

hnRNPM cooperates with BCAS2 to modulate alternative splicing during oocyte development

Corresponding Author: Dr Shumin Zhou

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)

This manuscript provides compelling and insightful data to reveal the crucial role of hnRNPM in pre-mRNA alternative splicing during oocyte development. Using a germ cell-specific knockout mouse model, the authors confirmed in vivo that HnRNPM deficiency results in complete infertility in female mice. Furthermore, the results of SCAN-seq and LACE-seq demonstrated that hnRNPM regulates pre-mRNA alternative splicing in oocytes by directly binding to transcripts. Additionally, IP-MS and RIP assays revealed that hnRNPM regulates alternative splicing in oocytes by promoting the binding of BCAS2 to pre-mRNA. Overall, the conclusion is robust, and the manuscript is well-written and clear in logic. I have the following comments.

- 1) IVM experiments confirmed that hnRNPM deficiency affects oocyte maturation. However, in the IVF assay, the authors only described that HnRNPM-deficient oocytes fail to complete cleave. It is unclear whether the maturation and fertilization rate of ovulated oocytes are affected. Whether the maturation phenotype is consistent between in vivo and in vitro? If not, please give an explanation.
- 2) The author introduces zygotic splicing activation (ZSA) in the background. What is the reason for the failure of zygotic cleavage in hnRNPM-deficient mice, is it due to the AS in oocytes or ZSA in zygotes? Whether knockdown of hnRNPM in zygotes using siRNA or Trim-Away affect embryonic development?
- 3) A previous study showed that hnRNPH1 recruits PTBP2 and SRSF3 to modulate alternative splicing in germ cells (Nat Commun. 2022 Jun 23;13(1): 3588.doi: 10.1038/s41467-022-31364-7), what is the relationship between hnRNPM and hnRNPH1? Are they co-localized in the oocytes?
- 4) Line 202-203, the author found most HnRNPM-deficient oocytes displayed abnormal pericentrin localization. Since pericentrin is a key MTOC marker, I suggest to evaluate whether the MTOCs were destroyed in hnRNPM-deficient oocytes before GVBD using pericentrin Immunofluorescence.
- 5) Whether hnRNPM CKO affect the protein level or the localization of BCAS2? In addition, BCAS2-deficient mice show more severe phenotype than hnRNPM-deficient mice, please discussion the difference.

Reviewer #2

(Remarks to the Author)

Zhou et al. investigate the role of heterogeneous nuclear ribonucleoprotein M (hnRNPM) in oocyte development, presenting its splicing regulatory role of maternal transcripts essential for meiosis. The authors demonstrate that the conditional knockout of hnRNPM in female germ cells leads to significant cytoplasmic defects, impaired meiotic maturation, and complete female infertility. Through single-cell amplification and sequencing of full-length RNAs using the Oxford nanopore platform (SCAN-seq), the study reveals that hnRNPM modulates alternative splicing of genes crucial for the meiotic cell cycle. Furthermore, the authors identified BCAS2 as an interacting partner of hnRNPM and propose a model where hnRNPM recruits BCAS2 to regulate the splicing of specific transcripts. The research is well-designed, and the findings are interesting, but several points could be strengthened to provide more solid evidence for the authors' conclusion.

Major:

1. Although the manuscript discusses the regulatory potential of hnRNP proteins, the introduction should explain why hnRNPM was chosen for study in the context of oocyte development.

2. In line 211, the authors report 89 novel transcripts linked to female infertility, yet Supplementary Fig. 3b shows ~6,000–7,000 novel isoforms per sample. Is female infertility specifically enriched, and were other pathways enriched in hnRNPM-deficient GV oocytes?
3. In Fig. 5b, it would be helpful to show gel images with both control and cKO oocytes.
4. In Fig. 6a, indicating the direction of splicing changes (e.g., increased vs. decreased inclusion) would be helpful.
5. In Fig. 6d and 6e, while the study convincingly demonstrates that hnRNPM depletion leads to alternative splicing of various transcripts, such as *Khdc3*, *Nlrp14*, *Cenph*, and *Zar1l*, it is not clear from the data presented whether these splicing changes result in significant alterations to the levels of corresponding intact proteins. Providing evidence, perhaps through Western blotting for some of these key affected proteins in hnRNPM-depleted oocytes, would more directly link the observed splicing defects to the downstream cellular phenotypes.
6. The analysis in Fig. 7f shows an overlap between hnRNPM-bound genes and differentially spliced genes. To provide more direct evidence for a regulatory role, the analysis could be extended to compare hnRNPM binding signals specifically on exons and introns that are alternatively spliced versus those unaffected by hnRNPM depletion.
7. A recent study cited by the authors (Zheng et al., 2024) demonstrated that hnRNPM protects against interferon response by repressing LINE-associated cryptic splicing. It would be a valuable addition to investigate whether the hnRNPM binding sites identified in oocytes are also enriched for LINE1 elements, and whether the key splice events affected by hnRNPM depletion are associated with these retrotransposons.
8. In Fig. 8d, the authors propose that hnRNPM recruits *Bcas2* to regulate splicing. To clarify the nature of this interaction, a co-IP experiment including an RNase treatment step is necessary. This would determine whether the interaction is a direct protein-protein association or is mediated by RNA.
9. In Fig. 8f, the study proposes a “strong functional synergy” between hnRNPM and *Bcas2* based on the overlap of 98 commonly affected alternative splicing genes and 57 identical splicing events in the respective knockout oocytes. Are these overlaps statistically significant? While suggestive, further evidence is needed to solidify this claim, or toning down is necessary.

Minor:

