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CNOT6L Couples the Selective Degradation of Maternal Transcripts to Meiotic Cell Cycle Progression in Mouse Oocyte

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 24th April 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all three referees express interest in the findings reported in your manuscript but they also raise a number of both technical and conceptual concerns that you will have to address before they can support publication in The EMBO Journal.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:
-> Include a more careful characterisation (and quantification) of the CNOT6L knockout (ref #1 and #2)
-> Extensively rewrite the manuscript, both for clarity and to better integrate the findings with the existing literature. Most importantly, you need to include - and discuss - a citation of the 2015 study by Ma et al that reported depletion of CNOT6L via knockdown. In addition, your own previous work on BTG4 is currently described in a way that partly questions its contribution to maternal mRNA clearance. In line with what the referees comments, I would strongly encourage you to discuss the relative contribution of the two distinct adapter proteins and to what extent they act sequentially in CCR4-NOT recruitment during early development.
-> Provide more conclusive evidence for oocyte stageing and the onset of a phenotype in the KO mice (refs #1 and #3). Here ref #3 also points out that more conclusive data is needed to support an

effect on aneuploidy.
-> Provide a clearer presentation of the experimental data, the details of how this data was acquired and the rationale for conducting the experiments in the first place.

REFEREE REPORTS

Referee #1:

The authors show the importance and mechanism of action of CCR4-NOT complex component CNOT6L in the destabilization of maternal transcripts in mouse meiotic maturation and early zygotic development. Interestingly, CNOT6L activity is limited to the meiotic resumption of oocytes, while another factor of maternal transcript degradation BTG4 acts in the maternal to zygotic transition. The authors present extensive in vivo and ex vivo experimental data that gives a very detailed view of the mechanism of stage specific degradation of maternal transcripts during oocyte maturation. This study greatly contributes to our understanding to the mechanisms of maternal mRNA destabilization. The claims in the paper are mostly substantiated and the technically the manuscript is more or less sound. I advocate publishing in EMBO Journal but I have some essential concerns that would need to be addressed first.
Major concerns:
1. The authors have generated a CNOT6L deficient mice by introducing a 10bp (not 11 as stated in text?) deletion with CRISPR/CAS9 system. This was validated through PCR amplification with specific primers on genomic DNA. However, more extensive validation of the successful depletion of CNOT6L is required. The authors need to perform a western blot on the expression of CNOT6L in Cnot6l -/- mice.
2. In the RNA seq data to what degree is the CNOT6L mRNA reduced in the Cnot6l -/- GV and MII oocytes?
3. It is interesting how such a global effect on transcript clearance can result only in severe subfertility. Redundancy could be one mechanism; the authors should discuss this at least.
4. The study shows that Cnot6l -/- females are severely subfertile. It is quite intriguing how after one or two litters the females completely lose fertility. This raises the questions whether there is abnormal folliculogenesis with age that leads to exhausting the pool of primordial follicles? This would be an additional phenotype that would at least need to be documented. Histological analysis of the ovaries in young and adult mice (28 weeks) with evaluation of the numbers of different stage follicles can provide essential information about the normal progression through folliculogenesis.
5. The authors claim that Cnot6l -/- females can develop fully grown GV stage oocytes but they do not offer any data showing number of normal GV oocytes obtained from WT and Cnot6l -/- females. GV oocytes normally consist of 90% of cells with surrounded nucleolus (SN) and only 10% having a non-surrounded nucleolus (NSN). It has been shown that only SN GV oocytes mature to competent MII oocytes that can be fertilized and support early embryo development. Thus a simple staining with DNA dye and evaluation of the GV oocytes in Cnot6l-/- females can confirm that the phenotype is during oocyte maturation rather than at an earlier stage. This is a very important point.
6. Relating to the point above, it is very important to simply determine when the molecular phenotype kicks in. Are there already deregulated genes in GV oocytes. The authors need to present simple pairwise scatter plots of gene expression between wild type and KO for each of the GV, MI, MII and zygote stages. This is an essential point as if there is already deregulated gene expression in GV, the focusing of the claim that CNOT6L regulates transcripts during meiotic maturation is misleading; the authors would need to state that CNOT6L also functions in the formation of the maternal transcriptome in this case.
7. For the scatter plots requested in point 6, the authors need to define the number of genes deregulated per developmental stage and should state the thresholds used in the analysis, i.e. state the fold change and statistical significance parameters used to define deregulated transcripts.
8. For the RNA-seq datasets, biological duplicates are presented. I think for the most important datasets, i.e. GV and MII, at least biological triplicates must be presented. This is key to a powerful statistical analysis where one can confidently identify deregulated transcripts.
9. The RNA-seq datasets have not been deposited in a repository. The raw data needs to be deposited and accession numbers for RNA-seq datasets provided in a revised manuscript. This is a

critical point.
10. Figure 4E shows only the overlap of genes destabilized in GV-MII in WT, Cnot6l-/- and Btg4-/- while it will be interesting to understand whether transcripts that are stabilized in Cnot6l-/- oocytes are those that must be destabilized. Therefore, what is the overlap of genes destabilized in WT GV-MII transition and the genes upregulated in Cnot6l-/- GV-MII transition?
11. In the abstract, introduction and discussion the authors assert that the physiological role of meiotic resumption-coupled mRNA degradation is undefined; as exemplified by the following statement: 'The oocyte maturation-accompanied mRNA decay is considered a prologue of MZT in mammals, but its cellular function and physiological importance have been inconclusive.'. I think the authors may want to moderate these claims in the light of published findings 1-4 that attest to the importance of RNA-degradation and especially if it turns out that CNOT6L-deficient oocytes already show defective gene expression.
12. The following statement requires revision: 'We showed that Cnot6l deletion yields a phenotype similar to that of deletion of TUT4 and -7 (key enzymes of mRNA uridylation) or of YTHDF2 (reader of mRNA m6A modification) in oocytes, including impaired spindle assembly, polar body extrusion, and maternal mRNA stabilization (Ivanova et al., 2017; Morgan et al., 2017; Qi et al., 2016). Therefore, our results support in vivo participation of CCR4-NOT in the degradation of uridylated and m6A-labeled maternal mRNAs during oocyte maturation.'. Combined TUT4 and 7-deficiency results in a problem in the formation of a functional maternal transcriptome that is distinct from meiotic maturation related degradation presented for CNOT6L-/- mice, however the meiotic defects observed are similar. This underscores the necessity of presenting the impact of CNOT6L-deficiency on the GV transcriptome. YTHDF2-deficiency has a normal GV but a defective MII transcriptome and the consequences are in mitotic divisions of the zygotes. The authors should be more precise in their discussion and understand if CNOT6L is required for the formation of the maternal transcriptome.
Minor concerns:
1. Page 5 line 133 the (Fig.S1C) should also include D and E.
2. In Figure 1C please specify the number of mattings per genotype.
3. In the paragraph on page 7 in lines 173,178 Fig.3A and Fig.3B are missing parentheses.
4. Page 8 line 206 the sentence is grammatically incorrect.
5. Page 8 line 218 the sentence is about MI but in parentheses (22%vs 76% of mRNA degrades...) 76% corresponds to the MII stage (line 214 and 215).
6. In the text are used both GV-MII transition and GV-to-MII transition if they mean the same thing please chose one and use it throughout the text consistently.
7. In Material and Methods the mouse strain is C57B6 but should be C57Bl6.
8. Figure 6E is missing the error bar or they are not visible.
9. Supplementary figure 1A the marker in red letters deletion counts for 10bp not 11bp as indicated.

10. Supplementary figure 3D, there are missing lines in the bar plot indicating mean or average value.
References
1. Su, Y. Q. et al. Selective degradation of transcripts during meiotic maturation of mouse oocytes. Dev. Biol. 1, 104-117 (2007).
2. Ma, J., Flemr, M., Strnad, H., Svoboda, P. & Schultz, R. M. Maternally recruited DCP1A and DCP2 contribute to messenger RNA degradation during oocyte maturation and genome activation in mouse. Biol Reprod 88, 11 (2013).
3. Ma, J., Fukuda, Y. & Schultz, R. M. Mobilization of Dormant Cnot7 mRNA Promotes Deadenylation of Maternal Transcripts During Mouse Oocyte Maturation. Biol. Reprod. 93, 48 (2015).
4. Ivanova, I. et al. The RNA m6A Reader YTHDF2 Is Essential for the Post-transcriptional Regulation of the Maternal Transcriptome and Oocyte Competence. Mol. Cell 67, 1059-1067.e4 (2017).

Referee #2:

General comments
Elimination of maternal mRNA across the oocyte-to-embryo transition is thought to play an important role in cytoplasmic reprogramming that eventually allows transfer of control of

development from maternal mechanisms to that of the newly formed embryo. The importance of this mRNA elimination process, however, has been difficult to fully test because the factors involved are not fully known. Here, the authors investigate the role of CNOT6L in this process. This is an exciting question they are addressing that would be of interest to a wide readership.
In this manuscript, the authors demonstrate that CNOT6L plays a pivotal role in oocyte maturation and female fertility through downregulation of a large number of maternal mRNAs during the transition from the GV to MII oocyte stage. By RNAseq analysis, they show that, in the presumed absence of CNOT6L, 76% of maternal mRNAs fail to be downregulated during oocyte maturation. In the absence of de novo transcription, this likely represents an important role for CNOT6Lin the elimination of maternal transcripts by mRNA decay. In support of this, they show that loss of Cnot6l leads to deadenylation of a subset of mRNAs regulated by CNOT6L. Using a polysome gradient to evaluate mRNAs being actively translated, the authors nicely show that a subset of mRNAs downregulated in the presence of CNOT6L indeed remain associated with ribosomes and likely continue to be translated.
Interestingly, mRNAs normally downregulated by CNOT6Lduring oocyte maturation are highly enriched for translational machinery and factors regulating translation. How this leads to meiotic arrest is not addressed, but it is almost certainly an indirect effect. (However, that Cnot6l plays a critical role in the turnover from maternal to embryonic translational machinery across the OET is an interesting possibility?). The authors also show that the mRNAs upregulated with Cnot6l knockout are enriched for AU-rich 3'UTR sequences and that a subset are associated with ZFP36L2. They show that translation of CNOT6L itself is likely upregulated during normal oocyte maturation in a 3'UTR CPE- and ERK1/2-dependent manner and can coexist on regulated mRNAs together with ZFP36L2. They argue that these CNOT6L activities are overlapping but yet distinct from those of BTG4/CNOT7 and propose that the 2 pathways might play distinct but complementary stage-specific roles to eliminate a significant portion of the maternal mRNA pool during oocyte maturation, which is an interesting possibility.
Major concerns
The authors present a large amount of data from well-designed experiments addressing this important question. We have a couple major concerns.
1. Cnot6l knockout mouse. The authors describe how Cnot6l was targeted and show evidence of the short 11 nt DNA deletion by PCR. However, critical data demonstrating significant knockdown or knockout of the CNOT6L protein is not provided. The efficiency of the proposed premature termination codon in inducing NMD must be demonstrated as it is possible a truncated form of Cnot6l protein could still be expressed with associated artifacts. Without these data, the authors and the readers are unable to draw conclusions as to the mechanism for the data presented and the role of CNOT6L.
2. Writing/presentation of data. While there is a large amount of interesting findings presented, the logic connecting the findings in the manuscript in its current form is difficult to follow and frustrating to read. It would be helpful if the authors can find a way to present it as a more logical, coherent story and to better synthesize the findings (both the details and the bigger picture) for the reader. As is, the data seem pieced together without a clear logical connection between some of the figures. Interesting directions are established (e.g., ZFP36L2, CPE regulation, BTG4 parallels) but then seem quickly abandoned for a different direction. For instance, the rationale for comparison to BTG4 is interesting with some backreading but not clearly presented for the reader and the stage-specific hypothesis for CNOT6/ZPF36L2 and CNOT7/BTG4 is exciting but not clearly presented or fully developed. The rationale for choosing to look at ZFP36L2 should be provided as well. Admittedly it is difficult to adequately introduce so many different topics but without it the rationale and chain of logic is lost.
3. Experimental details. Many details needed to understand and critically evaluate the findings are lacking. We have done our best to list what we could under minor concerns below but the list is not exhaustive.
4. Decay vs. deadenylation without decay. The authors cannot distinguish between these 2 possibilities with the experiments presented. This possible alternative explanation for their findings should be addressed in the discussion. And technically, the authors have not shown a direct effect on mRNA stability (i.e. by stability assays) for CNOT6L for any of the eliminated mRNAs although, in the absence of transcription, this is admittedly the most likely explanation.
5. Indirect effects. Unless the authors can connect the meiotic defects to specific defects in mRNA decay, that these are likely indirect/downstream effects should be addressed in the discussion. In addition, some of the eliminated mRNAs might be via downstream/indirect effects instead of direct targets of CNOT6L. Also, given that the KO is global, it is possible that effects of Cnot6l in other

