EMBO reports

PABPN1L Mediates Cytoplasmic mRNA Decay as a Placeholder during the Maternal-to-Zygotic Transition

Long-Wen Zhao, Hao Chen, Yezhang Zhu, Yun-Wen Wu, Shuai-Bo Pi, Lu Chen, Li Shen, and Heng-Yu Fan

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Corresponding author(s): Heng-Yu Fan (hyfan@zju.edu.cn)

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 27th Feb 2020

Dear Dr. Fan

Thank you for the submission of your research manuscript to our journal. I apologize for the delay in handling your manuscript, but we have only recently received the complete set of referee reports (copied below).

As you will see, all referees acknowledge that the findings are potentially interesting and consider the data overall compelling. However, the referees also point out several concerns regarding missing controls, missing information on the number of replicates or insufficient description of methods and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your

paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please follow the template below for the "Data Availability " section (see also <
https://www.embopress.org/page/journal/14693178/authorguide#dataavailability

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: NCBI Gene Expression Omnibus GSE46843
(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et

al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (technical or biological replicates), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

****

Referee #1:

This is a very clear, well written paper with beautiful data. I have only a few comments which hopefully will improve the paper further.

Main comments:
- What is the evidence that PABPN1L is indeed cytoplasmic? Could you use your antibody for IHC

on oocytes, or inject an mRNA with a tag for visualisation?

- The western data look clear and genuine, but at the very least a statement needs to be added stating how often experiments was repeated with comparable results. Given the widespread issues with westerns, it would be good practice to publish a replicate in the supplementary data.

Minor comments:

Line 54 "In these species, the MZT is accomplished during gastrulation when thousands of blastomeres have formed, and along these lines is otherwise called the "mid-blastula transition (MBT)" The mid-blastula transition occurs, as the name says, in the blastula stage, before gastrulation. In organisms where it is not, it is called the MZT.

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Line 62 and Line 95 ZGA: the term is not used frequently enough to merit abbreviation. Using too many abbreviations just makes this harder to read for the non-expert.

Figure 4A: this is a beautiful figure, but what was the rationale for treating the HeLa cells with cycloheximide? Presumably, the IP transcripts are endogenous transcripts, and therefore human and not mouse? This is suggested by the presence of human primers in supplementary table 6. If so, the gene names need altering to capitals. It would be desirable to have a no-poly(A) RNA control for this figure, just to show the PABPN1L/BTG4 binding is specific for poly(A) RNA. It needs stating in the legend that IP was with anti-HA, not with anti-Flag. It could be informative to see if PABPN1L is nuclear or cytoplasmic in HeLa cells using the same constructs for IHC.

Line 240 "ERK1/2-mediated phosphorylation and degradation of the cytoplasmic polyadenylation element-binding protein-1 (CPEB1) is a prerequisite for releasing the maternal mRNAs from translational dormancy (Uysal & Ozturk, 2019)." I don't think this is the correct reference. Weren't some of the authors of this paper themselves responsible for this discovery in mice and the Hake lab in Xenopus https://www.ncbi.nlm.nih.gov/pubmed/17344432 ?

Methods:

Line 493: the PAT method is described quite confusingly and doesn't make sense to me. Why use ligation to add oligodT to the anchor? This construct can quite easily be ordered as an oligo? What size of oligodT was used? As described, this is a very ancient version of the PAT, which primes anywhere in the poly(A) tail and shortens the products produced. Most laboratories now ligate an anchor to the end of the mRNA, use an oligodT-anchor oligo on which the poly(A) tail is used as a primer for DNA synthesis (eg with Klenow enzyme) or add do GI tailing with a terminal transferase (the authors used this previously). As the PAT data look nice, I suspect an anchor was added to the end of the mRNAs in some fashion and the description in the methods is an error.

Line 458: Why would one want to trim 150 base paired end reads to 50 bases? Was there a very terrible error rate? How many of the original reads mapped to the genome?

Several references are incomplete, eg Dai reference lacks page numbers and is wrong year (2019).

Referee #2:

Maternal-to-zygotic transition (MZT) involves the degradation of maternal transcripts/proteins and activation of embryonic genome (called zygotic genome activation), to support embryonic development. Of these two events, maternal decay is the perquisite for zygotic genome activation (ZGA). The Fan lab has been trying to understand this process using mouse models and have made several important discoveries regarding maternal decay during oocyte maturation and after fertilization. One of their recent findings is the identification of BTG4 as a licensing factor for MZT by promoting maternal mRNA degradation (Yu et al., 2016 NSMB).