1. In Fig. 1c and 1d, the study demonstrates that hnRNPM is absent from the GVBD stage through the zygote stage. It would be interesting to speculate on the functional implications of this dynamic expression. Is the dynamic expression during oocyte maturation important for ZSA? What might be the consequences if hnRNPM is consistently expressed during these stages?
2. In Fig. 2k and 2l, there appears to be a discrepancy between the dramatic development arrest shown in Fig. 2k and the moderate, albeit significant, changes in developmental rates presented in Fig. 2l. The visual evidence in Fig. 2k suggests a near-complete failure of *Hnrnp*-deficient oocytes to develop beyond the 2-cell stage after IVF. An explanation for this difference would strengthen the interpretation of the impaired developmental phenotype.
3. In Fig. 8g, the basis of claiming “increased exon skipping in *Cenpf*” could be more quantitatively supported.
4. Based on the study, we can hypothesize but cannot definitively conclude that human hnRNPM and *BCAS2* form the same interaction network to control splicing in human oocytes. While the RIP-qPCR analyses in Fig. 8n-p are suggestive, the splicing regulatory consequences in human transcripts tested here should be examined in a human cell model as well.
5. A minor but important point for clarity is the consistent use of nomenclature for genes, transcripts, and proteins. The study frequently uses human-centric or nomenclature for proteins (e.g., *BCAS2*) in a mouse model or mouse-centric nomenclature for transcripts (e.g., *Cenpf*, *Prc1*, and *Hdlbp* in Fig. 8) in a human model, while correctly italicizing mouse gene names (e.g., *Hnrnp*, *Bcas2*). Adhering to the correct organism-specific nomenclature throughout the manuscript would improve precision.
6. To better understand the specificity of the hnRNPM-*Bcas2* interaction, it would be informative to examine whether it also occurs at later developmental stages where both proteins are re-expressed, such as the 2-cell stage or a fully developed cell line. This would clarify if the interaction is unique to oogenesis or part of a more general splicing regulatory mechanism. While additional experiments are not necessary for this manuscript, the authors could consider discussing this point.
7. The color key is missing in Fig. 2f.
8. Should the column labels in Suppl Table 1b be KO-1 and KO-2?
9. A typo in line 408 “Notably, tThe meiotic”
10. In the affiliation, “8Sir Run Shaw Hospital” is likely to be “Sir Run Run Shaw Hospital.”

Reviewer #3

(Remarks to the Author)

The author studied the function of hnRNPM during oocyte development and thoroughly analyzed its molecular mechanism of action. The results demonstrate that hnRNPM is a key regulatory factor for the alternative splicing of pre-mRNA during oocyte development. hnRNPM recruits *BCAS2* to control the splicing of crucial pre-mRNA related to meiosis. These findings have significant academic value for understanding the regulation mechanisms of alternative splicing during oocyte development, and provide important references for understanding the pathogenesis of related diseases. The findings are innovative but still need to be further improved.

Major concerns:

1. *Stra8* Cre activation occurs at embryonic day 12.5. I am curious about the expression pattern of hnRNPM in oocytes during different developmental stages of the embryo and postnatally. If its expression level is high and it can be effectively knocked out after birth, does this indicate that it has no function in the establishment of primordial follicle pool and activation of primordial follicles? Additionally, previous studies have shown that *BCAS2* plays an important role in the first meiotic

division of oocytes. Does hnRNPM function independently of BCAS2 in early ovarian oocytes?

2. In oocytes and granulosa cells, BCAS2 exerts important regulatory functions by forming complexes with corresponding interacting proteins to influence the alternative splicing of precursor mRNAs. In this study, does the recruitment of BCAS2 by hnRNPM require the formation of a complex with PRP19 and CDC5L to perform splicing functions? If possible, provide relevant evidence, or discuss this in the revised version.

3. After the absence of hnRNPM in oocytes, how do the gene and protein levels of BCAS2, as well as its protein localization? The related results may be helpful. BCAS2 might undergo degradation or positional changes, which can also be reflected in the working model diagram.

4. In vitro OR in vivo experiments demonstrate whether Bcas2 knockdown or knockout affects the expression level and localization of hnRNPs, and alternative splicing of precursor mRNA regulated by hnRNPs?

5. Is BCAS2 cKO stra8 cre or vasa cKO? The authors should provide the breeding and acquisition process of BCAS2 cKO mice in the Materials and Methods section.

Minor concerns:

1. The abstract does not end with a period.

2. Line 116, revise 'cattle' for repetition.

3. Line 126, Ensure that 'tubulin' starts with an uppercase letter throughout the MS, including Figures and Legends.

4. Ensure that Stra8-Cre and Stra8-GFPcre are consistently used throughout the MS, e.g., Line 458, 465, Figure 2a, b, c and figure legends.

5. Gene writing should be uniform throughout the MS, e.g., Line 168, 312.

6. The controls in Figure 3c are WT? What's the difference between WT and CTRL?

7. Line 227, missed spaces should be added after 'site'.

8. Line 280-281, Revise repeated word usage according to the highlighted certain information in the Figure.

9. Ensure consistency between 'Western blot' and 'Western blotting' throughout the MS.

10. Modification of citation format in the main text, e.g., Line 52, 58, 70, 96, 312, 349-350, 373, 421.

11. References should be modified according to NC format, such as using both the full name and abbreviation of journal titles. ;

12. Figure 1a, 'Bovin' should be corrected.

13. The annotation information on the image is either left-aligned or centered, maintaining consistency; as shown in Figure 8e (which can be aligned similarly to the figure editing in Figure 1) it is left-aligned, see also Figures 1c, etc.

14. In the figures, should there be a space before the parentheses in the horizontal and vertical axes? Please ensure consistency across all figures by the author. For example, Figure 4b has a space, but other figures do not.

15. Figure S1, Modify the title of the figure legend to cover all key information in the figure; change the letters annotated in Figure S1 to black; 'Bovin' should be corrected.

16. The spacing between the upper and lower figures in Figure 5f should be adjusted, causing some letters to be covered by the lower figure; in Figure 5h, the Chinese text should be deleted or replaced.

17. In Figure legends, 'Error bars, mean $\pm$ SEM' should be revised to 'Error bars indicate SEM', OR 'Data are presented as mean $\pm$ SEM'.

18. Figure 3e, can the blue arrowhead be identified as mitochondrial aggregation? What are the morphological characteristics of mitochondrial aggregation? Modify the position of the yellow arrow (below).

19. In Figure 2e, DAPI signal should be added.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The positive responses satisfied all my concerns, I suggest the publication of this study.

Reviewer #2

(Remarks to the Author)

The authors have thoroughly revised the manuscript and successfully addressed all of our comments. We have no further concerns.

Reviewer #3

(Remarks to the Author)

My concerns have been addressed in the revised version.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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Dear reviewers,

Thank you for careful review of our manuscript entitled “hnRNPM recruits BCAS2 to modulate alternative splicing during oocyte development” (**NCOMMS-25-42705A**). We greatly appreciate your valuable comments and suggestions, which have significantly helped us revise and improve our paper. Your feedback has also provided important insights for guiding our further research.

We have carefully addressed all your comments and made every effort to revise the manuscript accordingly. The main corrections in the revised manuscript are highlighted in **yellow**, and our point-by-point responses to the reviewers' comments are presented in **blue text**.

Once again, on behalf of my co-authors, I sincerely thank you for your kind assistance in improving our manuscript. We deeply appreciate your efforts and apologize for taking up so much of your time. We look forward to your further suggestions.

Best regards,
Fei Sun
Heng-Yu Fan

The point-by-point response was listed below.