cell types could contribute to the phenotype.
More minor concerns
There are lots of potentially very interesting data presented but many details important for adequate evaluation by the reader are missing. This is a long list but provided with hopes it will aid the authors in revision.
1. Figure 1 and associated text
o 1A-B-- Authors argue Cnot6l is the most important CCR4 protein during oocyte maturation. This conclusion cannot be made based on mRNA levels as determined by qPCR. This is especially true in light of the fact that (as the authors point out themselves) that mRNA in the oocyte is accumulated and stored for later translational activation. While mRNA and protein levels might correlate in somatic cells (and this is arguable), the oocyte is unique and this cannot be assumed. If this claim is based on mouse phenotype, it needs to be qualified.
o 1B - If I am not mistaken, this is not a novel finding with respect to Cnot6l mRNA levels being significantly higher than Cnot6 in oocytes. Ok to leave for completeness but they need to appropriately reference Ma et al, 2015, somewhere.
o 1D - Quantification to show what % of MII oocytes have no polar body and/or distorted multipolar spindles should be included. Also, legend should explain arrows.
o 1E - The relative numbers of 1 cell embryos obtained from WT and KO (i.e., rates of fertilization) should be included. They are likely dramatically different but this has not been addressed. Are the "1 cell" cells shown zygotes? Cannot see PN.
o Lines 136-7 -- The authors claim ovaries of KO females are of normal histology but histology of ovary is not shown. H&E stain of ovarian section of adult cycling KO female should be shown with WT. Ovarian follicle counts in prepubertal vs. adults should also be provided as subfertility phenotype suggests premature depletion of follicles.
2. Figure 2
o 2A-B - The number of oocytes growing to GV stage comparable for KO vs. WT should be provided. It is important for the reader to be able to assess the degree to which there was also earlier effects on folliculogenesis, which was not addressed.
o 2C - Text states "majority" aneuploid but no quantification provided. Top2 and CREST are not described. Unclear what 20*2 means.
o 2F - Do not see TPX2 explained anywhere.
3. Figure 3
o In general, the significance of these experiments and what conclusions can be drawn are difficult to determine for anyone that is not a meiosis checkpoint expert. Additional explanation as to why these specific targets were chosen for investigation and what conclusions can be drawn from these findings and why are needed here.
o 3C-D -- There is no mention about what Crest is.
4. Figure 4
o 4A - Were there differences in mRNA levels in GVs at the start? These data are important to assess effects of Cnot6l during folliculogenesis.
o 4B - Not clear why it was hypothesized that mRNAs of a specific abundance would be targeted or what this adds. However, useful overall to see relative levels and comparison with Btg4.
5. Figure 5
o 5B-5D - If there was a rationale for choosing these specific genes to test, would be useful for the reader to know. For instance, knowing that you chose genes that you found to be regulated by Cnot6l but not Btg4 would help explain why you see little to no effect for Btg4 in 5C-D. If not, this should be briefly addressed.
o 5D - Actin should be included again as a negative control for comparison. Also curious that many of the mRNAs do not appear to be decreasing in abundance with deadenylation and/or with the transition from GV to MII?
6. Figure 6
o 6A - This data can be interpreted that CPEB1 protein is stablized in Cnot6-/- oocytes or increased due to mRNA stabilization. Seems they suggest protein stabilization here as a mechanism (phosphorylated but not degraded) and increased translation as a mechanism later in Fig. 8. Both might be trued but not addressed and spread over multiple figures. This entire last section feels disorganized and the logic connecting the experiments is not clear. Reader is left to fill in the gaps.
o 6B - Number of biological replicates not provided for here or for most experiments.
o 6B and Lines 287-8 - This conclusion is not supported as the authors do not show here that the increase in CNOT7 and cyclin B1 are due to CPEB action. And they need to provide references and explain they are looking at these proteins as they have been shown to be CPEB targets by others.

o 6G-H - Confusing that these are 2 separate figures but you have to look ahead to see the full legend as it is spread across both. Maybe make 1 figure and label stages instead?
7. Figure 7
o 7A-B - How were AREs defined/identified? Or the validated AREScore? Was one of the ARED databases used?
o 7D - It was really difficult to find that the IP here is to HA tag. It is only in one tiny label in the corner of the figure. Please add to the figure legend and Materials and Methods to make this clear as the experiment is not interpretable without this information.
o 7C- Also are the authors claiming that CNOT6L and CNOT7 are in distinct complexes? Otherwise, the potential significance of greater association with one CNOT6L relative to CNOT7 should be addressed in the text. Ideally, the reverse IP experiment would be done as well to confirm this finding.
8. Figure 8
o 8A-B - Similar experiments and conclusions have been published. Although these constructs might have been made to investigate the effect of Erk1/2 on Cnot6 translation, please review Ma et al.2015, from Schultz lab and reference as appropriate. Also, in the Schultz manuscript, the 3'UTR of Cnot6l has 3 CPE sequence and 1 HEX sequence, while Figure8A in this manuscript shows 4 CPE sequences. Unclear why there would be a difference?
o 8B-D - These results should be quantitated if possible over a large number of oocytes. Number of oocytes evaluated for each condition and number of experiments performed should also be provided.
9. Figure S1
o S1F - This would be useful earlier in the figure assuming the constructs are the same used for S1D-E. Also, in the related text, an explanation as to the significance of the catalytic site (I assume it is needed for deadenylation activity but this is never stated) and either a reference to a manuscript showing this mutation abolishes activity or to show loss of activity here. Same with N-terminal LRR domain. I assume there is a reference that can be cited to show interaction between Cnotl6 and Cnot7 and that this is the site of interaction. Otherwise, these 2 things need to be shown if they can't be referenced.
o S1E - No WT bar but reference in legend? Also, need to define what is meant by "rescue", "partial rescue" and "abnormal". Not sure how to interpret these data.
10. Figure S2
o Line 251 - "Remarkably, the transcripts of the first three categories were not enriched among the transcripts stabilized in Btg4-/- oocytes (Fig. S2E)." It is hard to understand the meaning of this sentence because GO terms in Cnot6-/- and Btg4-/- are nearly identical.
o Lines 226-7 -- The Cnot6l knockout caused more significant mRNA accumulation than the Btg4 knockout did (Fig. 4B; Fig. S2B). Again, not sure what this conclusion is based on as effect for Cnot6l and Btg4 in these figures look similar.
o S2B - Please explain the bars, lines, boxes, etc. in legend.
o S2C - Ratios are opposite here relative to text, i.e., MII/GV vs. GV/MII. Would be much more clear if consistent.
11. Figure S4
o S4B - Decreased oocyte maturation with ZFP36L2 depletion has been shown and published. Please review and reference Ball et al, 2014, and Dumdie et al, 2018, as appropriate.
12. Other comments
o Number of biological repeats not provided for each experiment. There is only a general statement in materials and methods that most experiments included 3 independent samples.
o Seems nonideal to normalize RNAseq data to a single spike in RNA although potentially ok with large shifts as seen here. Was a second level of normalization performed after normalization to the spike in? If so, this should be described. Also, authors should consider using ERCC cocktail in future experiments (Thermofisher catalog # 4456740).
o Line133 -- FigS1C doesn't show subfertility data?
o Lines 157-159 - Btg4 mouse seems to come from nowhere without rationale for the comparison or description of the mouse knockout.
o Line 233 - A lower limit needs to be provided for Cluster II (i.e., < 10 but > than what?)
o Line 235 - Do you mean MII/zygote > 10?
o Line 244 - the number 644 needs some context for the reader to determine if this is a large percentage or small percentage
o Line 281-283 - The sentence needs references.
o Line 286-288 -- needs references.

o Line 323 - which genes are related to spindle formation? This seems relevant.
o Lines 394-395 - Would suggest to describe importance of RNA modification instead of "hotness".
13. Strong statements. There are many statements in the manuscript that are unnecessarily and, often, inappropriately strong in our opinion. We have detailed some below but this is not an exhaustive list
o Line 165 and others - "Indispensible" is too strong without complete block in development.
o Lines 393-394 - Is this true? Schultz lab data on CNOT7 is not knockout but still genetic.
o Line 395 - "most suitable" is too strong
o Lines 406-8 - Overly speculative to extend results from this manuscript to a role for CNOT6L in decay of mRNAs modified by uridylation and/or M6A, particularly in the very first paragraph of the discussion.
o Line 431 - "universally acknowledged" is too strong
o Line 433 - as elsewhere in the manuscript, the mRNAs were upregulated but increased stability was not actually shown
o Line 439 - "solely" is too strong and this statement is not referenced
o Line 445 - "ensures" is too strong a conclusion for this proposed mechanism
o Line 484-481 - Concluding sentence seems awkward and a forced attempt to directly connect decay to meiotic arrest in our opinion.
o Lines 506-12. Important to include polyA tail lengths since relevant for translation efficiency in the oocyte. Also, were the mRNAs capped?
o Lines 514-18. Please provide RNA concentration, not just volume.
o Lines 618-631 - Please provide information as to the quality of the RNAseq data-reads, percent uniquely mapped reads, etc. Also, how was differential expression determined? Cutoffs? Q value?

Referee #3:

This manuscript addresses whether the Cnot6L subunit of the CCR4-NOT deadenylase complex regulates maternal mRNA degradation during oocyte maturation. Cnot6l knockout female mice are subfertile despite the presence of morphologically mature oocytes. Cnot6l knockout oocytes have meiosis I defects including a reduced rate of polar body extrusion. The knockout oocytes show impaired deadenylation and degradation of maternal mRNA, consistent with previous findings (Ma et al., 2015).

Comments:

1. My main concern regards some inconsistencies regarding the defects during meiotic cell cycle progression. The rate of polar body extrusion (PB1), i.e. the meiosis I division, is around 10% at 16 h after hCG in Cnot6l knockout oocytes (Fig 1D, S1D). However, 24 h after hCG more than 50% of the cells shown in Fig 1F have at least one polar body, although at this magnification it is not possible to discern between PB1 and PB2. Nevertheless, could the authors explain how so many cells have divided and indeed then again roughly 50% of embryos progress to the 2-cell stage, suggesting that at least 50% of oocytes had progressed to meiosis II in order to be fertilized and generate embryos.

2. The claim is made that "Although a small proportion of Cnot6l knockout oocytes released PB1 and developed to the MII stage (Fig. 2B), their chromosomes were mostly aneuploidy" (p. 6) (Fig. 2C). The legend of Fig. 2 says that numbers of chromosome pairs are indicated. What exactly is meant by chromosome pairs? The left (wt) and right (KO) panels show dyad chromosomes, whilst the middle panel (KO) is actually showing bivalent chromosomes. It is also not clear what "*2" indicates. Since loss of chromosomes can be a technical artifact of chromosome spreads, the claim that there is aneuploidy in the meiosis II eggs would need to be substantiated by a quantification and clear selection for MII eggs.

3. It is standard in the field to select mature oocytes that fulfill certain morphological criteria and undergo germinal vesicle breakdown (GVBD) within 60-120 min, depending on the mouse strain. Could the authors provide a rationale for why oocytes were selected for imaging if they underwent GVBD within 6 h after release? The problem with this is that potentially immature oocytes would be included in the experiment, which will have spindle defects such as those shown in Fig. 3A. To substantiate the claim that there are spindle defects, it would be necessary to rigorously select

mature oocytes that undergo GVBD within at most 2 h, provide time-lapse images starting with the GV state to show the typical surround-nucleolus (SN) configuration of mature oocytes and to quantify the defects in spindle assembly in a population of cells.

4. The SMC3 staining of bivalent chromosomes in Fig. 3C does not recapitulate the known the localization to the inter-chromatid axis. This raises some questions about the specificity of the antibody. It would be more appropriate to stain for meiotic cohesin.

5. Given that such few Cnot6l knockout oocytes undergo the meiosis I division, how were MI and MII oocytes selected for the RNA-seq experiments in Fig. 4? Is there a molecular marker within the data sets that can convincingly exclude the possibility that the MII oocytes are not contaminated with MI-arrested oocytes, which would contribute more mRNA and therefore appear to show defective mRNA degradation?

6. For the HPG experiments, the control of complete translational inhibition is missing to know to what extent the signal is specific (Fig. 6C).

7. The ERK1/ERK2 inhibition experiments are over-interpreted.

8. Previous work using an siRNA approach to knockdown CNOT7 and CNOT6L also showed an inhibition of mRNA deadenylation and found that CNOT6L is a dormant maternal mRNA (Ma et al., 2015). I am in favor of using the genetic knockout approach and would recommend that the authors more extensively compare and contrast their findings with this previous study.

1st Revision - authors' response 5th July 2018

Re: Manuscript EMBOJ-2018-99333

Referee #1:

1. The authors have generated a CNOT6L deficient mice by introducing a 10bp (not 11 as stated in text?) deletion with CRISPR/CAS9 system. This was validated through PCR amplification with specific primers on genomic DNA. However, more extensive validation of the successful depletion of CNOT6L is required. The authors need to perform a western blot on the expression of CNOT6L in Cnot6l -/- mice.

Response:

1) It should be 10 bp deletion, not 11 bp. We have corrected the labeling in Fig. S1A. Thanks to the reviewer for pointing it out.

2) We did not provide a western blot result in the first version of the manuscript because there are no CNOT6L-specific antibodies available. We have purchased a commercially available polyclonal CNOT6 antibody (Abcam, ab86209). This antibody recognizes both mouse CNOT6 and CNOT6L ectopically expressed in HeLa cells due to their high homology (Fig. EV1C). Based on the fact that expression of Cnot6l is more abundant than Cnot6 in mouse oocytes at the mRNA level, we used this antibody to detect CNOT6L protein expression in WT and *Cnot6l* null oocytes. The western blot result showed that the expected CNOT6/6L band was clearly detected in WT oocytes but its intensity was greatly reduced in Cnot6l null oocytes (Fig. EV1D), indicating that CNOT6L protein was successfully depleted in these oocytes. We hope that these new results have satisfied the reviewer's concern about the actual depletion of CNOT6L protein in the oocytes of our mutant mice.

