Maternal RNF114-mediated target substrate degradation regulates zygotic genome activation in mouse embryos

Shuai Zhou, Yueshuai Guo, Haifeng Sun, Lu Liu, Liping Yao, Chao Liu, Yuanlin He, Shanren Cao, Cheng Zhou, Mingrui Li, Yumeng Cao, Congjing Wang, Qianneng Lu, Wei Li, Xuejiang Guo and Ran Huo
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MS TITLE: Maternal RNF114 plays critical roles in activation of the early zygotic genome through promoting ubiquitin-mediated degradation of Chromobox protein CBX5

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

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

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, in which case, 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

In this study, Zhou and colleagues investigate the role of RNF114 in the maternal-to-zygotic transition. This work follows on from a previous study from the group (Yang et al. 2017) in which siRNA knockdown of Rnf114 in zygotes led to two-cell arrest. In this latest manuscript Zhou et al. generate Rnf114 knockout mouse models to show that maternal Rnf114 is essential to progress past the two-cell stage. Embryos lacking Rnf114 fail to activate the major wave of zygotic genome activation and have an accumulation of maternal transcripts. The authors then performed proteomic analysis of knockout embryos and identified 45 downregulated and 91 upregulated proteins, including CBX3 and CBX5 which they focused on further, demonstrating that CBX5 is a target of Rnf114 ubiquitination and that overexpression of Cbx5 by mRNA microinjection in zygotes lead to reduced preimplantation development and reduced major ZGA activation.

Comments for the author

Overall, the study is well conceived and very logical in its organisation and was a pleasure to read. The experiments have been performed well and appropriate controls used. My main comment is that I would like to see more data strengthening the link between Rnf114 and Cbx5 (and potentially other targets) as this is the weakest part of the manuscript. My major comments are:
1. The link between Rnf114 and Cbx5 needs further support. Ideally one would like to see a rescue experiment in which Cbx5 is knocked down by siRNA or similar in Rnf114 mutant oocytes or zygotes and the effect on the embryo development assessed. If Rnf114 is functioning by mediating the degradation of Cbx5 then inducing degradation of Cbx5 should rescue, partially at least, the phenotype of the Rnf114 mutant embryos. Is the level of overexpression in line with the proteomics? A qPCR would be useful to include here.

2. In the introduction the authors comment on data regarding Tab1, however this is not presented in the results section of the manuscript. Please move the presentation of this data from the discussion to the results section and integrate it into the manuscript. Were the experiments in supplemental figure 6 on Tab1 performed in normal or Rnf114 mutant embryos? How do the authors see Tab1 fitting into the mechanism between Rnf114 and Cbx5?

3. I would like to see more of a discussion on the other proteins that are misregulated in the Rnf114 null oocytes. How many of these overlap with the protoArray experiments performed in Yang 2017? Could the authors present the misregulated proteins in a main figure? How many of these have been linked to the MZT? Why did the authors decide to focus on Cbx3 and Cbx5 given they are not the most severely affected in terms of upregulation? The phenotype for Cbx5 overexpression is quite subtle compared to the Rnf114 phenotype. The authors speculate on this in the discussion but could some of the other hits from the proteomics analysis answer this? Perhaps this could be a way to introduce Tab1 which unfortunately was not detected in the proteomics, presumably due to its low abundance.

4. Given the biological roles for Cbx3/5 one would imagine that aspects of the nuclear structure/organisation would be affected in the overexpression embryos. Could the authors perform some stainings, for example H3K9me2/3, to determine whether there are global alterations in the embryos? Are there any changes that can be seen in the DAPI staining?

5. How as the normalisation done for the RNAseq analysis and was there any control for the total number of reads per cell? Please also show some quality control plots including the number of reads per cell and the number of genes detected per cell and comment on whether this is different

between the control and mutant embryos. Please also state in the results text how many cells/embryos were analysed.

I also have the following minor comments:
1. It would be helpful to have an initial figure showing the expression patterns of Rnf114 in preimplantation development as the authors refer to this in their previous work where it is hidden in a supplemental figure. One could also include expression patterns of Cbx3, Cbx5 and Tab1 in this figure to facilitate the biological interpretation of the data.

2. Please show individual datapoints in all the bar graphs and make sure that all western blots have the molecular weights marked.

3. Please quantify western blot in S1E as there is still some signal in the null oocytes. Are the low levels of mutant Rnf114 protein remaining in the mutant embryos catalytic inactive? For example, could the authors perform an in vitro assay using a RNF114 protein lacking the 81bp region that is deleted in the mouse?

4. Was the qPCR validation of the RNAseq analysis performed on separate biological samples or does this just represent a technical validation? This needs to be made clear in the text.

5. DAPI signal is very hard to see in Figure 2. One could consider greyscale for the individual panel as this has better visibility than blue on black.

6. Part of the western blot signal is missing in figure 5D. please show the entire band.

7. Why was H2O used as a control injection? Surely PBS would be better suited as it is isotonic?

8. Statistics are missing form figure 6F. please mark as ‘ns’ if not significant.
Thank you for this piece of work,
Melanie Eckersley-Maslin

Reviewer 2

Advance summary and potential significance to field

Zhou et al. report that Rnf114 mutant female mice are sterile. Although oocytes from these mice appear to be normal and can be fertilized, some developmental events after fertilization, which include zygotic genome activation (ZGA) and maternal mRNA/protein degradation, are severely impaired in Rnf114 maternal KO embryos, which possibly causes developmental arrest at the 2-cell stage. Given that RNF114 is a E3 ubiquitin ligase, the authors performed proteome analyses in addition to transcriptome analyses, and found that CBX5 and CBX3 protein levels are increased in Rnf114 KO oocytes as well as in Rnf114 maternal KO 2-cell embryos. Interestingly, overexpression of Cbx5 partially induced developmental arrest. Overall, the study is carried out in an appropriate manner and identify Rnf114 as an important maternal factor.

Comments for the author

However, before publication, further interpretation and discussion of the data as well as additional experiments, including some control experiments, would be necessary to support the authors’ conclusions.

Major points
1. The authors show that maternal RNAs do not decrease and the genes activated at minor ZGA are upregulated in Rnf114 m-/z+ 2-cell embryos. This suggests that developmental progression (or MZT) is delayed in Rnf114 m-/z+ embryos. Furthermore as conventional RNA-seq on 1-cell embryos is strongly biased to detect RNA from oocytes (maternal RNA), it is hard to conclude that minor ZGA is normal in Rnf114 m-/z+ 1-cell embryos from RNA-seq data. Thus, I suggest to perform EU labeling and phospho-pol II staining at the late 1-cell stage to examine whether minor ZGA is affected or not.

2. RNA-seq on MII oocytes detected more than 100 DEGs, and it is highly likely that PCA on MII oocyte data only can separate control and KO groups. However, this fact is not discussed at all in the text. Is there any DEGs related to maternal mRNA degradation or developmental progression? Similarly, is there any genes whose expression is consistently altered in proteome data?

3. Some embryos injected with Cbx5 mRNA showed developmental arrest but most of them developed normally (Fig. 6D). This suggests that the decrease in EU incorporation might be limited to some embryos, which cannot be easily seen by bar plots with SEM.
Please show all the raw data points in the graph as shown in Supplementary Fig. 6D.

4. It is unclear how much CBX5-FLAG is exogenously expressed relative to physiological CBX5 level. As CBX5 is upregulated ~1.4X higher in Rnf114 m-/z+ embryos, too much expression should be avoided.

5. The evidence that RNF114 promotes the degradation of CBX5 is not strong. At the very least, proteasome inhibitor treatment is necessary in the co-expression assay indicated in Fig. 5C-D, and data quantification should be performed.

6. The authors concluded that RNF114 is important to adjust the level of maternal factors and that ZGA is indirectly affected. Therefore, I would like the authors to reconsider the title of this paper.

Minor points
1. Although Sirt1 was used as one of the zygotic products, the evidence of this is unclear. As Sirt1 seems to be expressed in oocytes, more explanation would be necessary.

2. (line 143) Please explain the “HOM group”.

3. I would like the authors to reconsider the Discussion. For example, there are some explanation for the domains of RNF114 protein and insights into RNF114 based on previous studies, but these should be mentioned in Introduction. In addition, most of Discussion is used to merely summarize or repeat the obtained results.

Reviewer 3

Advance summary and potential significance to field

In Huo et al. the authors build on the prior paper from some in the same group, Yang et al EMBO reports 2017, where they showed the Rnf114 is a maternal effect gene using knockdown of Rnf114 in the zygote. Now using genetic knockout of Rnf114 the authors explore the role of Rnf114 in maternal to zygotic transition showing that in the absence of Rnf114 the embryos fail to progress past the 2 cell stage. Using microscopy and transcriptomics, they show that the Rnf114 maternal null embryos both fail to degrade the maternal RNA store, and do not undergo major zygotic genome activation. To tease apart the mechanism for this effect, they use proteomics in MII oocytes. They identify and validate CBX5 as a target for RNF114-mediated ubiquitynation and degradation. Cbx5 overexpression by microinjection has a modest effect on embryo development, but is similar to that of Rnf114 maternal deletion. Therefore the authors propose that Rnf114 is involved in required to degrade repressor proteins to enable maternal to zygotic transition and the major wave of zygotic genome activation.

I found this paper interesting. It is a significant extension to prior work, that reveals a role for protein degradation via RNF114 of at least CBX5 in the maternal to zygotic transition.

Comments for the author

There are some additional experiments along with some editorial changes necessary.
- The new Rnf114 KO needs further clarity. The deletion doesn’t cause a frameshift mutation but all the same appears to destabilise the protein levels. Furthermore, qRT-PCR showed a (not statistically significant) drop in RNA levels, whereas their RNAseq showed a striking and significant

reduction in RNA. Comment on this would help the reader understand the KO. Was the RNA depletion expected given that a nonsense mutation is not introduced?

- While there are very few DEGs at the 1 cell stage, what are the 13 DEG? Please include a list. Since detectably expressed genes at this stage are likely to be involved in early embryonic events, these 13 genes may themselves be required for major ZGA genes. This requires for additional explanation in the paper.
- For the proteomic analysis, it isn't explained why MII oocytes were chosen (rather than 2 cell embryos, for example). This should be added to aid the reader. In addition, the replicate numbers aren't present in the figure legend, so need to be included, as does comment about multiple testing correct (FDR referred to in methods but tables and text mention P value instead).
- Based on the proteomic analysis, it isn't clear why CBX3 and CBX5 were chosen for further analysis. There was GO term analysis of the list of proteins; however, given that the conclusion relies on the presence of repressor proteins in general in the RNF114 targeted proteins, beyond just CBX3/5, further comment on the some of the specific proteins that were found would be helpful. Further explanation is also required for the choice of specifically CBX3 and CBX5 to follow up is necessary. Were additional other repressors tried but did not validate?
- The mild phenotype with Cbx5 overexpression is promising, however the conclusions would be best supported using a rescue experiment. In this case, does Cbx5 knockdown on the Rnf114KO background rescue to a significant degree the effect of the Rnf114KO on embryo development and and ZGA (qRT-PCR)?
- The previous paper reported TAB1 as a target of RNF114. Here the authors present further work on TAB1 in the discussion and as supplemental figure, although it did not come out of their proteomic analysis. The authors need to firstly move this work to the results section, and more clearly introduce the TAB1 protein and their prior data. The published data shows a mild effect of Tab1 overexpression on embryo development (similar to Cbx5). Here they show that in 2 cell embryos TAB1 protein is higher in Rnf114KO, that there is lower transcription by EU staining, and inefficient ZGA. The authors should perform a rescue experiment, as above. In the future, it would be to test whether Tab1 and Cbx5 double knockdown can rescue the Rnf114KO defect in embryo development.
- For all the Western blot analyes, there are small changes seen in levels of proteins. The quantitation is helpful, however such small changes are hard to quantitate by Western blot. The use of an Odyssey system would be best here, as in the prior paper. In addition, all Western blot image sections are very small cuts of the gel, and sometimes don't include markers. In particular those in Figure 5B need repeating as the WB aren't clear.

Additional minor comments:
Figure 1A – N=9, does this refer to # plugs resulting in no litters, or number of females mated continually over 6 months. Please clarify.

