Cell Genomics Transparent Peer Review Record

Fancm has dual roles in the limiting of meiotic crossovers and germ cell maintenance in mammals

Author list

Vanessa Tsui, Ruqian Lyu, Stevan Novakovic, Jessica M. Stringer, Jessica E. M. Dunleavy, Elissah Granger, Tim Semple, Anna Leichter, Luciano G. Martelotto, D. Jo Merriner, Ruijie Liu, Lucy McNeill, Nadeen Zerafa, Eva Hoffmann, Moira K. O'Bryan, Karla Hutt, Andrew J. Deans, Jörg Heierhorst, Davis J. McCarthy, and Wayne Crismani

Summary

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Second round of review:	Number of reviewers: 2 Accepted : 2nd June 2023
Data freely available:	Yes
Code freely available:	Yes

Referees' reports, first round of review

Reviewer 1

In this study, the authors explored the function of FANCM in mouse fertility. By using large-scale crossover analyses with both single-gamete sequencing and pedigree-based bulk-sequencing datasets, they found that more COs are generated in FANCM mutant mice in spite of normal number of MLH1 foci in mid-pachytene cells, indicating that the increased CO in FANCM mutant mice are class II COs. They also found that FANCM deficiency severely perturbed gametogenesis in both male and female mice, which is partially attributed to the cGASSTING pathway. The experiments are well designed and performed. The manuscript is well written. The data provided in this manuscript basically supports their conclusion. However, a few points still need to be addressed.

Major points:

1. The title "Fancm regulates meiotic double-strand break repair pathway choice in mammals" is not appropriate for two reasons: 1) This paper showed that FANCM plays separable roles, namely anti-crossover function and promoting gametogenesis. However, the latter is not indicated in the title. 2) The concept of "pathway choice" is not that specific, as "pathway choice" could indicate the choice between HR and NHEJ, as well as the choice between SDSA and DSBR pathway. Thus, it should be more accurate and specific.
2. The spermatogenesis in FANCM mutant mice was partially rescued by String KO. However, the authors only showed that more germ cells are present in the double mutant mice. As FANCM deficiency not only caused germ cell loss, but also affected the sperm motility. The authors should also show whether the sperm motility could also be rescued in the double mutant mice. Actually, all the work on String KO mice is not that related to the DSB repair.
3. If some mid-pachytene cells were apoptotic, we should expect to see reductions in the subpopulations of pachytene and diplotene cells and the metaphase I-anaphase I cells in the XII stage tubules. However, the authors showed that they did not observe these reductions. The author should give explanation for this?
4. Detailed methods for quantification of daily sperm production should be provided.

5. It would be more plausible if the authors can provide some results regarding how FANCM functions in DSB repair and the generation of class II COs during meiosis of male mice.

Minor concerns:
1. Some background information about cGAS-STING should be provided in the introduction.
2. It would be better if the authors could indicate the number of COs produced in FANCM deficient mice and compare it with control mice.
3. The authors should perform TUNEL assay (a late stage of cell apoptosis) to confirm the apoptotic events (PAS-positive) in the mutant testes.
4. Line 7, no FANCH. The authors should reword this sentence.
5. Line 132, F1 strain was not defined at its first appearance.
6. Figure 1, the genotypes were not indicated.

Taken together, the conclusion drawn in this paper is of certain significance and the data provided basically support their conclusions.

Reviewer 2

In the paper by Tsui et al., the authors comprehensively describe the phenotype of mice lacking the Fanconi anemia gene fancm. The authors characterize for the first time the effect of fancm loss on meiotic crossovers utilizing technologies other than immunofluorescence to reveal that the genetic map length of fancm-/- mice is increased. This demonstrates that FANCM limits crossovers in mammals. These mice have reproductive defects and the authors analyzed different stages of gametogenesis to pinpoint the cause of these defects. They found that the excess crossovers are unlikely to cause gametogenesis defects. Therefore, FANCM in mammals has separable roles, one regulating the DSB repair pathway choice and other, less clear role on promoting genomic stability, maybe in spermatogonial precursors.