2. In the RNA seq data to what degree is the CNOT6L mRNA reduced in the Cnot6l -/- GV and MII oocytes?

Response: In the RNA-seq data, *Cnot6l* mRNA level significantly decreased in the *Cnot6l* null GV and MII oocytes. We have extracted the data and presented in revised Figure 4C.

3. It is interesting how such a global effect on transcript clearance can result only in severe subfertility. Redundancy could be one mechanism; the authors should discuss this at least.

Response: We have used 5 *Cnot6l* null females in the 32-week fertility test. Four of them were completely infertile. Only 1 female give birth to 2 pups during this period. Although the majority of

the *Cnot6l* null females were completely infertile, redundancy could certainly be one mechanism that caused only subfertility in some *Cnot6l* null mice. We have discussed this issue in the revised manuscript as the reviewer suggested.

4. The study shows that Cnot6l -/- females are severely subfertile. It is quite intriguing how after one or two litters the females completely lose fertility. This rises the questions whether there is abnormal folliculogenesis with age that leads to exhausting the pool of primordial follicles? This would be an additional phenotype that would at least need to be documented. Histological analysis of the ovaries in young and adult mice (28 weeks) with evaluation of the numbers of different stage follicles can provide essential information about the normal progression through folliculogenesis.

Response:

1) We appreciate the reviewer's good suggestion to check folliculogenesis in the ovaries of Cnot6l null females. Actually we have already done so, but did not include the data in the original manuscript, with the thought of avoiding potential distraction.
2) We did the histological analyses for ovaries of 3- and 6-month-old *Cnot6l* null mice. H&E staining results showed that all these ovaries contain multiple developing follicles and corpus lutea (revised Figure EV2A). Therefore, the *Cnot6l* null mice do not have a premature ovarian failure phenotype as the oocyte-specific *Ddb1* and *Dcaf1* knockout mice we reported before (Yu C et al., 2013 Science; Yu C et al., 2014 Mol Human Reprod). We did not quantify the follicle numbers for the whole ovary because this is quite a lot of work and the *Cnot6l* null mice have normal ovarian histology.
3) It is not surprise that *Cnot6l* knockout does not affect folliculogenesis because the *Cnot6l* mRNAs were dormant in GV stage-arrested mouse oocytes (Ma et al, 2015; and our current study). Only after meiotic resumption, MAPK cascade triggered their translation in maturing oocytes, and CNOT6L proteins begin to accumulate and catalyze mRNA deadenylation. This is a key mechanism that accelerates the mRNA turnover during oocyte meiotic maturation. We have further discussed this issue in the revised manuscript.

5. The authors claim that Cnot6l -/- females can develop fully grown GV stage oocytes but they do not offer any data showing number of normal GV oocytes obtained from WT and Cnot6l -/- females. GV oocytes normally consist of 90% of cells with surrounded nucleolus (SN) and only 10% having a non-surrounded nucleolus (NSN). It has been shown that only SN GV oocytes mature to competent MII oocytes that can be fertilized and support early embryo development. Thus a simple staining with DNA dye and evaluation of the GV oocytes in Cnot6l-/- females can confirm that the phenotype is during oocyte maturation rather than at an earlier stage. This is a very important point.

Response: We have done this experiment as the reviewer suggested. We collected cumulus-oocyte complexes (COCs) containing fully-grown GV oocytes from antral follicles of PMSG-primed *Cnot6l* null mice by needle puncturing under a stereoscope, and determined their chromatin configuration by DAPI staining. The results in revised Figure EV2 showed that similar numbers of COCs were harvested from ovaries of WT and *Cnot6l* null mice; more than 90% of GV oocytes from COCs of WT and *Cnot6l* null mice have a surrounded nucleolus (SN) as the reviewer indicated. These results further suggested that the oocyte development of Cnot6l KO mice is normal before meiotic maturation.

6. Relating to the point above, it is very important to simply determine when the molecular phenotype kicks in. Are there already deregulated genes in GV oocytes. The authors need to present simple pairwise scatter plots of gene expression between wild type and KO for each of the GV, MI, MII and zygote stages. This is an essential point as if there is already deregulated gene expression in GV, the focusing of the claim that CNOT6L regulates transcripts during meiotic maturation is misleading; the authors would need to state that CNOT6L also functions in the formation of the maternal transcriptome in this case.

Response: We have done this analysis as the reviewer suggested, and presented pairwise scatter plots of gene expression between WT and KO for each of the GV, MI, MII and zygote stages. The results in revised Figure 4D showed that relatively very small numbers of transcripts were up- or down-regulated for more than 5 folds in *Cnot6l* null oocytes at the GV stage; the numbers of upregulated and downregulated genes are not significantly different (78 vs. 71). In contrast, remarkably more transcripts were upregulated than downregulated in *Cnot6l* null oocytes at the MI (1678 vs. 18) and MII (1164 vs. 17) stages, as well as in zygotes derived from *Cnot6l* null oocytes (179 vs. 22). This trend still holds true when we reduced the threshold of analyses to transcripts with

fold changes > 2 (Fig. EV3C). These results strongly support out claim that CNOT6L specifically regulates transcripts during meiotic maturation.

7. For the scatter plots requested in point 6, the authors need to define the number of genes deregulated per developmental stage and should state the thresholds used in the analysis, i.e. state the fold change and statistical significance parameters used to define deregulated transcripts.
Response: For the scatter plot charts (Figure 4D and Figure EV3C), we indicated the number of genes upregulated and downregulated per developmental stage, statistical significance parameters, and the thresholds used in the analyses.

8. For the RNA-seq datasets, biological duplicates are presented. I think for the most important datasets, i.e. GV and MII, at least biological triplicates must be presented. This is key to a powerful statistical analysis where one can confidently identify deregulated transcripts.
Response: We appreciate the reviewer's comments that biological triplicates of RNA-seq data might be better. However, setting up new RNA-seq analyses might cause a significant delay of publishing these results. Our qRT-PCR results have already confirmed that the presented RNA-seq data are reliable. We also checked the recent papers that presented oocyte RNA-seq results (Babani M et al, Mol Cell, 2017, 68:1083-1094; Zhang B et al, Nature, 2016, 537:553-557; Zhao B et al, Nature, 2017, 542:475-478; Hendrickson PG et al, Nature Genetics, 2017, 49:925-934). These papers all presented RNA-seq results in biological duplicates, suggesting that biological duplicates are widely accepted in the field.

9. The RNA-seq datasets have not been deposited in a repository. The raw data needs to be deposited and accession numbers for RNA-seq datasets provided in a revised manuscript. This is a critical point.
Response: We have promised to the journal that we will deposite the original RNA-seq datasets and provide accession numbers before final publication of the revised manuscript. But it is too early to release the information for now because our manuscript has not been accepted for publication yet. This is allowed by the journal policy.

10. Figure 4E shows only the overlap of genes destabilized in GV-MII in WT, Cnot6l-/- and Btg4-/- while it will be interesting to understand whether transcripts that are stabilized in Cnot6l-/- oocytes are those that must be destabilized. Therefore, what is the overlap of genes destabilized in WT GV-MII transition and the genes upregulated in Cnot6l-/- GV-MII transition?
Response: As the reviewer suggested, we showed in revised Figure 4I the overlap of genes destabilized in WT GV-MII transition (FPKM > 1; fold change (GV/MII) > 10) and the genes upregulated in Cnot6l-/- GV-MII transition (FPKM > 1; fold change ($Cnot6l^{-/-}$/WT at MII) > 10).

11. In the abstract, introduction and discussion the authors assert that the physiological role of meiotic resumption-coupled mRNA degradation is undefined; as exemplified by the following statement: 'The oocyte maturation-accompanied mRNA decay is considered a prologue of MZT in mammals, but its cellular function and physiological importance have been inconclusive.'. I think the authors may want to moderate these claims in the light of published findings 1-4 that attest to the importance of RNA-degradation and especially if it turns out that CNOT6L-deficient oocytes already show defective gene expression.
Response: We appreciate the reviewers suggestion and have moderate these claims in the revised manuscript.

12. The following statement requires revision: 'We showed that Cnot6l deletion yields a phenotype similar to that of deletion of TUT4 and -7 (key enzymes of mRNA uridylation) or of YTHDF2 (reader of mRNA m6A modification) in oocytes, including impaired spindle assembly, polar body extrusion, and maternal mRNA stabilization (Ivanova et al., 2017; Morgan et al., 2017; Qi et al., 2016). Therefore, our results support in vivo participation of CCR4-NOT in the degradation of uridylated and m6A-labeled maternal mRNAs during oocyte maturation.'. Combined TUT4 and 7-deficiency results in a problem in the formation of a functional maternal transcriptome that is distinct from meiotic maturation related degradation presented for CNOT6L-/- mice, however the meiotic defects observed are similar. This underscores the necessity of presenting the impact of CNOT6L-deficiency on the GV transcriptome. YTHDF2-deficiency has a normal GV but a defective MII transcriptome and the consequences are in mitotic divisions of the zygotes. The

authors should be more precise in their discussion and understand if CNOT6L is required for the formation of the maternal transcriptome.
Response: We have provided new results in Fig. EV2 and Fig. 4D to show that CNOT6L is NOT required for the formation of the maternal transcriptome. We have also reworded these statements in the revised Discussion.

Minor concerns:
1. Page 5 line 133 the (Fig.S1C) should also include D and E.
Response: We labeled wrong here. It should be Fig. 1C instead of Fig. S1C. We have corrected this in revised manuscript.

2. In Figure 1C please specify the number of mattings per genotype.
Response: In this experiment, we maintained a *Cnot6l* null female (~6 weeks old) with a fertile WT male in each cage for 32 weeks, and recorded the pups. We did not count the number of mattings per genotype during this interval. It appears that the *Cnot6l* null female do not have matting problems, because when we mate the PMSG/hCG-injected *Cnot6l* null females with WT males, we always see plugs the next morning.

3. In the paragraph on page 7 in lines 173,178 Fig.3A and Fig.3B are missing parentheses.
Response: We have double checked the texts mentioned by the reviewer. It seems to us that parentheses should not be used here, because Fig.3A and Fig.3B are directly referred in the text. If we are wrong, the editor will still point it out during proof reading before publication.

4. Page 8 line 206 the sentence is grammatically incorrect.
Response: We have reworded this sentence in the revised manuscript.

5. Page 8 line 218 the sentence is about MI but in parentheses (22%vs 76% of mRNA degrades...) 76% corresponds to the MII stage (line 214 and 215).
Response: We have reworded this sentence in the revised manuscript.

6. In the text are used both GV-MII transition and GV-to-MII transition if they mean the same thing please chose one and use it throughout the text consistently.
Response: We have used "GV-to-MII transition" throughout the revised text consistently.

7. In Material and Methods the mouse strain is C57B6 but should be C57Bl6.
Response: We have corrected this. Thanks to the reviewer for pointing it out.

8. Figure 6E is missing the error bar or they are not visible.
Response: We have revised this panel to make the error bar visible.

9. Supplementary figure 1A the marker in red letters deletion counts for 10bp not 11bp as indicated.
Response: It should be 10 bp. We have corrected the labeling in Fig. S1A. Thanks to the reviewer for pointing it out.

10. Supplementary figure 3D, there are missing lines in the bar plot indicating mean or average value.
Response: We indicated average value in the revised figure EV4F.

Referee #2:

1. Cnot6l knockout mouse. The authors describe how Cnot6l was targeted and show evidence of the short 11 nt DNA deletion by PCR. However, critical data demonstrating significant knockdown or knockout of the CNOT6L protein is not provided. The efficiency of the proposed premature termination codon in inducing NMD must be demonstrated as it is possible a truncated form of Cnot6l protein could still be expressed with associated artifacts. Without these data, the authors and the readers are unable to draw conclusions as to the mechanism for the data presented and the role of CNOT6L.
Response: We appreciate that both Reviewer 1 and 2 raised the same important issue. Please see our detailed responses to Reviewer 1's comment #1.

2. Writing/presentation of data. While there is a large amount of interesting findings presented, the logic connecting the findings in the manuscript in its current form is difficult to follow and frustrating to read. It would be helpful if the authors can find a way to present it as a more logical, coherent story and to better synthesize the findings (both the details and the bigger picture) for the reader. As is, the data seem pieced together without a clear logical connection between some of the figures. Interesting directions are established (e.g., ZFP36L2, CPE regulation, BTG4 parallels) but then seem quickly abandoned for a different direction. For instance, the rationale for comparison to BTG4 is interesting with some backreading but not clearly presented for the reader and the stage-specific hypothesis for CNOT6/ZPF36L2 and CNOT7/BTG4 is exciting but not clearly presented or fully developed. The rationale for choosing to look at ZFP36L2 should be provided as well. Admittedly it is difficult to adequately introduce so many different topics but without it the rationale and chain of logic is lost.

