RNA Ligase RTCB regulates mRNA alternative splicing and is required for mouse oocyte development and maintenance

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Original submission

First decision letter

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MS TITLE: RNA Ligase RTCB-mediated mRNA Alternative Splicing is Required for Mouse Oocyte Development and Maintenance

AUTHORS: Hua Zhang, Jun-Chao Jiang, Hua-Nan Wang, Nai-Zheng Ding, and Heng-Yu Fan

I have now received all the referees' reports on the above manuscript, and have reached a decision. As you will see, the referees express considerable interest in your work, but also recommend a substantial revision of your manuscript, in particular the reviewers suggest, and I agree, that data should be provided for claims made in the manuscript, such as changes to mRNA abundance etc. If you are able to revise the manuscript along the lines suggested, which involves further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

Reviewer 1

Advance summary and potential significance to field

Yes.

Comments for the author

Zhang et al. studied the role of RNA Ligase RTCB in mRNA alternative splicing during mouse oocyte maturation and embryonic development. The authors demonstrate that RTCB expression is upregulated during oocyte maturation and embryonic stages, and the conditional mutant mice show POI and sterility phenotype. Further, they have shown that RTCB regulates the maternal mRNA splicing and specifically affect the DNA methylation and DNA damage repair transcripts which play a vital role in oocyte maturation and ZGA. This is an exciting manuscript; however, some aspects of the study need to be clarified, and more experiments are necessary to describe the manuscript's findings. Also, some issues with the writing must be addressed. Overall, the study is attractive, nicely focused and would benefit from revisions.

Major comments:
Introduction Fig1:
What is the specificity of RTCB staining? It seems some dots are all over the place. It would be good if the authors showed the knockout oocyte RTCB localization as a –ve control.
If the mRNA level of rtcb is reduced in MII compared to GV fig. 1 (a), how could the protein of rtcb be more at the MII stage? Maybe the quantification may solve the problem.
Fig2:

(A, B) Weight and size cannot be an accurate indicator of follicullogenesis. It should be supplemented with the no. of follicles/ oocyte count from both sets of animals. Then the result will be more conclusive.
In addition, the mechanism of premature ovarian failure caused by *Rtcb* deletion needs to be addressed. Or what is the reason behind follicle loss that should be addressed to make this paper strong?
Does the decline in the ovarian pool is due to the compromised DNA damage repair pathway? As the level of Rad50 mRNA IR increases.

In the discussion, authors stated that:
Line 287: perhaps the lack of RTCB affects the expression of some follicle activation-related transcripts, leading to premature ovarian failure. What are they, and it is crucial to validate their conclusion.

Fig3:
Line 132:
However, the proportion of SN configuration was only approximately 40% in the *Rtcb*-null oocytes (Fig. 3C and D), and the oocytes resume the meiosis shows WT levels of PBE. Why do authors see two populations? Any insights?
Is there any size difference in those 40% oocytes?

Fig5:
Balance of cyclin B1 to CDK1 ratio plays a vital role in controlling the CDK1 activity during long G2M in oocytes. Surprisingly, Fig5 F and G overexpression of CDK2 in WT shows a 100% GVBD rate. Don't they show premature chromosome segregation and aneuploidy?
How does the Rtcb regulate the p-T161-CDK1? Whereas the normal CDK1 levels comparable in both WT and control?

Minor comments:
The introduction does not describe the existing results like Sara Guckian Kosmaczewski's and Dang Vinh Do's papers describing the importance of alternative splicing and Rtcb role in oocysts. Please try to add the existing literature about the role of alternative splicing in oocyte

maturation and embryo development.
Line 98: *Rtcb* is highly expressed in germ cells – rewrite this as it only shows female oocyte stages and not the male counterpart.

Fig1A: Y-axis relative rtcb mRNA level. No need to show it in a separate box as labelled rtcb. Avoid the confusion Error bars are missing in the graph, like the brain without an error bar. Immunofluorescence Fig. 1 (D)
Figures are unclear; we can't distinguish the nuclear and cytoplasmic localization as seen in HeLa cells in Jurkin et al. 2014.
Line 528: Representative ovaries from 4-, 6-, 8-, and 10-week-old: Write the weeks 4,6,8
Line 169: The PCR products were used to run the DNA gel, the results confirmed that IR occurred on these transcripts (Fig. 4C): Sentence is not clear. Please rewrite it.
Fig 5K fig. labelling is not proper (I think) as in the first half of the western says FLAG tag which is not visible in western. – Did the authors microinject the only flag peptide in the oocytes?
Line 202,203, and 204.
This observation indicates that the failure of GVBD is mainly due to a decrease in CDK1 activity rather than the loss of cyclin B1 and CDC20 proteins. However, in fig 5(j), western says that the expression of Cyclin B1 and CDC20 proteins increases in rtcb k/out mice, not decrease. It is contradictory to what they wrote and what the western says.
Line 206: As a result, the GVBD rate of *Rtcb*-null oocytes overexpressing CDK1T14A, Y15F was approximately 70% (Fig. 5L). Please rewrite the sentence.

Line 252 **-** Unreported function of RTCB in meiosis in mouse oocytes – authors can use mouse oocyte maturation instead of oocyte meiosis (rewrite it).
Line 267 - decreased RAD50 protein levels may lead to GVBD failure – authors haven't shown any western for the RAD50. How they can be sure about the decrease in RAD 50 protein level. As they reported the Rad 50 transcription in fig 4(b)
Line 277 - GVBD failure, premature ovarian failure, fertilization - No aspect of fertilization was studied in this study.
The authors did not show any data related to the possible mechanism of immature ovarian failure. The author should check early meiosis prophase defects. For example, the loss of oocytes in ovaries defective synapsis takes around two months (Rinaldi et al., 2017 Mol cell). If possible, the authors should check for this case.

Reviewer 2

Advance summary and potential significance to field

Zhang et al. present an interesting study that highlights the vitality and complex nature of RNA processing mechanisms in female reproduction and early embryo development. Specifically, they examine the role of RNA terminal phosphate cyclase B (RtcB) at multiple critical junctures in oocyte growth and maturation, as well as during the transition to embryo. They claim that RtcB regulates a number of processes in this window of mouse gamete and embryo development, with oocyte-specific loss of RtcB leading to female infertility and premature ovarian failure. They propose this occurs due to the aberrant accumulation of maternal intron-containing transcripts, including those important for DNA methylation. They further demonstrate that RtcB-deficient oocytes imperfectly fail to resume meiosis, and partially rescue this resumption by exogenous expression of CDK1. Ultimately, they highlight the downstream effect of reduced oocyte developmental competence with loss of RtcB – oocytes either fail to undergo fertilization, progress through zygotic genome activation, or undergo development to the blastocyst stage.
The experiments conducted in this study are technically challenging and many are quite rigorous, which is notably difficult in a conditional knock-out system in which oocyte development is innately affected. Also the topic of RNA processing in development is an exciting and important one, particularly in this case the regulation of gene expression via alternative splicing during prolonged storage of maternal mRNA in mouse oocytes, and I believe there would be much enthusiasm for this subject matter.

Comments for the author

Zhang et al. present an interesting study that highlights the vitality and complex nature of RNA processing mechanisms in female reproduction and early embryo development. Specifically, they examine the role of RNA terminal phosphate cyclase B (*RtcB*) at multiple critical junctures in oocyte growth and maturation, as well as during the transition to embryo. They claim that *RtcB* regulates a number of processes in this window of mouse gamete and embryo development, with oocyte-specific loss of *RtcB* leading to female infertility and premature ovarian failure. They propose this occurs due to the aberrant accumulation of maternal intron-containing transcripts, including those important for DNA methylation. They further demonstrate that *RtcB*-deficient oocytes imperfectly fail to resume meiosis, and partially rescue this resumption by exogenous expression of CDK1. Ultimately, they highlight the downstream effect of reduced oocyte developmental competence with loss of *RtcB* – oocytes either fail to undergo fertilization, progress through zygotic genome activation, or undergo development to the blastocyst stage.

The experiments conducted in this study are technically challenging and many are quite rigorous, which is notably difficult in a conditional knock-out system in which oocyte development is innately affected. Also the topic of RNA processing in development is an exciting and important one, particularly in this case the regulation of gene expression via alternative splicing during prolonged storage of maternal mRNA in mouse oocytes, and I believe there would be much enthusiasm for this subject matter. However, there are a number of major concerns that must be addressed before publication is warranted in *Development:*

Major concerns:

1) At times, the narrative of the manuscript text is not supported by data within the figures. In other instances, conclusions drawn from experimental data are overstated. Four examples follow:

- Lines 160-161: The authors claim that "*Rtcb* deletion leads to IR accumulation and decreased global mRNA levels." Fig 3 demonstrates downregulation of many genes, but a global reduction of total mRNAs is not shown. For this, total reads in cKO and control oocytes must be quantified, with robust normalization to ERCC spike-ins.
- While DNMT1, DNMT3A, and DNMT3B appear to be reduced at the protein level (Fig 4D), only data for *Dnmt3b* is presented at the mRNA level (Fig 4B), and total mRNA appears to be non-significantly increased, rather than decreased, as would be expected given the authors hypothesis for regulation by *RtcB*. Analysis of mRNA levels for other Dnmt mRNAs, as well as a discussion of alternative proposed mechanisms, would clarify this finding.
- Line 226-227: "These results reveal that maternal RTCB is indispensable for fertilization and early embryo development." The authors demonstrate successful fertilization and development to the blastocyst stage (although reduced compared to controls). Deficits in developmental progression at this stage are likely due to a reduction in competence of the oocyte and claiming that this factor is indispensable for fertilization and embryo development is an overstatement.
- Line 234 states that "transcriptional activation was significantly suppressed" in *RtcB*-deficient oocytes. However, the authors cannot claim that transcriptional activation in the embryo is suppressed based on the expression of a handful of ZGA genes. While challenging, a transcriptional run-on assay would allow for this claim and significantly elevate these findings.

2) Often, unclear language is used, or not enough information is provided to discern experimental rationale, methods, rigor, and statistics.

- In Fig 2, the authors claim that oocyte-specific *RtcB* loss leads to premature ovarian failure at 8- 10 weeks. They display quantification of ovary weights at each time point, but do not clearly state how many mice were used for this analysis. Further, they claim a loss of primordial, primary, secondary, and antral follicles at 8-10 weeks, but only show one representative histology image from each stage. Quantification of follicle type at each time point would help to discern exactly what stage is affected, and providing total numbers would add necessary rigor to this result.
- In Fig 3C-D, the authors do not state in the main text, or in the methods, how they define SN vs NSN. Did they observe any oocytes in a partly surrounded nucleolus (pSN) pattern?
- More detail should be provided about the precise stage of oocytes that were isolated for RNA sequencing.

- Statistical analysis of their RNA sequencing experiments states that a fold change >2, p-value < 0.05, and FDR < 0.05 was used (Lines 142-144). It is very unlikely that the p-value and FDR are equivalent in a transcriptome-wide differential expression analysis. The authors should more clearly state if a false discovery correction was used, and if so, by which method.
- The authors use ERCCs as a tool to normalize total copy number. They should demonstrate how they used these synthetic RNAs to normalize, or that the levels of these RNAs were at least unchanged across their samples.
- What is the CASH software? The authors should provide a reference or detail its use in methods.
- Line 163-164: "The results showed that the expression levels of most of the transcripts decreased (Fig. 4A)." The authors should provide fold-change cutoffs or statistics to add robustness to this finding.