The paper overall is very well written and well referenced. Despite the number of experiments presented it is easy to follow and the logic for the experiments is presented very clearly. I particularly appreciate the inclusion of negative results in the paper, as they help to provide a complete picture of what might be happening in these mice.

I believe this is a paper that will be of great interest to the meiosis and DNA repair communities.

A few minor comments:

1) I might have missed it but I cannot easily find how many F1 or single gametes were sequenced.
2) From the genetic maps, it is clear that there are extra crossovers. I am curious whether the authors could present this data in a different way to understand how many extra crossovers are in a single gamete. What is the distribution? Are there F1 mice or gametes with a huge excess of crossovers or it is an even increase?
3) Any thoughts on how DNA replication might be affected in these mice?

Authors' response to the first round of review

Response to reviewers' comments

The reviewers' comments are formatted with italics and quotation marks. Our responses are in plain text.

Reviewer #1.

"In this study, the authors explored the function of FANCM in mouse fertility. By using large-scale crossover analyses with both single-gamete sequencing and pedigree-based bulk-sequencing datasets, they found that more COs are generated in FANCM mutant mice in spite of normal number of MLH1 foci in mid-pachytene cells, indicating that the increased CO in FANCM mutant mice are class II COs. They also found that FANCM deficiency severely perturbed gametogenesis in both male and female mice, which is partially attributed to the cGASSTING pathway. The experiments are well designed and performed. The manuscript is well written. The data provided in this manuscript basically supports their conclusion. However, a few points still need to be addressed."

Major points:

1. *"The title "Fancm regulates meiotic double-strand break repair pathway choice in mammals" is not appropriate for two reasons: 1) This paper showed that FANCM plays separable roles, namely anti-crossover function and promoting gametogenesis. However, the latter is not indicated in the title. 2) The concept of "pathway choice" is not that specific, as "pathway choice" could indicate the choice between HR and NHEJ, as well as the choice between SDSA and DSBR pathway. Thus, it should be more accurate and specific."*

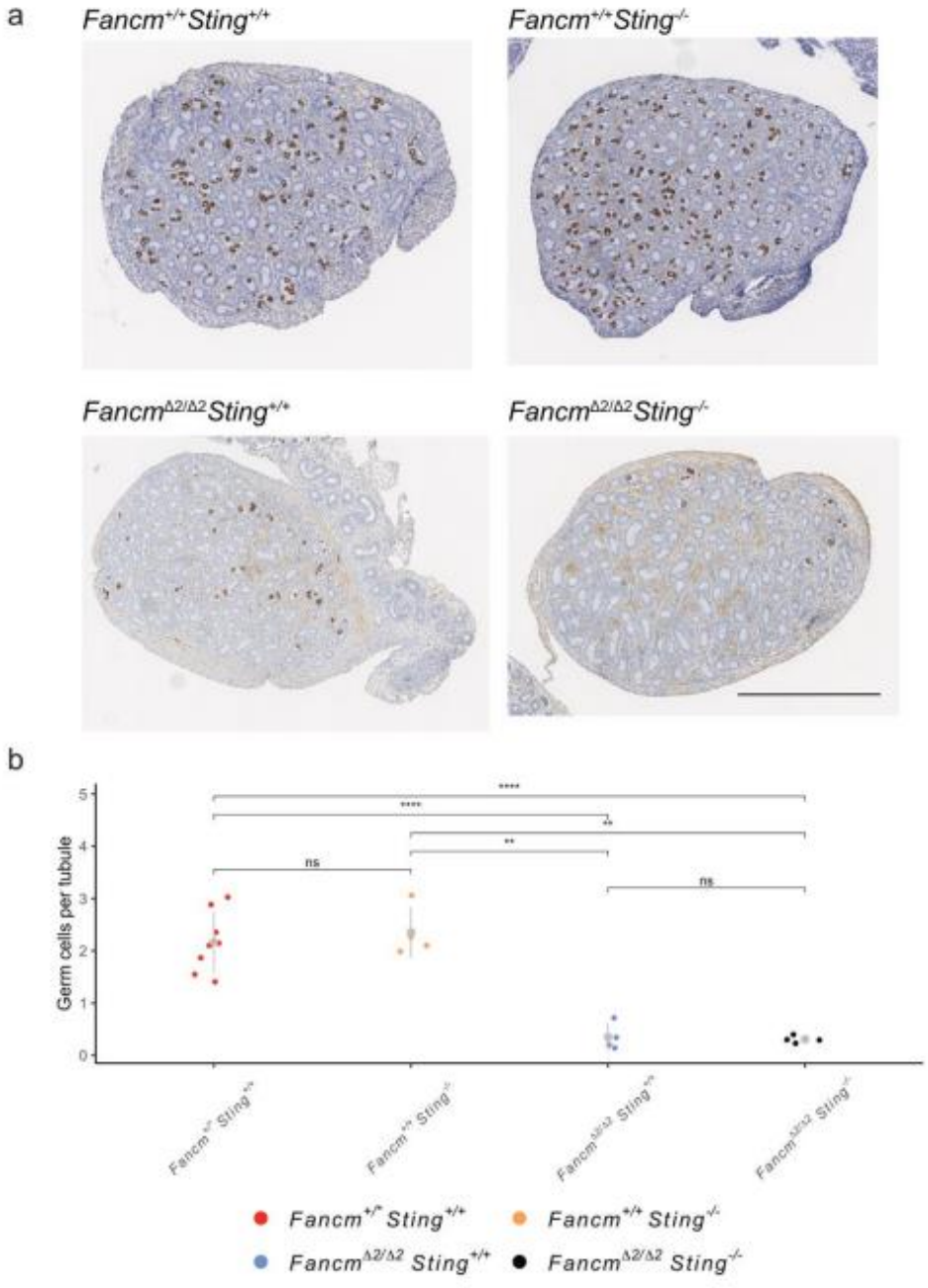
We agree with the reviewer that the manuscript title could be improved. We have changed it to "*Fancm* has dual roles in the limiting of meiotic crossovers and germ cell maintenance in mammals".