Reviewer #1 (Remarks to the Author)

This manuscript provides compelling and insightful data to reveal the crucial role of hnRNPM in pre-mRNA alternative splicing during oocyte development. Using a germ cell-specific knockout mouse model, the authors confirmed in vivo that HnRNPM deficiency results in complete infertility in female mice. Furthermore, the results of SCAN-seq and LACE-seq demonstrated that hnRNPM regulates pre-mRNA alternative splicing in oocytes by directly binding to transcripts. Additionally, IP-MS and RIP assays revealed that hnRNPM regulates alternative splicing in oocytes by promoting the binding of BCAS2 to pre-mRNA. Overall, the conclusion is robust, and the manuscript is well-written and clear in logic. I have the following comments.

Response: We sincerely thank Reviewer #1 for the positive and insightful comments on our manuscript, as well as for the constructive suggestions that have significantly helped us improve our work.

1) IVM experiments confirmed that hnRNPM deficiency affects oocyte maturation. However, in the IVF assay, the authors only described that *Hnrnpm*-deficient oocytes fail to complete cleave. It is unclear whether the maturation and fertilization rate of ovulated oocytes are affected. Whether the maturation phenotype is consistent between in vivo and in vitro? If not, please give an explanation.

Response: We sincerely thank the reviewer for this insightful suggestion. hnRNPM deficiency severely compromises both oocyte maturation and fertilization competence. Specifically, only ~30% of ovulated oocytes from *Hnrnpm* cKO mice reached the MII stage following hCG injection, confirming severe maturation defects (**Supplementary Fig. 2f-g**), a finding consistent with our in vitro results. In addition, *Hnrnpm*-deficient oocytes fail to form pronucleus (**Fig. 2k-l**), suggesting a fertilization failure. These results have been incorporated into the revised manuscript to provide a more comprehensive understanding of hnRNPM's critical role in oocyte development.

2) The author introduces zygotic splicing activation (ZSA) in the background. What is the reason for the failure of zygotic cleavage in hnRNPM -deficient mice, is it due to the AS in oocytes or ZSA in zygotes? Whether knockdown of hnRNPM in zygotes using siRNA or Trim-Away affect embryonic development?

Response: We thank the reviewer for this critical question. *Hnrnpm* deficiency severely impairs oocyte maturation, leading to completely failure of pronucleus formation (**Fig. 2k-l**). Since this defect occurs prior to the timing of ZSA, the failure of zygotic cleavage in *Hnrnpm*-deficient mice is primarily caused by defective AS in oocytes, rather than a failure of ZSA.

To directly evaluate the role of hnRNPM during ZSA, we performed siRNA-mediated knockdown in zygotes. This acute depletion did not block the first cleavage but resulted in a 2-cell arrest (unpublished), a phenotype consistent with ZSA failure, suggesting that hnRNPM is required for ZSA and development beyond the 2-cell stage.

3) A previous study showed that hnRNPH1 recruits PTBP2 and SRSF3 to modulate alternative splicing in germ cells (Nat Commun. 2022 Jun 23;13(1): 3588.doi: 10.1038/s41467-022-31364-7), what is the relationship between hnRNPM and hnRNPH1? Are they co-localized in the oocytes?

Response: We thank the reviewer for raising an interesting question regarding the potential functional relationship between hnRNPM and hnRNPH1. Both hnRNPM and hnRNPH1 belong to the heterogeneous nuclear ribonucleoprotein (hnRNP) family and function as key RNA-binding proteins to regulate pre-mRNA splicing. Our co-immunoprecipitation experiments demonstrate a physical interaction between hnRNPM and hnRNPH1 (**Fig. R1a**). Furthermore, immunofluorescence co-localization analysis reveals significant nuclear co-

localization of hnRNPM and hnRNPH1 in oocytes (**Fig. R1b**), providing cytological evidence supporting their potential functional collaboration in splicing regulation.

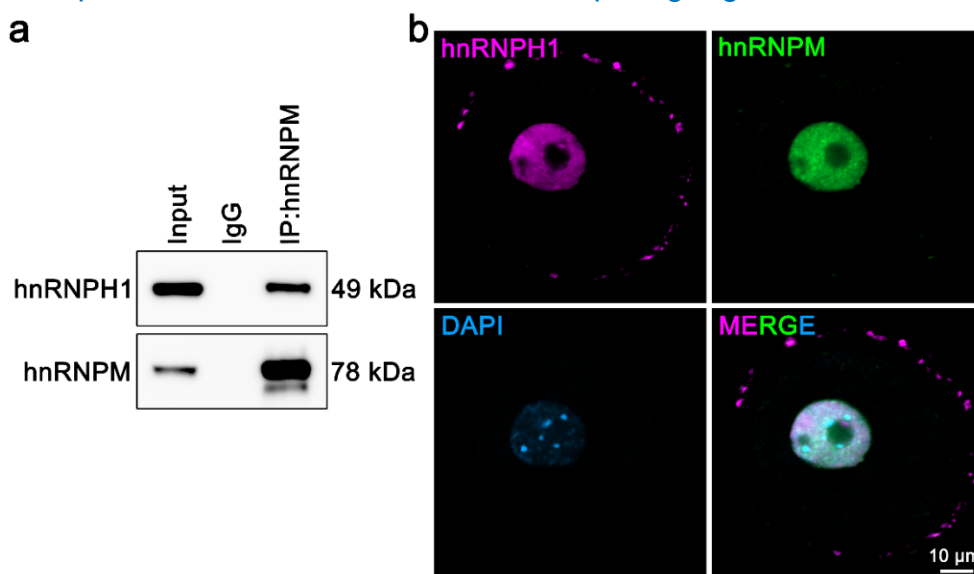


Fig. R1 hnRNPM interacts and colocalizes with hnRNPH1. **a** Co-immunoprecipitation assays analyze the interaction between hnRNPM and hnRNPH1. **b** Immunofluorescence staining of GV oocytes showing hnRNPH1 (purple) and hnRNPM (green).

4) Line 202-203, the author found most *HnRNpm*-deficient oocytes displayed abnormal pericentrin localization. Since pericentrin is a key MTOC marker, I suggest to evaluate whether the MTOCs were destroyed in hnRNPM-deficient oocytes before GVBD using pericentrin Immunofluorescence.

Response: We thank the reviewer for this excellent suggestion. To directly assess whether MTOCs are destroyed in *HnRNpm*-deficient oocytes before GVBD, we perform pericentrin immunofluorescence using GV-stage oocytes. Strikingly, single large pericentrin were present in controls but undetectable in most of *HnRNpm*-deficient oocytes (**Supplementary Fig. 2h-i**), suggesting a pre-existing MTOC defect prior to meiotic resumption.