In this study, the authors reported a key role of the polyA-binding protein Pabpn1l in maternal decay in mice and provided convincing evidence demonstrating that Pabpn1l not only acts as an adaptor between BTG4 and its target mRNAs, but also stabilizes BTG4 by preventing SCF-βTrCP1-mediated ubiquitination and degradation. The evidence including: 1) Pabpn1l female mice are infertile due to early embryonic arrest caused by defective maternal decay and ZGA (Figure 1-3); 2) Pabpn1l interacts with BTG4 C-terminus and this interaction is required for targeting BTG4 to its mRNA targets (Figure 4-5); 3) Pabpn1l stabilizes BTG4 by preventing it from ubiquitination by βTrCP1 (Figure 6); 4) key role of R171 and RRM in Pabpn1l polyA-binding activity.

In summary, the data presented are of high quality and convincing suitable for publication in EMBO Report. I only have a few minor comments:

For all RNA-seq analyses (i.e., Figure 2A and Figure 3A), could the authors also provide RPKMs of genes that are not differentially expressed? The authors used a relatively stringent cutoff (FC > 3) for DEG identification. Some genes with FC > 2 may be still informative, but are not included. For example, Figure 3 and S3 showed many know 2-cell embryo-specific transcripts such as Zscan4, Zfp352, and MuERVL are down-regulated in Pabpn1l-/+ embryos. Is their activator Dux also down-regulated? How about Dux, as a minor ZGA gene, expression in Pabpn1l-/+ zygote and 2-cell?

Fig. 3A showed 718 genes are down regulated. Considering that 269 genes are upregulated, the net down regulated genes are 449, which is much fewer than the roughly 2000 ZGA genes. However, the EU staining data presented in Fig. 3C suggest a major down-regulation (more than 50% of global transcription). The authors should give some explanation of this discrepancy.

Figure 3F, some embryos in Pabpn1l-/+ group is morphologically abnormal or may be still at 1-cell stage. It should be clarified in the legend or the labeling.

Dividing oocytes do not have nuclear membrane, thus PABPN1L is supposed to interact with BTG4 in the cytoplasm. Can the authors provide co-immunostaining of PABPN1L and BTG4 in oocytes/zygotes to confirm this?

Referee #3:

In the manuscript by Zhao et al. entitled "Nuclear Poly(A)-binding Protein 1-like (PABPN1L) Mediates Cytoplasmic mRNA Decay as a Placeholder during the Maternal-to-Zygotic Transition",

the authors characterize the function and requirement for an oocyte enriched polyadenosine RNA binding protein, PABPNL1. They create a Pabnl1 knockout mouse and define a key role for PABPNL1 in modulating RNA decay during MZT. The authors present data to support two modes for this regulation: 1) serving as an RNA binding module to recruit BTG4/CCR4-NOT to decay RNAs at MZT and 2) stabilizing BTG4 through this interaction.

Overall, the authors provide compelling evidence for a critical role for PABPNL1 in MZT/ZGA. The manuscript is very nicely laid out with clear conclusions and compelling results to support each conclusion. There are some aspects of specific results to support the model that need a few additional experimental controls.

While this is a presentation point, the manuscript is all presented in the past tense, including the conclusions drawn from the data presented here. This is very distracting as the style makes separating what was known prior to this study, which was not much, from the new results presented here challenging. This includes running titles for the sections within the Results section. The authors should present the results of this study in the present tense (see below for examples).

Specific Comments:

Figure 1 demonstrates the expression of PABPNL1 in mouse as well as the requirement in development. These results are convincing in defining a key role for PABPNL1.

Figure 2 shows results of RNA-Seq analysis of oocytes or zygotes. The authors detect little difference at the oocyte stage, but detect many transcripts that show a statistically significant increase in steady-state levels in the zygote. The authors validate the changes and provide evidence for a failure to shorten poly(A) tails, a process that precedes decay.

