858), please add how thresholds in different wild type plants compare.
18. I suggest adding to the discussion the effects epigenetic mutants on plant meiotic DSBs and how this knowledge relates to the current study.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have thoroughly addressed my questions and comments on the manuscript. In most cases, they implemented the modifications I suggested, and where this was not feasible or useful, they provided clear and convincing explanations. A particularly interesting new result is the analysis showing differences in the distribution of CO midpoints relative to SNPs in the mop1 background compared to WT (Fig. 3b).
The manuscript is substantially improved compared to the original version, and I have no further concerns aside from a few minor editorial suggestions:
• Line 244 – the phrasing "…this valley was higher…" is somewhat awkward; I suggest the clearer "…this valley was shallower…".
• Line 455 – it should read "ZYP1", not "ZIP1".

Reviewer #2

(Remarks to the Author)
The authors have thoroughly addressed my previous concerns by providing new data and analyses, which have substantially improved the revised manuscript. Overall, the study now presents a clearer and stronger contribution to the field.

Reviewer #3

(Remarks to the Author)
The authors have successfully addressed most of the previous reviewers' comments, the manuscript has been substantially improved.
Three minor comments that, in my opinion, still need to be addressed:
1. It is very interesting that ASY4 and ZIP4 are downregulated in mop1 mutants. I strongly encourage the authors to incorporate this finding into the Discussion. ASY1 and ZYP1 depletion in Arabidopsis results in crossover redistribution to subtelomeric regions – an effect that is similar to what the authors observed in mop1 in their previous study and in a number of chromosomes in this study. Could it be that crossover changes observed in mop1 result from both, depletion of the axis/SC components and CHH hypomethylation?
2. In the section 'Frequencies of A/T-associated motifs increase in female mop1, while G/C associated motifs decrease in male mop1 COs' the authors talk about decreased/increased frequencies of A/T and G/C motifs in male and female mop1 vs WT, but are these increases/decreases statistically significant? Please add statistical analysis.
3. Lines 474-475 – I am not sure I understand how a CO can be 'the same' if it is detected at a different chromosomal position. Please explain or rephrase.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have thoroughly addressed all my comments. Overall, this is a thorough study and a substantial contribution to the field.

RESPONSES TO THE EDITOR AND REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, Hasan et al. investigated the distribution of crossover (CO) events in the *mop1* mutant of maize in comparison to the wild type. MOP1 is considered the ortholog of RDR2 in Arabidopsis and plays a key role in the RdDM pathway. As a result, *mop1* mutants display significantly altered (mainly reduced) DNA methylation, particularly in the CHH context, and to a lesser extent in CG and CHG.

The authors generated and sequenced large BC1 populations for both wild-type and *mop1* genotypes, enabling high-quality and comprehensive mapping of CO locations in both male and female meiosis. They then conducted a multi-layered characterization of crossover patterns across genotypes and sexes. Their findings indicate that *mop1* mutants exhibit a reduction in COs during male, but not female meiosis, and that the distribution of COs differs substantially from that of the wild type in both sexes. Moreover, the authors report that COs in *mop1* more frequently occur in regions with high SNP density and show stronger associations with MITE transposable elements. Interestingly, the observed CO redistribution overlaps only marginally with DMRs, suggesting that reduced DNA methylation alone is not sufficient to render these regions permissive for recombination.

This work offers valuable insights not only into the recombination landscape of *mop1* mutants with impaired DNA methylation but also into crossover dynamics in wild-type maize, which have not previously been explored in such depth. I found the results regarding the relationship between crossovers, polymorphism density, and transposable element types particularly compelling. However, I also identified some conclusions that are not strongly supported by the presented data, and I have concerns regarding aspects of the methodology.

Response: Thank you for your positive evaluation and suggestions for this work.