In addition, we performed quantitative analysis of oocytes with pericentrin signals at 8 and 16 h after culture. Interestingly, MTOCs were detected in approximately 37.9% of cKO cells at 8 hours post-release, with the rate increasing to about 80.5% by 16 hours (**Supplementary Fig. 2h**). These findings indicate that hnRNPM deficiency leads to a delayed appearance of pericentrin signals in oocytes. Thank you once again for your constructive feedback.

5) Whether hnRNPM CKO affect the protein level or the localization of BCAS2? In addition, BCAS2-deficient mice show more severe phenotype than hnRNPM-deficient mice, please discussion the difference.

Response: We thank the reviewer for this insightful comment. To determine whether hnRNPM depletion affects BCAS2, we first examined the protein level and subcellular localization of BCAS2 in control and *Hnrnp*m-deficient oocytes. Our results clearly show that the protein level of BCAS2 was significantly reduced in *Hnrnp*m CKO oocytes compared to controls (**Fig. 8n**). However, the subcellular localization of the remaining BCAS2 protein did not appear to be altered. It still exhibited a nuclear localization pattern similar to that in control oocytes (**Supplementary Fig. 6g**).

The complete deficiency of BCAS2 likely abolishes its entire function, leading to a catastrophic failure in spliceosome assembly and mRNA processing, thus manifesting a more severe phenotype. In contrast, the loss of hnRNPM results in a partial reduction, but not a complete ablation, of BCAS2 protein. We propose that the residual BCAS2 in *Hnrnp*m-deficient oocytes retains a degree of functionality, thereby mitigating the phenotypic severity compared to a full BCAS2 knockout. We have now included a detailed discussion on this point in the revised manuscript (**Lines 362-365 & 474-479**).

Reviewer #2 (Remarks to the Author)

Zhou et al. investigate the role of heterogeneous nuclear ribonucleoprotein M (hnRNPM) in oocyte development, presenting its splicing regulatory role of maternal transcripts essential for meiosis. The authors demonstrate that the conditional knockout of hnRNPM in female germ cells leads to significant cytoplasmic defects, impaired meiotic maturation, and complete female infertility. Through single-cell amplification and sequencing of full-length RNAs using the Oxford nanopore platform (SCAN-seq), the study reveals that hnRNPM modulates alternative splicing of genes crucial for the meiotic cell cycle. Furthermore, the authors identified BCAS2 as an interacting partner of hnRNPM and propose a model where hnRNPM recruits BCAS2 to regulate the splicing of specific transcripts. The research is well-designed, and the findings are interesting, but several points could be strengthened to provide more solid evidence for the authors' conclusion.

Response: We sincerely appreciate the reviewer's thoughtful summary and positive feedback on our work. We agree that providing more robust evidence is essential and have therefore conducted extensive new experiments in accordance with the comments below.

In addition, We apologize for the lack of clarity regarding the novel transcripts mentioned in **Major Comments #2, #3, and Minor Comment #8**. In **Figure 5**, we presented novel transcript data only for WT1 and WT2, and not for KO1 and KO2, as the aim of this figure was to highlight previously overlooked transcripts present in normal oocytes, emphasizing a finding with broader biological relevance. In KO samples, a proportion of the novel transcripts represent abnormal isoforms resulting from dysregulated splicing, which is why they were not included them in the analysis presented in **Figure 5**. In contrast, the differentially expressed

transcripts in cKO groups—including novel transcripts—is comprehensively presented and interpreted in **Figure 6**. Collectively, **Fig. 5b-e** and **Supplementary Table 1b** present data exclusively from WT samples (detailed in **Major Comment #3 and Minor Comment #8**), and the identification of 89 novel transcripts linked to female infertility is also based on analysis of WT samples (detailed in **Major Comment #2**).

We deeply regret any confusion our original presentation may have caused and have thoroughly revised the relevant figures and text to improve clarity. We hope the current version satisfactorily addresses your concerns.

Major:

1. Although the manuscript discusses the regulatory potential of hnRNP proteins, the introduction should explain why hnRNPM was chosen for study in the context of oocyte development.

Response: We sincerely thank the reviewer for raising this important point. Our selection of hnRNPM for this study was based on the following compelling reasons. First, our research group has long been dedicated to investigating the functions of hnRNP family proteins in the reproductive system^{1–6}. In a previous systematic evaluation of hnRNP expression across multiple tissues, we observed that the expression of HNRNPM in the testis and ovary was significantly higher compared to other tissues (**Fig. R2**)⁷, suggesting a potentially critical role in germ cell development and reproductive physiology.

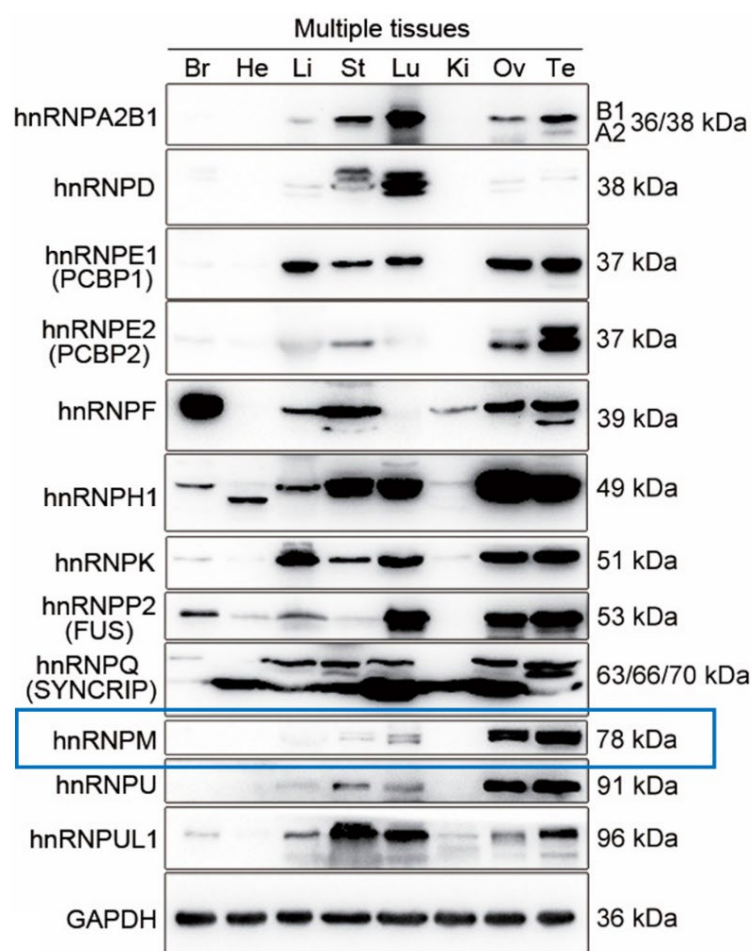


Fig. R2 Western blot analyses of the protein expression levels of 12 hnRNP family proteins in multiple mouse tissues⁷.