Minor points for Figure 2, but important:
The Figure, the Legend, and the text do not use precisely the same abbreviations/terminology for the oocyte (GV in the figure) and zygote, making the data a bit challenging to someone not familiar with these terms. Even the figure title is "Figure 2: Transcriptome analyses in Pabpn1l-deleted oocyte and embryos during the MZT." when the figure refers to GV and zygote.

In addition, the authors use the term "upregulated" or "downregulated" for the transcripts that increase or decrease, respectively. The term "upregulated" tends to infer an increase in expression (often by transcription), but what the method measures is steady-state transcript levels. Indeed, the authors argue that the change in transcript levels is due to a defect in decay, making the term "upregulation" even more confusing/inappropriate.

The authors next turn the stage of zygotic gene activation (ZGA) and examine the program of gene activation that occurs at the 2-cell stage (Figure 3).

This sentence explaining the results presented is very confusing, "Gene set enrichment analysis revealed that the decreased 167 transcripts belonged to those being activated during normal MZT but failed after maternal 168 Pabpn1-deletion: 419 of the 718 downregulated transcripts at the 2-cell stage (WT/Pabpn1l♀−/♂+ 169 {greater than or equal to} 3) were products of early zygotic genes being activated in the WT 2-cell 170 embryos (Fig. 3B)."

I think that the authors mean that the transcripts that are decreased relative to WT are due to a

failure to activate transcription in the Pabpn1 mice. More words are probably needed to clarify this point- perhaps a schematic in the figure would help to clarify the point. The Venn diagram currently shown in Figure 3B does not help very much to make this point clear. This point is very clear in Figure 3E, so perhaps the language just needs to be changed.

Overall, the data presented on the global transcription do provide evidence that the transcriptional program that occurs at ZGA is impaired in the mutant mice.

Figure 4 begins to address mechanism by exploring the idea that PABPNL1 interacts with BTG4 to target specific transcripts for CCR4-NOT-mediated decay. The authors employ HeLa cells for these biochemical studies. The first set of experiments employs Flag-tagged proteins and the only control is deletion of the PABPNL1 RRM. The authors should really employ another poly(A) binding protein, PABPN1 would be the most obvious one (especially since the authors show PABPN1 does not interact with BTG4 in Figure 4G), as a control. The RRM is a large domain of PABPNL1 so these data provide little evidence for specificity. There are also no immunoblots provided to demonstrate that the ΔRRM protein is expressed at comparable levels in HeLa cells under the conditions employed. There is an immunoblot shown in Figure 5C, but the context is different and the levels of ΔRRM PABPNL1 do appear lower than WT PABPNL1. There is an easy solution to this issues as additional controls that employ the Arg-171->Ala protein are actually presented in Figure S6. These data should be incorporated into the main text to shore up these results.

The authors then turn to defining the domain of BTG4 that interacts with PABPNL1. They map this interaction to a C-terminal domain and also show specificity because PABPNL1 shows no interaction with the related protein BTG2 (Figure 4E).

Figure 5 provides compelling in vivo rescue data. The only issue with the results presented here is that a control employed, Arg171->Ala variant comes out of nowhere with the statement (an essential RNA-binding residue in the RRM) with no reference or evidence to back up this statement. While a single amino acid change that impairs RNA binding is a far preferable control to a large deletion such as the ΔRRM employed (which the authors show here is expressed at lower levels than WT PABPNL1), there needs to be some basis for the choice of this particular variant. There is language later in the Results and a Supplemental Figure (S6) that argues based, primarily it seems on sequence conservation, that this residue is important for RNA binding. This information needs to be presented at this point in the manuscript. Indeed, there are structures of related poly(A) binding proteins (PDB# 2JWN), which might allow the authors to model the structure of the RRM domain in PABPNL1 and provide a more compelling argument for why this residue is likely to be critical for RNA bidning. Ideally, they could incorporate this PABPNL1 variant into the experiments presented in Figure 4A to provide a far more compelling control for that experiment than the current ΔRRM.

