Eif4enif1 haploinsufficiency disrupts oocyte mitochondrial dynamics and leads to subfertility

Yuxi Ding, Zequn He, Yanwei Sha, Kehkooi Kee and Lin Li
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MS TITLE: Eif4enif1 Haploinsufficiency Leads to Subfertility through Disrupted Oocyte Mitochondrial Dynamics

AUTHORS: Yuxi Ding, Zequn He, Yanwei Sha, Kehkooi Kee, and Lin Li

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 substantial revision of your manuscript before we can consider publication. 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 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. 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 referees' 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

Editor:

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This is a well-conceived, well-written manuscript using Eif4enif1-deficient mice to examine the mechanism by which loss of Eif4enif1 impairs fertility and document that Eif4enif1-haploinsufficiency significantly altered mitochondria-associated ribonucleoprotein domain (MARDO) distribution in mouse oocytes. It will stand the test of time as an excellent set of investigations.

Authors:

In this manuscript, the authors generate an Eif4enif1 heterozygous knockout mouse model to mimic the clinical mutations. Their major conclusions are: 1) Eif4enif1 haploinsufficiency significantly altered both the transcriptome and translatome in GV oocytes; and 2) Eif4enif1 haploinsufficiency resulted in dramatic changes of the distribution pattern of MARDO, respectively.

The authors observed follicle loss in aged ovary when they crossed Eif4enif1 heterozygous with WT mice.
Using a T & T dual sequencing, they also found abnormal mRNA and translation profiles in Eif4enif1 hetero oocytes. Following GO analysis, the authors studied mitochondrial dynamics and observed significant mitochondrial hyper fusion and ring-like pattern. The manuscript uniquely documents the molecular changes in Eif4enif1 deficient oocytes. The results are of considerable interest to the developmental biology community and provide significance insight into oocyte maturation and early embryo development.

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Major issues:

1. The authors did not use Eif4enif1 loxp mice for conditional knockout in oocytes. If possible, they could try to confirm the results in ZP3-Cre; Eif4enif1-loxp oocytes in the future.

2. Considering EIF4ENIF1 is not an oocyte-specific protein, did EIF4ENIF1 also control the mitochondrial distribution in somatic cells?

3. In a recently published paper “Germ cell-specific eIF4E1b regulates maternal mRNA translation to ensure zygotic genome activation” (Guanghui et al., Genes Dev, 2023, May), the authors also claimed the roles of eIF4E1b in oocyte translation. Looks like mammals recruit several eIF4E members to control the translation in oocytes. It would be better to add a section to discuss these proteins (eIF4E-BP1, eIF4E1b, CPEB1…).

4. Fig 3a, the authors did not explain why EIF4ENIF1 granules suddenly disappeared after GV stage.

5. The authors claimed both transcription and translation were regulated by EIF4ENIF1. But how? Did the authors try to figure out how EIF4ENIF1 regulate transcription?

6. Is mitochondria fission/fusion directly correlated with transcription/translation? It is hard to jump from major conclusion A to conclusion B.

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2. Fig 1c, compared to the unambiguous DDX6 staining, both granule structures and blurry background were observed in the EIF4ENIF1 staining images. Are EIF4ENIF1 only enriched in those bright granules (p-body)?

3. upper panel, Fig 1c, looks like the EIF4ENIF1 granules were only found in those large oocytes. How about the EIF4ENIF1 pattern in whole mount staining?

4. figure 3a What fraction of EIF4ENIF1 granules co-stained with MARDO? Were all EIF4Enif1 granules wrapped by mitochondria?

Reviewer 2

Advance summary and potential significance to field

This study generated a new POI model, an Eif4enif1 heterozygous mutant, and demonstrated that its haploinsufficiency causes POI, mitochondrial hyperfusion, alteration of MARDO, and impaired oocyte maturation and embryonic development. The authors further performed transcriptome and translatome analysis by a recently developed technology, called T&T-seq to highlight the abnormality. The results are clearly presented, and the manuscript is well written and has potential impact to clinical fields. I support the publication of this manuscript but have a few concerns before publication.

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Major comments

1. The structure of the title is "…leads to X through Y", which implicates that the cause of X is Y. However, this paper only shows the cooccurrence of X and Y in the mutant, and it remains unclear whether Y is really the cause of X in this mutant. Therefore, it is more appropriate to tone down the title, like 'Eif4enif1 haploinsufficiency disrupts oocyte mitochondrial dynamics and leads to subfertility'.

2. T&T-seq showed that both transcription and translation are widely upregulated. Intuitively, the upregulation of translation is reasonable, as Eif4enif1 has repressive functions to translation. However, why is transcription also upregulated? This brings to me a further question of whether T&T-seq enables quantitative comparison between WT and mutant samples without spike-in normalization. Discussion will be helpful to clarify this point as well as the possible mechanisms of transcriptional upregulation.

Minor point

1. Fig. 2D-2L The authors show abnormalities in mitochondrial dynamics in fully-grown GV-stage mutant oocytes, but the number of primordial follicles is reduced at 9m-old. How are the primordial follicles affected in the mutant?