3) Necessary controls were overlooked. In Fig 1G, the authors present a key finding, that oocyte-specific conditional knock-out females are infertile. They generate these mice by crossing *Rtcb*-floxed mice with oocyte *Gdf9*-Cre mice. However, as a control, it is important to show that the *Rtcb*-floxed females from which the conditional line was derived do not display impaired fertility, which could underly the infertility observed in resultant lines.

4) The western blot image in Fig 5J is hard to visualize, particularly for p-T161-CDK1, which is a critical finding. The image looks darker on the WT side and it is difficult to visualize the band.

Minor concerns:

- The title of "mouse oocyte development and maintenance" is a unclear. Also, while the authors demonstrate that *RtcB* is essential for oocyte and/or embryo developmental competence, they do not necessarily show that this is done through alternative splicing, as the title suggests.
- Grammar throughout the manuscript is poor, and language is unclear. Some examples as follows:
    - Line 41-42 "During the development from growing oocytes to fully grown oocytes, an increase in volume is coupled with active transcription events."
    - Line 44-45: "a single gene can generate multiple transcripts and protein variants, which significantly increases various transcriptomes and proteomes."
- Further discussion on the following topics would enhance the significance of the conclusions for the reader:
    - In Fig 2, the authors demonstrate a dramatically reduced number of follicles across 6-10 weeks. How do they propose that all follicular stages of growth disappeared in such a short period of ~4 weeks? Did they visualize cell death or corpora lutea?
    - Fig 3I demonstrates the various categories of splicing detected via their analysis. Intron retention is actually relatively infrequent compared to some others. Does this give the authors pause about the function of *RtcB* in oocytes? Do they have thoughts about potential other mechanisms through which *RtcB* might be acting in the oocyte?

Reviewer 3

Advance summary and potential significance to field

In this manuscript, Zhang et al. seek to understand how RNA terminal phosphate cyclase B (RTCB)-mediated RNA ligation influences mouse oocyte development. The authors conditionally depleted RTCB in early-stage oocytes using a Cre-Lox system and characterized oocyte growth and embryo development at various stages, from single-cell division to the zygotic genome activation (ZGA). They showed that loss of RTCB resulted in female mouse infertility, which was influenced by changes in alternative splicing patterns of the DNA replication machinery transcripts. While the findings are interesting within the context of female reproduction, the study would benefit from experiments that better solidify the conclusions being drawn in the manuscript.

Comments for the author

Major Comments

- The reader would benefit from a more detailed description of oogenesis (i.e., the stages of development) and the relationship to mRNA (i.e., expression and splicing) in the Introduction section.
- The definition of an exitron should be made clearer in the Introduction section. From my understanding, an exitron is not actually a regular/canonical retained intron. Based on the results of this study, it seems the authors are focusing on canonical intron retention events rather than exitron splicing. This is a main concern of this manuscript for us. This needs to be addressed in the abstract. As it is, it sounds that all the paper would talk about exitrons and based on what we read in the manuscript, authors are investigating regular IR.
- The authors should be sure to define abbreviations in figure legends and in the main text and minimize the number of abbreviations. As the manuscript is now, it is difficult to follow the flow on the story due to so many abbreviations.
- The conclusions being drawn from Fig. 1D are weak given the immunofluorescence images. The authors should perform a subcellular fractionation study and blot for RTCB to confirm with an orthogonal approach that RTCB is present in both cellular compartments.
- The use of a Cre-Lox system is a strong model for studying the developmental effects of RTCB depletion. The authors nicely showed that in mice lacking RTCB in the oocytes, the female mice were infertile (Fig. 1G). Important controls are missing, however, from the validation of the conditional knockout that should be included (e.g., Cre-negative line and a WT; Gdf9-Cre line).
- The authors claim that depletion of RTCB leads to decreased global mRNA levels (line 161); however, they did not directly quantify this. Given that the authors already collected RNA for sequencing analysis, a figure confirming that total mRNA abundance is reduced would be beneficial to the reader and support the claim being made.
- Line 162: authors stated that they analyzed 91 transcripts with IR changes; however in Fig. 3I they said that there were 42 IR events. I don't see how these two statements coexist.
- The authors posit that the observed IR in the RTCB-depleted cells leads to loss of protein expression. For the transcripts exhibiting IR, the authors should interrogate the rate of nonsense-mediated decay (NMD) of these candidates to confirm that the IR results in unstable mRNA that is rapidly turned over. This would also be an orthogonal approach to validate that RTCB depletion causes decreased mRNA level (see previous point).
- In Fig. 3I, the authors show the distribution of splicing changes when RTCB is depleted; however, the number of transcripts affected by IR is only 4% of the total changes occurring. Are DNA replication transcripts affected only in the cases of IR? What are the common families of transcripts being alternatively spliced in each class? The authors should elaborate on their findings here.
- The authors show a clean recovery of the GVBD rate when they overexpress CDK1. An explanation of how the GVBD rate was calculated and quantified (Fig. 5A) would benefit the reader. If there are representative images showing this process it would be helpful to include them as well.
- Authors need to describe the statistical analysis that has been performed in the RNA-seq analysis.

Minor Comments

- Authors need to follow the guidelines of gene symbols, protein symbols RNA symbols. Based on our understanding only gene symbols should be italicized.
RNAs should be on regular font.
- Avoid red-green combinations (color blind readers).
- The authors should be sure to define abbreviations in figure legends and in the main text (e.g., GV, GC, GCBD in Fig. 1).
- The authors need to include their sources when referencing previous studies. For example, the authors write "According to a previous study approximately 5% of tRNA genes in mice contain introns" (lines 71-72), but there is no corresponding citation. Similarly, in the Discussion section (lines 252-254)
the authors write "According to previous studies, IR occurs…and 16S rRNA splicing"
but there are no references.
- In the Abstract, "play" should be "plays" (line 34).

First revision

Author response to reviewers' comments

Reviewer 1:

Zhang et al. studied the role of RNA Ligase RTCB in mRNA alternative splicing during mouse oocyte maturation and embryonic development. The authors demonstrate that RTCB expression is upregulated during oocyte maturation and embryonic stages, and the conditional mutant mice show POI and sterility phenotype. Further, they have shown that RTCB regulates the maternal mRNA splicing and specifically affect the DNA methylation and DNA damage repair transcripts which play a vital role in oocyte maturation and ZGA. This is an exciting manuscript; however, some aspects of the study need to be clarified, and more experiments are necessary to describe the manuscript's findings. Also, some issues with the writing must be addressed. Overall, the study is attractive, nicely focused and would benefit from revisions.

Response: We appreciate the reviewer's positive assessment of our manuscript and constructive suggestions to improve the quality of our study. We thank the reviewer for pointing out that the study is attractive, nicely focused and the results are interesting. In this view, we have made extensive efforts to provide more experimental details, elaborate on our interpretations and conclusions, and improve the quality of the content during revision.

Major comments:

Fig 1: What is the specificity of RTCB staining? It seems some dots are all over the place. It would be good if the authors showed the knockout oocyte RTCB localization as a -ve control. If the mRNA level of rtcb is reduced in MII compared to GV fig. 1 (a), how could the protein of rtcb be more at the MII stage? Maybe the quantification may solve the problem.

Response:

5) As the reviewer suggested, we have done immunofluorescent staining of RTCB in the GV stage of oocytes of cKO mice. No RTCB signals were detected, demonstrating the specificity of the antibody. The results were added to the revised Fig. 1E.

6) Overall, our WB (Fig. 1C) showed RTCB protein level did not significantly change during oocyte maturation though its mRNA level was slightly decreased at MII stage (Fig. 1A-B). But protein levels in oocytes indeed did not strictly follow the mRNA pattern. It is common for maternal mRNAs that the translation rate increases due to mRNA polyadenylation after meiotic resumption n, leading to an elevation of protein levels (PMID: 34048556; PMID: 27993988).

Fig 2: (A, B) Weight and size cannot be an accurate indicator of follicullogenesis. It should be supplemented with the no. of follicles/ oocyte count from both sets of animals. Then the result will be more conclusive. In addition, the mechanism of premature ovarian failure caused by Rtcb deletion needs to be addressed. Or what is the reason behind follicle loss that should be addressed to make this paper strong? Does the decline in the ovarian pool is due to the compromised DNA damage repair pathway? As the level of *Rad50* mRNA IR increases.

Response: We supplemented the follicular count results of 6- and 8-week-old WT and *Rtcb*$^{oo-/-}$ mice. The results showed that the number of multi-layer secondary follicles and antral follicles in the ovary of 6-week-old *Rtcb*$^{oo-/-}$ mice decreased significantly (Fig. 2H and I), and the number of follicles at all levels in the ovary decreased significantly at 8 weeks. We further detected the γH2AX signal in oocytes on ovarian sections and found that the DNA damage signal was enhanced in *Rtcb*$^{oo-/-}$ oocytes (Fig. J and K), and obvious apoptosis signal was detected in follicles by TdT-mediated dUTP Nick-End Labeling (TUNEL) assay (Fig. L and M). These observations suggest that *Rtcb* deletion in oocytes leads to DNA damage and facilitated follicle atresia.

In the discussion, authors stated that: Line 287: perhaps the lack of RTCB affects the expression of some follicle activation-related transcripts, leading to premature ovarian failure. What are they, and it is crucial to validate their conclusion.

Response: We performed gene ontology (GO) analysis on the decreased transcripts in *Rtcb*$^{oo-/-}$ oocytes and found that they were mainly enriched in protein transport and DNA damage repair pathway (Fig 3G). From the current results, we think that *Rtcb* deletion leads to DNA damage in oocytes and further affects the protein exchange between oocytes and follicles, resulting in apoptosis of granulosa cells and premature ovarian failure. The original statement is not accurate. We have deleted this sentence in the revision.

Fig 3: Line 132: However, the proportion of SN configuration was only approximately 40% in the Rtcb-null oocytes (Fig. 3C and D), and the oocytes resume the meiosis shows WT levels of PBE. Why do authors see two populations? Any insights? Is there any size difference in those 40% oocytes?

Response:

1) We only calculated PB1 emission rates in oocytes that underwent GVBD, whereas most oocytes with NSN configuration were not able to undergo GVBD. We have clarified the calculation method in Fig. 5A.

2) On average the Rtcb-deleted oocytes were smaller in size than the WT oocytes, as shown in Fig. 3B. However, we could not distinguish the NSN and SN configurations in oocytes under the stereoscope because they are not stained with DAPI.

Fig 5: Balance of cyclin B1 to CDK1 ratio plays a vital role in controlling the CDK1 activity during long G2M in oocytes. Surprisingly, Fig5 F and G overexpression of CDK2 in WT shows a 100% GVBD rate. Don't they show premature chromosome segregation and aneuploidy?

Response: Actually, Fig. 5F and G are the results from superovulation assay, these oocytes were separated from the oviducts after hCG. I assume that the reviewer was referring to Fig. 5L, which shows GVBD rates after overexpression of the constitutively active CDK1. In a published study, Dr. Kui Liu's group has established a mouse model where they introduced a constitutively active form of CDK1 in meiotically incompetent small oocytes, their prophase arrest is interrupted by premature resumption of meiosis and DNA damage. In this paper, they observed premature condensation, but not segregation of chromosome in oocytes (Adhikari D, et al, Cell Res. 2016 Nov;26(11):1212-1225. PMID: 27767095).

How does the Rtcb regulate the p-T161-CDK1? Whereas the normal CDK1 levels comparable in both WT and control?