2. *"The spermatogenesis in FANCM mutant mice was partially rescued by String KO. However, the authors only showed that more germ cells are present in the double mutant mice. As FANCM deficiency not only caused germ cell loss, but also affected the sperm motility. The authors should also show whether the sperm motility could also be rescued in the double mutant mice. Actually, all the work on String KO mice is not that related to the DSB repair."*

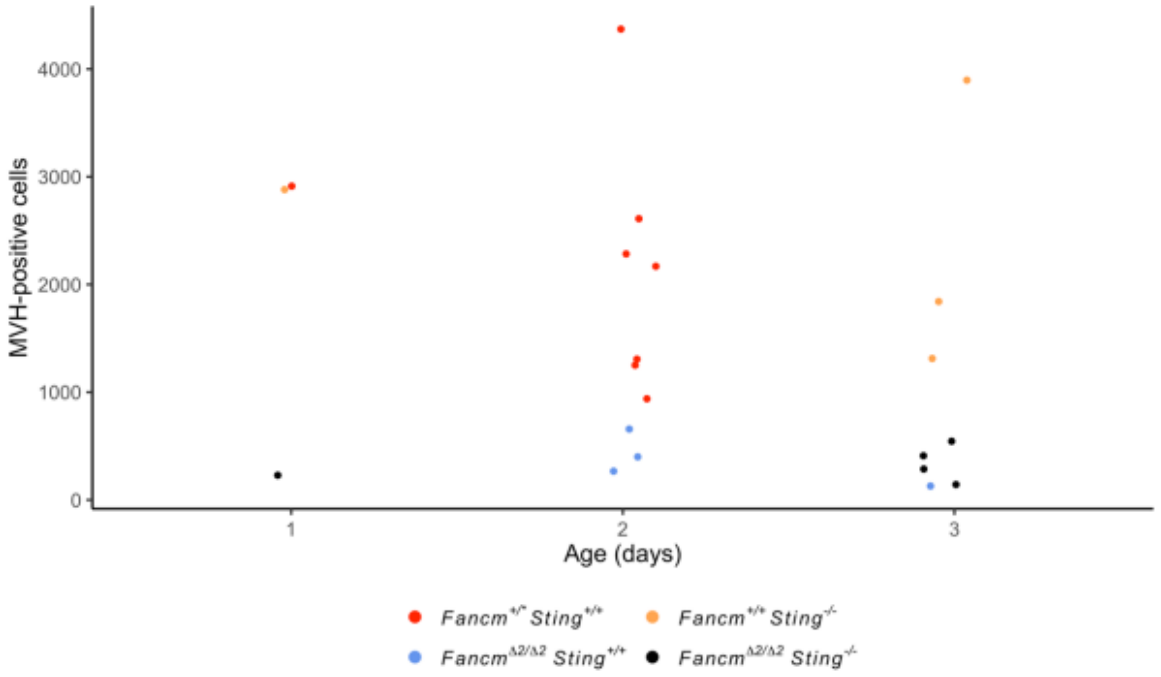
We agree that the interaction between Fancm and Sting could be better integrated into the manuscript. Therefore, we have performed a substantial experiment which determines at which broad developmental stages the STING pathway leads to a reduction in germ cells in the Fancm mutants i.e. during embryogenesis or postpartum. To achieve this, we collected newborn male pups to compare germ cell numbers between *Fancm* single mutant and *Fancm Sting* double mutant mice. The findings of this experiment were that there was no difference between the two genotypes suggesting that STING mediates a post-natal reduction in germ cell number in male mice lacking FANCM. We have added this additional Figure 6 and Supplementary Figure 20 in the manuscript [lines 429-436, 615-630].