2. Fig.4G-4H The scale of the heat map needs to be explained.

3. Line 313-325 Dilution rates of the primary and secondary antibodies are wanted.

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First revision

Author response to reviewers' comments

Dear reviewers,

Thank you for acknowledging the significance of our work. We have worked to address the issues according to the reviewer's suggestions and made point-by-point responses to them in the response letter. Please see the "decision letter231013.pdf" file in the file list for the full information as figures and tables get lost when pasted into this box. Thanks again for your efforts and patience.

Reviewer 1 Advance Summary and Potential Significance to Field:
Editor:

This is a well-conceived, well-written manuscript using Eif4enif1-deficient mice to examine the mechanism by which loss of Eif4enif1 impairs fertility and document that Eif4enif1-haploinsufficiency significantly altered mitochondria-associated ribonucleoprotein domain (MARDO) distribution in mouse oocytes. It will stand the test of time as an excellent set of investigations.

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The authors observed follicle loss in aged ovary when they crossed Eif4enif1 heterozygous with WT mice. Using a T & T dual sequencing, they also found abnormal mRNA and translation profiles in Eif4enif1 hetero-oocytes. Following GO analysis, the authors studied mitochondrial dynamics and observed significant mitochondrial hyper-fusion and ring-like pattern. The manuscript uniquely documents the molecular changes in Eif4enif1 deficient oocytes. The results are of considerable interest to the developmental biology community and provide significance insight into oocyte maturation and early embryo development. The authors should address the following issues:

We thank reviewer 1 for acknowledging the significance of our work. We have worked to address the issues according to the reviewer's suggestions. Please find below for our answers to the questions and the changes to the text.

Major issues:

1. The authors did not use Eif4enif1 loxp mice for conditional knockout in oocytes. If possible, they could try to confirm the results in ZP3-Cre; Eif4enif1-loxp oocytes in the future.
We thank the reviewer for raising this point. When designing the animal model for this study, we initially aimed to mimic the general *EIF4ENIF1*-deficient state of patients with heterozygous *EIF41ENIF1* mutations, which were systemic and not confined to germ cells. The systemic Eif4enif1 heterozygous mice appeared to be an optimal choice regarding both time and efficiency, using which we were finally able to find out that the most prominent abnormality upon Eif4enif1 arose in the oocytes.
Thanks for the suggestion of refining the models in future studies and we really hope so. The Zp3- and Gdf9-Cre, target-gene-loxp system offers great platforms to study gene functions solely in oocytes as they restrict the deletion of genes to this specific cell type and beyond specific developmental stages. Nevertheless, for the current research question, we believe that the current model has provided compelling evidence to support our findings. We do hope that our findings could be of inspiration to later studies that continue to look into the oocyte-specific function of the EIF4ENIF1 protein, and believe that the conclusions of these studies would further strengthen and expand our current results.

2. Considering EIF4ENIF1 is not an oocyte-specific protein, did EIF4ENIF1 also control the mitochondrial distribution in somatic cells?

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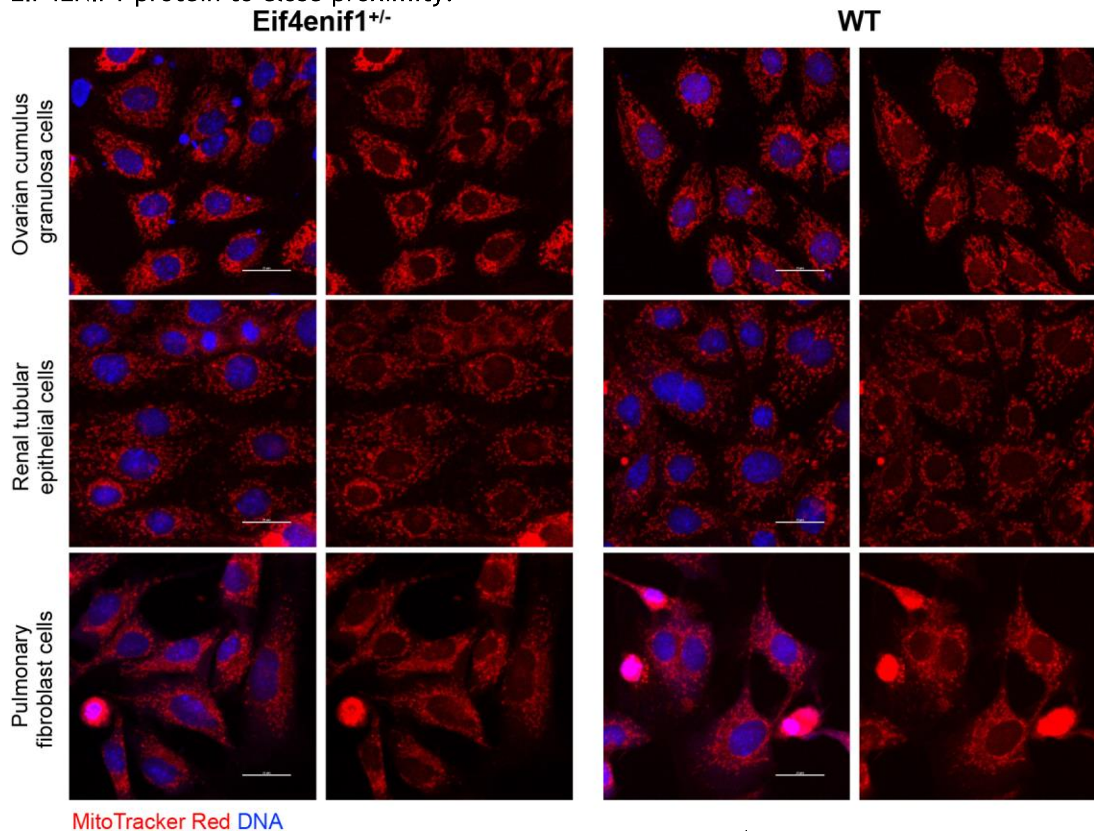
We thank the reviewer for raising this question. To explore whether *Eif4enif1* haploinsufficiency also affects mitochondrial distribution in somatic cells, we obtained ovarian cumulus granulosa cells, renal tubular epithelial cells, and pulmonary fibroblast cells from WT and Eif4enif1$^{+/-}$ female mice by cumulus-oocyte-complex (COC) and explant culture. MitoTracker Red staining was adopted to examine and compare the mitochondrial distribution between genotypes (Response Figure 1). To our observation, no significant alteration was observed in Eif4enif1$^{+/-}$ somatic cells of the selected cell types. It is assumed that EIF4ENIF1 may not be controlling mitochondria dynamics in somatic cells due to the lack of the oocyte-specific MARDO structure, which brings mitochondria and EIF4ENIF1 protein to close proximity.