Response: Dephosphorylation of threonine 14 and tyrosine 15 of CDK1 by the CDC25 phosphatases is a key step in the activation of the CDK1-cyclin B protein kinase (Morgan, et al. PMID: 7877684). We found that the levels of CDC25B protein were lower in cKO oocytes than those in WT oocytes (Fig. 6A). This may be the reason that causes the CDK1 activation defect in Rtcb cKO oocytes.

Minor comments:

The introduction does not describe the existing results like Sara Guckian Kosmaczewski's and Dang Vinh Do's papers describing the importance of alternative splicing and Rtcb role in oocysts. Please try to add the existing literature about the role of alternative splicing in oocyte maturation and embryo development.

Response: We are sorry for the oversight. We have added these references to the revised manuscript as the reviewer suggested.

Line 98: Rtcb is highly expressed in germ cells – rewrite this as it only shows female oocyte stages and not the male counterpart.

Response: We have rephrased this sentence as the reviewer suggested.

Fig 1A: Y-axis relative rtcb mRNA level. No need to show it in a separate box as labelled rtcb. Avoid the confusion Error bars are missing in the graph, like the brain without an error bar. Immunofluorescence Fig. 1 (D) Figures are unclear; we can't distinguish the nuclear and cytoplasmic localization as seen in HeLa cells in Jurkin et al. 2014.

Response: The immunofluorescence image of Rtcb-deleted oocytes was offered as a negative control. And we used the nuclear and cytoplasmic separation samples of 293T cells for WB, and the results showed that RTCB existed in both cellular compartments. (Revised Fig. 1E and F)

Line 528: Representative ovaries from 4-, 6-, 8-, and 10-week-old: Write the weeks 4,6,8

Response: We have corrected the inconsistency of time point description.

Line 169: The PCR products were used to run the DNA gel, the results confirmed that IR occurred on these transcripts (Fig. 4C): Sentence is not clear. Please rewrite it.

Response: We rephrased this sentence as the reviewer suggested.

Fig 5K fig. labelling is not proper (I think) as in the first half of the western says FLAG tag which is not visible in western. – Did the authors microinject the only flag peptide in the oocytes?
Response: Actually, we microinjected the in vitro transcribed mRNA products of the linearized empty vector, which encodes only the FLAG tag. The molecular weight of the protein product is too small to be detected in western blot. We have clarified this in the legend of revised Fig. 6F.

Line 202,203, and 204. This observation indicates that the failure of GVBD is mainly due to a decrease in CDK1 activity rather than the loss of cyclin B1 and CDC20 proteins. However, in fig 5(j), western says that the expression of Cyclin B1 and CDC20 proteins increases in rtcb k/out mice, not decrease. It is contradictory to what they wrote and what the western says.
Response: We have new experimental results in Fig. 6 demonstrating that CDK1 activitation was inhibited in Rtcb cKO oocytes, which causes of abnormal meiotic recovery. The expression of Cyclin B1 and CDC20 proteins were modestly increased in Rtcb cKO oocytes, may be caused by a mechanism that tries to overcome the defect of CDK1 activation but failed to rescue GVBD.

Line 206: As a result, the GVBD rate of Rtcb-null oocytes overexpressing CDK1T14A, Y15F was approximately 70% (Fig. 5L). Please rewrite the sentence. Line 252 - Unreported function of RTCB in meiosis in mouse oocytes – authors can use mouse oocyte maturation instead of oocyte meiosis (rewrite it).
Response: We rephrased these sentences during revision.

Line 267 - decreased RAD50 protein levels may lead to GVBD failure – authors haven't shown any western for the RAD50. How they can be sure about the decrease in RAD 50 protein level. As they reported the Rad 50 transcription in fig 4(b)
Response: We detected RAD50 protein levels in GV oocytes of WT and *Rtcb* cKO mice. Consistent with the RT-PCR results, RAD50 protein level decreased in *Rtcb*-deleted oocytes. This result was added to the revised Fig. 4E.

Line 277 - GVBD failure, premature ovarian failure, fertilization - No aspect of fertilization was studied in this study.
Response: We have rephrased this sentence to avoid inaccuracy. We have our revised manuscript edited by an academic English editing company (Endigo) to improve the language.

The authors did not show any data related to the possible mechanism of immature ovarian failure. The author should check early meiosis prophase defects. For example, the loss of oocytes in ovaries defective synapsis takes around two months (Rinaldi et al., 2017 Mol cell). If possible, the authors should check for this case.
Response: We appreciate the reviewer's comments. However, the *Gdf9-Cre* is only expressed in oocytes after primordial follicle formation, which occurs in mice approximately at day 1-3 after birth. In contrast, female germ cells go through early meiosis prophase before birth, at embryonic day 11-13 in mice. Therefore, *Rtcb* was not deleted in oocytes in the $Rtcb^{fl/fl}$;*Gdf9- Cre* mice until postnatal day 3. Theoretically, these mice should not have early meiosis prophase defects because *Rtcb* gene is intact at this stage.

Reviewer 2
Zhang et al. present an interesting study that highlights the vitality and complex nature of RNA processing mechanisms in female reproduction and early embryo development. Specifically, they examine the role of RNA terminal phosphate cyclase B (RtcB) at multiple critical junctures in oocyte growth and maturation, as well as during the transition to embryo. They claim that RtcB regulates a number of processes in this window of mouse gamete and embryo development, with oocyte-specific loss of RtcB leading to female infertility and premature ovarian failure. They propose this occurs due to the aberrant accumulation of maternal intron- containing transcripts, including those important for DNA methylation. They further demonstrate that RtcB-deficient oocytes imperfectly fail to resume meiosis, and partially rescue this resumption by exogenous expression of CDK1. Ultimately, they highlight the downstream effect of reduced oocyte developmental competence with loss of RtcB – oocytes either fail to undergo fertilization, progress through zygotic genome activation, or undergo development to the blastocyst stage. The experiments conducted in this study are technically challenging and many are quite rigorous, which is notably difficult in a

conditional knock-out system in which oocyte development is innately affected. Also the topic of RNA processing in development is an exciting and important one, particularly in this case the regulation of gene expression via alternative splicing during prolonged storage of maternal mRNA in mouse oocytes, and I believe there would be much enthusiasm for this subject matter. However, there are a number of major concerns that must be addressed before publication is warranted in Development:

Response: We appreciate the reviewer's positive assessment of our manuscript and constructive suggestions to improve the quality of our study. In response to the reviewer's suggestion that the quantification of global mRNA levels should be shown, we have provided additional RNA-seq analyses in *Rtcb* KO oocytes, made extensive efforts to provide more background information and experimental details, adjust the interpretations and conclusions, and improve the quality of our paper during revision.

Major concerns:

(1) At times, the narrative of the manuscript text is not supported by data within the figures. In other instances, conclusions drawn from experimental data are overstated. Four examples follow:

• Lines 160-161: The authors claim that "Rtcb deletion leads to IR accumulation and decreased global mRNA levels." Fig 3 demonstrates downregulation of many genes, but a global reduction of total mRNAs is not shown. For this, total reads in cKO and control oocytes must be quantified, with robust normalization to ERCC spike-ins.

Response: We are sorry for the oversight. We added the quantification results showing that total mRNA abundance is reduced in *Rtcb*-deleted oocytes in the revised Figure 3E. The results were normalized to ERCC spike-in.

• While DNMT1, DNMT3A, and DNMT3B appear to be reduced at the protein level (Fig 4D), only data for Dnmt3b is presented at the mRNA level (Fig 4B), and total mRNA appears to be nonsignificantly increased, rather than decreased, as would be expected given the authors hypothesis for regulation by RtcB. Analysis of mRNA levels for other Dnmt mRNAs, as well as a discussion of alternative proposed mechanisms, would clarify this finding.

Response: These data from RNA-seq have been added in revised Fig. 4D, as expected, the mRNA levels for other *Dnmt* mRNAs were decreased as well. We also discussed the potential alternative functions of RTCB in oocyte development and maternal-to-zygotic transition.

• Line 226-227: "These results reveal that maternal RTCB is indispensable for fertilization and early embryo development." The authors demonstrate successful fertilization and development to the blastocyst stage (although reduced compared to controls). Deficits in developmental progression at this stage are likely due to a reduction in competence of the oocyte and claiming that this factor is indispensable for fertilization and embryo development is an overstatement.

Response: We appreciate the reviewer's comments. We agree with the reviewer that deficits in developmental progression at this stage are likely due to a reduction in competence of the oocyte. We rephrased this sentence to avoid overstatement.

• Line 234 states that "transcriptional activation was significantly suppressed" in RtcB-deficient oocytes. However, the authors cannot claim that transcriptional activation in the embryo is suppressed based on the expression of a handful of ZGA genes. While challenging, a transcriptional run-on assay would allow for this claim and significantly elevate these findings.

Response: Thanks for reviewer's comments. However, transcriptional run-on assay is difficult for embroys. So we examined the overall transcriptional level in embryos by labelling newly synthesized RNAs with 5-ethynyl uridine (EU), and the results showed that the transcriptional activity in cKO embryos decreased compared with WT.

(2) Often, unclear language is used, or not enough information is provided to discern experimental rationale, methods, rigor, and statistics.

• In Fig 2, the authors claim that oocyte-specific RtcB loss leads to premature ovarian failure at 8- 10 weeks. They display quantification of ovary weights at each time point, but do not clearly state how many mice were used for this analysis. Further, they claim a loss of primordial, primary, secondary, and antral follicles at 8-10 weeks, but only show one representative histology image from each stage. Quantification of follicle type at each time point would help to discern exactly what stage is affected, and providing total numbers would add necessary rigor to this result.

Response:
1) We are sorry for the oversight. We used 8 ovaries for each genotype, and the number of ovaries were supplied in the revised Fig. 2B.
2) As reviewer's suggestion, we performed follicle counting on 6- and 8-weeks-old WT and *Rtcb*$^{oo-/-}$ ovaries. The results showed that the ovaries of 6-week-old *Rtcb*$^{oo-/-}$ mice (not stimulated by exogenous gonadotropins) contained similar numbers of primordial, primary, and bilayer secondary follicles relative to the WT mice, but fewer secondary and antral follicles with multilayer granulosa cells (Fig I). Futher, the number of follicles at all levels in the ovary decreased significantly at 8 weeks, which illustrate that lack of RTCB caused premature ovarian failure in female mice after 8 weeks of age.

In Fig 3C-D, the authors do not state in the main text, or in the methods, how they define SN vs NSN. Did they observe any oocytes in a partly surrounded nucleolus (pSN) pattern?
Response: We have added the definition of SN and NSN in the revised main text: 'And in this stage, the GV oocytes can be divides into two classes based on their chromatin configuration, some of them are non-surrounded nucleolus(NSN) oocytes, whose chromatin does not form a Hoechst-positive rim surrounding the nucleolus and the NSN oocytes have low developmental competence. The others are surrounded nucleolus(SN) oocytes, whose chromatin forms a Hoechst-positive rim surrounding the nucleolus, and the transcription in SN oocytes are silenced(Tan et al., 2009).' Yes, there are some oocytes in a partly surrounded nucleolus (pSN) pattern, and we included them in the NSN group for both WT and *Rtcb* knockout oocytes.

- More detail should be provided about the precise stage of oocytes that were isolated for RNA sequencing.

Response: We provided details about the stage of oocytes for RNA-seq in the revised Materials and Methods.