Furthermore, an independent study employing mRNA interactome capture (RIC) analysis on 50,000 fully-grown mouse oocytes identified hnRNPM as one of the core mRNA-binding proteins (RBPs) enriched in oocytes⁸. This finding indicates that hnRNPM may functionally engage in regulating the fate of maternal mRNAs in oocytes. Therefore, based on both its tissue-enriched expression pattern and functional evidence from oocyte-specific RBP profiling, we focused on hnRNPM to elucidate its potential functions in oocyte development and reproductive health. This description has been added to introduction (**Lines 84-90**).

2. In line 211, the authors report 89 novel transcripts linked to female infertility, yet Supplementary Fig. 3b shows ~6,000–7,000 novel isoforms per sample. Is female infertility specifically enriched, and were other pathways enriched in hnRNPM-deficient GV oocytes?

Response: We thank the reviewer for raising this important point regarding the specificity and breadth of pathway enrichment among the novel transcripts. Our initial identification of 89 novel transcripts linked to female infertility was based on a systematic review of published literature. However, this approach did not allow us to confirm whether these transcripts were

specifically enriched in female infertility or to analyze other potential pathways.

To address this, we performed a Gene Ontology (GO) enrichment analysis using the international standard database (<https://www.geneontology.org/>) to investigate the biological processes involving these novel transcripts. Strikingly, the top enriched processes were closely related to female infertility—including female meiosis chromosome segregation, regulation of fertilization and maternal-to-zygotic transition of gene expression (**Supplementary Fig. 3c**).

Interestingly, alongside the female-specific terms, one of the most strongly enriched terms was "male meiosis chromosome segregation" (**Supplementary Fig. 3c**), suggesting that hnRNPM's role in meiotic regulation may be conserved across sexes. Beyond gametogenesis, the analysis also revealed significant enrichment in other key biological processes, including regulation of stress granule assembly, tRNA surveillance and mRNA 3'-end processing by stem-loop binding and cleavage (**Supplementary Fig. 3c**), indicating that hnRNPM deficiency may influence a broader range of RNA metabolic functions.

In summary, GO enrichment analysis revealed that the novel transcripts are specifically associated with female infertility and are also enriched in pathways related to male meiosis and RNA processing.

3. In Fig. 5b, it would be helpful to show gel images with both control and cKO oocytes.

Response: We thank the reviewer for this suggestion. We apologize for the lack of clarity regarding the novel transcripts mentioned. We only presented representative data of WT oocytes, as the primary aim of **Fig. 5b-e** was to validate the effectiveness of our technical approach for oocyte samples. Therefore, we only presented representative data of WT oocytes.

In **Fig. 6**, we focused on comparing differences between Control and cKO groups, **including analysis of novel transcripts**. Among the four genes shown in **Fig. 5b-e**, alternative splicing differences of *Nlrp14* and *Zar1l* were observed. Accordingly, we have included gel images from both control and cKO oocytes for these two genes in **Fig. 6d-e** to directly demonstrate these differential splicing events.

We deeply regret any confusion our original presentation may have caused and have thoroughly revised the text to improve clarity. We hope the current version satisfactorily addresses your concerns.

4. In Fig. 6a, indicating the direction of splicing changes (e.g., increased vs. decreased inclusion) would be helpful.

Response: This is an excellent suggestion. We fully agree that indicating the direction of splicing changes will improve the clarity of Fig. 6a. Accordingly, the number of splicing events

with increased inclusion or decreased inclusion is now explicitly indicated in **Supplementary Fig. 4b**.

5. In Fig. 6d and 6e, while the study convincingly demonstrates that hnRNPM depletion leads to alternative splicing of various transcripts, such as *Khdc3*, *Nlrp14*, *Cenph*, and *Zar1l*, it is not clear from the data presented whether these splicing changes result in significant alterations to the levels of corresponding intact proteins. Providing evidence, perhaps through Western blotting for some of these key affected proteins in hnRNPM-depleted oocytes, would more directly link the observed splicing defects to the downstream cellular phenotypes.

Response: We thank the reviewer for raising this important point regarding the impact of splicing alterations. As suggested, we performed Western blot analysis to evaluate protein-level changes for two key affected candidates, CENPH and ZAR1L (KHDC3 and NLRP14 were not tested due to the lack of commercially available antibodies). The results confirm a slight downregulation of CENPH and a dramatic reduction in ZAR1L in hnRNPM-depleted oocytes (**Supplementary Fig. 4e**). These data provide direct evidence that the splicing defects caused by hnRNPM ablation lead to reduced expression of proteins, thereby bridging the observed molecular changes to potential phenotypic consequences.

6. The analysis in Fig. 7f shows an overlap between hnRNPM-bound genes and differentially spliced genes. To provide more direct evidence for a regulatory role, the analysis could be extended to compare hnRNPM binding signals specifically on exons and introns that are alternatively spliced versus those unaffected by hnRNPM depletion.

Response: We thank the reviewer for this excellent suggestion. Accordingly, we analyzed hnRNPM binding signals on exons and introns that are alternatively spliced versus those unaffected by hnRNPM depletion. Strikingly, hnRNPM binding was significantly enriched on the exons of transcripts that underwent alternative splicing upon its depletion, compared to those that were unaffected (**Supplementary Fig. 5e-f**). This provides much more direct evidence that hnRNPM binding at these specific locations is functionally responsible for the splicing regulation.

7. A recent study cited by the authors (Zheng et al., 2024) demonstrated that hnRNPM protects against interferon response by repressing LINE-associated cryptic splicing. It would be a valuable addition to investigate whether the hnRNPM binding sites identified in oocytes are also enriched for LINE1 elements, and whether the key splice events affected by hnRNPM depletion are associated with these retrotransposons.

Response: We thank the reviewer for this insightful suggestion. Accordingly, we performed the recommended analysis and found that hnRNPM exhibits no significant binding preference

for LINE1 elements (**Fig. R3**). We propose two possible explanations for this result. First, hnRNPM may not operate in oocytes through the same mechanism as reported by Zheng et al. Alternatively, the discrepancy may arise from methodological differences in sequencing. In the study by Zheng et al., nuclear RNA was used for sequencing, thereby enriching pre-mRNAs and increasing sequencing depth for nascent transcripts. In our study, however, the limited quantity of oocyte samples necessitated the use of total RNA, which is dominated by mature cytoplasmic mRNAs.