Figure 1B – clarify that these embryos were produced by IVF (the wording is we subsequently fertilized the eggs with wild-type sperm, so this is assumed).

Figure S2A needs to be referred to the text. Can there be more similarly sized sections chosen, with a greater zoom and labelled regions that are scored in S2B?

S2C, unclear what N=24 refers to, when there were >60 mice in each group?

S3C – were there replicates for qRT-PCR? It isn't clear how this qRT-PCR was performed and more detail is required. Was it independent of the original libraries?

CBX5 IF in Rnf114KO – the images show altered CBX5 staining levels but also altered distribution.

This should be quantitated and commented on.

Figure 6B what tissue was used for the WB analysis?

For the ubiquitination validation, could the authors choose a more relevant cell type to repeat their analysis e.g. 2-cell like ES cells, or germ cell tumour cell lines?

Throughout, quite old reviews are cited (often 20 years old). These should be updated to keep it current with the field.

Language – the manuscript needs careful editing as frequently the wording leads to confusion, or, reads as an overinterpretation. Here are some examples although there are other instances, so a thorough edit is required.

Line 107 – 'we subsequently fertilized Rnf114-/- oocytes with WT sperm' – clarify it was IVF Line 165 – consider rewording inactivated to failure to activate to be more descriptive for readers.

Line 210, don't need 'Above all'. Consider 'Firstly,'

Line 244 'embryos conjugated to RNF114-damaged oocytes and wild-type sperm' – this doesn't accurately explain the situation, instead say Rnf114 null oocytes were fertilized with wild-type sperm to create embryos.

Line 322 'scavenges transcriptional inhibitor's – this reads like an overinrepretation of the data as it is currently presented.

Line 323 - Maternal loss of RNF114 leads to repressors accumulation – to make this claim there would need to be more examples given from the list of upregulated genes, beyond CBX3 and CBX5, rather than just GO terms. Are there other good examples to highlight, even without further investigation?

First revision

Author response to reviewers' comments

Point-by-Point Response:
We thank all the reviewers for the time that they have taken to assess the acceptability of this manuscript and for their helpful comments and suggestions. We have taken all the concerns into consideration as we revised our manuscript. We wish that the reviewers will find the revised manuscript is a much more improved version. Followings are our point-by-point responses to the reviewers.

Reviewer Comments:

Reviewer 1:
In this study, Zhou and colleagues investigate the role of RNF114 in the maternal-to- zygotic transition. This work follows on from a previous study from the group (Yang et al. 2017) in which siRNA knockdown of Rnf114 in zygotes led to two-cell arrest. In this latest manuscript Zhou et al. generate Rnf114 knockout mouse models to show that maternal Rnf114 is essential to progress past the two-cell stage. Embryos lacking Rnf114 fail to activate the major wave of zygotic genome activation and have an accumulation of maternal transcripts. The authors then performed proteomic analysis of knockout embryos and identified 45 downregulated and 91 upregulated proteins, including CBX3 and CBX5 which they focused on further, demonstrating that CBX5 is a target of Rnf114 ubiquitination and that overexpression of Cbx5 by mRNA microinjection in zygotes lead to reduced preimplantation development and reduced major ZGA activation.

Reviewer 1 Comments for the Author:
Overall, the study is well conceived and very logical in its organisation and was a pleasure to read. The experiments have been performed well and appropriate controls used. My main comment is that I would like to see more data strengthening the link between Rnf114 and Cbx5 (and potentially other targets) as this is the weakest part of the manuscript. My major comments are:

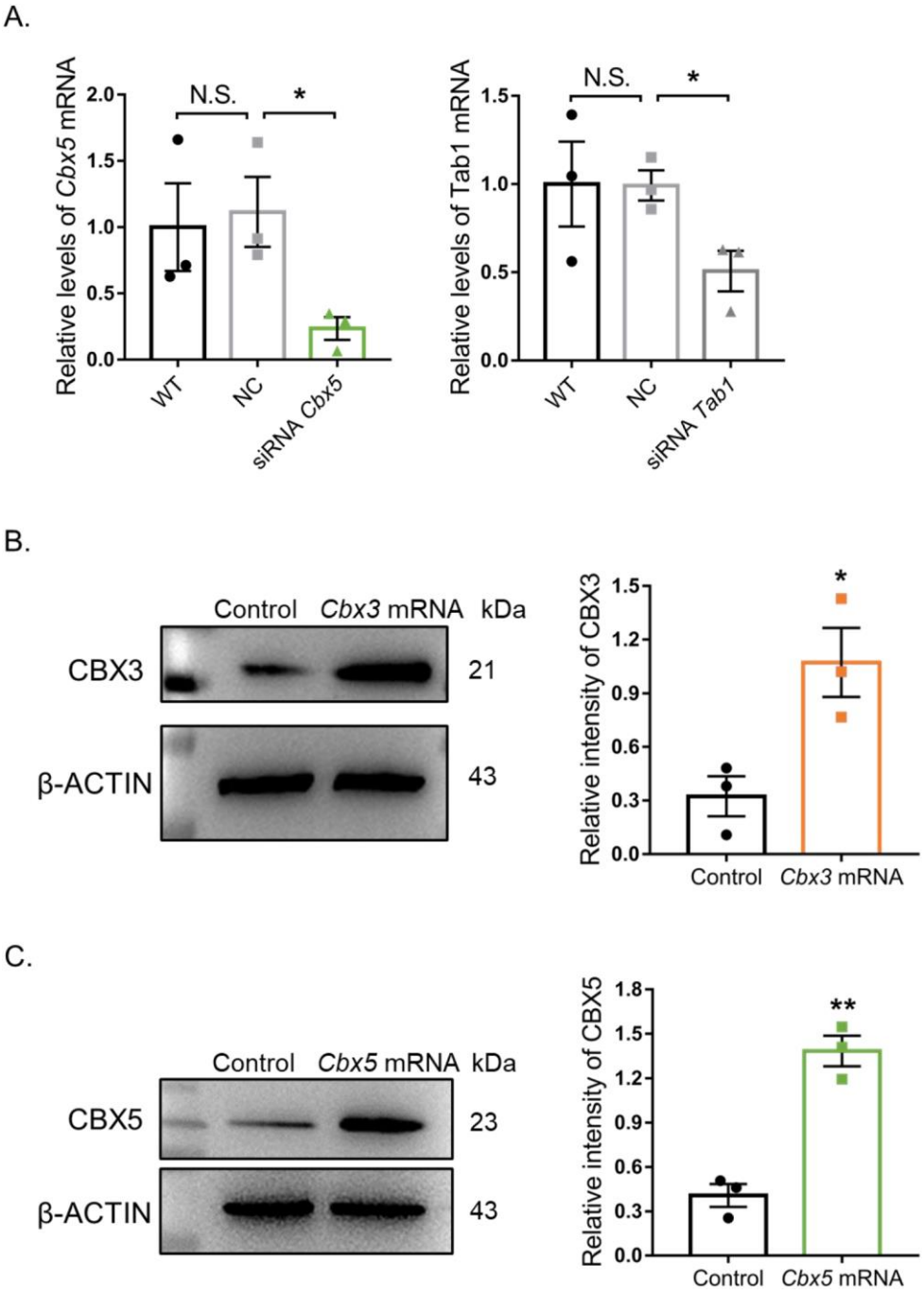
1. The link between Rnf114 and Cbx5 needs further support. Ideally one would like to see a rescue experiment in which Cbx5 is knocked down by siRNA or similar in Rnf114 mutant oocytes or zygotes and the effect on the embryo development assessed. If Rnf114 is functioning by mediating the degradation of Cbx5 then inducing degradation of Cbx5 should rescue, partially at least, the phenotype of the Rnf114 mutant embryos. Is the level of overexpression in line with the proteomics? A qPCR would be useful to include here.

Response:

We firstly appreciate the reviewer's affirmation for our work and thank him/her also for the critical comments.

According to this suggestion, we designed and synthesized specific siRNA for Cbx5 gene. In addition, considering the suggestion of another reviewer combinedly, we also ordered the Tab1 siRNA, which has been proven to be effective in our previous work. The negative control (NC) siRNA was also in line with the previous work [1]. Their knockdown efficiency was verified on wild type GV oocytes. (Figure A below). Then, we injected Cbx5 siRNA and Tab1 siRNA into the Rnf114$^{m-/z+}$ zygotes, respectively. We then observed the development of the embryos. The results showed that more maternal Rnf114 depleted embryos developed to the 4-cell stage after injecting with Cbx5 and Tab1 siRNA, compared to the control embryos. Further, although these embryos gradually blocked at subsequent developmental stages, there was still a few embryos could develop to the blastocyst stage (Figure 7B, C in revised manuscript). These results indicated that degradation of Cbx5 and Tab1 could partially rescue the phenotype of the Rnf114 mutant embryos.

As shown in Figure 6B in the first draft, exogenous overexpression of CBXs protein was detected in embryos using FLAG labeled antibodies, which had no corresponding bands in the control group. Therefore, it cannot accurately reflect whether the overexpression degree is consistent with the proteomics. During the progress of revising, we re-injected Cbx mRNAs into the wild-type zygotes, and collected 2-cell embryos to extract the protein, thus performed western blot analyses with antibodies against CBX3 and CBX5, instead of anti-FLAG antibody. After quantitative analysis, there is about 2.31-fold and 2.4-fold increase of CBX3 and CBX5 in the overexpressed embryos (Figure B, C below). The degree of exogenous overexpression was a bit higher than the results in the proteomics. However, it was close to the CBX protein level in mutant mouse oocytes, using the same detection method (Figure 4D, E in the revised manuscript). Considering the differences between the two quantify techniques, we believe that the overexpression level of the exogenous CBX proteins is within the range comparable to that of the Rnf114 mutant group. And in the revised version, Figure 6B has been replaced with this result.



*(A) Verification of knockdown efficiency of Cbx5 and Tab1 siRNA in GV oocytes. N = 3, independent replicates. (B, C) Immunoblot showing the exogenous protein levels of CBX3 (B) and CBX5 (C) in 2-cell embryos after microinjecting Cbx3-flag or Cbx5-flag mRNA. B-Actin was used as the loading control. N=3, independent replicates. All graphs are presented as the means ± SEM. N.S. = no significance, *P<0.05, **P<0.01, compared to the control group in the unpaired two-tailed t-test.*