- Statistical analysis of their RNA sequencing experiments states that a fold change >2, p-value < 0.05, and FDR < 0.05 was used (Lines 142-144). It is very unlikely that the p-value and FDR are equivalent in a transcriptome-wide differential expression analysis. The authors should more clearly state if a false discovery correction was used, and if so, by which method.

Response: It's a mistake during the writing. Actually we used FDR in this statistical analysis, and we deleted the p-value. We are very sorry for the oversight.

- The authors use ERCCs as a tool to normalize total copy number. They should demonstrate how they used these synthetic RNAs to normalize, or that the levels of these RNAs were at least unchanged across their samples.

Response: We described the use of ERCC as a tool to normalize total transcript copy numbers in the revised Materials and Methods: After sequencing, by comparing with ERCC reference genome, the relative overall content of total RNA of initial biological samples is deduced according to the different proportion of ERCC among different samples. If the ERCC ratio of sample 1 after comparison is A, the ERCC ratio of sample 2 is B, and the total RNA level of sample 1 is 1, the relative total RNA level of sample 2 is A / B.

- What is the CASH software? The authors should provide a reference or detail its use in methods.

Response: In the revised manuscript, we used another software rMATS, to detected the alternative splicing. And we provided a reference about rMATS in the revised Maternals and Methods.

- Line 163-164: "The results showed that the expression levels of most of the transcripts decreased (Fig. 4A)." The authors should provide fold-change cutoffs or statistics to add robustness to this finding.

Response: We provided fold-change cutoffs and more details about Fig. 4A in Table 2.

(3) Necessary controls were overlooked. In Fig 1G, the authors present a key finding, that oocyte-specific conditional knock-out females are infertile. They generate these mice by crossing Rtcb-floxed mice with oocyte Gdf9-Cre mice. However, as a control, it is important to show that the Rtcb-floxed females from which the conditional line was derived do not display impaired

fertility, which could underly the infertility observed in resultant lines.
Response: We appreciate the reviewer's comments. The Gdf9-Cre mouse strain has been widely used in the field of reproductive biology and has been proved to have normal fertility by itself (Lan ZJ, Xu X, Cooney AJ. Biol Reprod. 2004 Nov;71(5):1469-74. PMID: 15215191). The same *Rtcb* floxed mouse strain has been reported to have no reproductive defects in a published study (Li R, et al., Reproduction. 2021 Nov 10;162(6):461-472. PMID: 34591784). We have stated that the *Gdf9-Cre* and *Rtcb* floxed mice are fertile and cited these references in the revised manuscript.

(4) The western blot image in Fig 5J is hard to visualize, particularly for p-T161-CDK1, which is a critical finding. The image looks darker on the WT side and it is difficult to visualize the band.
Response: We used another p-T161-CDK1 antibody from Abcam and WB results showed, in agreement with previous conclusions, that p-T161-CDK1 was significantly reduced in the cKO oocytes (the antibody would detect p-T160-CDK3, i.e., the second band in the revised Fig. 6A, which was not significantly changed).

Minor concerns:
The title of "mouse oocyte development and maintenance" is a unclear. Also, while the authors demonstrate that RtcB is essential for oocyte and/or embryo developmental competence, they do not necessarily show that this is done through alternative splicing, as the title suggests.
Response: We appreciate the reviewer's comments and rephrased the title as 'RNA Ligase RTCB Regulates mRNA Alternative Splicing and is Required for Mouse Oocyte Development and Maintenance'. In this way, we weakened the claim that RTCB maintains oocyte and/or embryo developmental competence through alternative splicing. So far the best known biochemical function of RTCB is in mRNA alternative splicing. Therefore, it is logical to attribute its biological role in oocytes to changes of alternative splicing. As suggested by the reviewer, we also discussed alternative mechanisms by which RTCB functions in oocytes and regulates female fertility.

Grammar throughout the manuscript is poor, and language is unclear. Some examples as follows: o Line 41-42 "During the development from growing oocytes to fully grown oocytes, an increase in volume is coupled with active transcription events." o Line 44-45: "a single gene can generate multiple transcripts and protein variants, which significantly increases various transcriptomes and proteomes."
Response: We have our revised manuscript edited by an academic English editing company (Endigo) to improve the language.

Further discussion on the following topics would enhance the significance of the conclusions for the reader:
In Fig 2, the authors demonstrate a dramatically reduced number of follicles across 6-10 weeks. How do they propose that all follicular stages of growth disappeared in such a short period of ~4 weeks? Did they visualize cell death or corpora lutea?
Response: As reviewer suggested, we examined the signal of phosphorylated H2AX (γH2AX, a unrepaired DNA lesions marker) in oocytes on sections and found that in *Rtcb*$^{oo-/-}$, more than 50% of oocytes in secondary and antral follicles had obvious DNA damage signal (Fig 2J and K). And we found many follicles with TdT-mediated dUTP Nick-End Labeling (TUNEL) positive signals in cKO ovaries, indicating that granulosa cells underwent apoptosis.

Fig 3I demonstrates the various categories of splicing detected via their analysis. Intron retention is actually relatively infrequent compared to some others. Does this give the authors pause about the function of RtcB in oocytes? Do they have thoughts about potential other mechanisms through which RtcB might be acting in the oocyte?
Response: We performed go analysis on transcripts that underwent alternative splicing events other than intron retention and showed that these transcripts were mainly enriched in RNA associated, but did not contain transcripts related to DNA repair and epigenetic modification, as we found in IRFinder. In the case of intron retention, we believe that the result of IRFinder is more reliable. Indeed, RtcB might have another mechanism to affect alternative splicing in oocytes, but we currently lack direct evidence to illustrate this.

Reviewer 3:
In this manuscript, Zhang et al. seek to understand how RNA terminal phosphate cyclase B (RTCB)-mediated RNA ligation influences mouse oocyte development. The authors conditionally depleted RTCB in early-stage oocytes using a Cre-Lox system and characterized oocyte growth and embryo development at various stages, from single-cell division to the zygotic genome activation (ZGA). They showed that loss of RTCB resulted in female mouse infertility, which was influenced by changes in alternative splicing patterns of the DNA replication machinery transcripts. While the findings are interesting within the context of female reproduction, the study would benefit from experiments that better solidify the conclusions being drawn in the manuscript.
Response: We appreciate the reviewer's positive assessment of our manuscript and constructive suggestions to improve the quality of our study. In this view, we have made extensive efforts to provide more experimental details, elaborate on our interpretations and conclusions, and improve the quality of the content during revision.

Major Comments
- The reader would benefit from a more detailed description of oogenesis (i.e., the stages of development) and the relationship to mRNA (i.e., expression and splicing) in the Introduction section.

Response: We have added a more detailed description of oogenesis and the relationship to mRNA in the revised Introduction, as the reviewer suggested.

- The definition of an exitron should be made clearer in the Introduction section. From my understanding, an exitron is not actually a regular/canonical retained intron. Based on the results of this study, it seems the authors are focusing on canonical intron retention events rather than exitron splicing. This is a main concern of this manuscript for us. This needs to be addressed in the abstract. As it is, it sounds that all the paper would talk about exitrons and based on what we read in the manuscript, authors are investigating regular IR.

Response: To avoid confusion, we replaced 'exitron' with 'intron retention (IR) in the revised manuscript.

- The authors should be sure to define abbreviations in figure legends and in the main text and minimize the number of abbreviations. As the manuscript is now, it is difficult to follow the flow on the story due to so many abbreviations.

Response: As suggested by the reviewer, we have defined abbreviation in figure legends and main text when they first appear, and did the best to avoid using abbreviations.

- The conclusions being drawn from Fig. 1D are weak given the immunofluorescence images. The authors should perform a subcellular fractionation study and blot for RTCB to confirm with an orthogonal approach that RTCB is present in both cellular compartments.

Response: Because only a small number of oocytes were obtained from mice, the regular methods of nuclear and cytoplasmic separation are not applicable to oocytes. Therefore, we used the nuclear and cytoplasmic separation samples of 293T cells for WB, and the results showed that RTCB existed in both cellular compartments (revised Fig. 1F).

- The use of a Cre-Lox system is a strong model for studying the developmental effects of RTCB depletion. The authors nicely showed that in mice lacking RTCB in the oocytes, the female mice were infertile (Fig. 1G). Important controls are missing, however, from the validation of the conditional knockout that should be included (e.g., Cre-negative line and a WT; Gdf9-Cre line).

Response: We appreciate the reviewer's comments. The Gdf9-Cre mouse strain has been widely used in the field of reproductive biology and has been proved to have normal fertility by itself (Lan ZJ, Xu X, Cooney AJ. Biol Reprod. 2004 Nov;71(5):1469-74. PMID: 15215191). The same *Rtcb* floxed mouse strain has been reported to have no reproductive defects in a published study (Li R, et al., Reproduction. 2021 Nov 10;162(6):461-472. PMID: 34591784). We have stated that the *Gdf9-Cre* and *Rtcb* floxed mice are fertile and cited these references in the revised manuscript.

- The authors claim that depletion of RTCB leads to decreased global mRNA levels (line 161); however, they did not directly quantify this. Given that the authors already collected RNA for sequencing analysis, a figure confirming that total mRNA abundance is reduced would be

beneficial to the reader and support the claim being made.
Response: We are sorry for the oversight. We added the quantification results showing that total mRNA abundance is reduced in *Rtcb*-deleted oocytes in the revised Figure 3E. The results were normalized to ERCC spike-ins.

- Line 162: authors stated that they analyzed 91 transcripts with IR changes; however in Fig. 3I they said that there were 42 IR events. I don't see how these two statements coexist.

Response: We are sorry for the confusion. The 91 transcripts with IR changes were founded by IRFinder software, and the Fig.3J was detected by rMATS software, so the results about IR events were different. We have added references about this two software in the methods and main text.

- The authors posit that the observed IR in the RTCB-depleted cells leads to loss of protein expression. For the transcripts exhibiting IR, the authors should interrogate the rate of nonsense-mediated decay (NMD) of these candidates to confirm that the IR results in unstable mRNA that is rapidly turned over. This would also be an orthogonal approach to validate that RTCB depletion causes decreased mRNA level (see previous point).

Response: We appreciate the reviewer's comments. However, It is difficult for us to detect the NMD rate in oocytes directly, but we found that the intron retention of these transcripts affected their open reading frame, so these transcripts are likely to be degraded through NMD pathway. We have stated the potential involvement of nonsense-mediated decay pathway in the revised Discussion.

- In Fig. 3I, the authors show the distribution of splicing changes when RTCB is depleted; however, the number of transcripts affected by IR is only 4% of the total changes occurring. Are DNA replication transcripts affected only in the cases of IR? What are the common families of transcripts being alternatively spliced in each class? The authors should elaborate on their findings here.

Response: As reviewer suggested,we did the gene ontology (GO) enrichment analysis, and the results showed that the relevant transcripts are mainly enriched in RNA processing (revised Fig. S1), but did not contain transcripts related to DNA repair and epigenetic modification, as we found in IRFinder. RtcB might have another mechanism to affect alternative splicing in oocytes, but we currently lack direct evidence to illustrate this. We discussed this point in the revised Discussion.

- The authors show a clean recovery of the GVBD rate when they overexpress CDK1. An explanation of how the GVBD rate was calculated and quantified (Fig. 5A) would benefit the reader. If there are representative images showing this process, it would be helpful to include them as well.

Response: We have offered an illustration about how the GVBD and PBE rate was calculated in revised Fig. 5A.