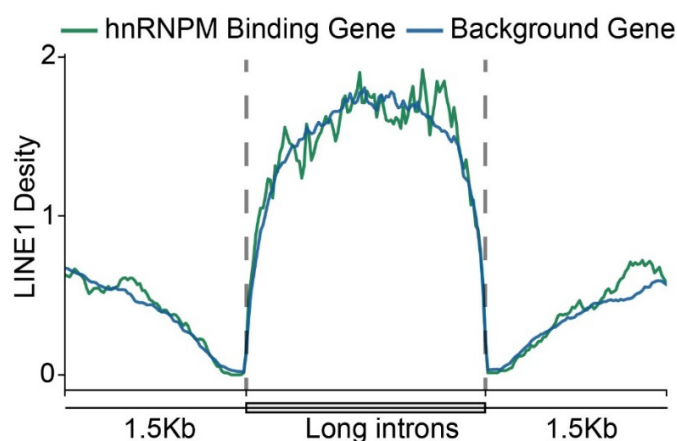


Fig. R3 Comparison of LINE1 intensity on long introns between hnRNPM-binding genes and background genes.

8. In Fig. 8d, the authors propose that hnRNPM recruits *Bcas2* to regulate splicing. To clarify the nature of this interaction, a co-IP experiment including an RNase treatment step is necessary. This would determine whether the interaction is a direct protein-protein association or is mediated by RNA.

Response: We thank the reviewer for raising this critical point. We fully agree that distinguishing between a direct protein-protein interaction and an RNA-facilitated one is essential. In direct response to this comment, we performed the suggested co-IP experiment with RNase treatment. As shown in **Fig. 8d**, the interaction between hnRNPM and BCAS2 remained intact following RNase treatment, indicating that their association is a direct protein-protein interaction and not dependent on RNA.

9. In Fig. 8f, the study proposes a “strong functional synergy” between hnRNPM and *Bcas2* based on the overlap of 98 commonly affected alternative splicing genes and 57 identical splicing events in the respective knockout oocytes. Are these overlaps statistically significant? While suggestive, further evidence is needed to solidify this claim, or toning down is necessary.

Response: We thank the reviewer for raising this important point regarding statistical significance. In direct response, we performed hypergeometric tests to rigorously evaluate

the overlap. The results revealed that both overlaps are overwhelmingly significant, with p-values of 2×10^{-27} for the 98 commonly affected genes and 4×10^{-27} for the 57 identical splicing events, indicating they are far beyond random chance. We have now included these statistical results in the revised manuscript (**Fig. 8f legend**).

Minor:

1. In Fig. 1c and 1d, the study demonstrates that hnRNPM is absent from the GVBD stage through the zygote stage. It would be interesting to speculate on the functional implications of this dynamic expression. Is the dynamic expression during oocyte maturation important for ZSA? What might be the consequences if hnRNPM is consistently expressed during these stages?

Response: We thank the reviewer for this interesting suggestion. Accordingly, we have incorporated the following paragraph to speculate on the functional implications of hnRNPM's dynamic expression pattern (**Lines 393-401**): "The specific absence of hnRNPM from the GVBD to zygote stages implies that its expression may be precisely regulated to ensure proper ZSA. We propose that this transient downregulation serves at least two critical functions. First, The absence of hnRNPM, a known splicing regulator, may protect the pre-deposited maternal mRNA pool from spurious or aberrant splicing. Second, its removal might be essential to 'reset' the splicing landscape, allowing the nascent zygotic transcriptome to be established under a new regulatory framework. Conversely, persistent hnRNPM expression potentially cause mis-splicing of both maternal transcripts and early zygotic genes, ultimately lead to failure of ZSA and subsequent embryonic developmental arrest."

2. In Fig. 2k and 2l, there appears to be a discrepancy between the dramatic development arrest shown in Fig. 2k and the moderate, albeit significant, changes in developmental rates presented in Fig. 2l. The visual evidence in Fig. 2k suggests a near-complete failure of Hnrnpm-deficient oocytes to develop beyond the 2-cell stage after IVF. An explanation for this difference would strengthen the interpretation of the impaired developmental phenotype.

Response: We thank the reviewer for noting the apparent discrepancy between Fig. 2k and 2l. In response, we wish to clarify that the developmental rates of the cKO group are indeed 0% at all stages, which is fully consistent with the phenotype shown in Fig. 2k. We acknowledge that in the original Fig. 2l, the cKO data trajectory was completely overlapped with the X-axis, potentially hindering visual interpretation. To address this issue, we have adjusted the axis scaling in the revised panel to clearly display the 0% trend above the baseline. We apologize for any confusion caused by the initial presentation and thank the reviewer for this opportunity to improve our figure.

3. In Fig. 8g, the basis of claiming “increased exon skipping in *Cenpf*” could be more quantitatively supported.

Response: We thank the reviewer for this important suggestion. In response, we have replaced the representative images in **Fig. 8g** to support the increased exon skipping in *Cenpf*.

4. Based on the study, we can hypothesize but cannot definitively conclude that human hnRNPM and BCAS2 form the same interaction network to control splicing in human oocytes. While the RIP-qPCR analyses in Fig. 8n-p are suggestive, the splicing regulatory consequences in human transcripts tested here should be examined in a human cell model as well.

Response: We thank the reviewer for this excellent suggestion. We agree that validating the splicing regulatory function of hnRNPM and BCAS2 on the identified human transcripts in a human cell model is crucial for strengthening our conclusions. Our new results clearly demonstrate that depletion of either hnRNPM or BCAS2 in human cell recapitulates the splicing changes of *PRC1* and *HDLBP* (**Supplementary Fig. 6f**). We also apologize that, despite extensive efforts, we were unable to successfully amplify *CENPF* in human cells under our experimental conditions. Nevertheless, the consistent and reproducible splicing alterations in *PRC1* and *HDLBP* upon *HNRNPM* or *BCAS2* knockdown strongly support the conclusion that the hnRNPM/BCAS2 network functions to regulate alternative splicing in human cells.

5. A minor but important point for clarity is the consistent use of nomenclature for genes, transcripts, and proteins. The study frequently uses human-centric or nomenclature for proteins (e.g., BCAS2) in a mouse model or mouse-centric nomenclature for transcripts (e.g. *Cenpf*, *Prc1*, and *Hdlbp* in Fig. 8) in a human model, while correctly italicizing mouse gene names (e.g., *Hnrnrm*, *Bcas2*). Adhering to the correct organism-specific nomenclature throughout the manuscript would improve precision.

Response: We thank the reviewer for highlighting this issue. We have carefully reviewed the entire manuscript and revised the nomenclature to adhere to organism-specific conventions. Human genes are now presented in uppercase italics (e.g., *BCAS2*), mouse genes in capitalized italics (e.g., *Bcas2*), and all protein names are in uppercase non-italics (e.g., BCAS2). These corrections have been made consistently throughout the manuscript.