The finding that FANCM is required for normal sperm motility was interesting but exploring its origins and whether it is related to the STING pathway is beyond the scope of this, already very large, study. The suggestion of further motility experiments would likely give only negative results given that we did not observe increased daily sperm production in *Fancm Sting* double mutant mice compared to *Fancm* single mutants.

New experiment and new Figure 6.



New Supplementary Figure 20.



3. *"If some mid-pachytene cells were apoptotic, we should expect to see reductions in the subpopulations of pachytene and diplotene cells and the metaphase I-anaphase I cells in the XII stage tubules. However, the authors showed that they did not observe these reductions. The author should give explanation for this?"*

In the original submission we have discussed this point and offered two hypotheses as to why the two distinct techniques returns two sets of quantitative data that cannot be fit into a simple model.

We wrote the following in the original Discussion on lines 579-597 and have kept this content on lines 601-613 of the revised submission.

"Direct analysis of markers of DSB formation and repair did not suggest a strong, or any, defect in meiotic DSB repair in the absence of FANCM. Similarly chromosome dynamics were unchanged when considering synapsis and chromosome segregation. Nevertheless, histological analyses showed unambiguous phenotypes when considering whole tubules, as opposed to individual meiocytes, such as complete absence of large groups of pachytene cells that would be found in comparable tubules in

the wild type. Considering these data together, it seems most plausible that any recombination defect that may exist in *Fancm*-deficient mice is mild, particularly given that daily sperm production is measured in the millions and litter sizes from male mutants are relatively unchanged. However, an alternative interpretation could be that a particularly mild recombination defect triggers a checkpoint arrest, which is reflected by the PAS-positive metaphase I-anaphase I cells."

4. *"Detailed methods for quantification of daily sperm production should be provided."*

In the original manuscript we have provided the following information for quantification of daily sperm production

"Testis daily sperm production (DSP), was determined as previously described [147]. Briefly harvested testes were weighed, snap-frozen and stored at -80°C. After thawing, testes were homogenized by sonication in DSP buffer (0.15 M NaCl, 0.01% NaN3, 0.05% Triton X-100) to lyse all cells except the condensed spermatid nuclei. The number of elongated spermatids per testis were determined used a haemocytometer and DSP estimated by dividing the total elongated spermatid number per testis by 4.84, which corresponds to the estimated number of days that an elongated spermatid remains within the testis [148]." [Lines 783-790]

We have provided more detailed methods for quantification of daily sperm production. This modification can be found in lines 818-843 of the manuscript in the Methods section:

"Testis daily sperm production (DSP), was determined as previously described (150). Briefly for each animal, one testis was harvested, weighed, snap-frozen and stored at -80°C. After thawing, the testis were homogenized by sonication using a Model 150VT ultrasonic homogenizer in DSP buffer (0.15 M NaCl, 0.01% NaN3, 0.05% Triton X-100) to lyse all cells except the condensed spermatid nuclei. Three 10 second pulses at 30% amplitude were applied with the sonicator.