Response Figure 1. MitoTracker staining of WT and Eif4enif1$^{+/-}$ somatic cells. Bar = 25μm.

3. In a recently published paper "Germ cell-specific eIF4E1b regulates maternal mRNA translation to ensure zygotic genome activation" (Guanghui et al., Genes Dev, 2023, May), the authors also claimed the roles of eIF4E1b in oocyte translation. Looks like mammals recruit several eIF4E members to control the translation in oocytes. It would be better to add a section to discuss these proteins (eIF4E-BP1, eIF4E1b, CPEB1…).

We really appreciate the reviewer for this suggestion. After an extended literature review, we have come up with a discussion section that looks into the involvement of eIF4Es (eIF4E1a/eIF4E1b/eIF4E2) and 4E-BPs (EIF4ENIF1/4E-BP1) in CPEB-related oocyte translation control:

"The crosstalk between eIF4Es (4Es) and 4E-binding proteins(4E-BPs) is essential in translation regulation. In eukaryotic cells, the universally expressing eIF4E participates in forming the eIF4F complex and helps with the recruitment of ribosome small units to the 5' cap of mRNAs (Kamenska, Simpson, et al., 2014). EIF4ENIF1(4E-T) is capable of binding both eIF4E1 and eIF4E2(Kamenska et al., 2016), whereas the latter is nearly absent in late oocytes and early embryos (Guo et al., 2023). Instead, a germ cell-specific eIF4E1 isoform, eIF4E1b, showed confined expression within the late oocyte to the early embryo time window (Guo et al., 2023; Minshall et al., 2007; Yang et al., 2023). In Xenopus oocytes, eIF4E1b, rather than 1a, is the only 4E isoform that was observed to interact with the CPEB complex which contains EIF4ENIF1 but not 4E-BP1(Minshall et al., 2007). In mouse GV oocytes, the cellular location of the eIF4E1b protein is similar to EIF4ENIF1(Guo et al., 2023), while we failed to observe co-localization of eIF4E with EIF4ENIF1 in oocytes at the GV stage using an antibody that should mainly target eIF4E1a, unlike in primordial oocytes or 293FT cells (Fig. S2C, S2D). It seems that eIF4E1b/EIF4ENIF1 is the specific 4E/4E-BP pair that takes part in the CPEB-

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involved translation control in late oocytes and early embryos. Given that EIF4ENIF1 is involved in oocyte mitochondria dynamics, it is worth exploring whether eIF4E1b and CPEB also take part in this process as well as their association with the MARDO.
(Please see line 277-295 for this content in the original text)

4. Fig 3a, the authors did not explain why EIF4ENIF1 granules suddenly disappeared after GV stage.
We thank the reviewer for raising this point. In immunostaining of EIF4ENIF1 in mouse fully-grown oocytes upon meiosis resumption, we observed that EIF4ENIF1 clustered into bright cloud-like structures (the MARDO) spanning the cytoplasm, while it solely surrounded the nucleus region at MI and disassembled at MII.
The loss of cytoplasm-spanning EIF4ENIF1 granules is most likely a combined result of mitochondria distribution change and protein degradation. It is assumed that EIF4ENIF1 is recruited to the mitochondria-surrounding region, and accompanies the mitochondria to surround the spindle at MI and disassociate at MII (instead of forming cytoplasmic clusters as in GV). In this process, the EIF4ENIF1 protein, abundance gradually decreases (Figure 3B,3C), which further contributes to the reduction in the granular signal. Cheng et al. suggested that the degradation of the ZAR1 protein also contributed to the disassembly of MARDO during meiotic resumption, since ZAR1 is essential for the phase separation process of MARDO(Cheng et al., 2022).