Figure 6 addresses an additional function of PABPNL1 in stabilizing BTG4 in oocytes. The data shown in Figure 6A is compelling and 100 ooctyes were required to collect the data. It would be preferable to see this result from more than one experiment with quantitation, but the amount of material required may be prohibitive. Perhaps this result is representative of multiple experiments. Subsequent experiments use transgenic cell lines, so this is really the key result. The authors address the nature of this regulation in Figure S5. They have one statement that the "Btg4 3'UTR was normally activated in maturing Pabpnl1-/- ooctyes." What they mean by "activated" here is not clear. Activation of a 3'UTR is not a common terminology. Probably what the authors mean is that the translational activation that is regulated by the 3'UTR of Btg4 is intact in the Pabpnl1-/- ooctyes. Figure 6B requires a control such as expression of PABPN1 to demonstrate that the

protection of BTG4 from rapid turnover is specific to PABPNL1. Alternatively, the authors mapped the domain near the C-terminus of PABPNL1 that binds to BTG4 so the prediction would be that this PABPNL1 variant would not alter the decay rate of BTG4. This critique also applies to Figure 6D, to demonstrate that the protection of BTG4 from ubiquitylation is specific to PABPNL1 or the interaction with PABPNL1.

Finally, the last figure employs thermal stability to explore RNA binding. This is not the most direct assay, but together with all the other data is reasonable. These data might have made more sense before the part of the manuscript about BTG4 stability (because the story moved away from RNA binding), but there is a great deal of data here. Perhaps the BTG4 protein regulation could have been reserved for another study, but this is what the authors present. The RNA binding studies only employ poly(A) RNA. Authors really should have included other sequences to provide evidence for specificity. Most poly(A) binding proteins have binding sites on the order of 10-20 nucleotides so the 20N having no effect may suggest that the change in thermal stability does not represent the direct binding to RNA. This protein has a single RRM similar to conventional PABPN1. These data are some of the lest convincing for the conclusions that the authors draw, although they are almost certainly correct that PABPNL1 binds polyadenosine, there are far more direct binding assay that could be employed.

The final paragraph of the Results, which addresses the Arg-171 residue is a bit confusing. The authors refer to another structure and key residues but do not clearly state how those residues relate to Arg-171 (Fig. S6B shows this but not obvious from the text). Regardless, this text should appear earlier in the manuscript when this PABPNL1 variant is first employed.

The model does a nice job of summarizing a great deal of information.

Presentation comments (examples):

Typically results should be stated in the present tense (presumably they are still true). For example, the summary paragraph of the Introduction should make statements in the present tense such as the following suggested change:

"Genetic deletion of Pabpn1l impaired the deadenylation and degradation of a subset of maternal mRNAs during the MZT."

Should really read:

"Genetic deletion of Pabpn1l impairs the deadenylation and degradation of a subset of maternal mRNAs during the MZT."

Even in the Results headers this is an issue:

"Maternal PABPN1L was essential for ZGA"

Presumably this important finding has not changed and was not known before the present study so the statement should be:

"Maternal PABPN1L is essential for ZGA"

The authors use the language on the bottom of page 8- "To confirm our hypothesis in vivo,". Scientists should always TEST hypotheses as they can only be tested, but never confirmed or proven. This is not to detract from the experiments, which are compelling, but a wording change required.

Re: EMBOR-2019-49956-T

Responses to reviewers' comments

Reviewer 1

This is a very clear, well written paper with beautiful data. I have only a few comments which hopefully will improve the paper further.

Main comments:

What is the evidence that PABPN1L is indeed cytoplasmic? Could you use your antibody for IHC on oocytes, or inject an mRNA with a tag for visualisation?

Response: We appreciate the reviewer's positive comments that our investigation is clear and the data are solid. The PABPN1L antibody is not effective enough to detect endogenous PABPN1L protein. We injected mRNAs encoding HA-PABPN1L in WT MII oocytes and zygotes as the reviewer indicated. After immunofluorescence of HA, PABPN1L displays notably cytoplasmic localization as compared with no treatment group at MII and zygote stage (Fig EV1B).

The western data look clear and genuine, but at the very least a statement needs to be added stating how often experiments was repeated with comparable results. Given the widespread issues with westerns, it would be good practice to publish a replicate in the supplementary data.

Response: For each result of western blot, at least three independent experiments were done with consistent results, and we chose the representative image for demonstration. As suggested by the reviewer, we have added the statement to claim the repeatability of the western blot results in the figure legend and provided the original uncropped images in Source Data part.

Minor comments:

Line 54 "In these species, the MZT is accomplished during gastrulation when thousands of blastomeres have formed, and along these lines is otherwise called the "mid-blastula transition (MBT)" The mid-blastula transition occurs, as the name says, in the blastula stage, before gastrulation. In organisms where it is not, it is called the MZT.