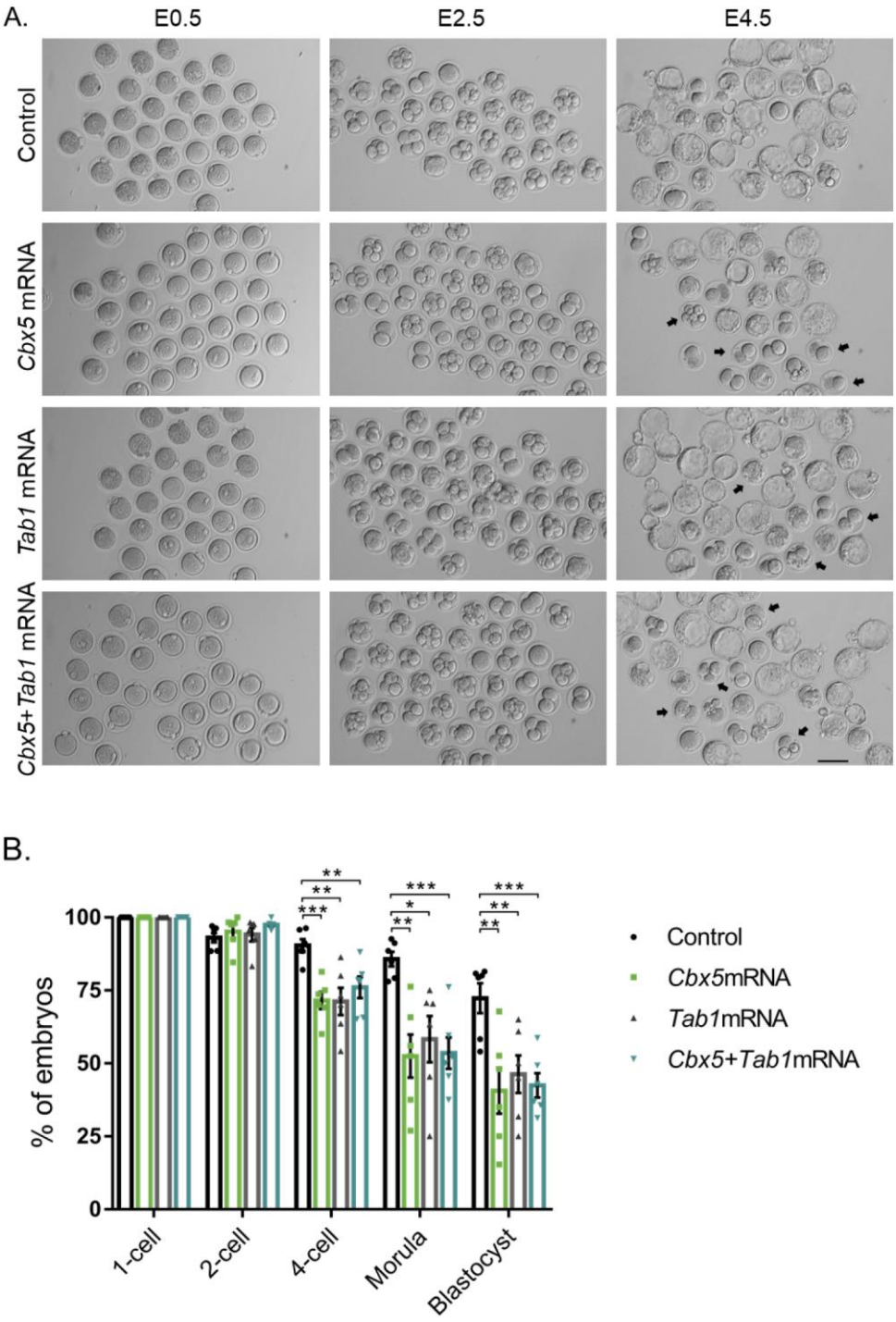
2. In the introduction the authors comment on data regarding Tab1, however this is not presented in the results section of the manuscript. Please move the presentation of this data from the Discussion to the Results section and integrate it into the manuscript. Were the experiments in supplemental figure 6 on Tab1 performed in normal or Rnf114 mutant embryos? How do the authors see Tab1 fitting into the mechanism between Rnf114 and Cbx5?

Response:

Thanks for this suggestion. In the revised version, we have transferred the relevant data to the Results section. In Figure S6, the TAB1 protein level assay was performed in Rnf114 mutant embryos and the experiments under TAB1 overexpression were performed in normal embryos. We

added the explanations in the manuscript. According to existing reports, TAK1 binding protein 1 (TAB1) binds to TGF-beta activating kinase (TAK1) to induce the autophosphorylation of TAK1 [2]. The TAB1-TAK1 complex phosphorylates substrates MKK and IKK in the cytoplasm and promotes translocation of AP-1 and NF-κB to the nucleus to regulate MAPK and NF-κB signaling pathways, which mediate cell differentiation, proliferation, survival, and immunity [3-5]. However, CBX5 is mainly involved in the formation of heterochromatin, histone modification and other biological processes in the nucleus [6]. Although, some study has revealed a few of factors involved in the MAPK and NF-κB pathway interact with CBX5 in the nucleus to regulate cell development [7, 8], no association between TAB1 and CBX5 has been reported.

Thanks for the reviewer to raising the question about how Tab1 fits into the mechanism between RNF114 and CBX5. This is indeed a question worthy of considering. To explore the relationship of TAB1 and CBX5 in the embryo with Rnf114 mutation, we injected Tab1 and Cbx5 mRNA into the zygote simultaneously. The development rate of embryos injected with Cbx5 and Tab1 mRNA was similar to that of embryos injected with Cbx5 and Tab1mRNA respectively (Figure A, B below). This result suggested that the simultaneous accumulation of TAB1 and CBX5 did not aggravate the blocking effect on embryos, although both TAB1 and CBX5 are substrates regulated by RNF114. Whether there is a link between these two proteins still remains to be further explored.



*(A) Representative DIC images of Cbx5-, Tab1- and Cbx5+Tab1-overexpressing embryos development. The arrows point to the embryos with developmental arrest. Scale bars = 100μm. (B) Percentage of embryos derived from overexpressed zygotes to reach the various preimplantation embryo stages. N = 6, total of 199-275 embryos/group. All graphs are presented as the means ± SEM. *P<0.05, **P<0.01, ***P < 0.001 compared to the control group in the unpaired two-tailed t-test.*

3. I would like to see more of a discussion on the other proteins that are misregulated in the Rnf114 null oocytes. How many of these overlap with the protoArray experiments performed in Yang 2017? Could the authors present the misregulated proteins in a main figure? How many of these have been linked to the MZT? Why did the authors decide to focus on Cbx3 and Cbx5 given they are not the most severely affected in terms of upregulation? The phenotype for Cbx5 overexpression is quite subtle compared to the Rnf114 phenotype. The authors speculate on this

in the discussion but could some of the other hits from the proteomics analysis answer this? Perhaps this could be a way to introduce Tab1 which unfortunately was not detected in the proteomics, presumably due to its low abundance.

Response:

We would like to thank the reviewer for bringing up this issue as it gives us the opportunity to discuss this point in detail. We have compared the misregulated proteins found in this study with the results of previous protoArray experiment, unfortunately, there is no overlap between the two experiments. We think there are three main reasons:

1) In the previous protoArray experiment, the species for the protein-chip database is humans, while mass spectrometry (MS) detection in this study was carried out on mice.

2) The coverage of protein microarray is limited. The protein microarray used in previous work is ProtoArray® Human Protein Microarrays v5.0. We compared 91 upregulated and 45 downregulated proteins in the MS results with the protein microarray database, and found that only 29 upregulated proteins and 10 downregulated proteins were existed in the protein microarrays.

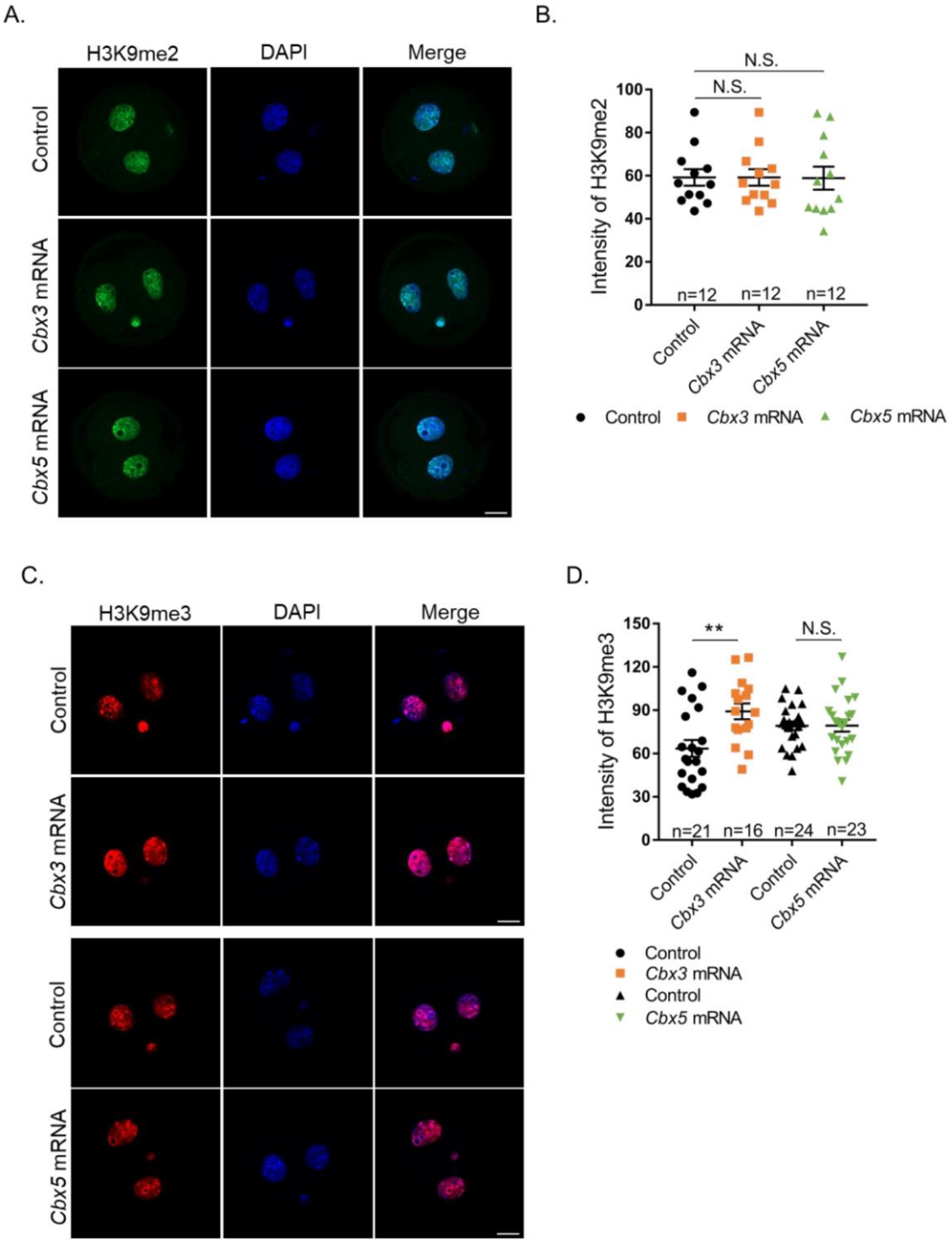
3) According to the criteria of positive signal in protein microarray screening (Z-Factor>0.5, Z-score≥2.0, Replicate spot CV<50%, Inter-assay CV<50% and CI P-Value<0.05), only two of the 29 upregulated proteins (RP9 and PRKAG2) meet the criteria, however they could not be recognized as potential substrates of RNF114 in the protoArray results due to the signal in E1/E2 control assay and mutant RNF114 assay is higher than the signal detected in WT RNF114 assay. On the other hand, the 13 potential substrates of RNF114 identified by protein microarray in the previous article were not in the differential protein list identified by MS, of which only 6 proteins were detected, but no difference. The other 7 proteins, including TAB1, were not detected in the MS maybe due to its low abundance, as the reviewer pointed out. Both of the two methods have some limitations, the difference in candidate proteins obtained by the two methods further suggested it is necessary to use various methods to explain the complex regulatory mechanism of RNF114 during the process of MZT.

In order to present the differential proteins on a main figure, we performed an interaction network analysis on the differential proteins (Figure 4B in revised manuscript). Then, A detailed search of the differentially expressed proteins in the mass spectrometry was conducted, and no direct correlation with MZT was found. Furthermore, we observed two enrichments of differential proteins associated with “Chromatin” and “Cytomembrane” in the interaction network analysis (Red box in Figure 4B in revised manuscript). We found that most of the proteins presented in the two clusters were upregulated. LYZ1, the most significantly changed protein in the “Cytomembrane” cluster, is a marker for Paneth cell, a type of intestinal epithelial cell [9]. LYZ1 is secreted to extracellular region to dissolve bacteria and stabilize the intestinal environment [10]. Some proteins pertaining to the Annexin family were also gathered in the “Cytomembrane” cluster, including ANXA1, ANXA2, ANXA3 and ANXA11. According to previous studies, these proteins are mainly related to embryo implantation and postimplantation development[11-15]. So far, there is no evidence to support that the above differential proteins are directly related to the MZT process. On the other hand, the structure and state of chromatin change dramatically during MZT in mice. Epigenetic changes occur in all dimensions of chromatin, including the alterations in DNA methylation, histone composition and modifications, the transform of chromatin accessibility and Topologically associating domains (TAD) in the higher structures of chromatin [16]. Thus, we focused on the proteins associated with “Chromatin”. Some histone subunits were disturbed in RNF114 null oocytes, with abnormal accumulation of Hist1h1e and H2afv and decreased content of Hist1h1t. It has been reported that different variants of histone 1 play the dominant role in different cell types. For example, Hist1h1t is a specific histone in male germ cell [17], whose decreased expression in mutant oocytes may not be explained temporarily. H1foo plays a leading role in oocytes[18], while it was no differences between control and mutant group in our proteomic results. Furthermore, histone 1 variants may have a compensatory effect [19]. H2afv, one of the histone 2 variants, plays a role in regulating the formation of heterochromatin and gene transcription in drosophila[20, 21]. But its role in mice is not yet clear. It needs to be further investigated whether the changes of individual histone variants are related to the development arrest caused by RNF114 deletion in the future. The factors binding to chromatin were also enriched in the interaction network. We focused on two proteins of the same family, CBX3 and CBX5, according to the terms of “nuclear heterochromatin”, “site of DNA damage”, “chromatin DNA binding” in GO analysis (Figure 4C in the revised manuscript). We have added the discussion about differentially expressed proteins into the revised version.