- Authors need to describe the statistical analysis that has been performed in the RNA-seq analysis.

Response: We described the statistical analysis that has been performed in the RNA-seq analysis, in the revised Materials and Methods.

Minor Comments

- Authors need to follow the guidelines of gene symbols, protein symbols, RNA symbols. Based on our understanding only gene symbols should be italicized. RNAs should be on regular font.

Response: According to the Guide to Nomenclature for Genes in Mouse and Rat, RNAs should be italicized.
http://www.informatics.jax.org/mgihome/nomen/short_gene.shtml

- Avoid red-green combinations (color blind readers).

Response: We have avoided red-green combinations in the revision, as the reviewer suggested.

- The authors should be sure to define abbreviations in figure legends and in the main text (e.g., GV, GC, GCBD in Fig. 1).

Response: In the revised manuscript we have paid attention to define all abbreviations in Figure

legends and in the main text when they first appear.

- The authors need to include their sources when referencing previous studies. For example, the authors write “According to a previous study, approximately 5% of tRNA genes in mice contain introns” (lines 71-72), but there is no corresponding citation. Similarly, in the Discussion section (lines 252-254) the authors write “According to previous studies, IR occurs…and 16S rRNA splicing” but there are no references.

Response: We are sorry for the oversights. We have carefully checked the manuscript and provided necessary references.

- In the Abstract, “play” should be “plays” (line 34).

Response: We have made corrections. Thanks to the reviewer for pointing our the error.

Second decision letter

MS ID#: DEVELOP/2022/200497

MS TITLE: RNA Ligase RTCB Regulates mRNA Alternative Splicing and is Required for Mouse Oocyte Development and Maintenance

AUTHORS: Hua Zhang, Jun-Chao Jiang, Yun-Wen Wu, Yuan-Song Yu, Hua-Nan Wang, Nai-Zheng Ding, and Heng-Yu Fan

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees agree the manuscript is of interest to the field. However, concerns on rigor the study remain. Primarily, concerns that controls were not run in the same experiment as test need to addressed, as to concerns of statistical analysis. In addition, detailed experimental methods need to be provided. Development takes rigor of the work being presented very seriously, thus, it is extremely important that all of the reviewers recommendations that address of the rigor of the analysis be addressed experimentally and with details in the text.If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will only provide this final round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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

Reviewer 1

Advance summary and potential significance to field

The authors have done a good job revising their manuscript and it is clearly improved. The clarity of the writing can still be improved (see below).

Comments for the author

Line 46: Through this mechanism, multiple transcripts and protein variants can be transferred and then translated from a single gene, which significantly enriches the transcriptomes and proteomes (Braunschweig et al., 2013; Stamm et al., 2005).

Through what mechanism?

Multiple transcripts and protein variants can be transferred where?

Which significantly enriches the transcriptomes and proteomes of what?

It contributes to mediating developmental transitions or cellular stress responses in some special cells or under certain conditions

And in this stage, the GV oocytes can be divides into two classes based on their chromatin configuration, some of them are non-surrounded nucleolus (NSN) oocytes, whose chromatin does not form a Hoechst-positive rim surrounding the nucleolus and the NSN oocytes have low developmental competence. The others are surrounded nucleolus (SN) oocytes, whose chromatin forms a Hoechst-positive rim surrounding the nucleolus, and the transcription in SN oocytes are silenced (Tan et al., 2009)

GV oocytes can be divided into two classes based on their chromatin configuration

Line 72: splicing after intron removal(Tanaka et al., 2011c).

removal – space

Line 73: contain introns(Wu and Hopper - Space

Fig1:

Immunofluorescence (IF) results showed that RTCB distributed in both the nucleus and cytoplasm of oocytes; Rtcb-deleted oocytes were used as a negative control (Fig. 1D).

Fig: 1E

Not necessary to mention that Rtcb-deleted oocytes were used as a negative control. This can go through in the writing.

Fig1 G

Good to use no of pups per mouse instead of no. of litter per mouse. Usually each litter consist no. of pups (range varies from strain to strain).

As a result, Rtcb –/– female mice were completely sterile (Fig. 1G)

Where is the explanation of the result? What do authors mean by as a result?

According to previous reports, the Gdf9-Cre and Rtcb-floxed mice are fertile (Lan et al., 2004; Li et al., 2021)

What do the authors want to say? If I am right both the single cre and floxed mice are fertile however the mutant is sterile?? Please write it properly to convey the message.

Line 150:

Rtcb deletion in oocytes leads to DNA damage and facilitated follicle atresia

What does the author mean by “facilitated follicle atresia?” Want to say DNA damage induced follicular atresia? Or unrepaired DNA damage induces follicular atresia?

Line 183: splicing events(Shen et al., 2014).

Space

Line 204:

The RT-qPCR products were used to run the DNA gel, the running results confirmed that IR occurred on these transcripts

Awkward sentence. Please rewrite.

Line 223:

The timepoints for counting the development rate and the calculation method of the corresponding population have been provided in Fig 5A.

Please insert the scheme in the running story. The sentence is completely isolated and not necessary.

Line: 286

embryos, ,

Line 288: Compare with Rtcb♀+/♂+ embryos, EU signals were decreased in Rtcb♀-/♂+ embryos (Fig 7H and I).

Compared

How about

EU signals were decreased in Rtcb♀-/♂+ embryos compared with Rtcb♀+/♂+

Line 306: rRNA splicing(Maughan

Line 315: we detected the apoptosis signal of granulosa cells on ovarian sections at this stage

Only GC's show TUNEL +ve? Or oocytes also?

Line 321: Accumulation of many IR-containing mRNAs in the GV stage, including DNA methylation (Mbd1, Dnmt3b) and DNA damage repair-related mRNA (Rad50).

If these transcripts from oocytes? How come the granulosa cells show apoptosis? Rewriting is necessary

Line 327: RAD50 is crucial for DNA damage repair, and decreased RAD50 protein levels may lead to GVBD failure

Please refer to the relevant publications

MBD1 and DNMT3B are critical for the regulation of DNA methylation

Please refer to the relevant publications

341 to 347: Please rewrite the paragraph

Line 377- 378 : To construct Rtcb conditional knockout mice with loxp flanking on both sides of the fourth exon, KO First mice were mated with mice expressing FLP recombinase to delete their neo cassette inserted between the FRT sites.

Poor prose. Please rewrite. KO First mice???

Line 463: after picking the cells into 350 mL lysis buffer supplied in the RNeasy micro kit (QIAGEN)

350 mL lysis buffer? Is it right?

Line 713: Illustration of the timepoints and groups when the rates were calculated for experiments in

Rephrase the sentence.

Reviewer 2

Advance summary and potential significance to field

Zhang et al. examines the role of RNA terminal phosphate cyclase B (RtcB) across the life of the oocyte and transition to embryo, and present data showing that RtcB regulates a number of processes in this window of mouse gamete and embryo development.

I still believe that there will be much enthusiasm for this subject and that it warrants publication in Development, and while I do think that a lot of the reviewers concerns were addressed in this revised manuscript, a few critical concerns still remain.

Comments for the author

Major concerns:
In a handful of instances, the authors have not demonstrated rigorous statistical and experimental methods to support their conclusions. Mainly:
(1) The authors claim an overall reduction in total mRNA in KO oocytes. In my initial review, I pointed out that normalization to spike-in controls is necessary to draw this conclusion. The authors mentioned that they did normalize to ERCC spike-ins with this revision, but the methods are still unclear. In the Methods (Lines 468-471), they state that "by comparing with ERCC reference genome, the relative overall content of total RNA of initial biological samples is deduced according to the different proportion of ERCC among different samples." This is not clear. Did they actually normalize the number of reads to the number of ERCC reads (functioning as the control sequences across all samples)? Or did they just compare the number of ERCC reads in each sample? They need to clarify that this was properly normalized to make the claim of a global reduction in mRNA, which, in my opinion, is a major finding of the manuscript.
(2) Figure 3E (Line 679): The authors present a statistical P-value <0.01.
However this analysis was likely done with a very large number of genes (likely all genes expressed in the dataset). Multiple testing correction needs to be done in this case before the authors can conclude a significant result.
(3) Figure 6A: The level of CDC25B does not visually appear reduced in depleted oocytes compared to WT, as stated in the text. Could the authors provide quantification to support their conclusion?
(4) Figure 7I: Can the authors please present the total number of embryos
(n) for this experiment? This is especially important given the borderline level of significance presented and the very strong claim of ZGA being partially suppressed. With a borderline level of significance, a high number of embryos should be stained to support a finding such as this.
In addition, there are a number of instances of mis-labeled figures or figures that are out of order within the main text, making it difficult for the reader to follow. Following are some instances I identified, but the manuscript should be proof-read diligently to ensure the figures are appropriately presented and labeled within the text.
a. Fig 1G is presented prior to Fig 1F in the text.
b. Fig 1E is not mentioned in the text at all. I assume it should be in Line 117 but it is not.
c. Line 122: Should be Fig 1H d. Line 138: Fig D and H should be Fig 2D and H e. Line 210-211: Should be Fig 4E f. Line 230: Fig 5H appears in the text prior to Fig 5F, G

Minor concerns:
(1) The abstract is difficult to read due to some issues with writing and the order in which data is presented.
a. For ease of reading, the order of data presented in the text should match that of the abstract. In the current abstract, follicle development is presented at the end, whereas in the text, it is an earlier finding.
b. Line 30: “In some special cells or under certain conditions” is unclear.
c. Line 32: What is the purpose of “mainly” here? Compared to what other function is splicing regulation the main function?
d. Line 34: “recovery” should probably be “resumption”.
e. Line 34: “the lack of” should probably be “loss of”.
(2) There are a number of errors in grammar, spelling, or word choice throughout the manuscript. A couple examples include (although not an exhaustive list):
a. Line 79: “divides” should be “divided”
b. Line 116: Should be “RTCB is distributed”
c. Line 319: “restoring” should possibly be “resuming”
d. Line 321-322: “RTCB may be related to the regulation” is unclear.
Possibly “The function of RTCB may involve regulating…” or “RTCB functions in the regulation of…”
e. Lines 321-325: This is a confusing run-on sentence.
f. Line 336: “defects are brought to the zygotes” should possibly be
“defects become evident at the zygote stage”.
g. Line 342: Should be “a large number of alternative splicing events were found”
h.
(3) The authors on multiple occasions state that they are “confirming a hypothesis”, which should be restated as “testing a hypothesis”.
a. Line 93 b. Line 258
(4) Line 110: “significantly” increased should not be stated unless statistics are provided, which they are not.
(5) Line 192-193: It is unclear how the authors are “confirming” that IR accumulation is leading to changes in total mRNA here. They appear to just be presenting the same data as a heatmap, which is not a confirmatory analysis.
(6) Line 237: Is CDK1 activity “low” or just reduced compared to WT?
(7) Figure 5G: The y-axis is not clear. Could the authors clarify what the
“rate of GV oocytes” is? Do they mean the proportion of oocytes that are GV?
(8) Line 339-340: The authors have demonstrated that, indeed, RTCB-regulated IR splicing is important at multiple junctures of female gamete development.
However, a discussion of the incomplete phenotype would enhance this section i.e., some of the KO embryos progress all the way to the blastocyst stage. Can the authors comment on the different defects that they’ve discovered in both the oocyte and embryo, and how these defects might allow for developmental progression of a small portion of these embryos to the blastocyst stage?
(9) Lines 341-347: The authors briefly discuss other types of alternative splicing events affected by RTCB that they discovered, and mention an inconsistency in splicing events that they discovered between alternative splicing algorithms. Is it possible that this is an artifact of the different algorithms used to assess alternative splicing? At the very least, they should briefly discuss these various algorithms, why each was chosen for their respective analysis, and if any possible artifacts of the algorithms exist.