5. The authors claimed both transcription and translation were regulated by EIF4ENIF1. But how? Did the authors try to figure out how EIF4ENIF1 regulate transcription?
We would like to thank the reviewer for raising this question, and this is what we are also trying to figure out. We have several assumptions for how *Eif4enif1* haploinsufficiency could affect the oocyte gene expression profile at the mRNA level.
The first possibility is due to the cumulative effects of *Eif4enif1* haploinsufficiency throughout oocyte development. Although we did not identify significant phenotypic changes in Eif4enif1-deficient oocytes earlier than the GV stage, such as the disassembly of P-body granules in primordial and primary follicles, it is likely that *Eif4enif1* haploinsufficiency starts to affect translation of down-stream genes from these early oocyte developmental stages, which may include transcription regulators. These downstream genes could further alter the oocyte transcriptome at later stages. In this way, Eif4enif1 haploinsufficiency might have indirectly regulated the GV-stage transcriptome by altering the translation of transcription regulators in earlier stages.
A second possible explanation is that it affects mRNA stability instead of directly regulating transcription. Cheng et al. reported that MARDO functioned in oocyte mRNA storage and decay. They observed that in *Zar1* (another RNA binding protein in MARDO)-knockout oocytes where the MARDO structure was completely disassembled, many mRNAs were prematurely lost (Cheng et al., 2022). *Zar1* is also known to repress mRNA translation in oocytes, whereas its deficiency causes a significant alteration in both oocyte translatome and transcriptome(Rong et al., 2019, Cheng et al., 2022). We assume that similar situations could be happening in *Eif4enif1*-haploinsufficient oocytes, where the MARDO distribution pattern was also significantly abnormal. It is possible that the composition of MARDO-stored mRNAs was altered upon this distribution abnormality, resulting in different mRNA storage and decay patterns, which ultimately led to a distinct transcriptome.
Another possible mechanism is through metabolic changes. Our results show that Eif4enif1 haploinsufficiency disrupts oocyte mitochondrial dynamics and impacts mitochondria function such as ATP production. It is well established that transcription is an energy-dependent process. ATP is involved in the functioning of the RNA Polymerase II enzyme (RNA Pol II) (Conaway and Conaway, 1988, Sawadogo and Roeder, 1984). Therefore, Eif4enif1 haploinsufficiency might affect RNA Pol II function by changing oocyte cellular ATP levels, disrupting the transcriptome.
Thank you for this constructive question and we have added relevant discussions to the discussion section. (Please see line 259-276 in the original text)

6. Is mitochondria fission/fusion directly correlated with transcription/translation? It is hard to jump from major conclusion A to conclusion B.
We thank the reviewer for pointing out that more details should be given to explain the transition from oocyte translation/transcription to mitochondrial dynamics.
No previous study has reported a direct correlation between oocyte transcription/translation and mitochondrial dynamics, but we did not link the two processes out of thin air. The conclusion of oocyte mitochondria hyperfusion was achieved step by step via a combination of trials including

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translation-transcription sequencing data analysis, downstream functional experiments, and literature reviewing.
When looking into the results of GV oocyte T&T data, we aimed to figure out what main biological processes were altered upon Eif4enif1 haploinsufficiency. The Gene Ontology (GO) analysis was thus adopted to examine the enriched pathways in differentially expressed genes (DEGs) between WT and mutants on both transcription and translation levels. Since EIF4ENIF1 is reported to repress translation in somatic cells and there were more up-regulated genes at both translation and transcription levels, we mainly focus on the pathways enriched by genes with increased expression in mutant oocytes. We examined the up-regulated pathways (which involved MAPK, apoptosis, mitochondria, etc.), and found that the mitochondria distribution pattern in mutant oocytes was abnormal when analyzing phenotypes related to mitochondrial function, which drew our attention towards the mitochondria direction.
After observing increased mt-DNA levels and ATP content in mutant oocytes, we further wondered what mitochondrial abnormality underlay these changes. By literature searching, we found that what we observed in mutant oocytes was very similar to the phenotype of cells with mitochondria hyperfusion (Response Table 1). Therefore, we subjected WT and mutant oocytes to electron microscopy, which confirmed our assumption that E*if4enif1*-haploinsufficient oocytes underwent mitochondrial hyperfusion.
We have revised the manuscript to make this part of the content more detailed and reasonable. Thanks for your suggestion. (please see line 167-172, 187-189 and Table S1)

Phenotype\condition	**Mitochondrial hyperfusion**	**E*if4enif1* haploinsufficiency**
mtDNA content	mtDNA accumulation (Yan et al., 2019)/ elevated but statistically non-significant (*Drp1* KO oocyte)(Adhikari et al., 2022)	Mt-DNA copy number increase
ATP content	Increase(Giacomello et al., 2020)/ elevated but statistically non-significant (*Drp1* KO oocyte)(Adhikari et al., 2022)	increase
Subcellular location	Perinuclear (MFN1/2 overexpression) (Wakai et al., 2014)	Perinuclear