Major Points:
1. The conclusion regarding a shift in COs from pericentromeric regions to chromosome arms lacks sufficient support. In Figure 1, this effect is illustrated only for a single chromosome (panel 1e), and Supplementary Fig 4 does not show a statistically significant redistribution. Similarly, analyses in Supplementary Figs 5–7 remain ambiguous. I suggest the authors simply state that there are changes in the local redistribution of crossovers relative to the wild type and not emphasize pericentromere to arm shift.

Response: We agree with the reviewer that the redistribution of COs from pericentromeric regions to chromosome arms in some chromosomes is not statistically significant for some chromosomes. We changed this into this shift was not significantly detected in either female or male *mop1* using our high-resolution CO maps in the revision.

2. To better capture the observed shifts in CO distributions, the authors should consider presenting correlations between the chromosomal distribution of COs across genotypes and sexes. Such analysis would offer a more comprehensive view of CO landscape changes.

Response: Thank you for the suggestion. We have now included this analysis in the revised Fig. 1c.

3. Line 217 onward: The comparison of sequence motif frequency across genotypes/sexes must rely on a shared motif dataset. Otherwise, it becomes a comparison of unrelated elements. I recommend merging all CO datasets from the four populations to identify a common set of motifs, and then quantifying the frequency of those motifs in each population separately.

Response: Thank you for the excellent suggestion. We have now merged all high-resolution COs into a single dataset, resulting in a total of 1,835 COs, and performed the motif analysis. The findings are even more intriguing. Our results show that the frequencies of the two A/T polymers increased in female *mop1*, while the frequencies of the three C/G polymers decreased in male *mop1* (see Fig. 2).

4. Transposon analysis (Figure 4d): While the general association between COs and MITEs is convincing, some aspects of the analysis require improvement. For example, in line 277, the authors state: "in female WT, 33.8% of COs within a 2 kb interval overlapped with MITEs." However, this percentage lacks context unless it is compared to a random expectation. The authors should determine what proportion of randomly selected 2 kb genomic windows would overlap with each TE type. A similar issue arises in the analysis of CO hotspots and TE association (line 306 onward).

Response: Thank you for the suggestion. In the revision, we randomly selected 1,835 regions matching the lengths of CO intervals and examined their associations with different types of TEs. Our analysis showed that only 6.9% of the random regions overlapped with MITEs (Fig. 4d). According to maize genome annotation, MITEs comprise less than 2% of the genome. Despite this small proportion, over 30% of COs overlapped with MITEs. We have also included random region comparisons for both CO hotspots and coldspots, see the updated Figs. 5c and 5f.

5. Hotspot Analysis: Although the definition of hotspots is inherently somewhat arbitrary, the relatively low threshold used in this study limits the value of comparing hotspot conservation across genotypes and sexes, due to potential sampling effects. For example, if the authors were to randomly split the 110 WT female BC1 individuals into two groups and identify hotspots separately in each, how much overlap would they observe between the resulting sets, even if the threshold was halved? Likely very little — hence, the authors' analysis of hotspot conservation is, in my view, uninformative. I propose a revised strategy similar to the one mentioned for motif analysis: pool all CO data across the four groups, define a common hotspot set, and then evaluate the frequency of hotspot usage in each population.

Response: Hotspots were defined as 5 kb regions with recombination rates (cM/Mb) at least five times higher than the genome-wide average, following the criteria described by Kianian et al. (2018). Specifically, the midpoint of each 5 kb region was used to query recombination rates in MaryMap. These rates were then compared to the genome-wide average to identify recombination hotspots. Therefore, it is challenging to apply the same strategy as used in the motif analysis. For each hotspot identified in one population, if the same hotspot was also identified in the other three populations, it would correspond to the exact same sequence with identical start and end positions.

Kianian PMA, *et al*. High-resolution crossover mapping reveals similarities and differences of male and female recombination in maize. *Nat Commun* 9, 2370 (2018).

Minor Comments:
- Line 293: Should read: "...in female WT, female *mop1*, male WT, and male *mop1*, respectively..."

Response: Thank you. The typo is fixed.