The number of elongated spermatids per testis were determined using a Neubauer haemocytometer. Samples were first briefly vortexed before 10 µL was loaded into the haemocytometer. The number of sperm per 10 µL was determined by counting the number of sperm heads contained within five 0.20 mm x 0.20 mm x 0.1 mm squares. For each animal two replicates were counted and averaged. The average number of sperm per 20 µl was multiplied by 50,000 to determine the average number of sperm per 1 ml and then multiplied by the volume of homogenate (1 mL + sample weight) to determine the number of sperm per homogenate. The number of sperm per testis was then calculated by first determining the number of sperm per gram of testis ((n/homogenate) / sample weight (g) = n/g [sperm/ g of testis]) and then multiplying this by the whole testis weight. The DSP of the testis was then calculated by dividing the number of sperm (n) per testis by the time divisor 4.84, which corresponds to the estimated number of days that an elongated spermatid remains with the testis (150).

These calculations can be expressed as follows:

1. (n/homogenate) / sample weight (g) = n/g [sperm/ g of testis]
2. (n/g) x testis weight (g) = total/testis [sperm/testis]
3. (total/testis) / 4.84 = DSP for 1 testis.

5. *"It would be more plausible if the authors can provide some results regarding how FANCM functions in DSB repair and the generation of class II COs during meiosis of male mice."*

We appreciate the reviewer's suggestion for additional direct evidence of Fancm's involvement in meiotic DSB repair and the limitation of class II crossovers in male mice. However, we would like to note that our study utilizes extensive novel genetic and bioinformatic approaches, as well as cytological data, to address these questions. Specifically, our results in Figure 1 demonstrate that crossover frequencies are modified in the absence of Fancm, as evidenced by three different techniques. In Figure 2, we show a different distribution of double crossovers in the mutant compared to wildtype, which is consistent with reduced interference or extra class II crossovers. We also observe no change in the numbers of MLH1 or MLH3 foci (Figure 3), which has been shown in a number of Fancm-deficient strains. These findings support our conclusion that Fancm operates in meiotic DSB repair and class II crossover limitation.

While we acknowledge that further experimentation, such as generating new mouse lines or reagents, could provide additional evidence, we believe that this is beyond the scope of this study and would require a significant investment of time and resources. Furthermore, the additional impact of such experiments would not be proportional to the extra time investment.

Minor points:

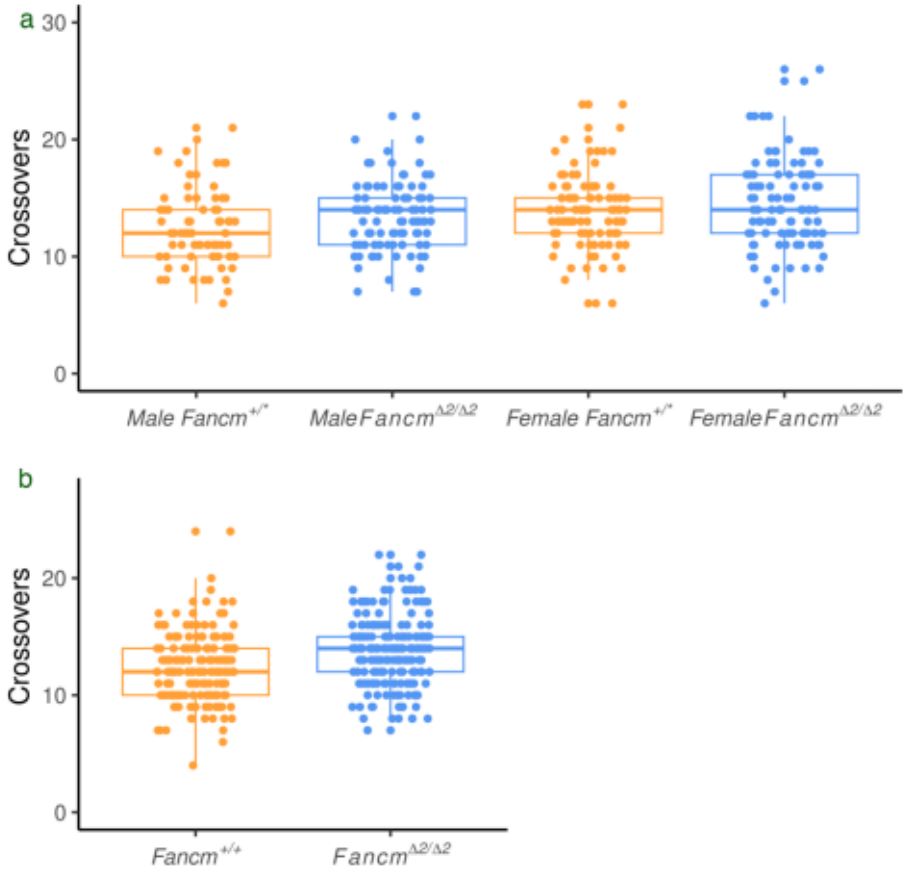
a. *"Some background information about cGAS-STING should be provided in the introduction."*