Response:

1) We appreciate the reviewer's good suggestions for better presentation of the results in this manuscript. In the revised manuscript, we presented the data as a more logical and coherent story: The analyses of mouse phenotypes were presented in Figures 1, 2, and 3. Guided by the phenotypes of *Cnot6l* knockout mice, we sequentially assessed the stability (Figure 4), polyadenylation (Figure 5), translation, and polysome binding (Figure 6) of maternal transcripts affected by *Cnot6l* KO. As logical extension of these findings, we studied the regulation of CNOT6L during mouse oocyte maturation: we provided evidence that the RNA-binding protein ZFP36L2 functions as a CNOT6L adaptor in targeting a subset of maternal transcripts (Figure 7); and the translation of *Cnot6l* itself is coupled to meiotic resumption by MAPK cascade (Figure 8). We have pointed out the rationale and logical connections in the last paragraph of the revised Introductions.
2) We provided the rationale for choosing to look at ZFP36L2 in the text related to the revised Figure 7.
3) To clearly present the hypothesis that CNOT6L-ZFP36L2 and CNOT7-BTG4 play stage-specific role in oocyte maturation and maternal-zygotic transition, we have provided a diagram illustration in the revised Figure 8. We believe that it helps the readers to follow the rationale and chain of logic.

3. Experimental details. Many details needed to understand and critically evaluate the findings are lacking. We have done our best to list what we could under minor concerns below but the list is not exhaustive.

Response: We have tried our best to address the reviewer's concerns below and have provided more experimental details as requested.

4. Decay vs. deadenylation without decay. The authors cannot distinguish between these 2 possibilities with the experiments presented. This possible alternative explanation for their findings should be addressed in the discussion. And technically, the authors have not shown a direct effect on mRNA stability (i.e. by stability assays) for CNOT6L for any of the eliminated mRNAs although, in the absence of transcription, this is admittedly the most likely explanation.

Response: We discussed this issue in the revised Discussion, as the reviewer suggested.

5. Indirect effects. Unless the authors can connect the meiotic defects to specific defects in mRNA decay, that these are likely indirect/downstream effects should be addressed in the discussion. In addition, some of the eliminated mRNAs might be via downstream/indirect effects instead of direct targets of CNOT6L. Also, given that the KO is global, it is possible that effects of Cnot6l in other cell types could contribute to the phenotype.

Response:

1) Because the degradation of so many maternal mRNAs were blocked or delayed after *Cnot6l* knockout, and because the functions of many proteins encoded by these maternal mRNAs were unknown, it is impossible for us to specifically connect the meiotic defects with the decay of defined mRNAs. Indirect/downstream effects certainly should play a role here. We addressed this issue in the revised Discussion, as the reviewer suggested.
2) As the reviewer pointed out, we could not rule out the possibility that some of the eliminated mRNAs might be via downstream/indirect effects instead of direct targets of CNOT6L. For instance, accumulation/stabilization of certain RNA-binding proteins (PABPC1L, CPEB1, MSY2, etc.) after *Cnot6l* knockout might indirectly prevent the decay of a subset of maternal

mRNAs. We also discussed this possibility in the revised manuscript.

3) Although the KO is global, our results did not support the possibility that effects of *Cnot6l* in other cell types could contribute to the phenotype. Ovarian histology, follicle development, and ovulation were normal in *Cnot6l* KO females, indicating that *Cnot6l* is dispensable in ovarian somatic cells. When fully grown GV oocytes were isolated and cultured *in vitro*, they failed to spontaneously develop to MII stage. Based on these evidence, we concluded that *Cnot6l* plays a cell autonomous function in maturing oocyte.

More minor concerns

1. Figure 1 and associated text

o 1A-B-- Authors argue Cnot6l is the most important CCR4 protein during oocyte maturation. This conclusion cannot be made based on mRNA levels as determined by qPCR. This is especially true in light of the fact that (as the authors point out themselves) that mRNA in the oocyte is accumulated and stored for later translational activation. While mRNA and protein levels might correlate in somatic cells (and this is arguable), the oocyte is unique and this cannot be assumed. If this claim is based on mouse phenotype, it needs to be qualified.

Response: We appreciate the reviewer's point that mRNA levels of *Cnot6* and *Cnot6l* in oocytes might not reflect their actual protein levels. As we have explained in responses to Reviewer 1's comment #1, there are no available antibodies that can specifically target CNOT6 or CNOT6L. On the other hand, our new western blot result using an antibody that recognizes both CNOT6 and CNOT6L showed that the CNOT6/6L level in oocytes was greatly decreased after *Cnot6l* knockout (revised Fig. EV1C-D), indicating that CNOT6L is indeed the dominant CCR4 protein form in mouse oocyte.

o 1B - If I am not mistaken, this is not a novel finding with respect to Cnot6l mRNA levels being significantly higher than Cnot6 in oocytes. Ok to leave for completeness but they need to appropriately reference Ma et al, 2015, somewhere.

Response: We have carefully read this paper (Ma et al, 2015). It only mentioned in the text that "we focused our attention on *Cnot6l* rather than *Cnot6* because our microarray data indicated that *Cnot6l* transcripts are far more abundant than *Cnot6* transcripts in oocytes with raw scores of 1,700 and 150, respectively", but did not actually compare the expression levels of CNOT6 and CNOT6L by specific experiments. Therefore, our result is not a repeat of this paper. We have cited this paper in the revised manuscript, as the reviewer suggested.

o 1D - Quantification to show what % of MII oocytes have no polar body and/or distorted multipolar spindles should be included. Also, legend should explain arrows.

Response: In revised Figure 1D-E, we have showed percentage of ovulated *Cnot6l* null oocytes have no polar body and/or distorted multipolar spindles. It appears that the *in vivo* matured *Cnot6l* null oocytes have a higher PB1 emission rate than those matured *in vitro*. This might be caused by two reasons: 1) the *in vivo* microenvironment supports oocyte meiotic maturation better than the *in vitro* culture system does; 2) Superovulation is an oocyte selection step by itself. Therefore, the oocytes that are less defective have a better chance to be ovulated by the *Cnot6l* KO mice. We also explained arrows in the legend of revised Fig. 1D.

o 1E - The relative numbers of 1 cell embryos obtained from WT and KO (i.e., rates of fertilization) should be included. They are likely dramatically different but this has not been addressed. Are the "1 cell" cells shown zygotes? Cannot see PN.

Response: We have showed the rates of fertilization in the revised Fig. EV2F-H. And results of confocal microscopy of zygotes were provided to show pronuclei (Fig. EV2G). It appears that although the Cnot6l null oocytes have severe defects of spindle assembly, most of them can be fertilized and form 2-3 PNs. We also changed "1-cell" into "zygote" to make it clear.

o Lines 136-7 -- The authors claim ovaries of KO females are of normal histology but histology of ovary is not shown. H&E stain of ovarian section of adult cycling KO female should be shown with WT. Ovarian follicle counts in prepubertal vs. adults should also be provided as subfertility phenotype suggests premature depletion of follicles.

Response: We appreciate that both Reviewer 1 and 2 raised the same important issue. Please see our detailed responses to Reviewer 1's comment #4.

2. Figure 2

o 2A-B - The number of oocytes growing to GV stage comparable for KO vs. WT should be provided. It is important for the reader to be able to assess the degree to which there was also earlier effects on folliculogenesis, which was not addressed.
Response: Again, both reviewer 1 and 2 requested the experimental result of GV oocyte count. Please see our detailed responses to Reviewer 1's comment #5.

o 2C - Text states "majority" aneuploid but no quantification provided. Top2 and CREST are not described. Unclear what 20*2 means.
Response:
1) Most *Cnot6l* knockout oocytes were arrested at the pre-MI stage, and only very small numbers of oocytes can release their polar body-1. As the result, it was very difficult for us to obtain enough numbers of MII oocyte from these mice for calculation of aneuploidy rates. However, as the reviewer requested, we have observed more *Cnot6l* KO oocytes and provided the quantification results in the revised Fig. 3D. We also described TOP2 and CREST in the legend of Fig. 2C.
2) "*2" in the original Fig. 2C indicates parental sister chromatid pairs attached to each other. We admit that this is a poor description and easy to cause confusion. Therefore, we revised the labeling of this panel, and only indicated the numbers of sister chromatid pairs (40).

o 2F - Do not see TPX2 explained anywhere.
Response: In the experiment of revised Fig. 2G, we use the tubulin-binding protein TPX2 as a marker of meiotic spindle. We could not use anti-tubulin antibody because this antibody and the anti-pericentrin antibody are both derived from mouse. Maternally accumulated microtubule nucleation factor (TPX2) are responsible for meiotic cell cycle progression and spindle assembly. We have explained this in the revised legend of Fig. 2G.

3. Figure 3
o In general, the significance of these experiments and what conclusions can be drawn are difficult to determine for anyone that is not a meiosis checkpoint expert. Additional explanation as to why these specific targets were chosen for investigation and what conclusions can be drawn from these findings and why are needed here.
o 3C-D -- There is no mention about what Crest is.
Response:
1) We have given additional explanation to the results related to Figure 3 in the revised text, as the reviewer suggested.
2) Anti-CREST is a polyclonal anti-centromere antibody widely used in the cell biology field. The acronym "CREST" refers to the five main features: calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia. (https://en.wikipedia.org/wiki/CREST_syndrome)

4. Figure 4
o 4A - Were there differences in mRNA levels in GVs at the start? These data are important to assess effects of Cnot6l during folliculogenesis.
Response: We appreciate that both Reviewer 1 and 2 raised the same important issue. Please see our detailed responses to Reviewer 1's comment #6.

o 4B - Not clear why it was hypothesized that mRNAs of a specific abundance would be targeted or what this adds. However, useful overall to see relative levels and comparison with Btg4.
Response: We suspected that the mRNAs with high abundance might be more important during oocyte maturation, and be specifically regulated by CCR4-NOT complex. Plus, it is a common transcriptome analyses reported in many papers.

5. Figure 5
o 5B-5D - If there was a rationale for choosing these specific genes to test, would be useful for the reader to know. For instance, knowing that you chose genes that you found to be regulated by Cnot6l but not Btg4 would help explain why you see little to no effect for Btg4 in 5C-D. If not, this should be briefly addressed.
Response: These genes were tested because the RNA sequencing results showed that they were significantly accumulated in Cnot6l but not in Btg4 knockout oocytes. We have clarified this in the revised text.

o 5D - Actin should be included again as a negative control for comparison. Also curious that many of the mRNAs do not appear to be decreasing in abundance with deadenylation and/or with the transition from GV to MII?

Response:

1) We have included actin in the PAT assay as a negative control for comparison.
2) To increase the sensitivity of PAT assay and get clear bands, we run the PCR with high cycle numbers. Therefore, we are afraid the PCRs are not quantitative, and the band intensity could not be used to determine the abundance of the target transcripts.

6. Figure 6

o 6A - This data can be interpreted that CPEB1 protein is stablized in Cnot6-/- oocytes or increased due to mRNA stabilization. Seems they suggest protein stabilization here as a mechanism (phosphorylated but not degraded) and increased translation as a mechanism later in Fig. 8. Both might be trued but not addressed and spread over multiple figures. This entire last section feels disorganized and the logic connecting the experiments is not clear. Reader is left to fill in the gaps.

Response: Because *Cpeb1* mRNA was degraded during maturation of WT oocytes, but was stabilized in *Cnot6l* null oocytes (Fig. 5B) , we detected CPEB1 protein level and showed that the protein level increased as well after *Cnot6l* knockout (Fig. 6A). This result has nothing to do with the results in Fig. 8, which addresses the translational regulation of *Cnot6l* mRNA itself. But anyway, we presented the data as a more logical and coherent story in the revised manuscript. We have pointed out the rationale and logical connections in the last paragraph of the revised Introductions.

o 6B and Lines 287-8 - This conclusion is not supported as the authors do not show here that the increase in CNOT7 and cyclin B1 are due to CPEB action. And they need to provide references and explain they are looking at these proteins as they have been shown to be CPEB targets by others.

Response: We provided references and explained in the revised manuscript that we looked at these proteins as they have been shown to be CPEB targets by others.

o 6G-H - Confusing that these are 2 separate figures but you have to look ahead to see the full legend as it is spread across both. Maybe make 1 figure and label stages instead?

Response: To avoid confusion, we separated the legend of Fig. 6G and 6H.

7. Figure 7

o 7A-B - How were AREs defined/identified? Or the validated AREScore? Was one of the ARED databases used?

Response: We identified ARE by looking for the known ARE sequence (AUUUA, Hudson et al, Nature Structural and Molecular Biology, 2004) in the 3'-UTR of transcripts. We did not use any databases.

o 7D - It was really difficult to find that the IP here is to HA tag. It is only in one tiny label in the corner of the figure. Please add to the figure legend and Materials and Methods to make this clear as the experiment is not interpretable without this information.

Response: We have re-labeled the Figure 7D, and added more information to the figure legend and the related manuscript text.

o 7C- Also are the authors claiming that CNOT6L and CNOT7 are in distinct complexes? Otherwise, the potential significance of greater association with one CNOT6L relative to CNOT7 should be addressed in the text. Ideally, the reverse IP experiment would be done as well to confirm this finding.

Response:

1) Based on current knowledge, people believe that CNOT6L and CNOT7 should be in the same functional complexes with full deadenylation activity. However, it is difficult to evaluate how stable the complex is. An explanation is that the interaction between CNOT6L and ZFP36L2 is stronger than that between CNOT6L and CNOT7. As a result, more CNOT6L than CNOT7 is coimmunoprecipitated with ZFP36L2. We have addressed this issue in the revised text.
2) We have done the reverse IP experiment as the reviewer suggested. The result in revised Figure 7C confirmed the finding that ZFP36L2 preferentially interacts with CNOT6L.