- Consider including a chromosomal distribution map of DMRs relative to COs. While the DMR data may originate from another publication, most readers will not be familiar with their distribution in *mop1* versus WT.

Response: The numbers of hyper- and hypomethylated DMRs are relatively small: 440 CG, 660 CHG, and 1,702 CHH hyper DMRs, and 2,749 CG, 4,140 CHG, and 12,162 CHH hypo DMRs. We attempted to plot the chromosomal distribution of these DMRs, but the patterns appeared dispersed. Instead, we focused on the distribution of both hyper and hypo DMRs within gene-associated regions, specifically, 2 kb upstream and downstream of genes and across gene bodies. As shown in Supplementary Fig. 32, the numbers of both hyper- and hypomethylated CHH DMRs form two distinct peaks near the transcription start sites (TSSs) and transcription termination sites (TTSs), which coincide with mCHH islands. This suggests that the *mop1* mutation primarily affects these island regions.

Reviewer #2 (Remarks to the Author):

In this manuscript, Hasan et al. investigates the effect of the *mop1* mutation on meiotic crossovers in the maize genome. They generated high-resolution maps of meiotic crossovers in male and female meiosis through NGS of backcrossed populations in wild-type and *mop1* maize. Their results are consistent with previous reports on high-resolution maps of meiotic DSBs or crossovers in various plant species, showing that crossovers are enriched in euchromatin, euchromatin-associated TEs near genes, and low-nucleosome-density regions with active histone marks, while being suppressed in retrotransposon-enriched pericentromeric regions.

Notably, the authors found that *mop1* led to a reduction in crossover frequency compared to the wild type. Moreover, *mop1* female meiosis reshaped the crossover landscape, leading to a preferential occurrence of crossovers in MITEs overlapping hypo DMRs in open chromatin regions. This study reinforces the importance of DNA methylation and the genetic and epigenetic state in shaping genome-wide meiotic crossover patterns.

However, the novelty of their findings appears somewhat limited, apart from the observed moderate reshaping of meiotic crossovers in *mop1*. Additionally, I have several critical concerns and comments that the authors need to address to improve the manuscript.

Response: Thank you for your valuable suggestions for improvement.

Comments:
Lines 164–169, Fig. 1d: Please provide a histogram of the high-resolution crossover mapping sites (i.e., the physical distance between two SNPs per crossover) and a histogram plot showing the number of crossovers per BCF1 individual.

Response: Please refer to Fig. 1c, 1d, Supplementary Fig. 5, and Supplementary Data 1.

Line 171: It does not appear that the authors aligned crossover sites to the complete maize genome (from telomere to telomere). Therefore, the phrase "with no COs observed in the centromeric regions" should be revised to "with no COs observed in the pericentromeric regions close to the centromeric regions."

Response: We have removed the phrase "with no COs observed in the pericentromeric regions close to the centromeric regions" from the revised manuscript.

Fig. 1c: The circular plot is not appropriate for comparing crossover landscapes between samples. Please provide XY plots for each chromosome to better visualize and compare crossover distributions.

Response: Thank you for the suggestion. In the revised manuscript, we have included an XY plot showing crossover distribution along the maize chromosomes (see Fig. 1c).

Lines 176-177 and Fig 1: In this manuscript, the authors did not explain why crossover rates are significantly reduced in *mop1* males but remain unchanged in females (Fig. 1d), even though this observation represents a major effect of *mop1* on meiotic crossovers. Given that *mop1* leads to a decrease in CHH methylation and may potentially affect gene expression via hypo-CHH methylation, the authors should examine the transcript levels of meiotic genes in wild-type and *mop1* meiocytes. In particular, the transcript level of HEI10, a dosage-dependent pro-crossover factor, should be investigated in wild-type and *mop1* mutants, as the levels of HEI10 mRNAs and protein abundance correlate with crossover number in Arabidopsis and mice.