Reviewer 3

Advance summary and potential significance to field

Overall, author addressed most of my previous comments.
The only two points that I would made are:
a. Minor: skipped exons is not the appropriate name of this type of splicing event. Those are "cassette exons" that depending on the conditions, developmental stage, patho-physiological contexts can be more included or more skipped. The term
"skipped exon" is referring to skipping and these are exons that can be both included or skipped depending on the context. "Cassette exons" is the right name of the alternative splicing type of event.

b. Major: This is related to one of my previous comments that I paste below with the response from the authors. I would leave this to the decision of the editor. I mentioned the lack of controls and authors referred to previous publications about those controls. In my personal opinion, those controls should still come from the same studies. They are controls from your studies, your approaches, protocols environmental conditions, researcher handling, etc.

The use of a Cre-Lox system is a strong model for studying the developmental effects of RTCB depletion. The authors nicely showed that in mice lacking RTCB in the oocytes, the female mice were infertile (Fig. 1G). Important controls are missing, however, from the validation of the conditional knockout that should be included (e.g., Cre-negative line and a WT; Gdf9-Cre line).
Response: We appreciate the reviewer's comments. The Gdf9-Cre mouse strain has been widely used in the field of reproductive biology and has been proved to have normal fertility by itself (Lan ZJ, Xu X, Cooney AJ. Biol Reprod. 2004 Nov;71(5):1469-74. PMID: 15215191). The same Rtcb floxed mouse strain has been reported to have no reproductive defects in a published study (Li R, et al., Reproduction. 2021 Nov 10;162(6):461-472. PMID: 34591784). We have stated that the Gdf9-Cre and Rtcb floxed mice are fertile and cited these references in the revised manuscript.

Comments for the author

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a. Minor: skipped exons is not the appropriate name of this type of splicing event. Those are "cassette exons" that depending on the conditions, developmental stage, patho-physiological contexts can be more included or more skipped. The term
"skipped exon" is referring to skipping and these are exons that can be both included or skipped depending on the context. "Cassette exons" is the right name of the alternative splicing type of event.
b. Major: This is related to one of my previous comments that I paste below with the response from the authors. I would leave this to the decision of the editor. I mentioned the lack of controls and authors referred to previous publications about those controls. In my personal opinion, those controls should still come from the same studies. They are controls from your studies, your approaches, protocols environmental conditions, researcher handling, etc.

The use of a Cre-Lox system is a strong model for studying the developmental effects of RTCB depletion. The authors nicely showed that in mice lacking RTCB in the oocytes, the female mice were infertile (Fig. 1G). Important controls are missing, however, from the validation of the conditional knockout that should be included (e.g., Cre-negative line and a WT; Gdf9-Cre line).
Response: We appreciate the reviewer's comments. The Gdf9-Cre mouse strain has been widely used in the field of reproductive biology and has been proved to have normal fertility by itself (Lan ZJ, Xu X, Cooney AJ. Biol Reprod. 2004 Nov;71(5):1469-74. PMID: 15215191). The same Rtcb floxed mouse strain has been reported to have no reproductive defects in a published study (Li R, et al., Reproduction. 2021 Nov 10;162(6):461-472. PMID: 34591784). We have stated that the Gdf9-Cre and Rtcb floxed mice are fertile and cited these references in the revised manuscript.

Second revision

Author response to reviewers' comments

Responses to Reviewers' Comments

Reviewer 1

Comment:
The authors have done a good job revising their manuscript and it is clearly improved. The clarity of the writing can still be improved (see below).

Response:
We appreciate the reviewer's careful assessment of our manuscript and constructive comments that have helped us to significantly improve the quality of our manuscript. In this view, as the reviewer suggested, we have sincere efforts to further assess and revise the manuscript to improve its over-all quality.

Comment:
Line 46: Through this mechanism, multiple transcripts and protein variants can be transferred and then translated from a single gene, which significantly enriches the transcriptomes and proteomes (Braunschweig et al., 2013; Stamm et al., 2005).
Through what mechanism?

Response:
The mechanism mentioned here refers to alternative splicing; our expression of the same may not be have been clear enough. Accordingly, the term "mechanism" has been replaced by "alternative splicing" in the revised version of the manuscript.

Comment:
Multiple transcripts and protein variants can be transferred where?

Response:
We apologize for the oversight on our part. We intended to mean transcription and not transfer, here. The mistake has been corrected in the revised manuscript accordingly.

Comment:
Which significantly enriches the transcriptomes and proteomes of what?

Response:
Thank you for pointing out the ambiguity in the text. We have replaced the ambiguous phrase with "which significantly enriches the diversity of transcriptomes and proteomes" in the revised manuscript.

Comment:
It contributes to mediating developmental transitions or cellular stress responses in some special cells or under certain conditions

Response: We rephrased the indicated sentence in the revised manuscript to avoid any misperception. The rephrased sentence is as follows:
"Specific transcripts with IR are expressed in specific cell types or situations and therefore control developmental transitions or cellular stress responses".

Comment:
Line 72: splicing after intron removal(Tanaka et al., 2011c). removal – space
Line 73: contain introns(Wu and Hopper – Space

Response:
Thank you for the detailed inspection of our manuscript. We have carefully corrected these over-sights in the revised manuscript.

Comment:
Fig1: Immunofluorescence (IF) results showed that RTCB distributed in both the nucleus and cytoplasm of oocytes; Rtcb-deleted oocytes were used as a negative control (Fig. 1D).
Fig: 1E Not necessary to mention that Rtcb-deleted oocytes were used as a negative control. This can go through in the writing.

Response:
We appreciate your comment. Accordingly, we have modified this statement in the revised manuscript.

Comment:
Fig1 G Good to use no of pups per mouse instead of no. of litter per mouse. Usually each litter consist no. of pups (range varies from strain to strain).

Response:
We apologize for the error. In fact, we actually intended to mention “no. of pups per mouse” instead of “no. litter per mouse,” as shown in the data. We have now corrected the error in the revised manuscript.

Comment:
As a result, Rtcb -/- female mice were completely sterile (Fig. 1G)
Where is the explanation of the result? What do authors mean by as a result?

Response:
The “result” mentioned here refers to the results of the fertility test, which is indeed ambiguous. Accordingly, we have deleted the phrase "As a result" in this part of the text in the revised manuscript. P.S. It should be noted that Fig 1G mentioned here should actually be Fig 1H, which we have also corrected in the revised manuscript.

Comment:
According to previous reports, the Gdf9-Cre and Rtcb-floxed mice are fertile (Lan et al., 2004; Li et al., 2021)
What do the authors want to say? If I am right both the single cre and floxed mice are fertile however the mutant is sterile?? Please write it properly to convey the message.

Response:
What we intent to convey is *Gdf9-Cre* or Flox sites alone will not affect the fertility of mice, while $Rtcb^{fl/fl}$;*Gdf9-Cre* female mice are infertile. We believe the over-emphasis here may have resulted in contextual ambiguity; therefore, we have deleted this sentence in the revised version of the manuscript.

Comment:
Line 150:
Rtcb deletion in oocytes leads to DNA damage and facilitated follicle atresia What does the author mean by “facilitated follicle atresia?” Want to say DNA damage induced follicular atresia? Or unrepaired DNA damage induces follicular atresia?

Response:
As shown in Fig 2, we intend to say that unrepaired DNA damage induced follicular atresia. We have revised the manuscript accordingly.

Comment:
Line 183: splicing events(Shen et al., 2014). Space

Response:
Thank you for detailed inspection of our manuscript. We have incorporated the space appropriately in the revised manuscript.

Comment:
Line 204:
The RT-qPCR products were used to run the DNA gel, the running results confirmed that IR occurred on these transcripts
Awkward sentence. Please rewrite.

Response:
We have rewritten the sentence and checked for other errors. The rephrased sentence is as follows:
“Visual assessment of RT-PCR products through agarose gel electrophoresis showed that IR events occur in these transcripts after RTCB depletion.”

Comment:
Line 223:
The timepoints for counting the development rate and the calculation method of the corresponding population have been
Please insert the scheme in the running story. The sentence is completely isolated and not necessary.

Response:
Thank you for the suggestion. We have inserted the scheme in the previous statement as follows: "by measuring the development rate at different time points in oocytes cultured *in vitro* (Fig. 5A)".

Comment:
Line: 286 embryos, ,

Response:
Thank you for the careful inspection of our manuscript. We have revised the error in the revised manuscript accordingly.

Comment:
Line 288: Compare with Rtcb +/+ embryos, EU signals were decreased in Rtcb −/+ embryos (Fig 7H and I).
Compared
How about
EU signals were decreased in Rtcb −/+ embryos compared with Rtcb +/+

Response:
Thank you for the suggestion. We have revised the text under consideration as suggested.

Comment:
Line 306: rRNA splicing(Maughan

Response:
We apologize for the oversight. We wish to state that we have checked and revised the manuscript accordingly.

Comment:

Line 315: we detected the apoptosis signal of granulosa cells on ovarian sections at this stage
Only GC's show TUNEL +ve? Or oocytes also?

Response:
According to our results, the TUNEL-positive signals were exclusively observed in GC's and not in oocytes.

Comment:
Line 321: Accumulation of many IR-containing mRNAs in the GV stage, including DNA methylation (Mbd1, Dnmt3b) and DNA damage repair-related mRNA (Rad50).
If these transcripts from oocytes? How come the granulosa cells show apoptosis? Rewriting is necessary

Response:
This sentence refers to the reason why GV oocytes from 6-week-old KO mice cannot resume meiosis. The IR of these transcripts was detected in the oocytes. We believe that the cause of apoptosis in GCs is the secondary effect of abnormal oocyte development on GCs, which has been reported previously (Sha QQ, et al., Cell. Mol. Life Sci.2020 August; 77(15):2997-3012.PMID: 31676962).

Comment:
Line 327: RAD50 is crucial for DNA damage repair, and decreased RAD50 protein levels may lead

to GVBD failure
Please refer to the relevant publications
MBD1 and DNMT3B are critical for the regulation of DNA methylation Please refer to the relevant publications

Response:
Thank you for your suggestion. We have incorporated the relevant references in the revised manuscript.

Comment:
341 to 347: Please rewrite the paragraph

Response: As per the reviewer's suggestion, we have rewritten the paragraph in the revised manuscript.

Comment:
Line 377- 378 : To construct Rtcb conditional knockout mice with loxp flanking on both sides of the fourth exon, KO First mice were mated with mice expressing FLP recombinase to delete their neo cassette inserted between the FRT sites.
Poor prose. Please rewrite. KO First mice???

Response:

We apologize for the confusion in phrasing of the text. As the reviewer mentioned, the original description was slightly redundant and incorrect. As a result, we have rewritten this section for better contextual flow and comprehension.

Comment:
Line 463: after picking the cells into 350 mL lysis buffer supplied in the RNeasy micro kit (QIAGEN)
350 mL lysis buffer? Is it right?

Response:
We apologize for this error. The correct unit is µL, and we have revised it accordingly in the revised manuscript.

Comment:
Line 713: Illustration of the timepoints and groups when the rates were calculated for experiments in
Rephrase the sentence.