8. Figure 8

o 8A-B - Similar experiments and conclusions have been published. Although these constructs might have been made to investigate the effect of Erk1/2 on Cnot6 translation, please review Ma et al.2015, from Schultz lab and reference as appropriate. Also, in the Schultz manuscript, the 3'UTR of Cnot6l has 3 CPE sequence and 1 HEX sequence, while Figure8A in this manuscript shows 4 CPE sequences. Unclear why there would be a difference?

Response:

1) Indeed the paper by Dr. Schultz's group (Ma et al, 2015) have reported the translational activation of dormant Cnot6l mRNA after meiotic resumption. We have cited this paper when describing our own results.
2) On the other hand, as also noticed by the reviewer, there are important differences between this paper of Schultz lab and our current study. They used a truncated 3'-UTR fragment (402 bp, which contained 3 CPEs) in the reporter experiment (Fig. 2D of Ma et al, 2015). We have noticed that there is an additional CRE close to the translation stop codon, which was not included in the *Cnot6l* 3'-UTR fragment cloned by Ma et al, 2015. Therefore, we used the 1597 bp *Cnot6l* 3'-UTR fragment containing 4 CREs in our reporter experiment (Fig. 8A-D). We have explained this in the revised text.

o 8B-D - These results should be quantitated if possible over a large number of oocytes. Number of oocytes evaluated for each condition and number of experiments performed should also be provided.

Response: We quantified the intensity of fluorescent reporters in Fig. 8B and D, and included these results in the revised Figure 8. In addition, the number of oocytes evaluated for each condition and number of experiments performed were also provided in the revised Figure 8 and legend.

9. Figure S1

o S1F - This would be useful earlier in the figure assuming the constructs are the same used for S1D-E. Also, in the related text, an explanation as to the significance of the catalytic site (I assume it is needed for deadenylation activity but this is never stated) and either a reference to a manuscript showing this mutation abolishes activity or to show loss of activity here. Same with N-terminal LRR domain. I assume there is a reference that can be cited to show interaction between Cnotl6 and Cnot7 and that this is the site of interaction. Otherwise, these 2 things need to be shown if they can't be referenced.

Response:

1) We illustrated the functional domains of CNOT6L earlier in the figure as the reviewer suggested, by changing Fig. S1F to S1C.
2) We have provided references for the CNOT6L catalytic site and the N-terminal LRR domain. Actually, result in Fig. S1G also indicated that the CNOT6L$^{\Delta LRR}$ failed to interact with CNOT7.

o S1E - No WT bar but reference in legend? Also, need to define what is meant by "rescue", "partial rescue" and "abnormal". Not sure how to interpret these data.

Response: We are sorry that the WT bar was missing in the original Figure S1E. We have fixed this error in the revised Figure S1E. We also defined the terms of "rescue", "partial rescue" and "abnormal" in the revised figure legend.

10. Figure S2

o Line 251 - "Remarkably, the transcripts of the first three categories were not enriched among the transcripts stabilized in Btg4-/- oocytes (Fig. S2E)." It is hard to understand the meaning of this sentence because GO terms in Cnot6-/- and Btg4-/- are nearly identical.

Response: We deleted this sentence to avoid confusion.

o Lines 226-7 -- The Cnot6l knockout caused more significant mRNA accumulation than the Btg4 knockout did (Fig. 4B; Fig. S2B). Again, not sure what this conclusion is based on as effect for Cnot6l and Btg4 in these figures look similar.

Response: This conclusion is based on the comparison of oocyte mRNA levels at the MII stage – the median mRNA level in Cnot6l knockout MII oocytes is slightly higher than that in Btg4 knockout MII oocytes. We have modified the manuscript to make it clearer.

o S2B - Please explain the bars, lines, boxes, etc. in legend.

Response: We have added the explanation of bars, lines, and boxes of this box plot in the figure legend.

o S2C - Ratios are opposite here relative to text, i.e., MII/GV vs. GV/MII. Would be much more clear if consistent.
Response: We have revised Figure S2C to make the ratio consistent between the figure and the text.

11. Figure S4
o S4B - Decreased oocyte maturation with ZFP36L2 depletion has been shown and published. Please review and reference Ball et al, 2014, and Dumdie et al, 2018, as appropriate.
Response: We have described the findings of these studies in the revised manuscript.

12. Other comments
o Number of biological repeats not provided for each experiment. There is only a general statement in materials and methods that most experiments included 3 independent samples.
Response: We stated in Materials and Methods that most experiments included 3 independent samples. Therefore we did not repeatedly make the same statement for each experiment. However, we specifically mentioned when 2 independent samples were used for RNA-seq experiments.

o Seems nonideal to normalize RNAseq data to a single spike in RNA although potentially ok with large shifts as seen here. Was a second level of normalization performed after normalization to the spike in? If so, this should be described. Also, authors should consider using ERCC cocktail in future experiments (Thermofisher catalog # 4456740).
Response: We thank the reviewer's suggestion and realize the limitation of using a single spike in RNA. In this study we did not perform a second level of normalization. Yes we will consider using ERCC cocktail in future experiments.

o Line133 -- FigS1C doesn't show subfertility data?
Response: This is a typing error. It should be Fig. 1C here. We have corrected this.

o Lines 157-159 - Btg4 mouse seems to come from nowhere without rationale for the comparison or description of the mouse knockout.
Response: We have added sentences and references here to explain the rationale for this comparison.

o Line 233 - A lower limit needs to be provided for Cluster II (i.e., < 10 but > than what?)
Response: We have provided a lower limit here ($1<$GV/MII ≤ 10 and $1<$ MII/zygote ≤ 10).

o Line 235 - Do you mean MII/zygote > 10?
Response: Yes, it should be MII/zygote > 10. We have corrected this error. Thanks to the reviewer for pointing it out.

o Line 244 - the number 644 needs some context for the reader to determine if this is a large percentage or small percentage
Response: Among the significantly degraded transcripts during GV-to-MII transition in WT oocytes, 644 (33.95%) specifically stabilized in Cnot6l–/– oocytes but not in Btg4–/– oocytes at the MII stage. We have revised this sentence as the reviewer suggested.

o Line 281-283 - The sentence needs references.
Response: We have provided a reference here.

o Line 286-288 -- needs references.
Response: We have provided a reference here.

o Line 323 - which genes are related to spindle formation? This seems relevant.
Response: *Birc5* and *Tubb4b* are related to both spindle formation and chromosome alignment. During mitosis and meiosis, survivin protein encoded by *Birc5* assembles with the chromosomal passenger complex and regulates chromosomal segregation. Survivin also plays an essential role in proper amphitelic kinetochore-spindle assembly. *Tubb4b* encodes a tubulin protein that is a structural protein of spindle microtubules. We have provided these information in the revised text.

o Lines 394-395 - Would suggest to describe importance of RNA modification instead of "hotness".

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

13. Strong statements. There are many statements in the manuscript that are unnecessarily and, often, inappropriately strong in our opinion. We have detailed some below but this is not an exhaustive list
o Line 165 and others - "Indispensible" is too strong without complete block in development.
Response: We have replaced "Indispensible" with "required" in the revised manuscript.

o Lines 393-394 - Is this true? Schultz lab data on CNOT7 is not knockout but still genetic.
Response:
1) We have reworded this sentence to avoid arguments.
2) We have carefully read this paper mentioned by the reviewer. In this paper, the authors reported that RNAi depletion of CNOT7 impaired mRNA decay. However, no oocyte maturation defects were observed after *Cnot7* RNAi depletion. This is in agreement with the report that *Cnot7* knockout does not affect female fertility, indicating that *Cnot8* may play a redundant role.

o Line 395 - "most suitable" is too strong
Response: We have changed this sentence to “The mature oocyte is a suitable cell type for these studies…”.

o Lines 406-8 - Overly speculative to extend results from this manuscript to a role for CNOT6L in decay of mRNAs modified by uridylation and/or M6A, particularly in the very first paragraph of the discussion.
Response: Reviewer 1 also asked us to revise this section about CNOT6L and mRNAs decay modified by uridylation and/or M6A. We have made revisions as described in our response to reviewer 1’s comment #12.

o Line 431 - "universally acknowledged" is too strong
Response: We have deleted the word "universally " in the revised manuscript.

o Line 433 - as elsewhere in the manuscript, the mRNAs were upregulated but increased stability was not actually shown.
Response: Technically, we have not shown a direct effect of CNOT6L on stability (i.e. by stability assays) of the eliminated transcripts. Nonetheless, in the absence of transcription during oocyte meiotic maturation, this is a reasonable deduction that the CNOT6L targeted mRNAs were destabilized and degraded after deadenylation. We have discussed this issue in the revised Discussion.

o Line 439 - "solely" is too strong and this statement is not referenced
Response: We have deleted the word "solely " in the revised manuscript.

o Line 445 - "ensures" is too strong a conclusion for this proposed mechanism
Response: We have replaced the word "ensures" with “mediates” in the revised manuscript.

o Line 484-481 - Concluding sentence seems awkward and a forced attempt to directly connect decay to meiotic arrest in our opinion.
Response: We deleted the last sentence to avoid over-statement.

o Lines 506-12. Important to include polyA tail lengths since relevant for translation efficiency in the oocyte. Also, were the mRNAs capped?
Response: We have provided the length of polyA tails (200-250 bp) in the revised Materials and Methods. The in vitro transcribed mRNAs were not capped before microinjection. Presumably they will be capped after being microinjected into to the ooplasm.

o Lines 514-18. Please provide RNA concentration, not just volume.
Response: We have provided the concentration of injected mRNAs in the revised Materials and Methods section.

o Lines 618-631 - Please provide information as to the quality of the RNAseq data-reads, percent uniquely mapped reads, etc. Also, how was differential expression determined? Cutoffs? Q value?

Response: We provided these information in the revised Supplemental Table S3 and S4.

Referee #3:

This manuscript addresses whether the Cnot6L subunit of the CCR4-NOT deadenylase complex regulates maternal mRNA degradation during oocyte maturation. Cnot6l knockout female mice are subfertile despite the presence of morphologically mature oocytes. Cnot6l knockout oocytes have meiosis I defects including a reduced rate of polar body extrusion. The knockout oocytes show impaired deadenylation and degradation of maternal mRNA, consistent with previous findings (Ma et al., 2015).

Response: We have carefully read this paper mentioned by the reviewer, and compared our results with the previous study.

1) In this paper, they did not study the function of CNOT6L in oocytes. The authors (Ma et al 2015) clearly stated in the paper: "we could not detect CNOT6L by immunoblotting or immunocytochemistry, which excluded pursuing functional studies using an siRNA approach because we would not be able to document our ability to inhibit the maturation-associated increase in CNOT6L protein ... … Therefore, we focused our attention on the role of Cnot7 mRNA recruitment during maturation."
2) In this paper by Ma et al, the authors reported that RNAi depletion of CNOT7 impaired mRNA decay. However, no oocyte maturation defects were observed after *Cnot7* RNAi depletion. This is in agreement with the report that *Cnot7* knockout does not affect female fertility (Berthet et al, Mol Cell Biol, 2004), indicating that *Cnot8* may play a redundant role.

Therefore, our current manuscript is an original study that contains novel results, not just "consistent with previous findings (Ma et al., 2015)".

1. My main concern regards some inconsistencies regarding the defects during meiotic cell cycle progression. The rate of polar body extrusion (PB1), i.e. the meiosis I division, is around 10% at 16 h after hCG in Cnot6l knockout oocytes (Fig 1D, S1D). However, 24 h after hCG more than 50% of the cells shown in Fig 1F have at least one polar body, although at this magnification it is not possible to discern between PB1 and PB2. Nevertheless, could the authors explain how so many cells have divided and indeed then again roughly 50% of embryos progress to the 2-cell stage, suggesting that at least 50% of oocytes had progressed to meiosis II in order to be fertilized and generate embryos.

Response:

1) We have evaluated PB1 emission rate in oocytes matured *in vivo*, and added the results in revised Figure 1. It appears that PB1s are visible for 40-50% oocytes ovulated by *Cnot6l* null females. This PB1 emission rate matches with the results of embryonic development, in which nearly 40% fertilized eggs developed to 2-cell stage. On the other hand, 90% of the *in vivo* matured *Cnot6l* null oocytes have spindle formation defect. This is most likely the reason that causes embryonic development failure after fertilization.
2) The *in vivo* matured *Cnot6l* null oocytes have a higher PB1 emission rate than those matured *in vitro*. This might be caused by two reasons: 1) the *in vivo* microenvironment supports oocyte meiotic maturation better than the *in vitro* culture system does; 2) Superovulation is an oocyte selection step by itself. Therefore, the oocytes that are less defective have a better chance to be ovulated by the Cnot6l KO mice. We have explained this in the revised manuscript.
3) The discrepancy between the *in vitro* PB1 emission rate and the *in vivo* 2-cell formation rate was actually not as serious as the reviewer said. Our results in original Fig. 2A showed that nearly 30% *Cnot6l* null oocytes released PB1 at 24 h after culture. Figure 2E showed that on average 40% embryos (not 50% as the reviewer described) derived from *Cnot6l* null oocytes developed to 2-cell stage *in vivo*.
4) The phenomenon we observed in *Cnot6l* null oocytes (fail to release PB1 *in vitro* but have better embryo developmental rate *in vivo*) was also reported in *Tut4/7* null oocytes by another recent important study (Morgan et al, Nature 2017). The *in vitro* cultured *Tut4/7* null oocytes (from $Tut4^{fl/fl};Tut7^{fl/fl};Zp3\text{-}Cre$ female mice) have a PB1 emission rate < 10% (Extended data Fig. 4c and d). However, nearly 30% embryos derived from these mice developed to 2~4-cell stages *in vivo* (Fig. 2d). These results all indicate that some zygotes derived from defective oocytes can still manage to divide once or twice *in vivo*.