Response: Thank you for the suggestion. In the revised manuscript, we performed transcriptomic analysis of immature anthers from *mop1* mutants and their sibling WT controls. We examined the expression of 41 genes known to be involved in the meiotic pathway. Among them, only *ASYNAPTIC4* (*ASY4*), a gene associated with the formation of the meiotic chromosome axis, was significantly downregulated. *HEI10* also showed reduced expression in *mop1* anthers, but the difference was not statistically significant (see Supplementary Fig. 35 and Supplementary Data 4). The underlying reason for the reduced CO rates observed in *mop1* males remains unclear and will be addressed in future studies, but we have added some discussion on this in the revision.

Line 198-201: The authors argued that they observed unique crossover peaks along Chr1, indicating variations. However, this claim is not supported by statistical analysis. Please provide statistical analysis and highlight the peaks or regions showing significant differences. The crossover frequency data along chromosomes should be carefully analyzed and compared between genotypes, as the number of individuals and crossovers is relatively low.

Response: Due to the nature of the recombination rate data generated by MareyMap, it is challenging to precisely define the start and end positions, as well as the height, of the identified peaks. As a result, performing statistical comparisons between these peaks is not straightforward. We have revised the text in the manuscript accordingly. Thank you for your understanding.

Lines 226-232: These sentences and Fig 3a regarding CO intervals are unclear. The crossover site is precisely determined by two SNPs between B73 and Mo17 via reciprocal exchange, indicating the positions of these SNPs and their physical distance on the genome. What is definition of CO intervals here? Have the authors analyzed 1 kb upstream and downstream of the SNPs from the crossover midpoint? Did the authors use all crossover sites or only high-resolution crossover sites here? Why not show a histogram of the physical distances (mean distance) between the two SNPs at crossover sites? Additionally, the authors could present a plot showing SNP density (SNPs per 1 kb) within 1 kb, 2 kb, 3 kb, or 10 kb flanking the crossover midpoint.

Response: Yes, the CO intervals were defined by the two closest SNPs between B73 and Mo17. There may be additional SNPs within these intervals based on the parental genome sequences of B73 and Mo17. However, due to the relatively low sequencing depth (~3X per BC_1 individual) in our BC_1 populations, some SNPs may not have been detected. For this analysis, we used only 1,835 high-resolution CO sites mapped within 2 kb intervals. Please refer to Fig. 3b for SNP density within 2 kb flanking the CO midpoints.

Lines 257-286: These observations are consistent with previous reports in Arabidopsis. The TEs associated with crossovers are preferentially located near gene start and end sites in Arabidopsis. The authors should present a plot showing the location and distribution of MITEs associated with crossovers around gene start and end sites. Additionally, why not plot all crossovers from the genotypes in this study around gene start and end sites within a 2 kb window?

Response: Thank you. Please refer to Supplementary Fig. 24.

Line 362-375 (Fig. 6c): These data and descriptions are related to the previously suggested plot showing crossovers and MITEs around gene start and end sites within a 2 kb window. DMRs could also be included in the same plot. The current proportion plot is unclear and difficult to interpret intuitively, along with the corresponding description in the text.

Response: Thank you. Please refer to Supplementary Figs. 24 and 32.

Line 398, 403 and 406: There is no statistically significant difference in interference. These sentences containing "weaker" and "greater" should be revised and clarified; otherwise, they might lead to misinterpretation.

Response: Thank you for pointing this out. We have revised this section to state “No significant changes in CO interference in *mop1* mutants” in the updated manuscript.

Reviewer #3 (Remarks to the Author):

This is a very exciting follow up study on the effects of *mop1* RdDM mutant on crossovers in maize. The authors use high-resolution crossover mapping and extensive bioinformatic analysis to show: 1) different effects of *mop1* on total crossover numbers in male and female meiosis; 2) excitingly, the authors find de novo crossovers in female *mop1* mutants. This study is original, novel and would be of interest to the meiosis and epigenetics research community. The methodology is sound, the work meets the expected standard in the field.