We have added more background on cGAS-STING in the Introduction to read [lines 84-90]:

"Genomic instability has been connected to the activation of innate immunity (Mackenzie et al, Heijink et al, bregnard et al). One way that innate immunity is activated is through the action of a protein called Cyclic GMP-AMP synthase (cGAS), which binds to double-stranded DNA in the cytosol and triggers a type I interferon response through another protein called Stimulator of Interferon Genes (STING) (Decout et al). However, the exact relationship between reduced fertility resulting from genomic instability and the activation of innate immunity is not yet fully understood."

b. *"It would be better if the authors could indicate the number of COs produced in FANCM deficient mice and compare it with control mice."*

We have added a supplementary figure (Supplementary Fig 5) which shows the distribution of number of crossovers detected in gametes and F1 mice.



Supplementary Figure 5. The number of crossovers detected in F1 samples and gametes from each genotype group. A. The number of crossovers detected in F1 samples. B. The number of crossovers detected in single gametes.

c. *"The authors should perform TUNEL assay (a late stage of cell apoptosis) to confirm the apoptotic events (PAS-positive) in the mutant testes."*

We have performed caspase immunolabelling of testes cross-sections (Supplementary Figure 15), which we believe is an appropriate assay to investigate germ cell apoptosis. TUNEL assays label DNA double-strand breaks which are present in high numbers in wildtype meiosis and could confound results in an apoptosis study.

d. *"Line 7, no FANCH. The authors should reword this sentence."*

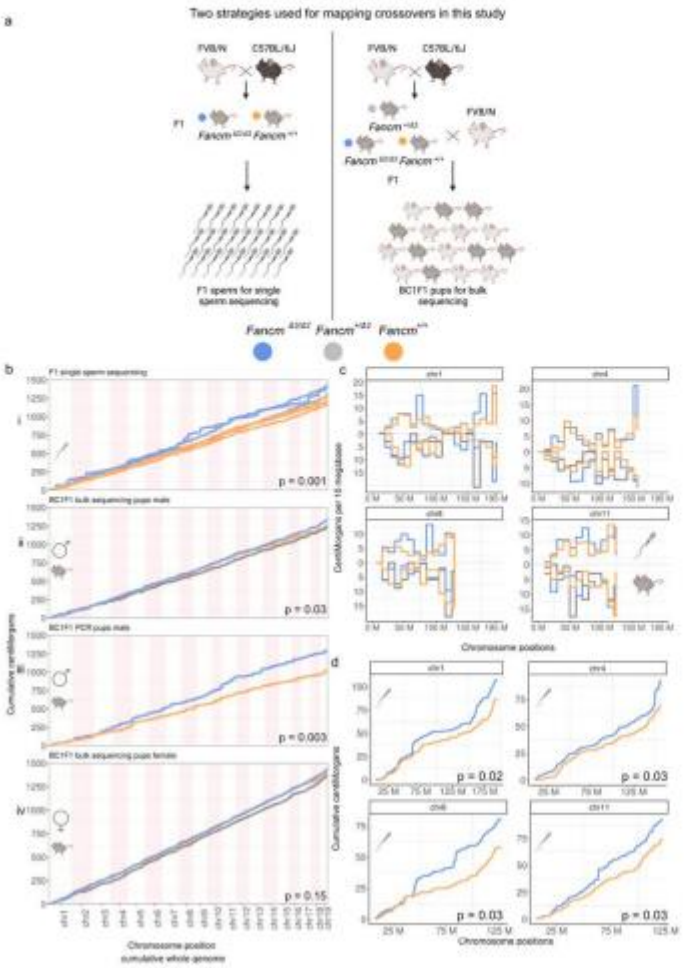
We understand that naming of FANC genes is not perfectly continuous through the alphabet. For the sake of clarity, we have reworded the sentence to read:
"There are 22 "FANC" genes, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN, FANCO, FANCP, FANCQ, FANCR, FANCS, FANCT, FANCU, FANCV, FANCW." [lines 6-10]