4. Given the biological roles for Cbx3/5 one would imagine that aspects of the nuclear structure/organisation would be affected in the overexpression embryos. Could the authors perform some stainings, for example H3K9me2/3, to determine whether there are global alterations in the embryos? Are there any changes that can be seen in the DAPI staining?

Response:

Thanks for your suggestion. We also concerned the role of the CBX family in the formation of heterochromatin and the regulation of inhibitory histone modifications [22, 23]. We examined the fluorescence level of H3K9me2/3 in 2-cell embryos overexpressing CBX3 or CBX5. The results showed that there was no significant change in H3K9me2 in the embryos after CBX3 overexpression, but the H3K9me3 increased slightly compared with the control group (Figure A-D, below). CBX5 overexpression did not affect H3K9me2 and H3K9me3 levels in 2-cell embryos (Figure A-D, below). Additionally, according to the results of DAPI staining, there were no significant changes in the size, shape, and color of the embryo nuclei (data not shown).



*(A, B) The staining of H3K9me2 (A) and the quantitation of H3K9me2 level (B) in 2-cell embryos with CBX3 and CBX5 overexpressed. (C, D) The staining of H3K9me3 (C) and the quantitation of H3K9me3 level (D) in 2-cell embryos with CBX3 and CBX5 overexpressed. Error bars represent SEM. N.S. = no significance, **P<0.01 in the unpaired two-tailed t-test. All scale bar = 20 μm.*

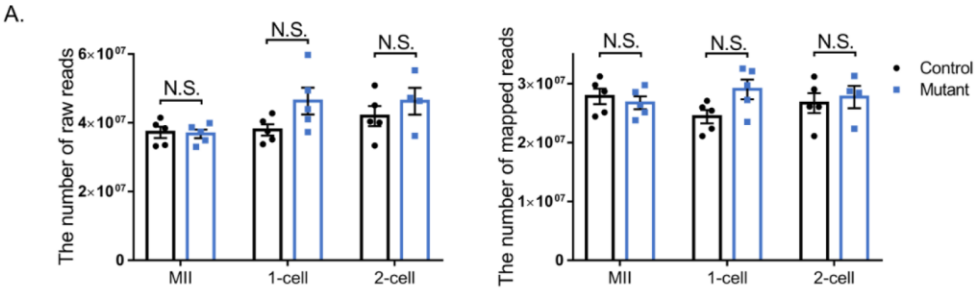
5. How as the normalization done for the RNAseq analysis and was there any control for the total number of reads per cell? Please also show some quality control plots including the number of reads per cell and the number of genes detected per cell and comment on whether this is different between the control and mutant embryos. Please also state in the results text how many cells/embryos were analysed.

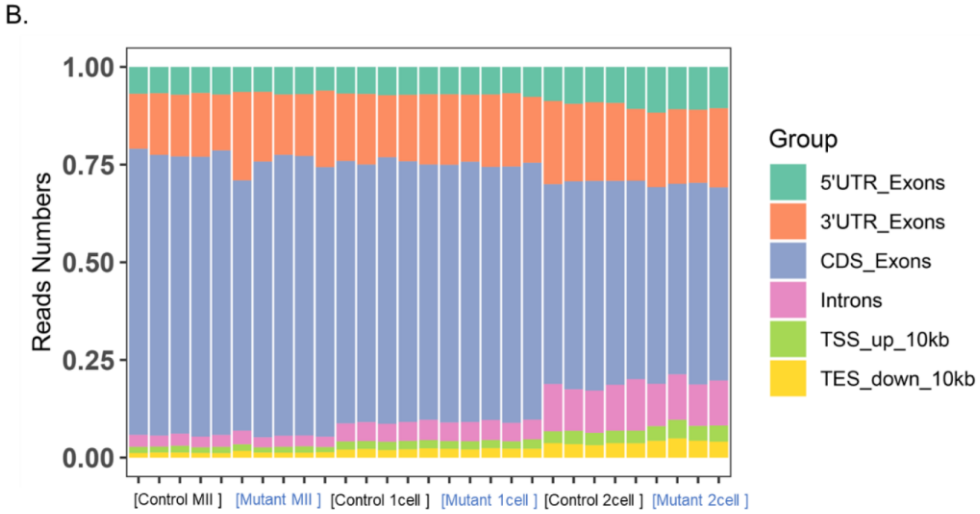
Response:

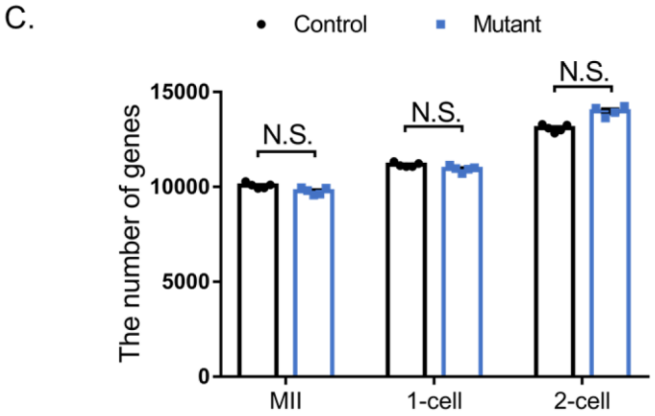
In our RNA-Seq data, the clean fastq files were mapped to GRCm38 Genome Reference using Hisat2

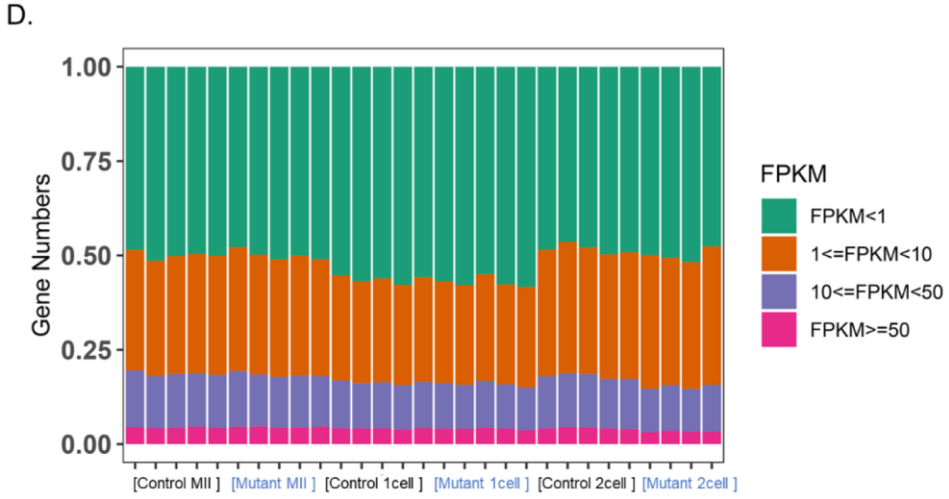
with default paraments. Samtools with parament -q 15 was used to ensure that only uniquely mapped reads were kept for further analysis. Although, we did not add exogenous control, like spike-in, in our samples, the numbers of raw and mapped reads in RNA-seq were similar between control and mutant group in different cell period (Figure A, below). We then distributed the mapped reads to genome features (like CDS exon, 5'UTR exon, 3'UTR exon, Intron, Intergenic regions) [24]. Although the reads distribution varied in differential cell period mainly due to CDS and intron's proportion, their distributions had no significant difference in the same period regardless of Control or Mutant *group (Figure B, below).*
For differential expression analysis, the gene expression counts were imported into R and normalized using size factors introduced by R package DESeq2. Differential expression genes (DEGs) with base mean>20, |log2FC|>1 and adjusted P-value <0.05 were identified by using result function in R package DESeq2 [25]. For heatmap or principal component analysis (PCA), the Fragments Per Kilobase Million (FPKM) was calculated to illustrate. The Figure C below show the average gene number in MII, 1cell and 2cell with FPKM >1 as the gene expression threshold.

Then we take the following thresholds for gene levels: no expression (FPKM < 1), low expression (1 ≤ FPKM < 10), medium expression (10 ≤ FPKM < 50), and high expression (FPKM ≥ 50) [26]. The percentage stack plot of gene expression level show that the cells in the same period have a similar expression level regardless of Control or Mutant group *(Figure D, below). All these data showed that the different between the control and mutant embryos was not introduced by transcriptome expression level and transcriptome distribution.*
We have stated the number of oocytes and embryos using to analysis in the revised Result text and put part of the quality control results in Supplementary Figure 3.









(A) The average number of raw and mapped reads in control and mutant group. N =4-5 independent replicates. (B) The distribution of mapped reads on the genome in each duplicate sample. (C)The average number of genes detected in control and mutant group, with FPKM >1 as the gene expression threshold. N =4-5 independent replicates. (D) The percentage stack plot of gene expression level in the duplicate samples. Graphs in (A) and (C) are presented as the means ± SEM.

N.S. = no significance in the unpaired two-tailed t-test.

Minor comments:

1. It would be helpful to have an initial figure showing the expression patterns of Rnf114 in preimplantation development as the authors refer to this in their previous work where it is hidden in a supplemental figure. One could also include expression patterns of Cbx3, Cbx5 and Tab1 in this figure to facilitate the biological interpretation of the data.
Response:
Thanks a lot for this suggestion. Since the expression patterns of RNF114 and TAB1 in oocytes and early embryos have been described in detail in previous manuscript, we did not repeat them in this work. According to your suggestion, we examined the expression patterns of CBX3 and CBX5 (Figure S5A, B in revised manuscript). The results showed that the expression level of CBX3 increased gradually with the development of embryos. Unlike CBX3, CBX5 maintained a high expression level during oocyte maturation, and decreased rapidly after fertilization and remained low expression until the blastocyst stage. The expression patterns of CBX proteins indicated that during preimplantation embryo development CBX5 undergoes degradation but not CBX3. This is consistent with our results, and indeed helps to explain the results that only CBX5 overexpression could mimic the phenotype of maternal RNF114 mutants.

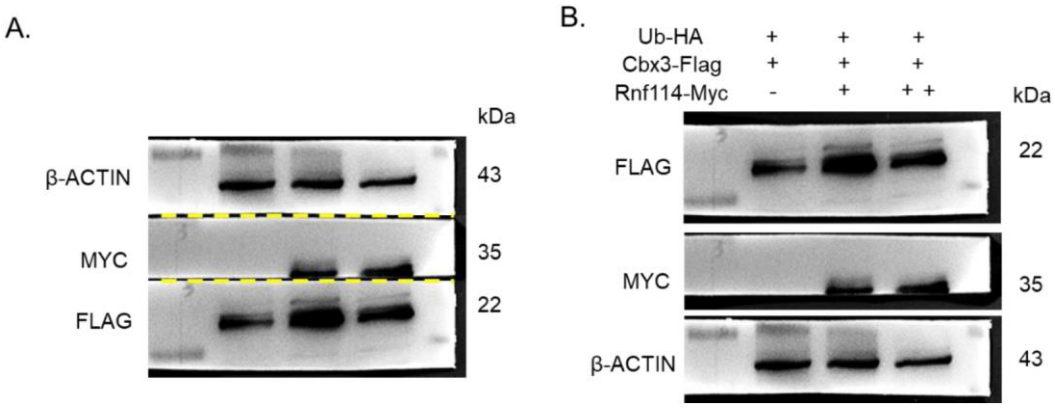
2. Please show individual datapoints in all the bar graphs and make sure that all western blots have the molecular weights marked.
Response:
Thanks for pointing out this. We have modified the bar graphs and western blots figures to show the individual datapoints and molecular weights accordingly in the revised version.