Response: Thank you for your positive comments and helpful suggestions for improvement.

My main comments/suggestions are:
1. I would like to see data and a discussion on gene expression in *mop1* mutants. How does loss of CHH in MITEs near genes in *mop1* affect proximal gene expression? Are any of these genes known meiotic factors (and, maybe, implicated in heterochiasmy)?

Response: In the revision, we performed transcriptomic analysis of immature anthers from *mop1* mutants and their sibling WT controls. We examined the expression of 41 genes involved in the meiosis pathway. Among them, only *ASYNAPTIC4* (*ASY4*), a gene involved in meiotic chromosome axis, was significantly downregulated in *mop1* mutants (Supplementary Fig. 35 and Supplementary Data 4). Additionally, we also compared DMRs, COs, and MITEs with DEGs. However, we did not find any COs near the 139 DEGs that contained DMRs and MITEs (see the revised text).

2. It would be great if authors added to the Results how many de novo crossovers per meiosis have been observed in female *mop1* on average (compared to the wild type total crossovers per meiosis that the authors observed in this study).

Response: Thank you for your comment. When we compared COs between WT and *mop1* in both female and male, only a small proportion (4.3%) of COs were shared between WT and *mop1* (see Supplementary Fig. 7). We do not believe these represent entirely new COs triggered by *mop1*. Rather, the global reduction of CHH methylation in *mop1* may have shifted the positions of these COs to adjacent chromosomal regions. Thus, even if they are the same COs, they appear at different mapped positions. Please see additional responses below.

3. This follows from my point above. What drives crossover redistribution to distal chromosome regions in *mop1*? If there are only very few (?) de novo crossovers in *mop1* in females – would this be enough to cause chromosome-wide crossover redistribution to the distal regions? And what would the mechanism be in males? I could not find (apologies if I missed it) in the previous paper by the authors or this manuscript the total percentage loss of CG and CHG methylation over the pericentromeric region (or genome/chromosome-wide) in *mop1*. Whilst I agree with the authors that CHH loss could attract active chromatin marks that could drive crossover redistribution (an exciting new possibility that needs to be explored beyond this study), I would like the authors to discuss whether an overall reduction of CG methylation over maize pericentromeres in *mop1* could drive crossover remodelling (similarly to Arabidopsis). In this case, CHH hypomethylation in MITEs would enable 'some new crossover sites' but would not necessarily drive the chromosome-wide crossover redistribution. It would also be good to report

the overall percentage loss of CHG methylation, although if CHG methylation is reduced, one would expect increased pericentromeric crossovers. In any case, I would like to see these reported and discussed.

Response: As shown in Supplementary Fig. 31 and previous publications (Li et al. 2015; Zhao et al. 2021), *mop1* does not cause a dramatic decrease in global CG and CHG methylation. Instead, it primarily removes CHH methylation at the edges of transposons (mCHH islands) near transcriptionally active genes. This loss of CHH methylation also leads to reductions in CG or CHG methylation in some regions (e.g., Fig. 6g). In contrast to *met1* and *ddm1* mutants, which have dramatic effects on both methylation and patterns of histone medication throughout plant genomes, the effects of *mop1* are relatively discrete and precisely targeted. Thus, the proximate effects of this mutant are likely to be local rather than global, although the overall effects can clearly be global. Despite these subtle effects, *mop1* targets regions that are abundant in the maize genome. There are approximately 631 million CHH cytosines and 26,137 mCHH islands (Li et al. 2015). It is possible that chromatin structure is altered in *mop1* mutants, causing COs identified in WT to shift to flanking regions, resulting in largely non-overlapping COs between WT and *mop1*. Further analysis of chromatin accessibility and histone modifications in *mop1* mutants would provide valuable insights. We have added additional discussion on this in the revision.

Li Q, *et al.* RNA-directed DNA methylation enforces boundaries between heterochromatin and euchromatin in the maize genome. *Proc Natl Acad Sci U S A* **112**, 14728-14733 (2015).