e. *"Line 132, F1 strain was not defined at its first appearance."*

We have defined F1 strain. It now reads "F1(FVB/N x C57BL/6J)". [line 142]

f. *"Figure 1, the genotypes were not indicated."*

We have indicated the genotypes more clearly in Figure 1 with a more prominent key showing genotypes and corresponding colours.



Reviewer #2.

"In the paper by Tsui et al., the authors comprehensively describe the phenotype of mice lacking the Fanconi anemia gene fancm. The authors characterize for the first time the effect of fancm loss on meiotic crossovers utilizing technologies other than immunofluorescence to reveal that the genetic map length of fancm-/- mice is increased. This demonstrates that FANCM limits crossovers in mammals. These mice have reproductive defects and the authors analyzed different stages of gametogenesis to pinpoint the cause of these defects. They found that the excess crossovers are unlikely to cause gametogenesis defects. Therefore, FANCM in mammals has separable roles, one regulating the DSB repair pathway choice and other, less clear role on promoting genomic stability, maybe in spermatogonial precursors.

The paper overall is very well written and well referenced. Despite the number of experiments presented it is easy to follow and the logic for the experiments is presented very clearly. I particularly appreciate the inclusion of negative results in the paper, as they help to provide a complete picture of what might be happening in these mice.
I believe this is a paper that will be of great interest to the meiosis and DNA repair communities."

We appreciate the reviewer for acknowledging the significance of our study and our manuscript.

Minor points:

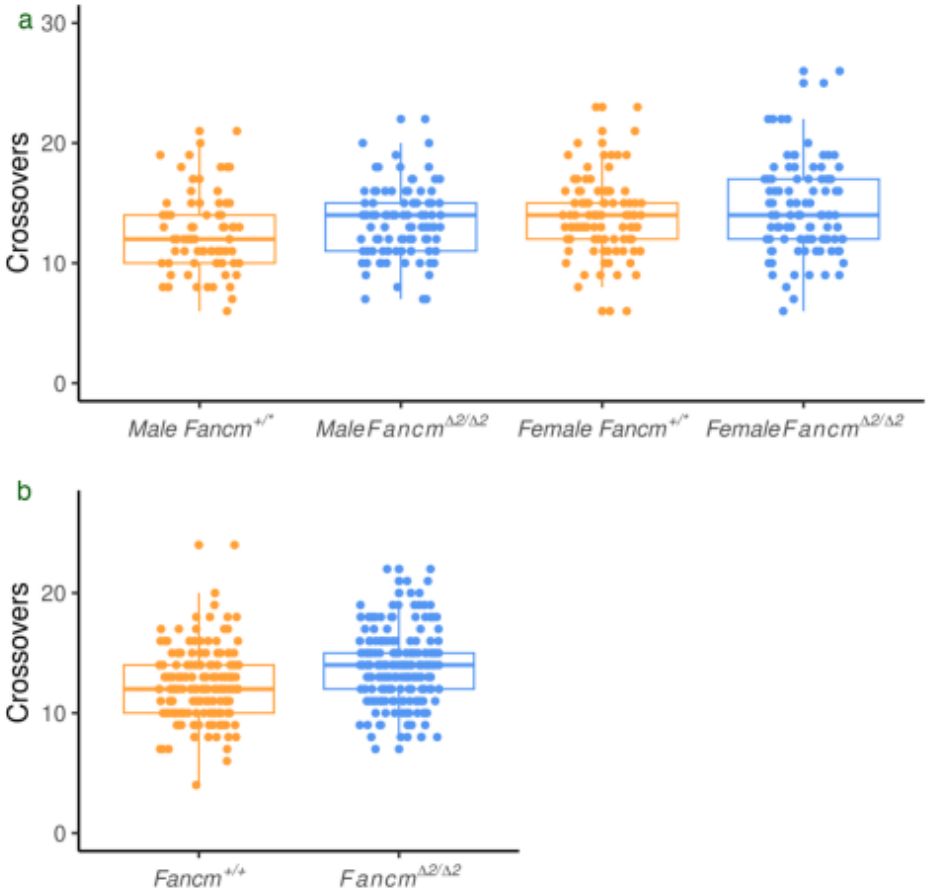
a. *"I might have missed it but I cannot easily find how many F1 or single gametes were sequenced."*