6. To better understand the specificity of the hnRNPM-Bcas2 interaction, it would be informative to examine whether it also occurs at later developmental stages where both proteins are re-expressed, such as the 2-cell stage or a fully developed cell line. This would clarify if the interaction is unique to oogenesis or part of a more general splicing regulatory

mechanism. While additional experiments are not necessary for this manuscript, the authors could consider discussing this point.

Response: We thank the reviewer for this insightful suggestion. To directly address whether the hnRNPM-BCAS2 interaction is specific to oogenesis or represents a more general mechanism, we performed additional experiments in HEK 293T cells. hnRNPM and BCAS2 also interact in this somatic cell line (**Fig. R4**). This new finding indicates that the hnRNPM-BCAS2 interaction is indeed a conserved feature of a broader splicing regulatory mechanism.

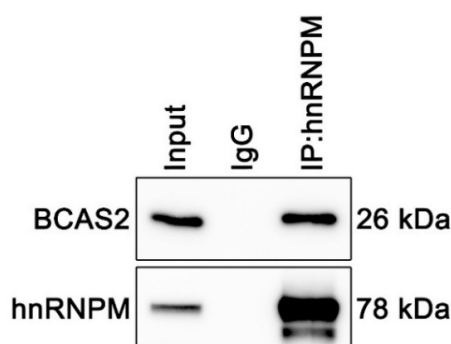


Fig. R4 Co-immunoprecipitation assays confirm the interaction of hnRNPM with BCAS2 in HEK 293T cells.

7. The color key is missing in Fig. 2f.

Response: Thank you for noticing this. We have now included the missing color key in **Fig. 2f** in the revised figure panel.

8. Should the column labels in Suppl Table 1b be KO-1 and KO-2?

Response: Thank you for your comment. We sincerely apologize for the lack of clarity in our previous description, which may have caused confusion. The purpose of analyzing novel transcripts in this study is to highlight previously overlooked transcripts present in normal oocytes, emphasizing a broader and more generalizable research finding. Since some novel transcripts detected in the KO samples may represent aberrant products resulting from gene deletions, we present data only for WT1 and WT2 in Suppl Table 1b to ensure a focus on authentic transcripts. We deeply regret any confusion our original presentation may have caused and have thoroughly revised the text to improve clarity. We hope the current version satisfactorily addresses your concerns.

9. A typo in line 408 “Notably, tThe meiotic“

Response: This has been corrected to “Notably, the meiotic...” in the revised manuscript.

10. In the affiliation, “8Sir Run Shaw Hospital” is likely to be “Sir Run Run Shaw Hospital.”

Response: We thank the reviewer for spotting this error. The affiliation has been corrected to “Sir Run Run Shaw Hospital.”

Reviewer #3 (Remarks to the Author)

The author studied the function of hnRNPM during oocyte development and thoroughly analyzed its molecular mechanism of action. The results demonstrate that hnRNPM is a key regulatory factor for the alternative splicing of pre-mRNA during oocyte development. hnRNPM recruits BCAS2 to control the splicing of crucial pre-mRNA related to meiosis. These findings have significant academic value for understanding the regulation mechanisms of alternative splicing during oocyte development, and provide important references for understanding the pathogenesis of related diseases. The findings are innovative but still need to be further improved.

Response: We thank the reviewer for the positive evaluation of our work and for the valuable suggestions. We have performed additional experiments and analyses to address the concerns as detailed below.

Major concerns:

1. Stra8 Cre activation occurs at embryonic day 12.5. I am curious about the expression pattern of hnRNPM in oocytes during different developmental stages of the embryo and postnatally. If its expression level is high and it can be effectively knocked out after birth, does this indicate that it has no function in the establishment of primordial follicle pool and activation of primordial follicles? Additionally, previous studies have shown that BCAS2 plays an important role in the first meiotic division of oocytes. Does hnRNPM function independently of BCAS2 in early ovarian oocytes?

Response: This is a great suggestion. We examined the expression of hnRNPM in oocytes across multiple developmental stages: from embryonic days (E13.5, E15.5, E17.5) to postnatal stages (P0, P3, P7) using immunofluorescence. In the embryonic stages (E13.5, E15.5, E17), the expression of hnRNPM in germ cells was weak and lower compared to Sertoli cells (**Supplementary Fig. 1d**). After birth (P0), hnRNPM expression in germ cells increased significantly (**Supplementary Fig. 1e**). In primordial and primary follicles at P3 and P7, the hnRNPM signal was notably stronger and exceeded the expression levels seen in Sertoli cells (**Supplementary Fig. 1e**).

To verify the knockout efficiency, we assessed hnRNPM expression in P0 oocytes from both control and cKO mice and confirmed that hnRNPM was completely absent in the germ cells of cKO mice (**Supplementary Fig. 2a**). Importantly, despite the perinatal expression of hnRNPM, its knockout had no effect on the number of primordial or primary follicles (**Fig. 2i**). This suggests that hnRNPM is dispensable for the establishment and activation of the

primordial follicle pool.

In addition, we sincerely appreciate your insightful comment regarding the potential interplay between hnRNPM and BCAS2, especially considering that previous studies have highlighted BCAS2's crucial role in the first meiotic division of oocytes. In contrast to BCAS2, conditional knockout of hnRNPM using *Stra8-Cre* did not lead to significant defects in the first meiotic division in either male or female germ cells⁹. This suggests that hnRNPM functions independently of BCAS2 in the regulation of meiosis, particularly during the first meiotic division. We have added these results and clarified this point in the Discussion (**Lines 468–474**).

2. In oocytes and granulosa cells, BCAS2 exerts important regulatory functions by forming complexes with corresponding interacting proteins to influence the alternative splicing of precursor mRNAs. In this study, does the recruitment of BCAS2 by hnRNPM require the formation of a complex with PRP19 and CDC5L to perform splicing functions? If possible, provide relevant evidence, or discuss this in the revised version.

Response: We thank the reviewer for this insightful comment. Our new data reveal that hnRNPM could interact with PRP19 and CDC5L (**Supplementary Fig. 6c-d**), suggesting its association with the entire PRP19/CDC5L/BCAS2 complex to coordinate splicing. These results provide a deeper mechanistic understanding for the role of hnRNPM in modulating the splicing machinery. We have included these new data and discussed their implications.

3. After the absence of hnRNPM in oocytes, how do the gene and protein levels of BCAS2, as well as its protein localization? The related results may be helpful. BCAS2 might undergo degradation or positional changes, which can also be reflected in the working model diagram.

Response: We thank the reviewer for this insightful suggestion. Our investigation showed that loss of hnRNPM led to reduced BCAS2 protein expression without affecting its subcellular localization (**Fig. 8n and Supplementary Fig. 6g**), suggesting that BCAS2 may undergo degradation. The working model has been updated accordingly to indicate that hnRNPM is necessary for maintaining normal BCAS2 protein levels (**Fig. 9**).