Zhao M, *et al.* The mop1 mutation affects the recombination landscape in maize. *Proc Natl Acad Sci U S A* **118**, (2021).

4. I would like to see a longer discussion of the implications of this finding for crop improvement – in maize and in crops in general

Response: Thank you. We have added further discussion in the revised manuscript.

Other comments:
Abstract:
1. Lines 33-34 – please rephrase ‘poorly understood’ to ‘no completely understood’ or something like this. There is a lot known about the regulation of chromosome segregation.

Response: We have changed ‘poorly understood’ to ‘not yet completely understood’.

2. Lines 71-73 – NCO can be formed from intermediates preceding dHJ – please correct

Response: Yes, we have edited the text into “Depending on the resolution pathway, they can be resolved into crossovers (COs) or, less commonly, non-crossovers (NCOs), although many NCOs are thought to result from synthesis-dependent strand annealing events that never form a dHJ.”

3. Lines 90-92 – instead of promoters, best to say transcription start and termination sites

Response: Thank you. We made the change.

4. Lines 96-98 – please rephrase for clarity – ‘male meiosis exhibits higher recombination frequencies at low temperatures’. Is this compared to high temperatures or females?

Response: We meant “compared to females” and have updated the text accordingly in the revision.

5. Lines 100-102 ‘CO numbers and distributions are similar’. Are the authors comparing males and females?

Response: Yes, this paper compared females and males of B73 and Mo17 hybrids. We edited the text to make it clear.

6. Line 120 and throughout the text – gene names should be in italic.

Response: Here, we use capitalized letters to denote the proteins encoded by the genes, rather than the genes themselves. Therefore, these names are not italicized. Nonetheless, we have carefully reviewed the entire manuscript to ensure that all gene names are italicized appropriately.

7. Lines 159-161 – could the authors please confirm that they used SNPs shared between 4 populations to map crossovers. If this was not the case, what was the reasoning behind it? What are the implications of using non-overlapping SNPs on crossover maps?

Response: We used the SNPs identified within each BC_1 population to construct high-resolution CO maps. However, we retained only those SNPs that were also confirmed by high-depth resequencing of the two parental lines. Additionally, the SNPs identified across the four BC_1 populations were largely shared. As shown in Supplementary Fig. 1 and Supplementary Table 1, 85% of the SNPs were common to all four populations.

8. Could the authors please add details of statistical tests they used throughout the study, either to the text and/or figure legends next to p-values. For example, line 176 says ‘COs per male meiosis were significantly reduced…’ – please add stats text and p-values in brackets here and in similar cases throughout the text.

Response: Thank you for the suggestion. In the revised manuscript, we have included the statistical tests and their *P* values in the figure legends.

9. Lines 182-193 – Figures 1e, Supplemental Fig 4-7 are good, but I suggest plotting crossovers on the (y -axis) against all maize combined chromosomes oriented from telomere to centromere (x-axis), for example, as in https://doi.org/10.15252/embj.2020104858 Figure 1c. This will allow the authors get their message across quicker. It will be also easier for the reader to interpret.

Response: Thank you for the suggestion. In the revised manuscript, we have included a figure plotting crossovers along the maize chromosomes (see Fig. 1c).

10. Conclusion: '*mop1* introduces new CO sites by removing DNA methylation from MITEs within 2 kb flanking regions of genes'. This is a very exciting finding. Could the authors please add a close up (e.g. a genome browser screenshot with nucleosome density, gene/TEs, polymorphisms, DNA methylation in three contexts, and key histone marks, e.g listed in lines 380-382) with a 'representative' new CO associated with MITEs in *mop1* (and how the same chromosome region looked in the wild type).

Response: This is a great suggestion. In the revised manuscript, we have included a genome browser figure illustrating gene/MITE structure, DNA methylation in all three contexts, key histone marks, and the associated CO (see Fig. 6g). While preparing this figure, we became even more confident in our data, as we identified numerous similar cases.