Thanks for highlighting that the sample size information is not easily available. We previously put the information in the legend text under Figure 1. We have now revised the text under the section "Fancm limits meiotic crossovers in mammals" to include the sample sizes of F1 and single gamete experiments. The revised text now reads:

"with 1-5X whole-genome coverage for each BC1F1 pup (total n = 372 with 200 from female F1 and 172 from male F1) [Lines 164-165].... with single-gamete (haploid) sequencing in F1 males (total n = 408 gametes from 3 animals per genotype)." [Line 178]

b. *"From the genetic maps, it is clear that there are extra crossovers. I am curious whether the authors could present this data in a different way to understand how many extra crossovers are in a single gamete. What is the distribution? Are there F1 mice or gametes with a huge excess of crossovers or it is an even increase?"*

We added a new Supplementary figure (5) which shows the distribution of the number of crossovers detected from gametes and F1 samples. We noticed that the increase of crossovers in the mutant group was not driven by a subset of mutant samples with excess crossovers but a shift in mean crossovers across samples in the group. Further, with the data that we have – and visualisations in Figure 1b, c, d and Supplementary figures 5, 6 – there is not an extreme subset of gametes or genomic regions that we can detect that contribute to the phenotypic difference between *Fancm+/+* and *Fancm-/-*, but rather what we feel is a more general genome-wide average increase in crossover rate.



Supplementary Figure 5. The number of crossovers detected in F1 samples and gametes from each genotype group. A. The number of crossovers detected in F1 samples. B. The number of crossovers detected in single gametes.

c. *"Any thoughts on how DNA replication might be affected in these mice?"*

We interpret this to comment to be referring to somatic DNA replication and repair as opposed to meiotic S-phase:

Fancm and its orthologues are translocases that can remodel stalled replication forks during DNA replication that could occur due to endogenous processes or chemotherapy-induced DNA inter-strand crosslinks. Fancm proteins achieve these functions through a variety of partners such as the BLM/Top3a/RMI1-2 complex and the FA core complex.

Based on data here showing that our mice have the traditional phenotypes of Fancm knocked out (Sup. Fig. 4), and previous studies, we would assume that replication-associated defects would be typical of cells that lack a gene from the Fanconi anaemia pathway. While there are mechanisms that provide a level of redundancy with the FA pathway, e.g. NEIL3, some cell types do not appear to be able to full overcome a lack of a functional FA pathway. An example of this is primordial germ cell during embryogenesis in mice.

Referees' report, second round of review

Reviewer 1

I have read the revised manuscript and the reply to the reviewer's comments. The authors answered almost all of my questions and concerns, and I am mostly satisfied with their answers and revisions. Thus, I suggest this manuscript can be accepted for publication now.

Reviewer 2

Addressed all concerns I had.

Authors' response to the second round of review

N/A