Response:
Thank you for your suggestion. We have rewritten this sentence as follows: "Schematic diagram of oocyte culture *in vitro* and calculation method of oocyte germinal vesicle breakdown (GVBD) and polar body emission (PBE) rate".

Reviewer 2
Comment:
Zhang et al. examines the role of RNA terminal phosphate cyclase B (RtcB) across the life of the oocyte and transition to embryo, and present data showing that RtcB regulates a number of processes in this window of mouse gamete and embryo development.

I still believe that there will be much enthusiasm for this subject and that it warrants publication in Development, and while I do think that a lot of the reviewers concerns were addressed in this revised manuscript, a few critical concerns still remain.

Response:
We truly appreciate the reviewer's careful assessment of our manuscript and constructive suggestions to improve the quality of our manuscript. We would also like to thank the reviewer for pointing out that several of the concerns raised by other reviewers have been adequately addressed in the revised version of the manuscript. Accordingly, we have made sincere efforts to address the

remaining concerns of the reviewer and attempted to improve the quality of the manuscript to the best of our abilities.

Comment:
Major concerns:

In a handful of instances, the authors have not demonstrated rigorous statistical and experimental methods to support their conclusions. Mainly:

1) The authors claim an overall reduction in total mRNA in KO oocytes. In my initial review, I pointed out that normalization to spike-in controls is necessary to draw this conclusion. The authors mentioned that they did normalize to ERCC spike-ins with this revision, but the methods are still unclear. In the Methods (Lines 468-471), they state that "by comparing with ERCC reference genome, the relative overall content of total RNA of initial biological samples is deduced according to the different proportion of ERCC among different samples." This is not clear. Did they actually normalize the number of reads to the number of ERCC reads (functioning as the control sequences across all samples)? Or did they just compare the number of ERCC reads in each sample? They need to clarify that this was properly normalized to make the claim of a global reduction in mRNA, which, in my opinion, is a major finding of the manuscript.

Response:
Thank you for this insightful suggestion. As the reviewer's suggestion, we wish to clarify that we have normalized the number of reads to the number of ERCC reads. We have also described the ERCC spike-ins method in more detail as follows: If the ERCC ratio of sample 1 after comparison is A, the ERCC ratio of sample 2 is B, the total RNA level of sample 1 is 1, and the relative total RNA level of sample 2 is A/B."

Comment:
2) Figure 3E (Line 679): The authors present a statistical P-value <0.01. However this analysis was likely done with a very large number of genes (likely all genes expressed in the dataset). Multiple testing correction needs to be done in this case before the authors can conclude a significant result.

Response:
We have used "Relative mRNA copy" instead of the "Total Reads," which is not a suitable title. This result was obtained by comparing the overall mRNA levels of WT and KO mice after normalization to ERCC spike-ins. Statistical analysis was performed between the WT and KO groups (the P-value came from three biological replicates in each group), and not the comparison of different genes.

Comment:

3) Figure 6A: The level of CDC25B does not visually appear reduced in depleted oocytes compared to WT, as stated in the text. Could the authors provide quantification to support their conclusion?

Response:
We appreciate the reviewer's suggestion and accordingly quantified the WB results using ImageJ. The results showed that the level of CDC25B in depleted oocytes was reduced by 57 % as compared with that in WT.

Comment:
4) Figure 7I: Can the authors please present the total number of embryos (n) for this experiment? This is especially important given the borderline level of significance presented and the very strong claim of ZGA being partially suppressed. With a borderline level of significance, a high number of embryos should be stained to support a finding such as this.

Response:
We apologize for this oversight and truly appreciate the reviewer's insightful comment. We have provided the total number of embryos in the revised manuscript.

Considering the difficulty of obtaining *Rtcb* $^{♀-/♂+}$ 2-cell and the criticality of the experiment, we performed two independent immunofluorescence experiments and procured the data shown in Fig.7I after normalization with WT.

Comment:
In addition, there are a number of instances of mis-labeled figures or figures that are out of order within the main text, making it difficult for the reader
to follow. Following are some instances I identified, but the manuscript should be proof-read diligently to ensure the figures are appropriately presented and labeled within the text.

a. Fig 1G is presented prior to Fig 1F in the text.
b. Fig 1E is not mentioned in the text at all. I assume it should be in Line 117 but it is not.
c. Line 122: Should be Fig 1H
d. Line 138: Fig D and H should be Fig 2D and H
e. Line 210-211: Should be Fig 4E
f. Line 230: Fig 5H appears in the text prior to Fig 5F, G

Response:
We sincerely apologize for the mislabelled figures. We have proof-read the complete manuscript and corrected all the figures-associated oversights very carefully as per the reviewer's suggestion and ensured that all the figures are properly labelled and presented within the text.

Comment:
Minor concerns:

(1) The abstract is difficult to read due to some issues with writing and the order in which data is presented.
a. For ease of reading, the order of data presented in the text should match that of the abstract. In the current abstract, follicle development is presented at the end, whereas in the text, it is an earlier finding.
b. Line 30: "In some special cells or under certain conditions" is unclear.
c. Line 32: What is the purpose of "mainly" here? Compared to what other function is splicing regulation the main function?
d. Line 34: "recovery" should probably be "resumption".
e. Line 34: "the lack of" should probably be "loss of".

Response:
We truly appreciate the reviewer's suggestions and have modified the manuscript accordingly.
3) We have changed the order of data presented in the Abstract to match that of the data presented in the text.
4) We replaced the sentence with "IR is recognized as a key mechanism in the regulation of gene expression" and updated the references to the original sentence in the introduction.
5) To avoid the confusion caused by the usage of the word "mainly", we have deleted it in the revised version of the manuscript.
d and e) Thank you for the suggestion. We have revised the text under consideration as suggested.

Comment:
(2) There are a number of errors in grammar, spelling, or word choice throughout the manuscript. A couple examples include (although not an exhaustive list):
a. Line 79: "divides" should be "divided"
b. Line 116: Should be "RTCB is distributed"
c. Line 319: "restoring" should possibly be "resuming"
d. Line 321-322: "RTCB may be related to the regulation" is unclear. Possibly "The function of RTCB may involve regulating…" or "RTCB functions in the regulation

of…"
e. Lines 321-325: This is a confusing run-on sentence.
f. Line 336: "defects are brought to the zygotes" should possibly be "defects become evident at the zygote stage".
g. Line 342: Should be "a large number of alternative splicing events were found"

Response:

We sincerely apologize for the grammatical errors. We have now not only made the corresponding modifications, as suggested by the reviewer, but also ensured that the rest of the manuscript has been carefully corrected for other errors. We thank the reviewer for carefully reviewing our manuscript and helpful suggestions for improving the quality of our revised manuscript.

Comment:
(3) The authors on multiple occasions state that they are "confirming a hypothesis", which should be restated as "testing a hypothesis".
a. Line 93
b. Line 258

Response:
As the reviewer's suggestion, we have replaced "confirming" with "testing" at the specified lines in the revised manuscript.

Comment:
(4) Line 110: "significantly" increased should not be stated unless statistics are provided, which they are not.

Response:
As the reviewer's suggestion, we removed the term "significantly" from relevant portions of the text.

Comment:
(5) Line 192-193: It is unclear how the authors are "confirming" that IR accumulation is leading to changes in total mRNA here. They appear to just be presenting the same data as a heatmap, which is not a confirmatory analysis.

Response:
As the reviewer has mentioned, the word "confirm" may not be appropriate here. We intend to express here that the expression of most transcripts in which IR occurs decreases, which is caused by IR accumulation. We have used the term "determine" instead of "confirm" at relevant portions of the text, accordingly.

Comment:
(6) Line 237: Is CDK1 activity "low" or just reduced compared to WT?

Response:
Based on the results, the activity of CDK1 was reduced as compared to that of WT, and we have revised the subtitle accordingly in the revised manuscript.

Comment:

(7) Figure 5G: The y-axis is not clear. Could the authors clarify what the "rate of GV oocytes" is? Do they mean the proportion of oocytes that are GV?

Response:
The proportion of oocytes that are GV is exactly what we intended to express. We have modified this section in the revised manuscript to avoid confusion.

Comment:
(8) Line 339-340: The authors have demonstrated that, indeed, RTCB-regulated IR splicing is important at multiple junctures of female gamete development. However, a discussion of the incomplete phenotype would enhance this section, i.e., some of the KO embryos progress all the way to the blastocyst stage. Can the authors comment on the different defects that they've discovered in both the oocyte and embryo, and how these defects might allow for developmental progression of a small portion of these embryos to the blastocyst stage?

Response:
1) First, as we mentioned in the discussion, RTCB deletion affects epigenetic modification, thus affecting the developmental potential of oocytes, but this effect is not fatal. Therefore, some oocytes can still develop GVBD; however, defects still exist in these oocytes.

2) We have found that there was no abnormality in the ovaries of 4-week-old *Rtcb*$^{oo-/-}$ mice as compared with that of WT mice, indicating that the first wave of follicle activation was normal, and this portion of oocytes may suffer less damage.
Consequently, very few of these oocytes can develop into blastocysts after fertilization.

Comment:
(9) Lines 341-347: The authors briefly discuss other types of alternative splicing events affected by RTCB that they discovered, and mention an inconsistency in splicing events that they discovered between alternative splicing algorithms. Is it possible that this is an artifact of the different algorithms used to assess alternative splicing? At the very least, they should briefly discuss these various algorithms, why each was chosen for their respective analysis, and if any possible artifacts of the algorithms exist.

Response:
We appreciate the insightful comments of the reviewer. We wish to clarify that we have used different tools to achieve different purposes. To detect transcripts containing intron retention, we have used IRFinder, which detects more IR events. The purpose of using rMATS was to determine whether other types of alternative splicing are affected. As mentioned in the IRFinder article (PMID:28298237), MISO has low detection sensitivity for IR events, and the Bayes model MISO (and rMATS) used for identifying alternative splicing is not applicable for IR detection, which may be the reason for the difference.

Reviewer 3
Comment:
Overall, author addressed most of my previous comments.
The only two points that I would made are:
a. Minor : skipped exons is not the appropriate name of this type of splicing event. Those are "cassette exons" that depending on the conditions, developmental stage, patho-physiological contexts can be more included or more skipped. The term "skipped exon" is referring to skipping and these are exons that can be both included or skipped depending on the context. "Cassette exons" is the right name of the alternative splicing type of event.
b. Major : This is related to one of my previous comments that I paste below with the response from the authors. I would leave this to the decision of the editor. I mentioned the lack of controls and authors referred to previous publications about those controls. In my personal opinion, those controls should still come from the same studies. They are controls from your studies, your approaches, protocols, environmental conditions, researcher handling, etc.

Response:
We sincerely appreciate the reviewer's careful assessment of our manuscript and the constructive suggestions to improve the quality of our study.
(1) We agree with the reviewer's suggestions regarding the name of the exon skipped or included events. However, in the rMATS tool that we have used in our study, this type of event was called skipped exon. To avoid confusion, we have used the term from the relevant study (PMID:25480548).
(2) Thank you again for your comment. The wild type (WT) mice mentioned in the manuscript were *Rtcb*$^{fl/fl}$ mice. All wild-type mice used in our experiments were littermates of conditional knockout mice. The Gdf9-Cre mouse strain has been widely used in the field of

reproductive biology and has been proven to have normal fertility, as previously reported (Lan ZJ, Xu X, Cooney AJ. Biol Reprod. 2004 Nov;71(5):1469-74. PMID: 15215191).