Response Table 1. Comparison between phenotypes observed upon mitochondrial hyperfusion and *Eif4enif1* haploinsufficiency

Minor issues:

1. Fig 1a, the authors examined the expression pattern of EIF4ENIF1 in fetal ovarian stages (E13.5-3dpp), but did not mention the phenotype in the fetal stages. In other words, did the loss of Eif4enif1 affect cyst breakdown and primordial follicle formation?
We appreciate the reviewer for raising this point. We were also interested in the phenotype of the fetal and newborn ovaries.
We performed immunostaining to examine the germ cyst breakdown and primordial process in WT and *Eif4enif1*$^{+/-}$ 0dpp ovaries and did not observe significant abnormalities in the mutants (Response Figure 2). This result is consistent with the phenotype of normal follicle number in young mutant mice.

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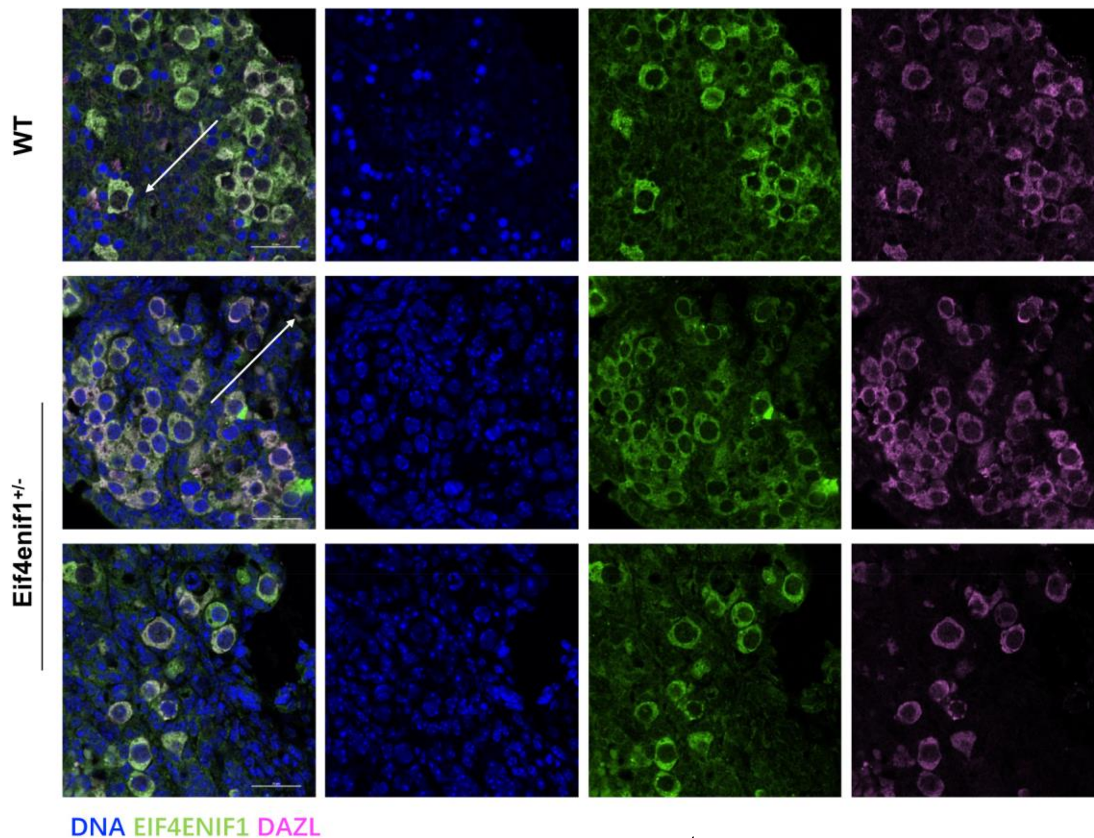

Response Figure 2. Immunostaining of WT and *Eif4enif1*$^{+/-}$ 0dpp mice ovaries. White arrows show the direction of germ cyst disassembly. The lower panel shows the detached primordial follicles in the mutant ovary. Bar = $25\mu m$.

2. Fig 1c, compared to the unambiguous DDX6 staining, both granule structures and blurry background were observed in the EIF4ENIF1 staining images. Are EIF4ENIF1 only enriched in those bright granules (p-body)?

We thank the reviewer for raising this question. According to our observations in ovarian section immunofluorescence, the EIF4ENIF1 protein resides not only within P-body-like granules, but also in diffused cytoplasmic form in early-stage germ cells. i.e., it is not only enriched within P-body-like granules.

3. Upper panel, Fig 1c, looks like the EIF4ENIF1 granules were only found in those large oocytes. How about the EIF4ENIF1 pattern in whole mount staining?

We thank the reviewer for raising this question. We examined the EIF4ENIF1 expression pattern in different cell types within the ovary. The average EIF4ENIF1 expression is higher in DAZL-positive germ cells than in gonadal somatic cells (Response Figure 3). P-body-like granular structures were present in germ cells both within and detached from the germ cysts (Response Figure 2), but were not observed in somatic cells.