3. Please quantify western blot in S1E as there is still some signal in the null oocytes. Are the low levels of mutant Rnf114 protein remaining in the mutant embryos catalytic inactive? For example, could the authors perform an in vitro assay using a RNF114 protein lacking the 81bp region that is deleted in the mouse?
Response:
Thanks for your suggestion. We quantified the western blot in S1E, and the result showed that there was still residual RNF114 in the Rnf114 mutant oocytes, whose level decreased 8-fold compared with control ovaries (Figure S1E in revised manuscript). Proteome analysis also showed that although the expressional level of RNF114 was most downregulated, about 7.5-fold change, a weak signal of RNF114 was detected in the mutant oocytes. The 81bp deletion leads to the destruction of the RING domain in RNF114 (as shown in Fig S1A). In fact, we have tested the E3 ligase of RNF114 protein with destroyed RING domain by in vitro ubiquitination system in the previous work, and the results verified that the RING domain mutant caused RNF114 lost catalytic activity [1].Based on it, we believed that the few remaining RNF114 protein is not bioactive in mutant oocytes.

4. Was the qPCR validation of the RNAseq analysis performed on separate biological samples or does this just represent a technical validation? This needs to be made clear in the text.
Response:
Thanks for your suggestion. The qPCR validation of the RNA-seq analysis was performed on separate biological samples. We have added explanation in the text.

5. DAPI signal is very hard to see in Figure 2. One could consider greyscale for the individual panel as this has better visibility than blue on black.
Response:
Thanks for pointing this out. We have made the modifications in Figure 2 in the revised manuscript.

6. Part of the western blot signal is missing in figure 5D. please show the entire band.
Response:
Thanks for pointing this out. The three strips in previous Figure 5D (now Figure 5C in revised version) are from the same PVDF membrane, and the cut position is close to 35kDa (Figure A, below). Thus, the results in Figure 5C are the entire band (Figure B, below). Combined with other reviewers' suggestions, we have adjusted all the pictures in Figure 5.



(A) The picture showing that these WB results come from the same PVDF membrane. The yellow dotted lines represented the location of the cut. (B) The full pictures of WB results.

7. Why was H2O used as a control injection? Surely PBS would be better suited as it is isotonic?
Response:
We used H2O as the control injection because in vitro transcribed mRNAs were diluted in H2O referring to some similar studies [27, 28]. The injection amount of the exogenous mRNAs was too small and we believed that the osmotic pressure would not be affected.

8. Statistics are missing form figure 6F. please mark as 'ns' if not significant.
Response:
Thanks for pointing this out. We have made the change in previous Figure 6F (now Figure 6G in revised version).

Reviewer 2:
Zhou et al. report that Rnf114 mutant female mice are sterile. Although oocytes from these mice appear to be normal and can be fertilized, some developmental events after fertilization, which include zygotic genome activation (ZGA) and maternal mRNA/protein degradation, are severely impaired in Rnf114 maternal KO embryos, which possibly causes developmental arrest at the 2-cell stage. Given that RNF114 is a E3 ubiquitin ligase, the authors performed proteome analyses in addition to transcriptome analyses, and found that CBX5 and CBX3 protein levels are increased in Rnf114 KO oocytes as well as in Rnf114 maternal KO 2-cell embryos. Interestingly, overexpression of Cbx5 partially induced developmental arrest. Overall, the study is carried out in an appropriate manner and identify Rnf114 as an important maternal factor.

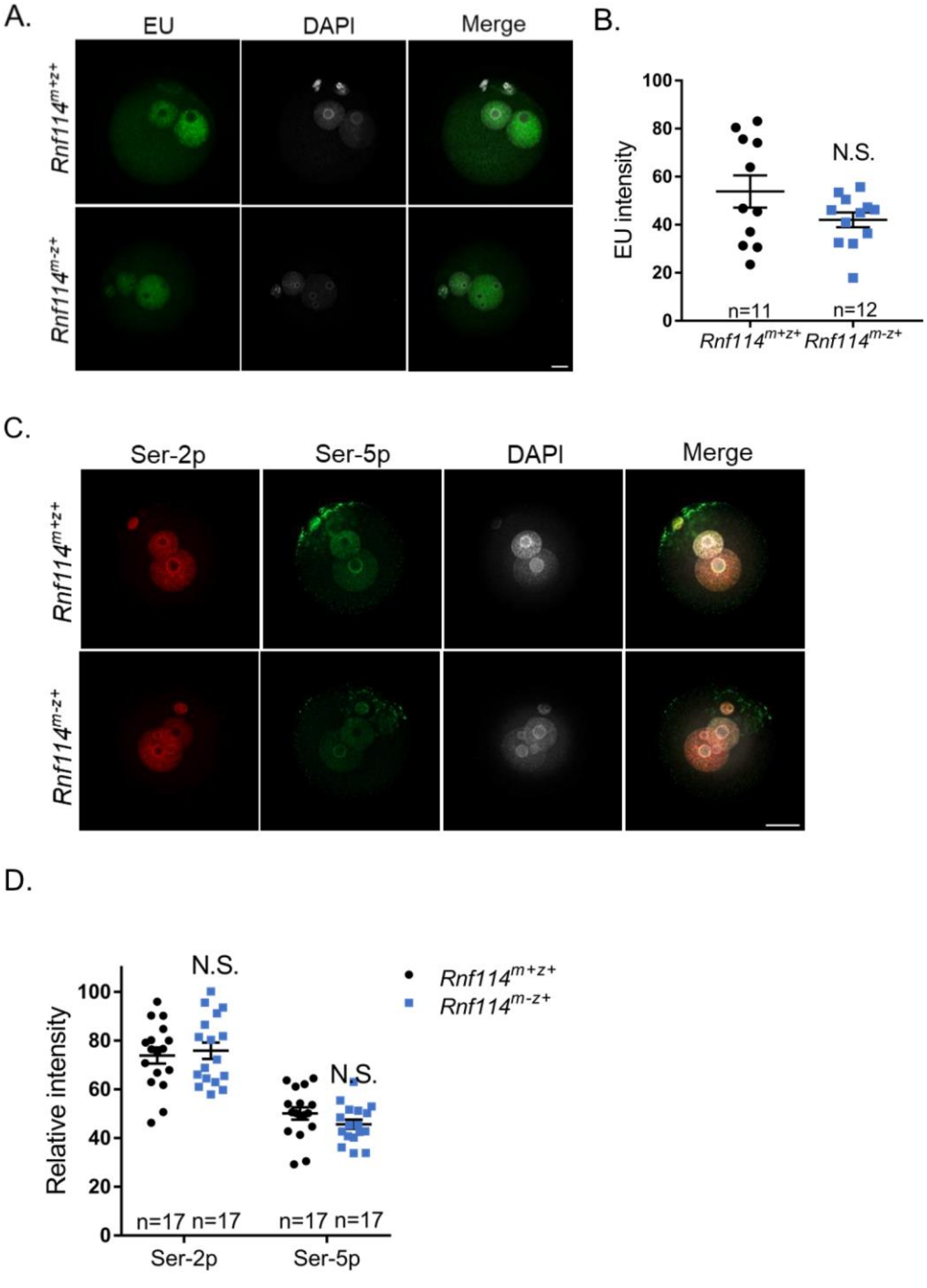
Reviewer 2 Comments for the Author:
However, before publication, further interpretation and discussion of the data as well as additional experiments, including some control experiments, would be necessary to support the authors' conclusions.

Major points
1. The authors show that maternal RNAs do not decrease and the genes activated at minor ZGA are upregulated in Rnf114$^{m-/z+}$ 2-cell embryos. This suggests that developmental progression (or MZT) is delayed in Rnf114$^{m-/z+}$ embryos. Furthermore, as conventional RNA-seq on 1-cell embryos is strongly biased to detect RNA from oocytes (maternal RNA), it is hard to conclude that minor

ZGA is normal in Rnf114$^{m-/z+}$ 1-cell embryos from RNA-seq data. Thus, I suggest to perform EU labeling and phospho-pol II staining at the late 1-cell stage to examine whether minor ZGA is affected or not.

Response:

Thanks for this suggestion, as per the reviewer's suggestion we analyzed the EU labeling and phospho-pol II staining at the late 1-cell stage in Rnf114$^{m-/z+}$ embryos. We found that there was no significant change in the EU level of Rnf114$^{m-/z+}$1-cell embryos compared with the control embryos (Figure A, B below). Furthermore, the staining of Pol II Ser-2p and Ser-5p (Figure C, D below) in Rnf114^{m-z+} 1-cell embryos also showed normal level. These results indicate that deletion of maternal RNF114 does not affect the minor ZGA. We have added these results to the Supplementary Figure 4 in the revised manuscript.



(A, B) EU staining (A) and quantification of this staining (B) in Rnf114^{m+z+} and Rnf114^{m-z+} 1-cell embryos. (C) Phosphorylation levels of serine moieties at positions 2 and 5 of Pol II for Rnf114^{m+z+} and Rnf114^{m-z+} 1-cell embryos. (D) Quantification of Ser-2p and Ser-2p staining. Error bars represent SEM. N.S. = no significance in the unpaired two-tailed t-test. All scale bar = 20μm.

2. RNA-seq on MII oocytes detected more than 100 DEGs, and it is highly likely that PCA on MII oocyte data only can separate control and KO groups. However, this fact is not discussed at all in the text. Is there any DEGs related to maternal mRNA degradation or developmental progression? Similarly, are there any genes whose expression is consistently altered in proteome data?

Response:

Following this advice, we further analyzed DEGs and added relevant description in the Discussion section. As shown in Figure 3C, there were 66 upregulated genes and 72 downregulated genes on the stage of MII. We found these DEGs in control groups were mainly concentrated in the range of FPKM < 10 (87.68%), which was the standard of low expression [26]. This may mean that even small fluctuations could make a big difference in the case of low expression. The disappearance of nearly all MII DEGs in 1-cell stage further illustrated the instability of difference in genes with low abundance. Principal component analysis (PCA) algorithm is used for reducing the dimensionality of high-through-out sequence data while retaining most of the variation in the data set.[29] Therefore, these genes could not distinguish mutant group from the control in PCA analysis. To learn more about these DEGs, we firstly performed GO analysis for upregulated and downregulated genes respectively, but no item was enriched (FDR<0.05). Then, the DEGs were searched and consulted one by one. With the exception of RNF114, no direct association between these DEGs and maternal RNA degradation and early embryo development has been found. We found that only two genes overlapped between transcriptome and proteome at MII stage, RNF114 and KAT14, both of which were downregulated. It has been reported that Kat14 knockout affected histone acetylation, leading cell development block in the G2/M phase, and mouse embryo arresting at E8.5 days [30, 31]. Therefore, the downregulation of KAT14 may not be the key factor caused RNF114 deleted embryo arrest at the 2-cell stage.

3. Some embryos injected with Cbx5 mRNA showed developmental arrest but most of them developed normally (Fig. 6D). This suggests that the decrease in EU incorporation might be limited to some embryos, which cannot be easily seen by bar plots with SEM. Please show all the raw data points in the graph as shown in Supplementary Fig. 6D.

Response:

Thanks for pointing this out. The raw data points of EU incorporation on embryos with CBX overexpression were shown as Figure 6G in the revised manuscript.

4. It is unclear how much CBX5-FLAG is exogenously expressed relative to physiological CBX5 level. As CBX5 is upregulated ~1.4X higher in Rnf114 m-/z+ embryos, too much expression should be avoided.

Response:

We thank the reviewer for pointing out this. In previous Figure 6B, we verified the exogenous CBX3 and CBX5 expression using anti-FLAG antibody. Now combined with the reviewer 1's suggestion, we used antibodies of CBX3 and CBX5 to verify the overexpression level, and the results showed that the exogenous expression level of the target protein was upregulated ~2.4X. The degree of exogenous overexpression was a bit higher than the results in the proteomics. However, it was close to the CBX protein level in mutant mouse oocytes, using the same detection method (Figure 4D, E in the revised manuscript). Considering the technical differences between WB and proteomic quantitative methods, we believe that the exogenous CBX5 expression level is basically comparable to the physiological level. The results were replaced in Figure 6B in the revised manuscript.

5. The evidence that RNF114 promotes the degradation of CBX5 is not strong. At the very least, proteasome inhibitor treatment is necessary in the co-expression assay indicated in Fig. 5C-D, and data quantification should be performed.