2. The claim is made that "Although a small proportion of Cnot6l knockout oocytes released PB1 and developed to the MII stage (Fig. 2B), their chromosomes were mostly aneuploidy" (p. 6) (Fig.

2C). The legend of Fig. 2 says that numbers of chromosome pairs are indicated. What exactly is meant by chromosome pairs? The left (wt) and right (KO) panels show dyad chromosomes, whilst the middle panel (KO) is actually showing bivalent chromosomes. It is also not clear what "*2" indicates. Since loss of chromosomes can be a technical artifact of chromosome spreads, the claim that there is aneuploidy in the meiosis II eggs would need to be substantiated by a quantification and clear selection for MII eggs.

Response:

1) We specified in the revised legend of Fig. 2 that "numbers of paired sister chromatids are indicated".
2) "The middle panel (KO) is actually showing bivalent chromosomes" because the bivalent chromosomes in KO oocytes failed to separate after in vitro maturation culture. "*2" in the original Fig. 2C indicates parental sister chromatid pairs attached to each other. We admit that this is a poor description and easy to cause confusion. Therefore, we revised the labeling of this panel, and only indicated the numbers of sister chromatid pairs (40).
3) We agree with the reviewer that "loss of chromosomes can be a technical artifact of chromosome spreads", but the WT and KO groups have the same chance of chromosome loss on slides. We provided the quantification results of clearly selected MII oocytes in revised Fig. 2D.

3. It is standard in the field to select mature oocytes that fulfill certain morphological criteria and undergo germinal vesicle breakdown (GVBD) within 60-120 min, depending on the mouse strain. Could the authors provide a rationale for why oocytes were selected for imaging if they underwent GVBD within 6 h after release? The problem with this is that potentially immature oocytes would be included in the experiment, which will have spindle defects such as those shown in Fig. 3A. To substantiate the claim that there are spindle defects, it would be necessary to rigorously select mature oocytes that undergo GVBD within at most 2 h, provide time-lapse images starting with the GV state to show the typical surround-nucleolus (SN) configuration of mature oocytes and to quantify the defects in spindle assembly in a population of cells.

Response: We are sorry for the confused description in the Methods section. In our live cell imaging experiment (Fig. 3A), oocytes underwent GVBD within 2 h (NOT 6 h) after release were selected for imaging. In our original Fig. 3A, we just showed the imaging results started from the 6 h time point to save space. As the reviewer requested, we added time-lapse images starting with the GV state to show the typical surround-nucleolus (SN) configuration of mature oocytes, in the revised Fig. 3A. In all other experiments involves the *in vitro* oocyte maturation, we also selected to use oocytes that undergo GVBD within at most 2 h. We clarified this issue in the revised Materials and Methods section (Live cell imaging).

4. The SMC3 staining of bivalent chromosomes in Fig. 3C does not recapitulate the known the localization to the inter-chromatid axis. This raises some questions about the specificity of the antibody. It would be more appropriate to stain for meiotic cohesin.

Response:

1) We have done the SMC3 staining of bivalent chromosomes again. Our new results in revised Fig. 3C did recapitulate the known localization of cohesion to the inter-chromatid axis. Thanks to the reviewer for pointing it out.
2) We totally agree with the reviewer that it would be ideal to stain for a meiosis-specific cohesin. In fact we have tried a commercially available antibody against RED8 (an meiosis-specific cohesin subunit), but it did not work well for immunofluorescence.

5. Given that such few Cnot6l knockout oocytes undergo the meiosis I division, how were MI and MII oocytes selected for the RNA-seq experiments in Fig. 4? Is there a molecular marker within the data sets that can convincingly exclude the possibility that the MII oocytes are not contaminated with MI-arrested oocytes, which would contribute more mRNA and therefore appear to show defective mRNA degradation?

Response:

For the RNA-seq experiments in Fig. 4, we collect WT and *Cnot6l* null oocytes at 0, 8, and 16 h after *in vitro* culture. At these time points, the WT are at GV, MI and MII stages. We are sure that the WT MII oocytes are not contaminated with MI-arrested oocytes because all the oocytes showed the presence of polar body-1 at this time point. We took samples only follow the time course, and did not specifically select MII oocytes from the KO group.

It is well established that the mouse GV oocytes will spontaneously develop to MII stage at 12-16 h in culture. However, the *Cnot6l* null oocytes failed to do so. Therefore, we collected WT and KO oocyte samples developed for the same time interval under the same condition, and analyzed the dynamic changes of their maternal transcriptome. This analyses helps us to understanding the function of CNOT6L and underlying mechanisms that regulate oocyte maturation.
Indeed a very small portion (~10%) of *Cnot6l* null oocytes have weaker meiosis defects and are able to release PB1, but they are not representative of this group. If we specifically selected these MII oocytes from the KO group for RNA-seq analyses, the key information might lose. Logically, the MI-arrest is the consequence of defective mRNA degradation in *Cnot6l* null oocytes, but not a course that we observed defective mRNA degradation after *Cnot6l* knockout.

6. For the HPG experiments, the control of complete translational inhibition is missing to know to what extent the signal is specific (Fig. 6C).

Response: We have done a negative control experiment by detecting HPG signal in MII oocytes treated with the translation inhibitor cycloheximide (CHX). The results showed that HPG signal was very weak in CHX-treated oocytes. We have added the new experimental results in the revised Figure EV4C and D.

7. The ERK1/ERK2 inhibition experiments are over-interpreted.

Response: We summarize the ERK1/2-related experimental results and our interpretations as follows:

1) Inhibition of ERK1/2 activation by U0126 blocked oocyte maturation-coupled translation activation of Cnot6l 3-UTR (Figure 8C and D). We interpreted that ERK1/2 triggered CNOT6L protein accumulation during oocyte meiotic maturation.
2) Forced activation of CCR4-NOT components partially rescued the oocyte maturation defects caused by ERK1/2 inhibition (Figure 8E and Figure S5D). Therefore, we interpreted that CNOT6L and other CCR4–NOT components are important downstream effectors of ERK1 and ERK2 in the regulation of spindle assembly and meiotic cell cycle progression in oocytes.

8. Previous work using an siRNA approach to knockdown CNOT7 and CNOT6L also showed an inhibition of mRNA deadenylation and found that CNOT6L is a dormant maternal mRNA (Ma et al., 2015). I am in favor of using the genetic knockout approach and would recommend that the authors more extensively compare and contrast their findings with this previous study.

Response: We have carefully read this paper mentioned by the reviewer, and compared our results with the previous study.

3) In this paper, they did NOT "use an siRNA approach to knockdown CNOT6L and showed an inhibition of mRNA deadenylation" as the reviewer said. Instead, the authors stated in the paper: "we could not detect CNOT6L by immunoblotting or immunocytochemistry, which excluded pursuing functional studies using an siRNA approach because we would not be able to document our ability to inhibit the maturation-associated increase in CNOT6L protein ... … Therefore, we focused our attention on the role of Cnot7 mRNA recruitment during maturation."
4) In this paper, the authors reported that RNAi depletion of CNOT7 impaired mRNA decay. However, no oocyte maturation defects were observed after *Cnot7* RNAi depletion. This is in agreement with the report that *Cnot7* knockout does not affect female fertility (Berthet et al, Mol Cell Biol, 2004), indicating that *Cnot8* may play a redundant role.

2nd Editorial Decision 7th August 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by all three original referees and their comments are shown below. As you will see, while ref #1 is generally satisfied with the revision, refs #2 and #3 raise a number of remaining points that have to be addressed before publication.

Most of these points relate to data presentation, description and interpretation and should be straightforward to address within a relatively short time frame. Ref #3's point about staging and the validity of the RNA-seq analysis is more severe but will in any case need to be clarified before the study can be published. Another important point that is raised by both refs #2 and #3 is the need to

properly cite and discuss the existing literature (with constructive examples provided by both referees).

Given the overall positive recommendations from the referees I would like to invite you to submit a second revised version in which you address the remaining referee concerns as well as the following editorial issues.

-> In your response to ref #3, point #4, you mention that a repeated analysis gave a result that contradicts the previous repeat of the same experiment but agrees with the expectations from the literature. Could you please elaborate on the reproducibility of this data and discuss why the repeat experiment yielded a different outcome than the original one?

-> There are several instances where the referees have asked for clarification of labeling/experimental strategy in order to understand the presented data (eg Ref #2's comments on fig 2F and fig 3). Please make sure that such information is available to the reader in the main manuscript file and not hidden in the legend. Furthermore, we require that statistics and number of replicas are specified in the legends for all individual experiments, meaning that is not sufficient to simply state a general number of replicas in the materials and methods section.

-> I would like to emphasise that it is requirement from our side that all RNA-seq data is made available to the referees during peer review and that it's an oversight from our side that this was not requested upon initial submission of your manuscript. You will therefore have to provide a database accession number for the RNA-seq data in the revised version of the manuscript (although please notice that this can be as a private link that is only made publically available upon publication).

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE REPORTS

Referee #1:

My main concerns have been address and I fully endorse the manuscript for publication.

I still believe that more replicates would improve the dataset to more confidently define CNOT6L targets.

Referee #2:

Summary:

In this manuscript, the authors demonstrate that CNOT6L plays a pivotal role in oocyte maturation and female fertility through downregulation of a large number of maternal mRNAs during the transition from the GV to MII oocyte stage leading to severe subfertility and defects in meiosis during maturation. defects. mRNAs normally downregulated by CNOT6L during oocyte maturation are highly enriched for translational machinery and factors regulating translation. The authors also show that the mRNAs upregulated with Cnot6l knockout are enriched for AU-rich 3'UTR sequences and that a subset are associated with ZFP36L2 but not BTG4 suggesting stage-specific pathways. In the revision, the authors have largely addressed the concerns raised but a few concerns remain.

Major comments:

1. Demonstration of successful CNOT6L2 KO. Description and validation of KO is still not adequate.
a. Protein level. I appreciate that a specific antibody is not commercially available. However, there are still still concerns with Western data presented.

i. No protein sizes are indicated in the image.
ii. MI is a strange stage to show. The authors need to show levels at GV and MII, the stages that encompass the stages analyzed in their other analyses. This is especially true for GV since they claim based on mRNA levels that CNOT6L is not significantly expressed before oocyte maturation and, therefore, can't play a major role in folliculogenesis. Also important to show CNOT6L has not accumulated in MII in the KO since they conclude that translation significantly increases during maturation.
iii. Authors should show entire gel to show that a truncated version of the protein is not made since only a 10nt deletion and mRNA is reduced but still present.
b. mRNA
i. Authors show mRNA levels from RNAseq data in 4C but why not show here with validation?
ii. Why not validate this result by qPCR as well (as done for some other genes elsewhere) since vital to manuscript?
c. DNA
i. Reader cannot evaluate PCR in EV1B because positions of GT-R1 and GT-R2 primers they state are used to validate are not shown in the EV1A figure. Other primer locations are shown instead.
ii. Authors still did not correct all instances of 11 bp deletion in manuscript. See line 132 and EVF1 legend for at least 2 examples.

2. Potential effect during folliculogenesis
a. The authors, like Ma et al, show increased translation during maturation. However, they cannot confidently evaluate or state that CNOT6L is not expressed in the growing or GV stage oocyte without an antibody. Therefore, authors should take care not state too strongly that it is dormant and not expressed before oocyte maturation and that it cannot play a role during growth.
b. To the contrary, they show that >800 maternal mRNAs are affects 2-fold or more by the GV stage. While this is fewer than across oocyte maturation, this is not an insignificant effect.
c. Therefore, a potential effect during folliculogenesis needs to be discussed.

3. Novelty of findings
a. Lines 127-130. I agree that the expression analysis in Ma et al, 2015, was not nearly as extensive as what is done here. However, as the authors point out in their rebuttal, it has been previously shown that Cnot6l mRNA levels are higher in the oocyte. This should be acknowledged and referenced. From Ma et al and author's rebuttal: "We focused our attention on Cnot6l rather than Cnot6 because our microarray data indicated that Cnot6l transcripts are far more abundant than Cnot6 transcripts in oocytes with raw scores of 1,700 and 150, respectively. "
b. I agree with R3 #8 comment in that siRNA KD of Cnot6l and analysis of deadenylation defect has already been partially done despite the author's claims to the contrary. See Ma et al, 2015, figures S2 and S3. I also favor the KO approach used here but agree that the authors need to acknowledge and reference these previous findings in the Results section and compare and contrast their own findings to these already published in the Discussion.
c. Similarly, both Ball, Rodriguez et al, 2014, and Dumdie, Cho et al, 2018, previously demonstrated ZFP36L2 depletion results in defects in oocyte maturation. Again, the authors need to acknowledge and reference these previous findings in the Results section and compare and contrast their own findings to these already published in the Discussion.