We appreciate the reviewer's suggestion of applying whole-mount staining in the ovary sample as it can provide a panoramic view of the protein expression distribution. However, since we are observing subcellular RNP granules, images of high resolution (100X) would be required, which, together with the relatively large size of an ovary, would necessitate optimization of tissue clearing and abundant image stitching. Consequently, the result file might be of very large size and difficult to handle. Therefore, we used tissue sections to analyze the EIF4ENIF1 expression pattern, which could also reveal its distribution among different cell types and different parts of the ovary but appeared to be of lighter data size and more labor-saving.

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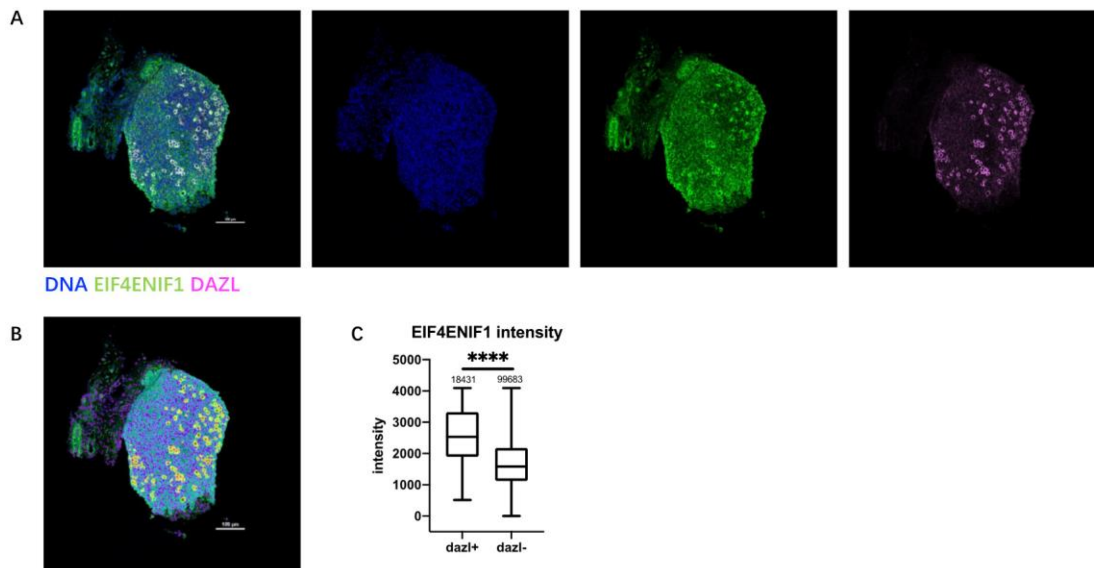
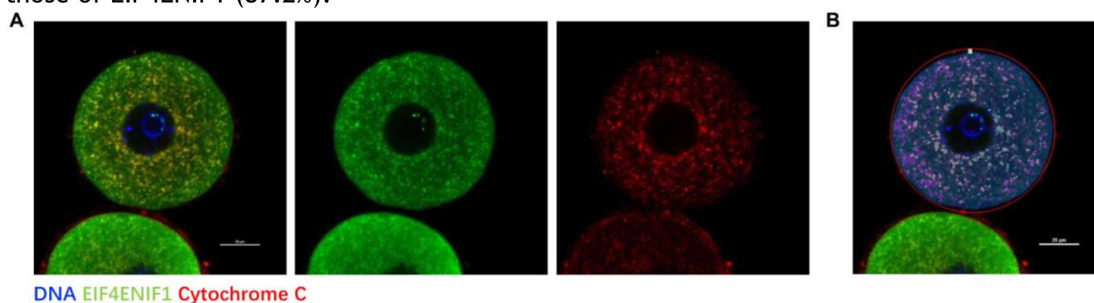


Response Figure 3. EIF4ENIF1 expression in WT 0dpp mouse ovaries.
(A) immunostaining of EIF4ENIF1 and germ cell marker DAZL in a WT 0dpp mouse ovary. Bar = 100 μm.
(B) illustration of threshold-based selection of DAZL positive (yellow) and DAZL negative (cyan) regions.
(C) box plot of EIF4ENIF1 fluorescent intensities of DAZL positive (yellow) and DAZL negative (cyan) regions. Each point stands for a pixel within the selected region. ****: P < 0.0001.

4. Figure 3a What fraction of EIF4ENIF1 granules co-stained with MARDO? Were all EIF4Enif1 granules wrapped by mitochondria?

We thank the reviewer for raising this question. MARDO and mitochondria both adopted a cloud-like appearance in SN GV ooplasm, whose exact boundaries are hard to define. According to our observation and (Cheng et al., 2022), EIF4ENIF1 highly co-localizes with mitochondria (Figure 6A, 6B) and MARDO protein ZAR1 (Cheng et al., 2022). We performed a threshold-based selection of EIF4ENIF1- and mitochondria-positive regions in the ooplasm (which could not be 100% accurate due to the blurry boundaries of both) to measure the portion of the EIF4ENIF1 signal that surrounds the mitochondria (Response Figure 4). 201 EIF4ENIF1 and 220 Cytochrome C (representing mitochondria) were identified in the analysis, among which 192 Cytochrome C spots overlap with those of EIF4ENIF1 (87.2%).