Response:

Thanks for this suggestion. We treated the cells with proteasome inhibitor MG132 for co-

expression analysis. This has been described in the Methods section. According to this suggestion, we conducted a quantitative analysis of WB in Fig. 5C-D, and the results confirmed that RNF114 promoted the degradation of CBX5 (Figure 5C, D in the revised manuscript).

6. The authors concluded that RNF114 is important to adjust the level of maternal factors and that ZGA is indirectly affected. Therefore, I would like the authors to reconsider the title of this paper.
Response:
We carefully considered the reviewers' comments and now revised the title to "Maternal RNF114-mediated target substrates degradation regulates zygotic genome activation in mouse embryos".

Minor points
1. Although Sirt1 was used as one of the zygotic products, the evidence of this is unclear. As Sirt1 seems to be expressed in oocytes, more explanation would be necessary.
Response:
We chose SIRT1 as the detection marker of major ZGA referring to the study of Raghavendra Nagaraj et al [32]. Now we have added the necessary explanation in the text.

2. (line 143) Please explain the "HOM group".
Response:
We apologize for the misrepresentation. The "HOM group" means "Mutant group". We have corrected it in the text.

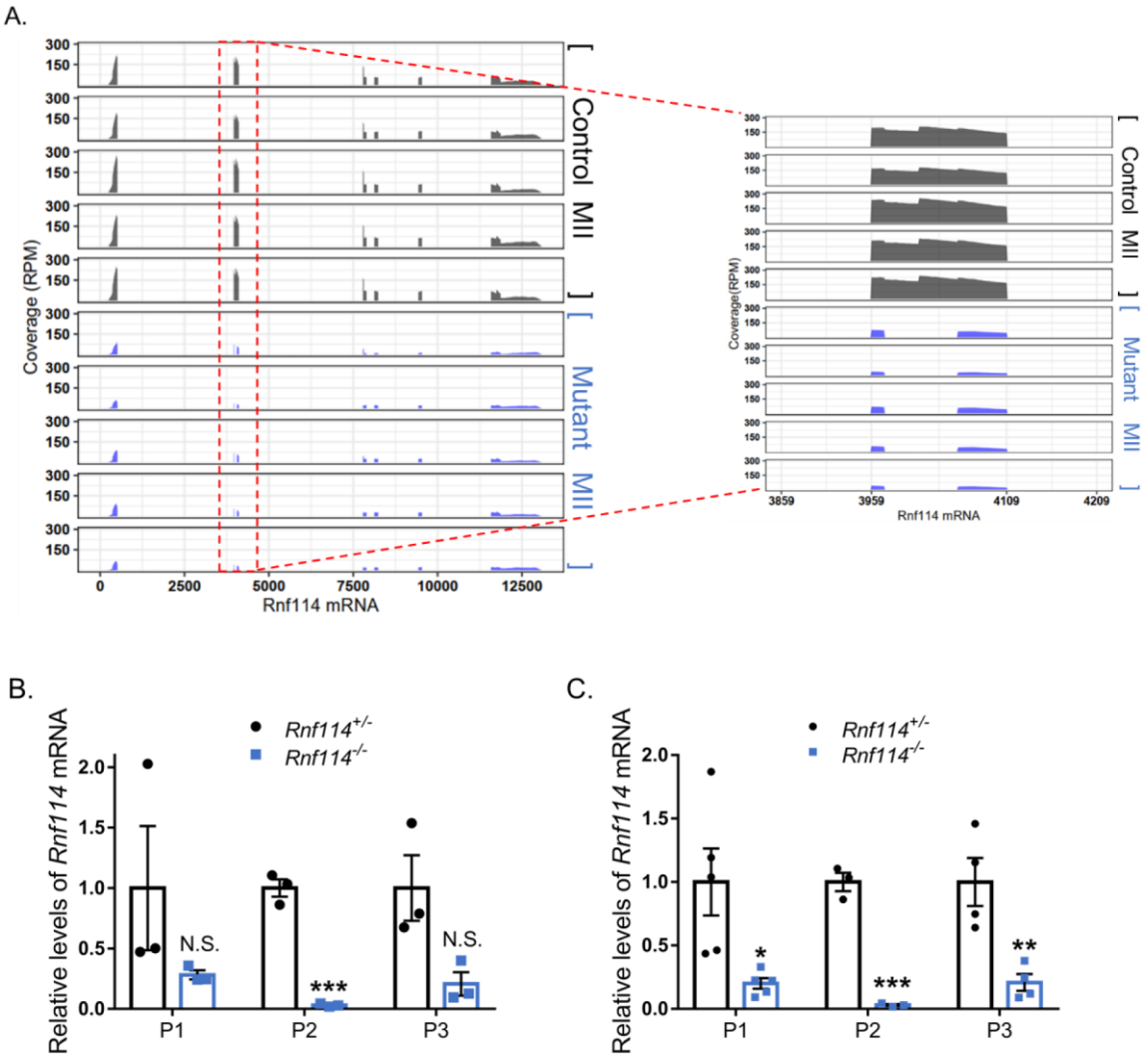
3. I would like the authors to reconsider the Discussion. For example, there are some explanations for the domains of RNF114 protein and insights into RNF114 based on previous studies, but these should be mentioned in Introduction. In addition, most of Discussion is used to merely summarize or repeat the obtained results.
Response:
Thanks for pointing out this question. We have modified the Discussion section according to the reviewer's suggestion. The basal description of RNF114 protein was moved to the Introduction section and we simply summarized the obtained results in Discussion section.

Reviewer 3:
In Huo et al. the authors build on the prior paper from some in the same group, Yang et al EMBO reports 2017, where they showed the Rnf114 is a maternal effect gene using knockdown of Rnf114 in the zygote. Now using genetic knockout of Rnf114 the authors explore the role of Rnf114 in maternal to zygotic transition showing that in the absence of Rnf114 the embryos fail to progress past the 2-cell stage. Using microscopy and transcriptomics, they show that the Rnf114 maternal null embryos both fail to degrade the maternal RNA store, and do not undergo major zygotic genome activation. To tease apart the mechanism for this effect, they use proteomics in MII oocytes. They identify and validate CBX5 as a target for RNF114-mediated ubiquitynation and degradation. Cbx5 overexpression by microinjection has a modest effect on embryo development, but is similar to that of Rnf114 maternal deletion. Therefore, the authors propose that Rnf114 is involved in required to degrade repressor proteins to enable maternal to zygotic transition and the major wave of zygotic genome activation.
I found this paper interesting. It is a significant extension to prior work, that reveals a role for protein degradation via RNF114 of at least CBX5 in the maternal to zygotic transition.

Reviewer 3 Comments for the Author:
There are some additional experiments along with some editorial changes necessary.

-The new Rnf114 KO needs further clarity. The deletion doesn't cause a frameshift mutation but all the same appears to destabilise the protein levels. Furthermore, qRT- PCR showed a (not statistically significant) drop in RNA levels, whereas their RNAseq showed a striking and significant reduction in RNA. Comment on this would help the reader understand the KO. Was the RNA depletion expected given that a nonsense mutation is not introduced?
Response:
Thanks for pointing out this key question. We have validated the Rnf114 mutation efficiency from mRNA and protein perspectives. The destruction of RING domain in RNF114 protein may result in its degradation, due to the key domains are important for stability of the protein itself [33, 34].

However, Rnf114 mRNA instability was unexpected. We compared the segments of Rnf114 that were detected in RNA-seq between Rnf114$^{+/-}$ and Rnf114$^{-/-}$ oocytes. The fragments of Rnf114 were decreased overall and the knockout fragment was almost undetectable in mutant oocytes (Figure A, below). Moreover, In the qRT-PCR, P1 and P3 showed a downward trend in mutant oocytes, despite a high degree of dispersion between the control data (Figure B, below). Now we repeated qRT-PCR for P1 and P3 and the results showed the drop in Rnf114 mRNA was statistically significant in Rnf114$^{-/-}$ oocytes (Figure C, below). We have changed the graphs in Supplementary Figure 1D in the revised manuscript. In fact, we do not yet have a clear explanation of this phenomenon. However, the degradation of mutant mRNA was demonstrated in some gene-edited animals [35, 36]. The specific regulatory mechanism remains to be further explored.



*(A) Transcription level of Rnf114 showing low abundance in Rnf114$^{-/-}$ MII oocytes, especially at knockout fragment. (B) The previous qRT-PCR results about Rnf114 mRNA levels primed by P1, P2, and P3 in Rnf114$^{+/-}$ and Rnf114$^{-/-}$ MII oocytes. N = 3, independent replicates. (C) The revised qRT- PCR results about Rnf114 mRNA levels primed by P1, P2, and P3 in Rnf114$^{+/-}$ and Rnf114$^{-/-}$ MII oocytes. N = 3-5, independent replicates. All graphs are presented as the means ± SEM. N.S. = no significance, *P<0.05, **P<0.01, ***P < 0.001 in unpaired two-tailed t-test.*

-While there are very few DEGs at the 1 cell stage, what are the 13 DEG? Please include a list. Since detectably expressed genes at this stage are likely to be involved in early embryonic events, these 13 genes may themselves be required for major ZGA genes. This requires for additional explanation in the paper.

Response:

Thanks for your suggestion. We have added the list of 13 DEGs at 1 cell stage to the

supplemental material (Supplementary table 2). We also presented these DEGs in a list here (Table below) and reviewed the published researches to get the knowledge of them. On the whole, these 13 genes were not reported to be involved in Major ZGA. Nine of the 13 DEGs were downregulated, and no direct correlation was found with embryonic development except Rnf114. Three of the 4 genes that were upregulated contained zinc finger domains, but no studies on them have been reported. The explanation was added in the discussion section.

Gene	Log_2FC	Adjusted *P*-value
Zfp990	1.763190415	5.70E-08
Zfp988	1.362256952	0.00239597
8430426J06Rik	1.362868097	0.0072766
Zfp985	1.028867098	0.01365432
Rnf114	-1.612307012	1.03E-26
5830417I10Rik	-1.977591963	5.57E-10
Mef2b	-1.892835964	2.20E-08
Hnrnpa0	-1.567451538	7.89E-08
Tmem92	-1.539239505	0.00366383
Btbd17	-1.01094257	0.00838686
Cep250	-1.123844261	0.00963794
Cachd1	-1.058947925	0.02334101
Chst9	-1.363576313	0.03404546

The list of DEGs from RNA-seq at 1-cell embryo stage.

-For the proteomic analysis, it isn't explained why MII oocytes were chosen (rather than 2 cell embryos, for example). This should be added to aid the reader. In addition, the replicate numbers aren't present in the figure legend, so need to be included, as does comment about multiple testing correct (FDR referred to in methods but tables and text mention P value instead).

Response:

Thanks for your helpful suggestions. We are so sorry for our negligence that we did not illustrate the replicate numbers in the figure legend. The number of replications per set of samples is 3. MII oocytes were selected for proteomic analysis mainly for two points. On the one hand, the deletion of RNF114 may lead to a large number of abnormal protein metabolism during oocyte maturation, thus affecting embryonic development. On the other hand, we focused on the maternal role of RNF114 in the study. The experimental scheme was that wild-type sperm fertilized to $Rnf114^{-/-}$ or $Rnf114^{+/-}$ oocytes to form embryos by IVF. If the embryos were selected for proteomic analysis, the influence of paternal genomic products on the results is difficult to rule out. We have added this explanation in the manuscript.

We have revised the less rigorous description in the figure legends. We also have reviewed and commented the testing in the manuscript, due to different statistical methods were used in different analyses.

Based on the proteomic analysis, it isn't clear why CBX3 and CBX5 were chosen for further analysis. There was GO term analysis of the list of proteins; however, given that the conclusion relies on the presence of repressor proteins in general in the RNF114 targeted proteins, beyond just CBX3/5, further comment on the some of the specific proteins that were found would be helpful. Further explanation is also required for the choice of specifically CBX3 and CBX5 to follow up is necessary. Were additional other repressors tried but did not validate?