4. In vitro OR in vivo experiments demonstrate whether *Bcas2* knockdown or knockout affects the expression level and localization of hnRNPs, and alternative splicing of precursor mRNA regulated by hnRNPs?

Response: We thank the reviewer for these insightful questions. Our data show that *Bcas2* knockout reduces hnRNPM protein levels without affecting its subcellular localization (**Supplementary Fig. 6h-i**). Together with the finding that loss of hnRNPM decreases BCAS2 protein expression (**Major comment 3**), suggesting that hnRNPM and BCAS2 likely form a

complex and are mutually dependent for protein stability. Furthermore, loss of *Bcas2* in oocyte recapitulates the aberrant splicing of hnRNPM targets transcripts (**Fig. 8g-k**), indicating that BCAS2 and hnRNPM collaborate closely in regulating alternative splicing. These data have been included and discussed in the revised manuscript.

5. Is BCAS2 cKO *stra8* cre or *vasa* cKO? The authors should provide the breeding and acquisition process of BCAS2 cKO mice in the Materials and Methods section.

Response: We apologize for this omission. We have now added the detailed information on the breeding strategy to obtain *Bcas2*^{flox/Del}; *Vasa*-Cre (designated *Bcas2* cKO) mice in the Materials and Methods section (**Lines 520-521**).

Minor concerns:

1. The abstract does not end with a period.

Response: Thank you for pointing this out. We have added a period at the end of the abstract to ensure correct punctuation.

2. Line 116, revise 'cattle' for repetition.

Response: Corrected.

3. Line 126, Ensure that 'tubulin' starts with an uppercase letter throughout the MS, including Figures and Legends.

Response: Thank you for bringing this up. We have corrected to "Tubulin" throughout the manuscript, figures, and legends.

4. Ensure that *Stra8*-Cre and *Stra8*-GFPcre are consistently used throughout the MS, e.g., Line 458, 465, Figure 2a, b, c and figure legends.

Response: Thank you for pointing this out. We have carefully revised the manuscript to ensure that "*Stra8*-Cre" are used consistently throughout the manuscript, figures, and legends.

5. Gene writing should be uniform throughout the MS, e.g., Line 168, 312.

Response: We sincerely thank the reviewer for this careful observation. We have now standardized the gene and protein nomenclature throughout the text by adhering to the following format: italicized, first-letter-capitalized for mouse genes (e.g., *Hnrnp*, *Bcas2*); italicized, all-capitals for human genes (e.g., *HNRNPM*, *BCAS2*); and non-italicized for proteins (e.g., hnRNPM, BCAS2).

6. The controls in Figure 3c are WT? What's the difference between WT and CTRL?

Response: We apologize for the inconsistency in the genotype annotation and have standardized it to "CTRL". "WT" in Figure 3c refers to the control oocytes from *Hnrnp*^{flox/+} mice, which are phenotypically wild-type.

7. Line 227, missed spaces should be added after 'site'.

Response: Thank you for pointing this out. We have added the necessary spaces after the word “site” as suggested.

8.Line 280-281, Revise repeated word usage according to the highlighted certain information in the Figure.

Response: Corrected.

9.Ensure consistency between 'Western blot' and 'Western blotting' throughout the MS.

Response: Thank you for pointing this out. We have standardized to "Western blot" throughout the manuscript.

10.Modification of citation format in the main text, e.g., Line 52, 58, 70, 96, 312, 349-350, 373, 421.

Response: We thank the reviewer for careful reading and have adjusted the citation format in the main text to ensure consistency with the required style.

11.References should be modified according to NC format, such as using both the full name and abbreviation of journal titles.

Response: Thank you for pointing this out. We have thoroughly reformatted the entire reference list according to the Nature Communications guidelines

12.Figure 1a, ‘Bovin’ should be corrected.

Response: We thank the reviewer for careful reading. We have corrected the typo “Bovin” to “Bovine” in Figure 1a and any other relevant sections.

13.The annotation information on the image is either left-aligned or centered, maintaining consistency; as shown in Figure 8e (which can be aligned similarly to the figure editing in Figure 1) it is left-aligned, see also Figures 1c, etc.

Response: Thank you for your valuable advice. We have adjusted Figure 8e to match the alignment style in Figure 1 (left-aligned), as well as other figures that may require alignment adjustments.

14.In the figures, should there be a space before the parentheses in the horizontal and vertical axes? Please ensure consistency across all figures by the author. For example, Figure 4b has a space, but other figures do not.

Response: Thank you for your comments. We have checked all axis labels and ensured consistency.

15.Figure S1,Modify the title of the figure legend to cover all key information in the figure; change the letters annotated in Figure S1 to black; ‘Bovin’ should be corrected.

Response: We thank the reviewer for careful reading. We have modified the figure legend title to be more comprehensive. Additionally, we have changed the annotated letters to black in Figure S1 and correct the typo "Bovin" to “Bovine” as suggested.

16.The spacing between the upper and lower figures in Figure 5f should be adjusted, causing some letters to be covered by the lower figure; in Figure 5h, the Chinese text should be

deleted or replaced.

Response: Thank you for your important suggestion. We have adjusted the spacing between the upper and lower figures in Figure 5f to prevent overlap. We have also removed the Chinese text in Figure 5h as reviewer's suggestion.

17. In Figure legends, 'Error bars, mean $\pm$ SEM' should be revised to 'Error bars indicate SEM', OR 'Data are presented as mean $\pm$ SEM'.

Response: Thank you for your valuable feedback. We have checked and corrected all Figure legends.

18. Figure 3e, can the blue arrowhead be identified as mitochondrial aggregation? What are the morphological characteristics of mitochondrial aggregation? Modify the position of the yellow arrow (below).

Response: Thank you for this interesting and important question. The structure indicated by the blue arrowhead can indeed be identified as mitochondrial aggregation. Mitochondrial aggregation is currently not clearly defined in the literature. Based on our research experience¹⁰, it is characterized by the clustering of multiple mitochondria—typically more than ten—tightly grouped together without being separated by cytoplasmic lattice structures. In addition, as suggested, we have adjusted the position of the yellow arrow to improve labeling accuracy. We sincerely appreciate your careful observation and hope this response addresses your concerns.

19. In Figure 2e, DAPI signal should be added.

Response: Thank you for bringing this up. We have added the DAPI (white) signal to Figure 2e as suggested.

Reviewer #4 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: On behalf of my co-authors and myself, I would like to extend our sincere gratitude for your thorough assessment and helpful suggestions for our manuscript.