Response: We appreciate the reviewer's opinion and we have revised this sentence in the main text.

Line 73 "prolonged" is the incorrect word here, as it refers to spans of time, not size. "elongated" would be correct.

Response: We have corrected it in the main text.

Line 62 and Line 95 ZGA: the term is not used frequently enough to merit abbreviation. Using too many abbreviations just makes this harder to read for the non-expert.

Response: We thank the reviewer for pointing this out for improving the readability of our manuscript. We counted the number of this term and found as many as 10 in the text. To follow the author guidelines of *EMBO Reports*, we provided the full name of ZGA when it is first displayed.

Figure 4A: this is a beautiful figure, but what was the rationale for treating the HeLa cells with cycloheximide? Presumably, the IP transcripts are endogenous transcripts, and therefore human and not mouse? This is suggested by the presence of human primers in supplementary table 6. If so, the gene names need altering to capitals. It would be desirable to have a no-poly(A) RNA control for this figure, just to show the PABPN1L/BTG4 binding is specific for poly(A) RNA. It needs stating in the legend that IP was with anti-HA, not with anti-Flag. It could be informative to see if PABPN1L is nuclear or cytoplasmic in HeLa cells using the same constructs for IHC.

Response:

1) In Fig 4A, the HeLa cells were not treated with cycloheximide. Maybe the reviewer was mistaken? But we indeed treated cells with this drug in experiments presented in Fig 7B. The treatment of cycloheximide is to inhibit *de novo* protein synthesis, and we could then measure the post-translational stability of the indicated proteins by collecting HeLa cells at different time points (Sha et al., 2018).

2) The IP transcripts in Fig 4A were indeed endogenous human transcripts. We have corrected the gene names for capitals in Table EV6 and Fig 4A.

3) We agree with the reviewer that it would be desirable to have a no-poly(A) RNA control (such as the mRNAs encoding histones) for this figure. However, due to the significant disruption that is being caused by the COVID-19 pandemic, our laboratory has been shut down, and we will have difficulty in meeting the timeline of submitting the revised manuscript if we wait to add this new result. Concerning the Poly(A) binding specificity of PABPN1L, we have generated both point mutation Arg-171-mutation and RRM-deletion truncation as the negative control, and the results highlight the importance of PABPN1L's RNA-binding ability via Poly(A) for BTG4. Also, we have tested the PABPN1L's binding specificity to Poly(A) using *in vitro* thermal shift assays, and the no-poly (A) RNA showed clear negative results (Fig 6A, left panel), which indicated that PABPN1L cannot interact with no-poly(A) RNA. Thus, we believed that our current results have addressed the reviewer's concern. Due to the COVID-19 situation, we ask the reviewer to kindly consider if the manuscript can be accepted without adding this control.

4) We have added the description of IP with anti-HA antibody in the legend of Fig 4A.

5) We performed immunofluorescence in HeLa cells transfected with plasmid expressing HA-tagged PABPN1L (Fig EV3A). The localization of FLAG-PABPN1L also displayed cytoplasmic distribution, similarly to that in oocytes or zygotes (Fig EV1B).

Line 240 "ERK1/2-mediated phosphorylation and degradation of the cytoplasmic polyadenylation element-binding protein-1 (CPEB1) is a prerequisite for releasing the maternal mRNAs from translational dormancy (Uysal & Ozturk, 2019)." I don't think this is the correct reference. Weren't some of the authors of this paper themselves responsible for this discovery in mice and the Hake lab in Xenopus https://www.ncbi.nlm.nih.gov/pubmed/17344432 ?
Response: Thanks for pointing this out, now we have corrected the references to support the claim.

Line 493: the PAT method is described quite confusingly and doesn't make sense to me. Why use ligation to add oligodT to the anchor? This construct can quite easily be ordered as an oligo? What size of oligodT was used? As described, this is a very ancient version of the PAT, which primes anywhere in the poly(A) tail and shortens the products produced. Most laboratories now ligate an anchor to the end of the mRNA, use an oligodT-anchor oligo on which the poly(A) tail is used as a primer for DNA synthesis (eg with Klenow enzyme) or add do GI tailing with a terminal transferase (the authors used this previously). As the PAT data look nice, I suspect an anchor was added to the end of the mRNAs in some fashion and the description in the methods is an error.
Response: According to the reviewer's suggestion, we provided an upstate and detailed description of the PAT assay in Materials & Methods. This is a modified methods that are more sensitive in detecting poly(A) tails of small amount of mRNAs than the methods we used in our previous publications The mRNA terminal ligation efficiency was low in the previous methods, maybe because the concentration of mRNAs extracted from oocytes was too low.