Response:

Thanks a lot for this suggestion. In combination with the suggestion of reviewer 1, we performed an interaction network analysis on the differentially expressed proteins (Figure 4B). The result showed that CBX3 and CBX5 appeared in one of the enriched clusters, which related to chromatin. In addition, CBX3 and CBX5 were also included in the enriched GO terms - "nuclear

heterochromatin", "site of DNA damage" and "chromatin DNA binding", etc., thus we focused on these two proteins in the further study. The detailed explanation could be found in the reply for Reviewer 1 and necessary explanation has been added in the manuscript. Moreover, we noticed some repressors was included in the upregulated proteins. It is reported that HES1 acts as a transcriptional suppressor to prevent Ser2 phosphorylation and extension of RNA polymerase II, which inhibits CCXL1 transcription [36]. PUM2 is an RNA-binding protein that attenuates translation elongation by forming the PUF-Ago-eEF1A complex [37]. The elimination of RTF2 from stalled replisomes promotes DNA replication process and maintains genome integrity [38, 39]. However, these repressors were scattered throughout the interaction analysis and related items were not significant in GO analysis (Figure 4B, Supplementary table 5 in revised manuscript). The upregulation of these repressor proteins may also be the reason of embryo development arrest and ZGA disorder in maternal RNF114 mutant embryos, which need to be further studied in the future.

-The mild phenotype with Cbx5 overexpression is promising, however the conclusions would be best supported using a rescue experiment. In this case, does Cbx5 knockdown on the Rnf114KO background rescue to a significant degree the effect of the Rnf114KO on embryo development and and ZGA (qRT-PCR)?

Response:

Thanks for this suggestion. Combined with the reviewer 1's suggestion, we conducted rescue experiments on maternal RNF114 mutant embryos by knocking down Cbx5. A part of mutant embryos could develop to the blastocyst stage by Cbx5 knockdown (Figure 7B, C in the revised manuscript). Furthermore, the treated embryos were collected and the influence on major ZGA was verified by qRT-PCR. The result showed that all the selected major ZGA genes were increased to various degrees in the Cbx5 knockdown group, although only part of them were increased significantly (Figure 7D in the revised manuscript).

-The previous paper reported TAB1 as a target of RNF114. Here the authors present further work on TAB1 in the discussion and as supplemental figure, although it did not come out of their proteomic analysis. The authors need to firstly move this work to the results section, and more clearly introduce the TAB1 protein and their prior data. The published data shows a mild effect of Tab1 overexpression on embryo development (similar to Cbx5). Here they show that in 2 cell embryos TAB1 protein is higher in Rnf114KO, that there is lower transcription by EU staining, and inefficient ZGA. The authors should perform a rescue experiment, as above. In the future, it would be to test whether Tab1 and Cbx5 double knockdown can rescue the Rnf114KO defect in embryo development.

Response:

Thanks a lot for this suggestion. We have transferred TAB1 data to the Results section, and conducted a rescue experiment of TAB1 on Rnf114^{m-z+} embryos. The reviewer 1 also raised the similar problem. As the results shown in the reply to reviewer 1, knockdown of Tab1 by specific siRNA could partially rescue the phenotype of the Rnf114 mutant embryos (Figure 7B, C in the revised manuscript). A part of embryos can development beyond 2-cell stage and the expressional level of major ZGA genes were increased partly after Tab1 knockdown (Figure 7D in the revised manuscript). However, the relationship between TAB1 and CBX5 still remains to be further explored as we discussed in the reply to reviewer 1.

-For all the Western blot analyes, there are small changes seen in levels of proteins. The quantitation is helpful, however such small changes are hard to quantitate by Western blot. The use of an Odyssey system would be best here, as in the prior paper. In addition, all Western blot image sections are very small cuts of the gel, and sometimes don't include markers. In particular those in Figure 5B need repeating as the WB aren't clear.

Response:

Thank you for pointing out this. In this study, we used the HRP linked secondary antibodies for Western blot, while the Odyssey system needs to use fluorescent dye labeled secondary antibody. Due to the limitations of experimental materials, it is difficult to repeat all the Western blot experiments under the Odyssey system in a short time. In the revised manuscript, we performed quantitative analyses for most of the WB results. The gray scale of WB blots was measured by Image J and calculated the ratio with the loading control for statistics. In addition, molecular weights were marked on all WB images now. The result of previous Figure 5B (now Figure 5A in revised manuscript) was repeated and replaced by clearer pictures.

Additional minor comments:
Figure 1A – N=9, does this refer to plugs resulting in no litters, or number of females mated continually over 6 months. Please clarify.
Response:
N=9 means the number of females mated continually over 6 months. We have clarified it in the legend of Figure 1.

Figure 1B – clarify that these embryos were produced by IVF (the wording is we subsequently fertilized the eggs with wild-type sperm, so this is assumed).

Response:
Thanks for pointing out this question. The main way we got embryos in this article was IVF, which has been described in the Methods section. We have revised the description in the text to make it more accurate.

Figure S2A needs to be referred to the text. Can there be more similarly sized sections chosen, with a greater zoom and labelled regions that are scored in S2B?
Response:
Thanks for this suggestion. We have modified the pictures, added a greater zoom and labelled regions.

S2C, unclear what N=24 refers to, when there were >60 mice in each group?
Response:
N=24 means the number of statistics. More than 60 mice in each group were counted for ovulation.

S3C – were there replicates for qRT-PCR? It isn't clear how this qRT-PCR was performed and more detail is required. Was it independent of the original libraries?
Response:
Yes, there were three replicates for the qRT-PCR detection. Now the method was described more detail in the Method section. It was independent of the RNA-seq libraries.

CBX5 IF in Rnf114KO – the images show altered CBX5 staining levels but also altered distribution. This should be quantitated and commented on.
Response:
Thanks for your concern and suggestion. CBX5, also named heterochromatin protein 1 alpha (HP1a), has been reported to localized mainly in the nucleus. Based on the results of the CBX5 expression during preimplantation embryo development, which has been showed in the reply to reviewer 1, the expressional level of CBX5 at 2-cell stage is very low. We carefully compared the fluorescence images of the Rnf114^{m-z+} and control embryos, and we thought that major difference is the signal of CBX5 in the nucleus was obviously increase in the Rnf114^{m-z+} 2-cell embryos, while the weak signal in the cytoplasm may be the background signal.

Figure 6B what tissue was used for the WB analysis?
Response:
In Figure 6B, we used 2-cell embryos for the WB analysis. Now this figure has been replaced according to the opinions of other reviewers, the antibodies were changed while the samples were still 2-cell embryos.

For the ubiquitination validation, could the authors choose a more relevant cell type to repeat their analysis e.g. 2-cell like ES cells, or germ cell tumour cell lines?
Response:
Thanks for this suggestion. For the ubiquitination validation, we referred to the method of verifying the ubiquitination of substrate protein by Yimei Jin et al. [37]. They also identified an E3 ubiquitin enzyme in oocytes and then validated substrate ubiquitin in 293T cell lines. Furthermore, we found that the RNF114 protein has high homology in humans and mice. We thought the results on 293T cells were somewhat representative and we would like to repeat the

analysis in other cell types as the reviewer suggested in the future.

Throughout, quite old reviews are cited (often 20 years old). These should be updated to keep it current with the field.

Response:

Thanks for pointing out this. According to the reviewer's suggestion, we have updated some of the references.

References

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Second decision letter

MS ID#: DEVELOP/2021/199426

MS TITLE: Maternal RNF114-mediated target substrates degradation regulates zygotic genome activation in mouse embryos

AUTHORS: Shuai Zhou, Yueshuai Guo, Haifeng Sun, Lu Liu, Liping Yao, Chao Liu, Yuanlin He, Shanren Cao, Cheng Zhou, Mingrui Li, Yumeng Cao, Congjing Wang, Qianneng Lu, Wei Li, Xuejiang Guo, and Ran Huo

Thank you for submitting your revised manuscript to Development. Your manuscript has been re-reviewed by the same three reviewers who originally reviewed your manuscript. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The reviewers are overall satisfied with your revised manuscript, and therefore we would like to publish your manuscript in Development, pending some minor amendments.

Specifically,
1. please attend the request of reviewer 3, to add the two protein markers in your western blots, above and below the corresponding molecular weight of the protein analysed.

2. please attend the text corrections requested by reviewer 1 and clarify the rationale behind the use of MG132 in the Main text of the manuscript, as requested by reviewer 2.

3. please provide an alternative blot image for MYC in Fig. 5C, as requested by reviewer 2.

Lastly, reviewer 1 recommends some english language editing in the discussion, and I would ask you to please take this into consideration for the preparation of your final manuscript.

Since these are all minor corrections, I would hope to receive your manuscript in the next 3 weeks.

Reviewer 1

Advance summary and potential significance to field

This is a revised version of a manuscript I previously reviewed investigating the role of maternal RNF114 in regulating major ZGA in mouse embryos.

Comments for the author

I thank the authors for all their hard work in addressing the previous concerns and points raised by the reviewers. I am satisfied with the extra work that has been done and find this manuscript has been improved and is a pleasure to read. I fully support publication and have no other major comments.

two minor scientific comments need to be fixed before publication:
- I can’t see any signal for EU staining in Figure S6C bottom panels for the Tab1 mRNA injected embryos, yet the quantification in Figure S6D shows that there is still signal intensity. Perhaps a more representative embryo image could be shown in Figure S6C.

- Regarding the PCA analysis where mutant and WT oocytes are not separated by PC1 or PC2, I wonder if other principle components are able to separate out these samples. The statement in the discussion on line 297/298 is perhaps too strong as only PC1 and PC2 are analysed in this study and the PCA analysis was done on all genes not on the oocyte DEGs as this sentence implies. It could perhaps be reworded to reflect this e.g. “ Therefore, although the number of DEGs at MII stage was more than 100, the mutant group was unable to be distinguished from the control by principle component 1 or 2 in the PCA analysis.”

A few minor editorial comments to improve readability:
- Title: should read ‘Maternal RNF114-mediated target substrate degradation..’ not ‘substrates’
- It would be helpful to include a brief description of RNF114 in the abstract, e.g. insert “the ubiquitin E3 ligase Ring Finger protein 114 (RNF114)” on line 26
- Line 72 should read: A previous study.. (rather than Previous study…)
- Line 210 should read: changed (rather than change)

- Line 245 should read: “in a previous study…”
- Line 246 should read: “embryonic development, was discovered”
- Referencing needs to be consistent – sometimes author-date is used, sometimes numbering. This should be updated to use the referencing style used by the journal.

Reviewer 2

Advance summary and potential significance to field

The authors appropriately responded to the comments from the reviewers and conducted some additional experiments, and I would like to appreciate the authorsÂ’ effort to revise the manuscript. Although I think the revised manuscript is now suitable for publication in Development, I want to give only a few minor comments/advices before its final acceptance:

Comments for the author

1. It is regrettable that the authors have not included an alternative blot image for MYC in Fig. 5C (the reviewer 1 already pointed out). The image is not quantitative and is not suitable to be put on a paper. As the authors conducted the experiments at least three times, I think it is easy to replace the image.

2. Fig. 5D – The authors’ response was not convincing. I understand that MG132 was necessary for the assay for Fig. 5A-B, yet it is difficult to understand why MG132 is treated for the assay in Fig. 5C-D because the authors tested a hypothesis that CBX5 might be degraded by RNF114. Please clarify this point in the main text or Materials & Methods.

Reviewer 3

Advance summary and potential significance to field

The authors have dealt with most of my prior concerns. The additional rescue experiments and explanations have enhanced the manuscript.

Comments for the author

Just two small final requests. The authors note they have now included the protein markers, however it is usual to include the two markers surrounding the protein that has been detected by Western blot. This would add confidence for each of the Western blots shown in the paper. Secondly, I think the paper needs to be edited in terms of English language usage - the discussion in particular could be streamlined and made simpler to understand.

Second revision

Author response to reviewers' comments

Point-by-Point Response:
We thank all reviewers for taking the time to read our manuscripts and reply, and for providing further comments. We have taken all the concerns into consideration as we revised our manuscript. We wish that the reviewers will find the revised manuscript is a much more improved version. Followings are our point-by-point responses to the reviewers.

Reviewer Comments:

Reviewer 1 Comments for the Author:
I thank the authors for all their hard work in addressing the previous concerns and points raised by the reviewers. I am satisfied with the extra work that has been done and find this manuscript has

been improved and is a pleasure to read. I fully support publication and have no other major comments.