11. A figure of a fine-mapped de novo crossover would be nice to see too.

Response: Please refer to the response above and Fig. 6g.

12. I find top panel of 4b confusing. Is there a way to replace with a clearer plot?

Response: We replaced the bar graph with a line graph. Please see the updated Fig. 4b.

13. I find Figure 6a hard to interpret.

Response: We have simplified Fig. 6a by including only male data in the main figure and moving the female data to Supplementary Fig. 30, since the methylation data are from immature anthers.

14. Is there a correlation between the loss of CHH in a MITE, changes in the proximal gene expression and probability of a de novo crossover at this MITE?

Response: As explained above and in the text, only a small proportion of differentially expressed genes (DEGs) had nearby differentially methylated regions (DMRs). Specifically, 15 (3.9%) and 41 (10.6%) DEGs contained CG and CHG DMRs, respectively, within these regions. A total of 83 (21.6%) DEGs harbored CHH DMRs nearby, the majority of which were hypomethylated. We also examined whether MITEs and COs overlapped with the DMRs of DEGs and found no COs near any of these 139 DEGs. These results suggest that removal of CHH methylation at MITEs that introduce COs near genes does not necessarily alter gene expression.

15. I suggest rephrasing lines 443-444 stylistically to say that methylation is removed in mop1 mutant allowing deposition of active histone marks (rather than 'mop1 removes methylation and deposits active histone marks').

Response: Changed as suggested.

16. Lines 454-456 – the authors propose that 'rather than altering the overall chromatin structure of the chromosomes, mop1 specifically modifies the chromatin state of particular regions, such

as MITEs near genes'. Given the extensive bioinformatic analysis, can this information now be extracted from the DNA methylation/histone ChIP data and presented in the paper?

Response: We would very much like to obtain this data. However, the available histone ChIP-seq datasets were not generated from meiocytes in *mop1* mutants. As noted above, isolating female meiocytes in maize remains technically challenging. Even in male meiocytes, performing ChIP-seq requires a substantial amount of starting material (1–5 g). Currently, our lab is adopting a more recent technique, Cleavage Under Targets and Release Using Nuclease (CUT&RUN), to profile histone modifications. We hope to use this method to investigate the histone marks associated with MITEs and COs in the near future.

17. Lines 465-473 – a similar threshold has been found in other plants (e.g. https://doi.org/10.15252/embj.2020104858), please add how thresholds in different wild type plants compare.

Response: We have added additional discussion and relevant citations in the revised manuscript.

18. I suggest adding to the discussion the effects of epigenetic mutants on plant meiotic DSBs and how this knowledge relates to the current study.

Response: Thank you for the suggestion. We have now added more discussion in the revised manuscript.

RESPONSES TO THE REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have thoroughly addressed my questions and comments on the manuscript. In most cases, they implemented the modifications I suggested, and where this was not feasible or useful, they provided clear and convincing explanations. A particularly interesting new result is the analysis showing differences in the distribution of CO midpoints relative to SNPs in the *mop1* background compared to WT (Fig. 3b).
The manuscript is substantially improved compared to the original version, and I have no further concerns aside from a few minor editorial suggestions:
• Line 244 – the phrasing "…this valley was higher…" is somewhat awkward; I suggest the clearer "…this valley was shallower…".

Response: Revised as suggested.

• Line 455 – it should read "ZYP1", not "ZIP1".

Response: Thank you. The typo is fixed.

Reviewer #2 (Remarks to the Author):

The authors have thoroughly addressed my previous concerns by providing new data and analyses, which have substantially improved the revised manuscript. Overall, the study now presents a clearer and stronger contribution to the field.

Response: Thank you for your comments.