Line 458: Why would one want to trim 150 base paired end reads to 50 bases? Was there a very terrible error rate? How many of the original reads mapped to the genome?
Response: We apologize for the typo. We have corrected the error in the revised Materials & Methods. The Table EV4 showed the mapping efficiency of each sample in RNA-seq results.

Several references are incomplete, eg Dai reference lacks page numbers and is wrong year (2019).
Response: We have rechecked the references cited in our manuscript and double confirmed our citations are corrected insected using Endnote X9.

Reviewer 2

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In this study, the authors reported a key role of the polyA-binding protein Pabpn1l in maternal decay in mice and provided convincing evidence demonstrating that Pabpn1l not only acts as an adaptor between BTG4 and its target mRNAs, but also stabilizes BTG4 by preventing SCF-βTrCP1-mediated ubiquitination and degradation. The evidence including: 1) Pabpn1l female mice are infertile due to early embryonic arrest caused by defective maternal decay and ZGA (Figure 1-3); 2) Pabpn1l interacts with BTG4 C-terminus and this interaction is required for targeting BTG4 to its mRNA targets (Figure 4-5); 3) Pabpn1l stabilizes BTG4 by preventing it from ubiquitination by βTrCP1 (Figure 6); 4) key role of R171 and RRM in Pabpn1l polyA-binding activity.

In summary, the data presented are of high quality and convincing suitable for publication in EMBO Report. I only have a few minor comments:

For all RNA-seq analyses (i.e., Figure 2A and Figure 3A), could the authors also provide RPKMs of genes that are not differentially expressed? The authors used a relatively stringent cutoff (FC > 3) for DEG identification. Some genes with FC > 2 may be still informative, but are not included. For example, Figure 3 and S3 showed many know 2-cell embryo-specific transcripts such as Zscan4, Zfp352, and MuERVL are down-regulated in Pabpn1l-/+ embryos. Is their activator Dux also down-regulated? How about Dux, as a minor ZGA gene, expression in Pabpn1l-/+ zygote and 2-cell?

Response: We appreciate the reviewer's rigorously comments on our study, and have seriously addressed his/her questions as detailed below.

1) According to the reviewer’s suggestion, we classified the genes into 3 groups with corresponding labels (upregulated ↑, unchanged—and downregulated ↓) based on FPKMs with a threshold of fold change = 2 or 1/2 at each stage (Table EV5).

2) We agree with the reviewer that it is interesting to detect *Dux* activation in 2-cell embryos by RT-qPCR, and as expected, the *Dux* is also downregulated in $Pabpn1l^{♀-/♂+}$ embryos (Appendix Fig S1A).

Fig. 3A showed 718 genes are downregulated. Considering that 269 genes are upregulated, the net down regulated genes are 449, which is much fewer than the roughly 2000 ZGA genes. However, the EU staining data presented in Fig. 3C suggest a major down-regulation (more than 50% of global transcription). The authors should give some explanation of this discrepancy.

Response: Due to the differences of RNA-seq procedures, including sequencing technology and limited numbers of embryos from KO animals, we are unable to ensure our screened "ZGA genes" as same as previous reported. For the accuracy of the analysis, the genes with FPKM < 1 in all samples were excluded which may cause the loss of the lower expressed genes. On the other hand, the "ZGA genes" in this RNA-seq with stringent threshold for gene set enrichment analysis, also guaranteed that our conclusion "maternal PABPN1L is essential for ZGA" is reliable.

Figure 3F, some embryos in Pabpn1l-/+ group is morphologically abnormal or may be still at 1-cell stage. It should be clarified in the legend or the labeling.

Response: We apologize for the wrong labeling. We have corrected the error in the Fig 3F, and added description in the corresponding Figure legend.