4. From response to R3 #5 comment re samples for RNAseq: "We took samples only follow the time course, and did not specifically select MII oocytes from the KO group." This needs to be somehow be stated in the manuscript-to simply call them GV, MI and MII is misleading since the KO oocytes did not reach these developmental stages. I assume this is true for all of the immunofluorescence evaluations as well? If so, this needs to be made clear. Some images are labeled as hours after meiotic release, others as GV/MI/MII.

5. There are still many figures without statistical analyses to support the author's conclusions. This list might not be complete:
a. 4A - looks different but stats should be performed
b. 4B and EV3B - Looks similar. Stats are needed to support the author's claim that the median mRNA level in Cnot6l-/- MII-oocytes is higher than that in Btg4-/- MII-oocytes
c. 4C - looks different but stats should be performed
d. 4F - no statistics provided
e. 5B - Some error bars are missing, no stats are provided

f. 8G - No bar for WT PB2 emission? Missing error bar on last bar? No statistics to support conclusion.
g. EV1H - No stats provided to support conclusion
h. EV4F - No stats and no median bar for MI and MII WT oocytes

Minor comments:

6. There remain multiple typos and grammatical errors throughout the manuscript.

7. Fig 4G and 4H - I do not understand why the total number of transcripts that are decreased 10 fold in WT appears to be different in these 2 figures? And can it be just a coincidence that some of the numbers are exactly the same (i.e., 1253, 644, 9)? Seems there might be a mistake here.

8. Fig 8H - I like the addition of a model figure. However, is there evidence that each of those factors on the left are translated downstream of CPEB? Also, figure implies they are not expressed before oocyte maturation and I am not sure that is the case. The authors have not shown this and I don't see it referenced anywhere

9. Fig EV1C - shouldn't left hand side label be CNOT6/6L for Hela as well?

10. Lines 94-95. "This activation is transient because the polyadenylated maternal mRNAs are quickly targeted for degradation (Yu, Ji et al., 2016b)." To my knowledge rapid decay of polyadenylated maternal mRNA has not been shown to be a general phenomenon.

11. Line 237- EV not S?

12. Line 280 - Changes during maturation and fertilization were evaluated

13. Line 345 - I would say modestly at best (not moderately)

14. Discussion
a. In my opinion, the Discussion does not effectively synthesize the findings or add any significant perspective/depth for the reader to put the findings in context of what is already known in the field.
b. Lines 470-78 - This discussion paragraph remains speculative and still seems unrelated and out of place.
c. Lines 480-1 - This is not true in light of Ma et al.
d. Lines 488-90 - Not sure how this claim can be made without an antibody available to evaluate CNOT6L protein levels?

Referee #3:

The authors have argued several points. However, at least one major concern remains. The RNA-seq experiments (Fig 4) are comparing WT and Cnot6l null oocytes at 0, 8 and 16 h time points after in vitro culture. Importantly, these are not comparable samples because the two groups are in different stages of meiosis. The Cnot6l null oocytes have a lower rate of GVBD than WT oocytes, thus the 8 h sample could be comparing pure meiosis I diakinesis oocytes in WT with mixed diakinesis and GV oocytes in Cnot6l. The discrepancy between samples is even greater at 16 h where WT oocytes are in meiosis II whereas 90% of the Cnot6l null oocytes remain arrested in meiosis I. It is therefore difficult to conclude whether the mRNA degradation defect is primarily due to lack of Cnot6l or a secondary consequence of arresting oocytes in meiosis I. Do the authors know whether an artificial arrest in meiosis I still allows timely mRNA degradation or whether this depends on progression to meiosis II?

In addition, chromosome spreads are subject to many technical artifacts that can only be limited by spreading all cells at the same time and in the same drop, which is usually not feasible and is unlikely to have been done here. It is therefore important to score spreads according to hypoploidy, euploidy and hyperploidy. The authors have the data at hand and it should therefore be straightforward to replace Fig 2D with a proper scoring of ploidy.

Lastly, I considered it important to show the GV time point of the live-cell imaging experiments to demonstrate that the meiosis I-arrested Cnot6l null oocyte in Fig. 3A has a mature SN chromatin configuration. The image presented is rather blurry and it is thus difficult to score it unambiguously, but it appears to be either an intermediate SN/NSN or immature NSN stage oocyte. Could the authors replace this time course with an unambiguous mature SN oocyte so that it is possible to better judge the spindle defect and meiosis I arrest.

2nd Revision - authors' response 9th September 2018

Point-to-point Responses to the Reviewers' Comments:

Referee #2:
Major comments:
1. Demonstration of successful CNOT6L2 KO. Description and validation of KO is still not adequate.
a. Protein level. I appreciate that a specific antibody is not commercially available. However, there are still still concerns with Western data presented.
i. No protein sizes are indicated in the image.
Response: We have provided original images of electrophoretic gels and blots as a "source data file", according to the editorial policy of EMBO J. Molecular weight markers were included in these images.

ii. MI is a strange stage to show. The authors need to show levels at GV and MII, the stages that encompass the stages analyzed in their other analyses. This is especially true for GV since they claim based on mRNA levels that CNOT6L is not significantly expressed before oocyte maturation and, therefore, can't play a major role in folliculogenesis. Also important to show CNOT6L has not accumulated in MII in the KO since they conclude that translation significantly increases during maturation.
Response:
1) The purpose of this experiment is to show that CNOT6L protein is indeed deleted in the oocytes of Cnot6l null mice, in response to the reviewers' previous requests. Therefore MI is a good stage to show, because Cnot6l transcripts undergo translational activation after GVBD, and the KO oocytes start to show developmental defects at this stage. GV oocytes were not used because CNOT6 is also expressed in these oocytes. Therefore we could not evaluate the deletion effect of CNOT6L.
2) In our opinion, MI and MII have no difference in this respect. As long as a gene is knocked out (a null allele), it is unlikely it will be expressed at any stages. In addition, there is another reason preventing us from detecting CNOT6L in the MII oocytes: both reviewers argued that most Cnot6l null oocytes are arrested at MI and failed to develop to MII. Therefore, if we compare the CNOT6L protein levels in WT and Cnot6l null oocytes at 16 h after in vitro culture (the time point when WT oocytes develop to MII), reviewers may concern that this is not a good time point to make the comparison because WT and KO oocytes were not at the same stage.

iii. Authors should show entire gel to show that a truncated version of the protein is not made since only a 10nt deletion and mRNA is reduced but still present.
Response: We have provided original images of entire electrophoretic gels and blots as a "source data file", according to the editorial policy of EMBO J.

b. mRNA
i. Authors show mRNA levels from RNAseq data in 4C but why not show here with validation?
ii. Why not validate this result by qPCR as well (as done for some other genes elsewhere) since vital to manuscript?
Response: We showed mRNA levels from RNA-seq data as requested by the comment 2 or Reviewer 1: "In the RNA seq data to what degree is the CNOT6L mRNA reduced in the Cnot6l -/- GV and MII oocytes?". This time we also showed the qRT-PCR results in the revised Fig. 4C, as requested here by reviewer 2.

c. DNA

i. Reader cannot evaluate PCR in EV1B because positions of GT-R1 and GT-R2 primers they state are used to validate are not shown in the EV1A figure. Other primer locations are shown instead.
ii. Authors still did not correct all instances of 11 bp deletion in manuscript. See line 132 and EVF1 legend for at least 2 examples.
Response: We have updated the primer information in Fig. EV1A, the legend of Fig. EV1B, and Appendix Table S1. We corrected all instances of 11 bp deletion in manuscript. We thank the reviewer for pointing out these errors.

2. Potential effect during folliculogenesis
a. The authors, like Ma et al, show increased translation during maturation. However, they cannot confidently evaluate or state that CNOT6L is not expressed in the growing or GV stage oocyte without an antibody. Therefore, authors should take care not state too strongly that it is dormant and not expressed before oocyte maturation and that it cannot play a role during growth.
Response: We agree with the reviewer that we should not state too strongly that CNOT6L protein is not expressed before oocyte oocyte maturation, and have revised the Discussion accordingly. On the other hand, we have presented results showing that follicle growth defects were not observed in *Cnot6l* knockout mice (Figure EV2).

b. To the contrary, they show that >800 maternal mRNAs are affects 2-fold or more by the GV stage. While this is fewer than across oocyte maturation, this is not an insignificant effect.
c. Therefore, a potential effect during folliculogenesis needs to be discussed.
Response: We agree with the reviewer that the levels of some maternal transcripts were affected by *Cnot6l* knockout in GV oocytes. On the other hand, we have presented results showing that follicle growth defects were not observed in *Cnot6l* knockout mice (Figure EV2). These facts and a potential effect during folliculogenesis were discussed as suggested by the reviewer.

3. Novelty of findings
a. Lines 127-130. I agree that the expression analysis in Ma et al, 2015, was not nearly as extensive as what is done here. However, as the authors point out in their rebuttal, it has been previously shown that Cnot6l mRNA levels are higher in the oocyte. This should be acknowledged and referenced. From Ma et al and author's rebuttal: "We focused our attention on Cnot6l rather than Cnot6 because our microarray data indicated that Cnot6l transcripts are far more abundant than Cnot6 transcripts in oocytes with raw scores of 1,700 and 150, respectively. "
Response: We acknowledged and referenced this in the revised manuscript. “This is in agreement with a previous report that *Cnot6l* transcripts were approximately 3 folds more abundant than *Cnot6* transcripts in mouse oocytes based on microarray data (Ma et al., 2015)”

b. I agree with R3 #8 comment in that siRNA KD of Cnot6l and analysis of deadenylation defect has already been partially done despite the author's claims to the contrary. See Ma et al, 2015, figures S2 and S3. I also favor the KO approach used here but agree that the authors need to acknowledge and reference these previous findings in the Results section and compare and contrast their own findings to these already published in the Discussion.
Response: We acknowledged and referenced this previous finding in the Results section under the subtitle “CNOT6L deletion impaired maternal mRNA clearance during oocyte maturation”, and also compared our own findings with these results in the Discussion.

c. Similarly, both Ball, Rodriguez et al, 2014, and Dumdie, Cho et al, 2018, previously demonstrated ZFP36L2 depletion results in defects in oocyte maturation. Again, the authors need to acknowledge and reference these previous findings in the Results section and compare and contrast their own findings to these already published in the Discussion.
Response: We have cited these two papers in the last version of our manuscript. As suggested by the reviewer, we further acknowledged these previous findings in the Results section, and compared our own results with these findings in the Discussion.

4. From response to R3 #5 comment re samples for RNAseq: "We took samples only follow the time course, and did not specifically select MII oocytes from the KO group." This needs to be somehow be stated in the manuscript-to simply call them GV, MI and MII is misleading since the KO oocytes did not reach these developmental stages. I assume this is true for all of the immunofluorescence evaluations as well? If so, this needs to be made clear. Some images are

labeled as hours after meiotic release, others as GV/MI/MII.
Response:
1) We made the statement in the text related to the RNA seq result section that we took samples only follow the time course, and did not specifically select MII oocytes from the KO group.
2) For the immunofluorescence evaluations, we did selected MII oocytes from the KO group, because PB1 can be clearly visualized by confocal microscopy. Actually we indicated the PB1s of the WT and *Cnot6l* null oocytes in these figure panels.
3) To make the labeling consistent, we indicated the hours after meiotic release in addition to the developmental stages (GV/MI/MII) throughout the figures.

5. There are still many figures without statistical analyses to support the author's conclusions. This list might not be complete:
a. 4A - looks different but stats should be performed
Response: Figure 4A shows relative mRNA copy number. There are only biological duplicates for the RNA-seq datasets in this study. However, Statistical analysis requires at least biological triplicates samples. So we are sorry that we cannot provide statistical analysis for this panel.

b. 4B and EV3B - Looks similar. Stats are needed to support the author's claim that the median mRNA level in Cnot6l-/- MII-oocytes is higher than that in Btg4-/- MII-oocytes
Response: We have included p values in the two figures.

c. 4C - looks different but stats should be performed
Response: We showed both the RNA-seq and the qRT-PCR results in the revised Fig. 4C, and included p values in all panels.

d. 4F - no statistics provided
Response: Figure 4F shows relative mRNA dynamic tendency of three gene clusters on the basis of heatmap, which is in Figure 4E. However, the datasets of heatmap in Figure 4E are merged data of biological duplicate samples. Therefore, we are sorry that we cannot provide statistical analysis for this panel. We also checked the recent papers that presented oocyte heatmap or mRNA dynamic tendency results (Hendrickson PG et al, Nature Genetics, 2017, 49:925-934 (Figure 1d); Zhang B et al, Nature, 2016, 537:553-557 (Figure 1b); Zhao B et al, Nature, 2017, 542:475-478 (Figure 1a and 2a);. All these results are merged data according to biological replicate samples. They did not provide statistical analysis as well. These cases suggest that this way of presenting is acceptable in the field.

e. 5B - Some error bars are missing, no stats are provided
Response: We have included p values in all panels. Some error bars are very short because the levels of some transcripts were very low at the MII stage.

f. 8G - No bar for WT PB2 emission? Missing error bar on last bar? No statistics to support conclusion.
Response:
1) None of the WT oocytes spontaneously released PB2 in this experiments. So there was no bar for WT PB2 emission.
2) We have included error bars and p values in this panel.

g. EV1H - No stats provided to support conclusion
Response: We have included p values in the revised Figure EV1H.

h. EV4F - No stats and no median bar for MI and MII WT oocytes
Response: We have included p values in this figure. In addition, we changed the color of median bars to red, as median bars in MI and MII WT oocytes almost overlap with lower quartile bars, and are difficult to distinguish.