Third decision letter

MS ID#: DEVELOP/2022/200497

MS TITLE: RNA Ligase RTCB Regulates mRNA Alternative Splicing and is Required for Mouse Oocyte Development and Maintenance

AUTHORS: Hua Zhang, Jun-Chao Jiang, Yun-Wen Wu, Yuan-Song Yu, Hua-Nan Wang, Nai-Zheng Ding, and Heng-Yu Fan

I have now received all the referees reports on the above manuscript, and have reached a decision. The overall evaluation is positive and we would like to publish a revised manuscript in Development. However, as reviewer 2 suggests, there are textual edits that need to be made to further clarify the study. I do not expect to send the manuscript back to the reviewers, however, it is imperative that you address the normalization of total RNA in the text or change the language to reflect what is being presented. Please attend to the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have done a good job revising their manuscript and it is clearly improved and ready for acceptance. Overall, the authors addressed most of my previous comments.

Comments for the author

The authors have done a good job revising their manuscript and it is clearly improved and ready for acceptance. Overall, the authors addressed most of my previous comments.

Reviewer 2

Advance summary and potential significance to field

Zhang et al. examines the role of RNA terminal phosphate cyclase B (RtcB) across the life of the oocyte and transition to embryo, and present data showing that RtcB regulates a number of processes in this window of mouse gamete and embryo development.

Comments for the author

In this second revision, the grand majority of my concerns (as well as other reviewers' concerns) were addressed. Two critical concerns remain for this reviewer:
(1) The authors claim an overall reduction in total mRNA in KO oocytes. In both of my prior reviews, I pointed out that normalization to spike-in controls is necessary to draw this conclusion. The authors mentioned that they normalized to ERCC spike-ins with the first revision, and the subsequent revision makes another attempt to explain their normalization strategy. I acknowledge the authors efforts to clarify their methods. However, it is still unclear to me how normalization was conducted. The authors state that the relative RNA content of a sample (Sample 2 in their example) is calculated by dividing the ERCC ratio of another sample (their Sample 1) by the ERCC ratio of that same sample (Sample 2). This is simply calculating a ratio between ERCC values of each sample, and is incorrect with respect to directionality in this specific example. In the example

the authors give, I would assume that the simplified normalization of Sample 1, given an ERCC value of A and a total RNA content of 1, would result in a normalized total RNA content of 1/A, which could then be compared with other samples to determine the relative RNA content in KO vs WT oocytes.
Alternatively, if ERCC reads are approximately equal between all samples, or if the differential expression of ERCC reads between KO and WT is unchanged, this would provide evidence that differential amplification of RNA between samples has not occurred.
I want to point out that I only wish to be sure that the finding the authors present is robust and supports their claim, otherwise this claim should not be made. To make the claim of a global reduction in mRNA in KO oocytes, they must demonstrate either (i) clear and appropriate methodology explaining their normalization strategy, or (ii) no change in ERCC expression between sample groups. I do not think that this concern should inhibit publication of this manuscript, but rather, that this result should not be presented if this concern is not addressed.
(2) There remain a number of errors in grammar and word choice throughout, with and the manuscript should be edited for clarity prior to publication. A non-exhaustive list follows:

- Abstract, Line 31. RTCB is not introduced prior to stating KO mouse findings.
- Abstract, Line 33-34. In line 33, “regulates” is used, and in line 34, “regulated” is used. Throughout the paper, please consider tense for ease of reading.
- Abstract, Line 35. “In contrast” here does not make sense. Prior results in oocytes devoid of RTCB were also presented.
- Line 75. “required” should be “requires”
- Line 78. “removed from the follicle environment” is unclear.
- Line 119. “on” should be “in”
- Line 219. “measuring the development rate” is not clear.
- Line 225. “oocytes could be ovulated” is also unclear.
- Line 269. “When most” should probably be “While most”
- Line 299. “transition” should be “transitions”.
- Line 329. “difficulty fertilizing” should likely be “difficulty with fertilization”
- Line 344. Use of the word “however” here is not appropriate.
- Line 345. “A series of factors” is odd phrasing here.
- Line 349-350. “leads to structural disorder of the ER” could instead be re-written for clarity to “leads to structural defects within the ER”.
- Line 354. “has a potential targeting function” is unclear.
- Line 355. “targeted treatment site” is unclear.
- Line 356. “a direction for treating potential reproductive diseases” is unclear.
- Line 357. “are functional” is vague. Can you please be more specific about what you mean here?

Reviewer 3

Advance summary and potential significance to field

Authors did acknowledge my comments but did not make changes. I would defer to the editor for the decision at this point.

Comments for the author

Authors did acknowledge my comments but did not make changes. I would defer to the editor for the decision at this point.

Third revision

Author response to reviewers' comments

Reviewer 1

The authors have done a good job revising their manuscript and it is clearly improved and ready for acceptance. Overall, the authors addressed most of my previous comments.

Response:

Thank the reviewer for the constructive suggestions, which helped us improve the quality and preciseness of the text. I'm glad that the reviewers have approved our revision.

Reviewer 2

Zhang et al. examines the role of RNA terminal phosphate cyclase B (RtcB) across the life of the oocyte and transition to embryo, and present data showing that RtcB regulates a number of processes in this window of mouse gamete and embryo development.

Response:

Thank the reviewer for the insightful and detailed suggestions in the first and second rounds of review. These suggestions play a very important role in improving the quality of the text and the preciseness of the article. We revised the text as much as possible according to these suggestions and replied to some questions.

Comment:

In this second revision, the grand majority of my concerns (as well as other reviewers' concerns) were addressed. Two critical concerns remain for this reviewer:

1) The authors claim an overall reduction in total mRNA in KO oocytes. In both of my prior reviews, I pointed out that normalization to spike-in controls is necessary to draw this conclusion. The authors mentioned that they normalized to ERCC spike-ins with the first revision, and the subsequent revision makes another attempt to explain their normalization strategy. I acknowledge the authors efforts to clarify their methods. However, it is still unclear to me how normalization was conducted. The authors state that the relative RNA content of a sample (Sample 2 in their example) is calculated by dividing the ERCC ratio of another sample (their Sample 1) by the ERCC ratio of that same sample (Sample 2). This is simply calculating a ratio between ERCC values of each sample, and is incorrect with respect to directionality in this specific example. In the example the authors give, I would assume that the simplified normalization of Sample 1, given an ERCC value of A and a total RNA content of 1, would result in a normalized total RNA content of 1/A, which could then be compared with other samples to determine the relative RNA content in KO vs WT oocytes.

Alternatively, if ERCC reads are approximately equal between all samples, or if the differential expression of ERCC reads between KO and WT is unchanged, this would provide evidence that differential amplification of RNA between samples has not occurred.

I want to point out that I only wish to be sure that the finding the authors present is robust and supports their claim, otherwise this claim should not be made. To make the claim of a global reduction in mRNA in KO oocytes, they must demonstrate either (i) clear and appropriate methodology explaining their normalization strategy, or (ii) no change in ERCC expression between sample groups. I do not think that this concern should inhibit publication of this manuscript, but rather, that this result should not be presented if this concern is not addressed.

Response:

We are sorry for the confusion caused by the method description of normalization using ERCC, and we are still trying to clarify this method. We revised the method of ERCC normalization in the new revision, as follows:

First, 10 oocytes with equal amount of ERCC were subjected to each RNA-seq library construction. And in RNA-seq data normalization, sequencing reads were mapped to ERCC reference to obtain the percentage of ERCC reads in total reads. Then gene expression levels were normalized by multiplying the raw FPKM values by a normalization factor (normalization factor = percentage of ERCC in WT oocytes / percentage of ERCC in the sample).

We very much agree that the conclusion drawn in the manuscript should be robust. However, at present, our method description of the results of the global mRNA level reduction in KO oocytes is

still not accurate enough. Considering the rigor of the experimental method, we deleted this conclusion (Fig.3E) in the revised version, and we think it has little impact on the main conclusions of the article.

Comment:

2) There remain a number of errors in grammar and word choice throughout, with and the manuscript should be edited for clarity prior to publication. A non-exhaustive list follows:

- Abstract, Line 31. RTCB is not introduced prior to stating KO mouse findings.

Response:

We sincerely appreciate the reviewer's careful assessment of our manuscript. We have added this point.

- Abstract, Line 33-34. In line 33, "regulates" is used, and in line 34, "regulated" is used. Throughout the paper, please consider tense for ease of reading.

Response:

We have checked the grammar errors in the manuscript for ease of reading.

- Abstract, Line 35. "In contrast" here does not make sense. Prior, results in oocytes devoid of RTCB were also presented.

Response:

We have replaced the "In contrast" with "Moreover".

- Line 75. "required" should be "requires"

Response:

According to the comments of the reviewers, we made modifications.

- Line 78. "removed from the follicle environment" is unclear.

Response:

We have rewritten the sentence as "stimulated by a gonadotrophin signal or released from an inhibitory follicular environment".

- Line 119. "on" should be "in"

Response:

According to the comments of the reviewers, we made modifications.

- Line 219. "measuring the development rate" is not clear.

Response:

The specific details of this sentence are mentioned in Figure Legends and Fig.5A. We do not think it is necessary to explain too much here.

- Line 225. "oocytes could be ovulated" is also unclear.

Response:

We have rewritten the sentence as "oocytes could be ovulated after hCG injection".

- Line 269. "When most" should probably be "While most"

Response:

Thanks for the suggestion of the reviewers, we have revised it.

- Line 299. "transition" should be "transitions".

Response:

According to the comments of the reviewers, we made modifications.

- Line 329. "difficulty fertilizing" should likely be "difficulty with fertilization"

Response:

Thanks for the suggestion of the reviewers, we have revised it.

- Line 344. Use of the word "however" here is not appropriate.

Response:

We have replaced the "however" with "In addition".

- Line 345. “A series of factors” is odd phrasing here.

Response:
Thanks for the suggestion of the reviewer, we have revised the sentence as “Different mRNA degradation pathway” to avoid ambiguity here.

- Line 349-350. “leads to structural disorder of the ER” could instead be re-written for clarity to “leads to structural defects within the ER”.

Response:
Thanks for the suggestion of the reviewer, we have revised it.

- Line 354. “has a potential targeting function” is unclear.

Response:
We have revised this sentence as “has potential other substrates”.

- Line 355. “targeted treatment site” is unclear.
- Line 356. “a direction for treating potential reproductive diseases” is unclear.

Response:
Considering that the reviewer thinks it will cause confusion, we deleted it in the revised version.

- Line 357. “are functional” is vague. Can you please be more specific about what you mean here?

Response:
This is a summary sentence. The specific function of transcripts in GV has been mentioned in the previous discussion. To avoid confusion, we changed this sentence into a summary of our research by the reviewer “Our study examined the role of *Rtcb* across the life of the oocyte and transition to embryo”.

Reviewer 3

Authors did acknowledge my comments but did not make changes. I would defer to the editor for the decision at this point.

Response:
Thanks to the reviewers for their comments, we supplemented the WT in the manuscript to strengthen the preciseness of our article.

Fourth decision letter

MS ID#: DEVELOP/2022/200497

MS TITLE: RNA Ligase RTCB Regulates mRNA Alternative Splicing and is Required for Mouse Oocyte Development and Maintenance

AUTHORS: Hua Zhang, Jun-Chao Jiang, Yun-Wen Wu, Yuan-Song Yu, Hua-Nan Wang, Nai-Zheng Ding, and Heng-Yu Fan
ARTICLE TYPE: Research Article

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