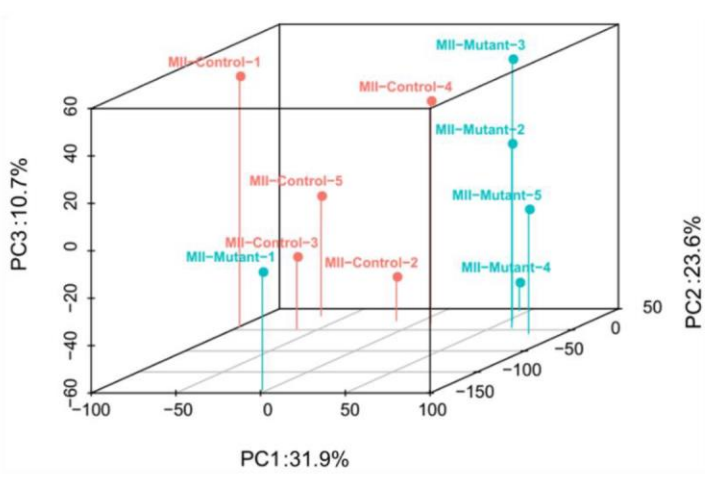
two minor scientific comments need to be fixed before publication:
- I can't see any signal for EU staining in Figure S6C bottom panels for the Tab1 mRNA injected embryos, yet the quantification in Figure S6D shows that there is still signal intensity. Perhaps a more representative embryo image could be shown in Figure S6C.

Response:
We firstly appreciate your affirmation for our revisions, and thank you for this suggestion. Now we have chosen more representative images of EU staining for the control and Tab1 mRNA injected embryos, and replaced them in Figure S6C.

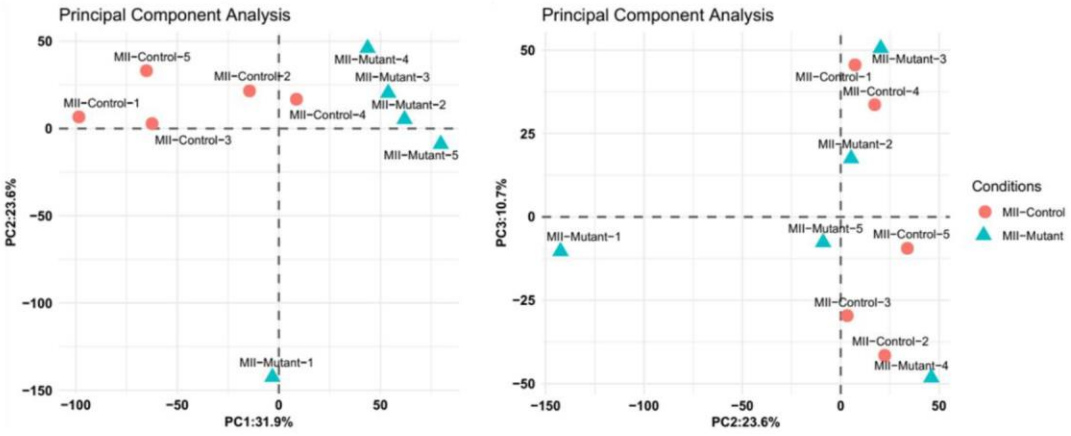
- Regarding the PCA analysis where mutant and WT oocytes are not separated by PC1 or PC2, I wonder if other principle components are able to separate out these samples. The statement in the discussion on line 297/298 is perhaps too strong as only PC1 and PC2 are analysed in this study and the PCA analysis was done on all genes not on the oocyte DEGs as this sentence implies. It could perhaps be reworded to reflect this e.g. " Therefore, although the number of DEGs at MII stage was more than 100, the mutant group was unable to be distinguished from the control by principle component 1 or 2 in the PCA analysis."

Response:
Thanks for your suggestion. We performed further three-dimensional PCA analysis on control and mutant groups at MII stage separately. Here, we show the diagrams including principal component (PC) 1, PC2 and PC3 (Figure A, B below). It could be found that although the control (WT) and mutant groups are separated to a certain extent, they cannot be completely distinguished from each other by PC1, PC2 and PC3, which represented 31.9%, 23.6% and 10.7% variance.
We are sorry for the less rigorous description. Now we have reworded it according to your suggestion.

A.



B.



(A) A score plot of the first three principal components for control and mutant groups at MII stage using FPKM. (B) The score plots of the first two principal components (left) and the second and third principal components (right) analysis.

A few minor editorial comments to improve readability:
- Title: should read 'Maternal RNF114-mediated target substrate degradation..' not 'substrates'

- It would be helpful to include a brief description of RNF114 in the abstract, e.g. insert "the ubiquitin E3 ligase Ring Finger protein 114 (RNF114)" on line 26

- Line 72 should read: A previous study. (rather than Previous study…)

- Line 210 should read: changed (rather than change)

- Line 245 should read: "in a previous study…"

- Line 246 should read: "embryonic development, was discovered"

- Referencing needs to be consistent – sometimes author-date is used, sometimes numbering. This should be updated to use the referencing style used by the journal.
Response:
Thank you very much for your meticulous advices. We have corrected these errors accordingly and thoroughly reviewed our manuscript to reduce such problems.

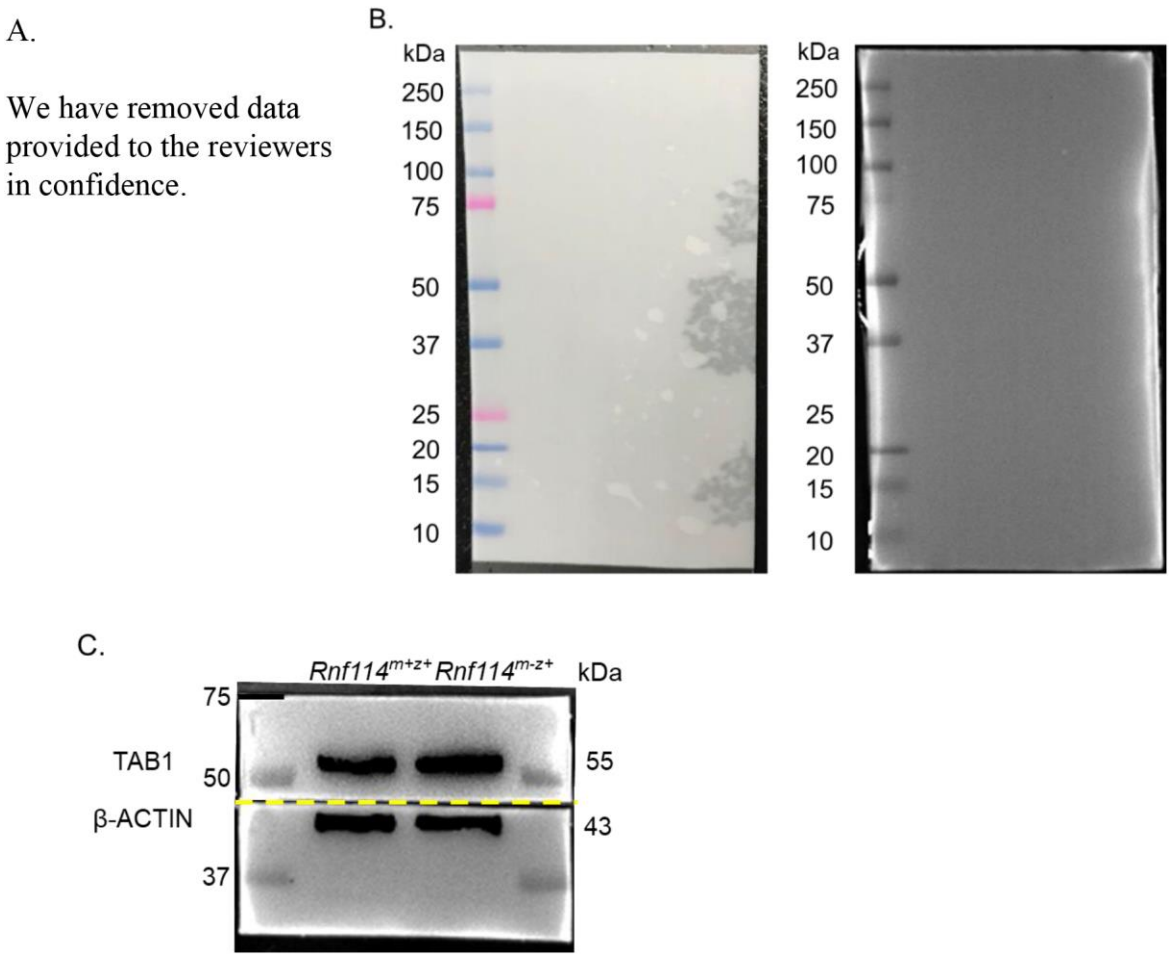
Reviewer 2 Comments for the Author:
1. It is regrettable that the authors have not included an alternative blot image for MYC in Fig. 5C (the reviewer 1 already pointed out). The image is not quantitative and is not suitable to be put on a paper. As the authors conducted the experiments at least three times, I think it is easy to replace the image.
Response:
Thanks a lot for this suggestion. We are sorry for it and now we have replace the blot image. Since the three replicate experiments were conducted independently, we replaced all the images in the Fig. 5C to make it uncontroversial. At the same time, we also replaced Fig. 5D with a better result.

2. Fig. 5D – The authors' response was not convincing. I understand that MG132 was necessary for the assay for Fig. 5A-B, yet it is difficult to understand why MG132 is treated for the assay in Fig. 5C-D because the authors tested a hypothesis that CBX5 might be degraded by RNF114. Please clarify this point in the main text or Materials & Methods.
Response:
Thank you for pointing out this. We sincerely apologize for any misunderstanding caused by our unclear statement. MG132 was indeed only used in the ubiquitination assay in Fig. 5A-B, and not used in the degradation experiments, which results showed in Fig. 5C-D. We have clarified this point in the main text and Materials and Methods.

Reviewer 3 Comments for the Author:
Just two small final requests. The authors note they have now included the protein markers, however it is usual to include the two markers surrounding the protein that has been detected by Western blot. This would add confidence for each of the Western blots shown in the paper. Secondly, I think the paper needs to be edited in terms of English language usage - the discussion in particular could be streamlined and made simpler to understand.
Response to the first point:
Thank you for pointing out this. According to this suggestion, we have replaced part of the WB pictures in the revised manuscript to include the upper and lower markers at the same time. However, it still needs to be explained as follows:
We used pre-stained protein marker (Bio-Rad Cat. #1610394) for Western blot analyses. The figure A below is the standard band of this marker (10-250kD). Although the PVDF membrane showed the same bands as the specification, we usually could not capture the obvious 25kD and 75kD bands during exposure imaging (Figure B, below). Due to the predicted molecular weights of several proteins examined in this study (RNF114, CBX5 an CBX3) are around 25kD, we marked the position of 25kD on WB images by a gray solid line according to the electrophoresis mobility and the faint

traces. And we also marked the position of 75kD in revised Fig. S6A.
In addition, in order to efficiently utilize the protein samples, sometimes we cut the western blot membrane into multiple pieces to simultaneously probe for different protein. In Fig. S6A, the band of TAB1 (55kD) is close to β-ACTIN (43kD), the cut position is under 50kD, so the WB picture of β-ACTIN only included one marker-37kD.

To show it clearly, we placed the original image as the below Figure C, with the cutting line.



(A) We have removed data provided to the reviewers in confidence. (B) The pictures showing that the protein marker on the PVDF membrane was in line with its specification under the white light (left), but the 25kD and 75kD bands were usually not clear in the exposed image (right). (C) The origin WB image of TAB1 and β-ACTIN in Fig. S6A. The yellow dotted lines represented the location of the cut.

Response to the second point:
Thanks for your suggestion. We have edited the manuscript in terms of English language usage, in particular the Discussion section, and wish it is more streamlined and simpler to be understood now.

Third decision letter

MS ID#: DEVELOP/2021/199426

MS TITLE: Maternal RNF114-mediated target substrate degradation regulates zygotic genome activation in mouse embryos

AUTHORS: Shuai Zhou, Yueshuai Guo, Haifeng Sun, Lu Liu, Liping Yao, Chao Liu, Yuanlin He, Shanren Cao, Cheng Zhou, Mingrui Li, Yumeng Cao, Congjing Wang, Qianneng Lu, Wei Li, Xuejiang Guo, and Ran Huo
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.