6. There remain multiple typos and grammatical errors throughout the manuscript.
Response: The original manuscript has been edited by an English Editing Service of Editage. We are sorry that some new errors might have been generated during the revision of the manuscript. We have carefully checked typos and grammatical errors again.

7. Fig 4G and 4H - I do not understand why the total number of transcripts that are decreased 10 fold in WT appears to be different in these 2 figures? And can it be just a coincidence that some of the numbers are exactly the same (i.e., 1253, 644, 9)? Seems there might be a mistake here.
Response:

1) We are sorry that there is a calculation mistake in Figure 4I. We have corrected this. In the revised panels, the total number of transcripts that are decreased 10 fold in WT is the same between Figure 4G and Figure 4I. Thanks to the reviewer for pointing it out.
2) It is not a coincidence that some of numbers are the same. These numbers (1253, 644, 9) between Figure 4G and Figure 4H show the same gene sets. For example, 1253 is the number of transcripts decreased 10 fold in WT oocytes but stabilized in *Cnot6l*$^{-/-}$ and *Btg4*$^{-/-}$ oocytes during GV-to-MII transition in both panels. 644 is the number of transcripts decreased 10 fold in WT and *Btg4*$^{-/-}$ oocytes but specifically stabilized in *Cnot6l*$^{-/-}$ oocytes during GV-to-MII transition. 9 is the number of transcripts decreased 10 fold in WT and *Cnot6l*$^{-/-}$ oocytes but specifically stabilized in *Btg4*$^{-/-}$ oocytes during GV-to-MII transition.

8. Fig 8H - I like the addition of a model figure. However, is there evidence that each of those factors on the left are translated downstream of CPEB? Also, figure implies they are not expressed before oocyte maturation and I am not sure that is the case. The authors have not shown this and I don't see it referenced anywhere
Response:
1) We provided references for meiotic maturation-coupled translational activation of *Btg4*, *Cnot6l*, *Cnot7*, and *Zfp36l2* in revised legend of Fig. 8H.
2) CPEB1 is a general activator of maternal transcripts during meiotic resumption in both *Xenopus* and mouse oocytes. We stated the CPEB1-mediated translational activation of maternal transcripts and provided references in revised legend of Fig. 8H. The references we provided have reported that *Btg4*, *Cnot6l*, and *Cnot7* all contained CPE in their 3'-UTRs and were downstream of CPEB in mouse oocytes.

9. Fig EV1C - shouldn't left hand side label be CNOT6/6L for Hela as well?
Response: We agree with the reviewer and have changed the label in Fig. EV1C to CNOT6/6L.

10. Lines 94-95. "This activation is transient because the polyadenylated maternal mRNAs are quickly targeted for degradation (Yu, Ji et al., 2016b)." To my knowledge rapid decay of polyadenylated maternal mRNA has not been shown to be a general phenomenon.
Response: We have deleted this sentence in light of the reviewer's opinion.

11. Line 237- EV not S?
Response: Yes, it should be "EV1F-H" instead of "S1F-H". We have correct this. Thanks to the reviewer for pointing it out.

12. Line 280 - Changes during maturation and fertilization were evaluated
Response: We agree with the reviewer that the description was not accurate. We changed this sentence to "We then divided the genes that were degraded during the meiotic maturation and fertilization of WT oocytes (GV/zygote>10 in WT) into three clusters" in the revised text.

13. Line 345 - I would say modestly at best (not moderately)
Response: We revised this sentence as the reviewer suggested.

14. Discussion
a. In my opinion, the Discussion does not effectively synthesize the findings or add any significant perspective/depth for the reader to put the findings in context of what is already known in the field.
Response: We further revised the Discussion section and discussed the new issues raised by the reviewers.

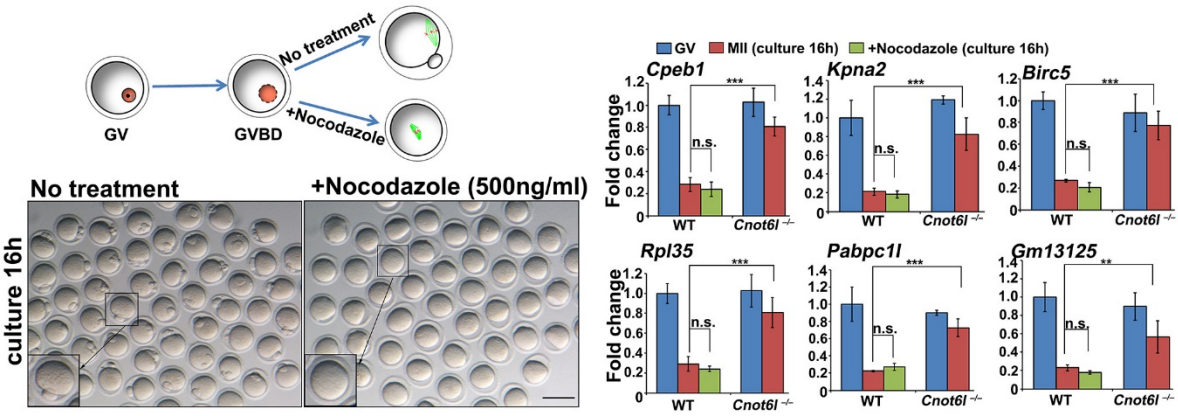
b. Lines 470-78 - This discussion paragraph remains speculative and still seems unrelated and out of place.
Response: In the revised Discussion, we deleted most contents of this paragraph and removed some sentences to the following paragraph.

c. Lines 480-1 - This is not true in light of Ma et al.
Response: In respect to the reviewer's opinion, we changed the sentence to "our study is the first report of gene knockout of *Cnot6l*…".

d. Lines 488-90 - Not sure how this claim can be made without an antibody available to evaluate CNOT6L protein levels?
Response: This claim is supported by *Cnot6l* 3'-UTR reporter assay in the Fig. 8 of this manuscript, and in the Figure 2D of Ma et al, 2015. We agree with the reviewer that we need to be cautious to make this clam without directly evaluating the endogenous CNOT6L protein level. We have reworded this sentences as: "Results of *Cnot6l* 3'-UTR reporter assay showed that the translation of maternal *Cnot6l* transcripts was activated after meiotic resumption, by an MAPK cascade-dependent mechanism in maturing oocytes."

Referee #3:
The RNA-seq experiments (Fig 4) are comparing WT and Cnot6l null oocytes at 0, 8 and 16 h time points after in vitro culture. Importantly, these are not comparable samples because the two groups are in different stages of meiosis. The Cnot6l null oocytes have a lower rate of GVBD than WT oocytes, thus the 8 h sample could be comparing pure meiosis I diakinesis oocytes in WT with mixed diakinesis and GV oocytes in Cnot6l. The discrepancy between samples is even greater at 16 h where WT oocytes are in meiosis II whereas 90% of the Cnot6l null oocytes remain arrested in meiosis I. It is therefore difficult to conclude whether the mRNA degradation defect is primarily due to lack of Cnot6l or a secondary consequence of arresting oocytes in meiosis I. Do the authors know whether an artificial arrest in meiosis I still allows timely mRNA degradation or whether this depends on progression to meiosis II?
Response: We appreciate the reviewer's insightful comments and explain as follows:

1) The reviewer's major concern was that some GV oocytes might contaminate the RNA-seq samples collected at the 8 and 16 h time points in the *Cnot6l* KO group. This is not a problem because we discarded all GV oocytes at 2 h after culture, in both WT and *Cnot6l* KO groups. Therefore, when we collected RNA-seq samples at 8 and 16 h after culture, we would not mix diakinesis and GV oocytes in the *Cnot6l* KO group. We realized that we did not clearly describe this key step in the main text and therefore caused the reviewer's concern, and will emphasize in the revised text that we have removed all GV oocytes at 2 h after culture before we collected RNA-seq samples at 8 and 16 h.
2) In addition, the reviewer's comment that "The *Cnot6l* null oocytes have a lower rate of GVBD than WT oocytes" was not accurate. As we presented in Fig. 2A, the *in vitro* cultured *Cnot6l* KO oocytes have a similar rate and dynamics of germinal vesicle breakdown (GVBD) as the WT control oocytes. Therefore, we would not encounter the situation that "the 8 h sample could be comparing pure meiosis I diakinesis oocytes in WT with mixed diakinesis and GV oocytes in *Cnot6l*".
3) The reviewer's another major critic is that "whether an artificial arrest in meiosis I still allows timely mRNA degradation or whether this depends on progression to meiosis II?" We have performed an additional experiment to answer this question. We artificially arrest the maturing oocytes in meiosis I by treatment of nocodazole, which is a widely used microtubule disruptor. Then we detected the degradation of selective mRNAs (the same mRNAs we detected in *Cnot6l* null oocytes) in these oocytes by quantitative RT-PCR. The results showed that while the degradation of these mRNAs were impaired by *Cnot6l* deletion, they are not affected by nocodazole treatment. These are strong evidence that the delayed mRNA decay we observed in *Cnot6l* null oocytes was primarily due to lack of *Cnot6l* instead of a secondary consequence of arresting oocytes in meiosis I. Because the current manuscript is already lengthy, we did not add these results to the manuscript. Instead, we are preparing another manuscript focusing on the effect of cell cycle progression on maternal mRNA decay.



In addition, chromosome spreads are subject to many technical artifacts that can only be limited by spreading all cells at the same time and in the same drop, which is usually not feasible and is unlikely to have been done here. It is therefore important to score spreads according to hypoploidy, euploidy and hyperploidy. The authors have the data at hand and it should therefore be straightforward to replace Fig 2D with a proper scoring of ploidy.
Response: We replaced Fig. 2D with scoring of ploidy as the reviewer suggested.

Lastly, I considered it important to show the GV time point of the live-cell imaging experiments to demonstrate that the meiosis I-arrested Cnot6l null oocyte in Fig. 3A has a mature SN chromatin configuration. The image presented is rather blurry and it is thus difficult to score it unambiguously, but it appears to be either an intermediate SN/NSN or immature NSN stage oocyte. Could the authors replace this time course with an unambiguous mature SN oocyte so that it is possible to better judge the spindle defect and meiosis I arrest.
Response:

1) We have carefully selected healthy looking fully grown oocytes containing well developed GV for live-cell imaging, and have taken some oocytes from the same batch of samples for confocal microscopy, so that we can see the chromatin configuration of the oocytes more clearly. The confocal microscopy results in Figure EV2E showed that more than 90% of the oocytes collected from Cnot6l KO mice have a mature SN chromatin configuration.
2) We are sorry that the images taken by live-cell imaging experiments were blurry. This is a technical problem caused by image stacking; we have to do imaging stacking to observe the spindles due to the thickness of the oocyte spindles. Nonetheless, the Cnot6l null oocyte presented in Fig. 3A appears to have a similar chromatin configuration as the WT control oocyte, indicating that the experiments of control and KO groups were parallel and comparable.

Accepted 10th October 2018

Thank you for submitting a revised version of your manuscript.

Your manuscript has now been seen once more by ref #3 (given the concerns about GV and oocyte staging and the implications for the overall conclusiveness of the study) and this person is satisfied with your clarifications and now recommends publication of your study (comments included below). I am therefore pleased to inform that your manuscript has been accepted for publication in The EMBO Journal. However, before we can go on to transfer your files for production I have to ask you for a few editorial points/clarifications.

-> In your response to the concerns from referee 2 you mention that you cannot perform statistical analysis since the data derives from two biological replicas. While I fully agree with your explanation I would encourage you to make the nature of the data more clear in the figure by plotting the values as two separate data series rather than a range (as is currently done).

REFEREE REPORTS.

Referee #3:

The addition of timings in the figure helps. So it is fine from my side to publish.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Heng-Yu Fan
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99333

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Each experiment included at least three independent samples and was repeated at least three times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We have stated that "No statistical method was used to predetermine sample size." in the Materials and Methods section
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	We have stated that "The experiments were randomized and were performed with blinding to the conditions of the experiments." in the Materials and Methods section
For animal studies, include a statement about randomization even if no randomization was used.	We have stated that "The experiments were randomized and were performed with blinding to the conditions of the experiments." in the Materials and Methods section
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Not applicable.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	No such methods used.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells were in healthy conditions but were not tested for mycoplasma contamination.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Wild type ICR strain mice were obtained from the Zhejiang Academy of Medical Science, China. All mutant mouse strains had an ICR background. Mice were maintained under SPF conditions in a controlled environment of 20-22ºC, with a 12/12 h light and dark cycle, 50-70% humidity, and food and water provided ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal care and experimental procedures were conducted in accordance with the Animal Research Committee guidelines of Zhejiang University.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We have promised to the journal that we will deposite the original RNA-seq datasets and provide accession numbers before final publication of the revised manuscript. But it is too early to release the information for now because our manuscript has not been accepted for publication yet. This is allowed by the journal policy.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Not applicable.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable.

G- Dual use research of concern

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