Reviewer #3 (Remarks to the Author):

The authors have successfully addressed most of the previous reviewers' comments, the manuscript has been substantially improved.
Three minor comments that, in my opinion, still need to be addressed:

1. It is very interesting that ASY4 and ZIP4 are downregulated in *mop1* mutants. I strongly encourage the authors to incorporate this finding into the Discussion. ASY1 and ZYP1 depletion in Arabidopsis results in crossover redistribution to subtelomeric regions – an effect that is similar to what the authors observed in *mop1* in their previous study and in a number of chromosomes in this study. Could it be that crossover changes observed in *mop1* result from both, depletion of the axis/SC components and CHH hypomethylation?

Response: Thank you for the suggestion. We agree that this is an interesting observation and represents an important direction for future studies. We have incorporated this information into both the Results and Discussion sections.

2. In the section 'Frequencies of A/T-associated motifs increase in female mop1, while G/C associated motifs decrease in male mop1 COs' the authors talk about decreased/increased frequencies of A/T and G/C motifs in male and female *mop1* vs WT, but are these increases/decreases statistically significant? Please add statistical analysis.

Response: Thank you for the suggestion. We consulted with Dr. Jin Zhang from the Department of Statistics at Miami University and performed a binomial test. The results are as follows: the frequencies of the two A/T polymers were markedly increased in female *mop1* compared to female WT, and the frequencies of the top three C/G motifs were reduced in male *mop1*. However, these changes were not statistically significant based on the *q* value (the adjusted p value under the Benjamini–Hochberg correction), although the raw *p* values for all five comparisons were significant.

motif_ID	Sex	WT_%	mop1_%	WT count	WT total	*mop1* count	*mop1* total	*p*_value	*q*_value	Significant FDR0.05
1-ACGACGACGACGACGWGGVCS	Female	28.3	28.1	113	399	88	313	1.0000	1.0000	FALSE
1-ACGACGACGACGACGWGGVCS	Male	36.5	31.2	124	340	119	382	0.0334	0.1668	FALSE
2-GVGRGRRRGAGRGRGRGRRRGRRGRVGRVG	Female	43.1	44.1	172	399	138	313	0.7323	0.9154	FALSE
2-GVGRGRRRGAGRGRGRGRRRGRRGRVGRVG	Male	43.2	37.7	147	340	144	382	0.0300	0.1668	FALSE
3-CAGCAGCAGCAGCAGCAGCNSS	Female	33.3	31.0	133	399	97	313	0.4015	0.7652	FALSE
3-CAGCAGCAGCAGCAGCAGCNSS	Male	35.9	29.1	122	340	111	382	0.0055	0.1096	FALSE
4-TATATATATWTWWWWWWAWWWWWAWTTA	Female	30.6	37.1	122	399	116	313	0.0141	0.1409	FALSE
4-TATATATATWTWWWWWWAWWWWWAWTTA	Male	29.7	29.3	101	340	112	382	0.9109	0.9588	FALSE
5-AAAWAAAAARAAAAARAAWRARWAHWWRW	Female	37.6	43.1	150	399	135	313	0.0471	0.1852	FALSE
5-AAAWAAAAARAAAAARAAWRARWAHWWRW	Male	41.2	42.7	140	340	163	382	0.5676	0.8219	FALSE

3. Lines 474-475 – I am not sure I understand how a CO can be 'the same' if it is detected at a different chromosomal position. Please explain or rephrase.

Response: Thank you for pointing this out. Our intent was to convey that the underlying CO events may not be entirely new in *mop1*, but rather appear shifted in position. The global reduction of CHH methylation in *mop1* may influence chromatin structure or positioning, which in turn can alter the apparent genomic coordinates of COs when mapped to the reference genome. Thus, even if the COs occur in essentially the same local regions, they may be detected at slightly different positions depending on chromatin context and mapping resolution. We have revised the text to clarify this point.

RESPONSES TO THE REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed all my comments. Overall, this is a thorough study and a substantial contribution to the field.

Response: Thank you for your constructive comments and suggestions, which helped us significantly improve both the analysis and the manuscript.