Response Figure 4. Co-localization of EIF4ENIF1 and mitochondria.
(A) immunostaining of EIF4ENIF1 and mitochondria (Cytochrome C) in a represented SN GV oocyte. Bar = 20 μm.
(B) illustration of threshold-based selection of EIF4ENIF1 (magenta)- and mitochondria (yellow)-positive regions.

***** Reviewer 2

Advance Summary and Potential Significance to Field:

This study generated a new POI model, an Eif4enif1 heterozygous mutant, and demonstrated that its haploinsufficiency causes POI, mitochondrial hyperfusion, alteration of MARDO, and impaired oocyte maturation and embryonic development. The authors further performed transcriptome and translatome analysis by a recently developed technology, called T&T-seq to highlight the abnormality. The results are clearly presented, and the manuscript is well written and has potential

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We thank reviewer 2 for acknowledging the content and potential clinal impact of our work. We have worked to address the issues according the reviewer's suggestions. Please find below for our answers to the questions and the changes to the text.

Major comments
1. The structure of the title is "…leads to X through Y", which implicates that the cause of X is Y. However, this paper only shows the cooccurrence of X and Y in the mutant, and it remains unclear whether Y is really the cause of X in this mutant. Therefore, it is more appropriate to tone down the title, like 'Eif4enif1 haploinsufficiency disrupts oocyte mitochondrial dynamics and leads to subfertility'.
We thank the reviewer for this constructive advice. We have re-considered the contents of the research and agree with the reviewer that it is indeed more appropriate to tone down the title. We like the title that was provided by the reviewer and have changed the title accordingly. Thanks for your title suggestion.

2. T&T-seq showed that both transcription and translation are widely upregulated. Intuitively, the upregulation of translation is reasonable, as Eif4enif1 has repressive functions to translation. However, why is transcription also upregulated? This brings to me a further question of whether T&T-seq enables quantitative comparison between WT and mutant samples without spike-in normalization. Discussion will be helpful to clarify this point as well as the possible mechanisms of transcriptional upregulation.
We thank the reviewer for raising this point. we appreciate the ERCC spike-in approach as it enables absolute quantification and provides more accurate normalization among samples of highly different expression profiles. However, the WT and mutant GV oocytes compared in the study were of the same cell type and did not show significant structural differences, which led to the assumption that the transcriptome of WT and mutant should still be relatively similar in a panoramic view. Moreover, only data from the same omics were compared in this study, which excluded the systematic errors brought by library construction differences. We examined the top 50 highly-expressed genes in all transcriptome samples, and no special expression preference between genotypes was observed (Response figure 5), indicating that highly expressed genes were not significantly altered upon *Eif4enif1* haploinsufficiency. Therefore, we believe that our current relative-quantitative normalization approach could offer persuasive comparisons between WT and mutant samples.
In fact, transcriptome changes were also observed in oocytes with deficiencies of other translation regulators (Yang et al., 2023, Cheng et al., 2022). In the work of Yang et al, the ERCC spike-in normalization was performed for normalization of the single oocyte RNA-seq data, while Cheng et al performed relative quantification with TMM-normalized CPM values, indicating that these transcriptome changes might be independent of normalization methods.
We would like to thank the reviewer for the question of how Eif4enif1 haploinsufficiency could affect the oocyte gene expression profile at the mRNA level, which we are also looking into. Below are several assumptions that we have for this phenomenon.
The first possibility is due to the cumulative effects of *Eif4enif1* haploinsufficiency throughout oocyte development. Although we did not identify significant phenotypic changes in Eif4enif1-deficient oocytes earlier than the GV stage, such as the disassembly of P-body granules in primordial and primary follicles, it is likely that *Eif4enif1* haploinsufficiency starts to affect translation of down-stream genes from these early oocyte developmental stages, which may include transcription regulators. These downstream genes could further alter the oocyte transcriptome at

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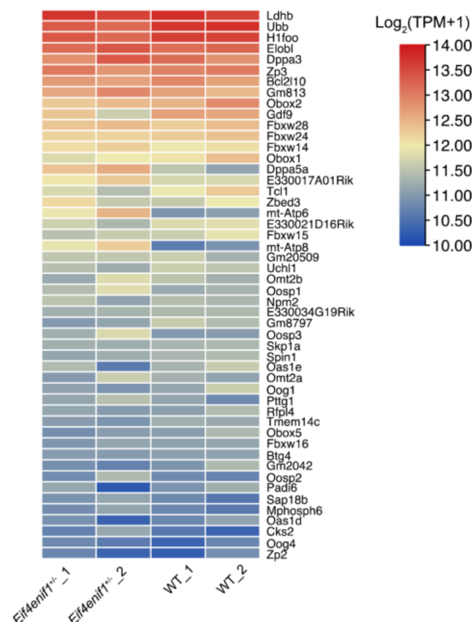
later stages. In this way, Eif4enif1 haploinsufficiency might have indirectly regulated the GV-stage transcriptome by altering the translation of transcription regulators in earlier stages.
A second possible explanation is that it affects mRNA stability instead of directly regulating transcription. Cheng et al. reported that MARDO functioned in oocyte mRNA storage and decay. They observed that in *Zar1* (another RNA binding protein in MARDO)-knockout oocytes where the MARDO structure was completely disassembled, many mRNAs were prematurely lost (Cheng et al., 2022). *Zar1* is also known to repress mRNA translation in oocytes, whereas its deficiency causes a significant alteration in both oocyte translatome and transcriptome(Rong et al., 2019, Cheng et al., 2022). We assume that similar situations could be happening in Eif4enif1-haploinsufficient oocytes, where the MARDO distribution pattern was also significantly abnormal. It is possible that the composition of MARDO-stored mRNAs was altered upon this distribution abnormality, resulting in different mRNA storage and decay patterns, which ultimately led to a distinct transcriptome.
Another possible mechanism is through metabolic changes. As is shown in our results, Eif4enif1 haploinsufficiency disrupts oocyte mitochondrial dynamics and impacts mitochondrial functions such as ATP production. It is well established that transcription is an energy-dependent process. ATP is involved in the functioning of the RNA Polymerase II enzyme (RNA Pol II) (Conaway and Conaway, 1988, Sawadogo and Roeder, 1984). Therefore, Eif4enif1 haploinsufficiency might affect RNA Pol II function by changing oocyte cellular ATP levels, disrupting the transcriptome.
Thank you for this constructive suggestion and we have added relevant discussions to the discussion section. (Please see line 259-276). A paragraph was also added in the method section to explain the analysis details of the RNA-seq-related data. (Please see line 518-525)



Response figure 5. Heatmap of the top50 highly expressed genes in all transcriptome samples.

Minor point
1. Fig. 2D-2L
The authors show abnormalities in mitochondrial dynamics in fully-grown GV-stage mutant oocytes, but the number of primordial follicles is reduced at 9m-old. How are the primordial follicles affected in the mutant?
We appreciate the reviewer for pointing this out. We indeed observed a reduced total follicle number, including primordial follicles, in 9-month-old Eif4enif1-haploinsufficient female mice. Although we originally did not focus on reproductive aging-related processes and therefore, unfortunately, do not have further experimental evidence to closely examine the relevant mechanism, we assume that the middle-age follicle loss might be a result of increased susceptibility of aged germ cells towards detrimental exposure such as oxidative damages (Wang et al., 2020). In the static primordial follicle pool, *Eif4enif1* deficiency might cause harm that accumulates with time and further increases damage susceptibility, leading to exacerbated cell loss. Thanks for mentioning and we believe that this would be a good topic for our future research.

2. Fig.4G-4H

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The scale of the heat map needs to be explained.
We thank the reviewer for raising this point. We checked the data and confirmed that the expression data in Figure 4G and 4H were converted into log2(TPM+1) and standardized (i.e., calculated of Z-scores) by rows before projection onto the color bars. We have amended figure 4G,4H and the relevant figure legends (Please see line 789-790). A relevant paragraph was also added in the method section to explain the analysis details of the RNA-seq-related data. (Please see line 518-525)

3. Line 313-325
Dilution rates of the primary and secondary antibodies are wanted.
We thank the reviewer for this suggestion. For immunofluorescence, primary antibodies were diluted at 1:100, except for mouse anti-DAZL (1:25), and mouse anti-cytochrome C (1:25). For Western blot, primary antibodies were diluted at 1:1000 for rabbit anti-EIF4ENIF1(Abcam), and 1:2000 for mouse anti-α-tubulin. Secondary antibodies were diluted at 1:1000 for immunofluorescence, and 1:2000 for western blot analysis.
We have added the specific dilution rates of primary or secondary antibodies for their respective usages.
(Please see line 357-360, 367-368)

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Second decision letter

MS ID#: DEVELOP/2023/202151

MS TITLE: Eif4enif1 Haploinsufficiency Disrupts Oocyte Mitochondrial Dynamics and Leads to Subfertility

AUTHORS: Yuxi Ding, Zequn He, Yanwei Sha, Kehkooi Kee, and Lin Li
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

I have carefully reviewed the revised manuscript and the authors' responses to reviewers' comments, and I am pleased to report that the authors have made significant improvements to the manuscript.

The revisions have addressed the concerns and suggestions raised by reviewer 2 and me. And they have enhanced the clarity, quality, and overall contribution of the work. The manuscript is now well-structured, and the content is robust, making it suitable for publication in Development. I believe that this work will be a valuable addition to the reproductive field.

Comments for the author

I appreciate the authors' diligence in addressing our suggestions. I strongly recommend the publication of the revised manuscript without further delay. I look forward to seeing this valuable contribution in print.

Reviewer 2

Advance summary and potential significance to field

NA

Comments for the author

All of my concerns were addressed.
*line 266-267. Citation is missing.

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