Dividing oocytes do not have nuclear membrane, thus PABPN1L is supposed to interact with BTG4 in the cytoplasm. Can the authors provide co-immunostaining of PABPN1L and BTG4 in oocytes/zygotes to confirm this?

Response: The PABPN1L antibody is not effective enough to detect endogenous PABPN1L protein. Instead, we injected mRNAs encoding HA-PABPN1L in WT oocytes and zygotes. After immunofluorescence of HA, PABPN1L displays notably cytoplasmic localization as compared with no treatment group at MII and zygote stage (Fig EV1B). In addition, because both the BTG4 and PABPN1L antibodies are rabbit-derived, we are unable to perform the co-immunostaining of PABPN1L and BTG4.

Reviewer 3

In the manuscript by Zhao et al. entitled "Nuclear Poly(A)-binding Protein 1-like (PABPN1L) Mediates Cytoplasmic mRNA Decay as a Placeholder during the Maternal-to-Zygotic Transition", the authors characterize the function and requirement for an oocyte enriched polyadenosine RNA binding protein, PABPNL1. They create a Pabnl1 knockout mouse and define a key role for PABPNL1 in modulating RNA decay during MZT. The authors present data to support two modes for this regulation: 1) serving as an RNA binding module to recruit BTG4/CCR4-NOT to decay RNAs at MZT and 2) stabilizing BTG4 through this interaction.

Overall, the authors provide compelling evidence for a critical role for PABPNL1 in MZT/ZGA. The manuscript is very nicely laid out with clear conclusions and compelling

results to support each conclusion. There are some aspects of specific results to support the model that need a few additional experimental controls.

While this is a presentation point, the manuscript is all presented in the past tense, including the conclusions drawn from the data presented here. This is very distracting as the style makes separating what was known prior to this study, which was not much, from the new results presented here challenging. This includes running titles for the sections within the Results section. The authors should present the results of this study in the present tense (see below for examples).
Response: We appreciate the reviewer for the positive comments and valuable suggestions for improving the readability of our manuscript. We have corrected the tense of our results into the present tense.

Specific Comments:

Figure 1 demonstrates the expression of PABPNL1 in mouse as well as the requirement in development. These results are convincing in defining a key role for PABPNL1.

Figure 2 shows results of RNA-Seq analysis of oocytes or zygotes. The authors detect little difference at the oocyte stage, but detect many transcripts that show a statistically significant increase in steady-state levels in the zygote. The authors validate the changes and provide evidence for a failure to shorten poly(A) tails, a process that precedes decay.

Minor points for Figure 2, but important:
The Figure, the Legend, and the text do not use precisely the same abbreviations/terminology for the oocyte (GV in the figure) and zygote, making the data a bit challenging to someone not familiar with these terms. Even the figure title is "Figure 2: Transcriptome analyses in Pabpn1l-deleted oocyte and embryos during the MZT." when the figure refers to GV and zygote.
Response: We apologize for the confusion of labeling. The oocyte includes both GV and MII stage oocyte, and the embryo includes both zygote and 2-cell stage embryo. In view of the different samples used in different experiments, we kept the title the same, but have reworded them to the specific stages case by case in the Figures and legends.

In addition, the authors use the term "upregulated" or "downregulated" for the transcripts that increase or decrease, respectively. The term "upregulated" tends to infer an increase in expression (often by transcription), but what the method measures is steady-state transcript levels. Indeed, the authors argue that the change in transcript levels is due to a defect in decay, making the term "upregulation" even more confusing/inappropriate.

Response: Thanks for the comments. We have changes the term "upregulated" and "downregulated" into “increase” and “decrease” in the revised manuscript.

The authors next turn the stage of zygotic gene activation (ZGA) and examine the program of gene activation that occurs at the 2-cell stage (Figure 3).

This sentence explaining the results presented is very confusing, "Gene set enrichment analysis revealed that the decreased transcripts belonged to those being activated during normal MZT but failed after maternal Pabpn1-deletion: 419 of the 718 downregulated transcripts at the 2-cell stage (WT/Pabpn1l♀−/♂+ greater than or equal to 3) were products of early zygotic genes being activated in the WT 2-cell embryos (Fig. 3B)."

I think that the authors mean that the transcripts that are decreased relative to WT are due to a failure to activate transcription in the Pabpn1 mice. More words are probably needed to clarify this point- perhaps a schematic in the figure would help to clarify the point. The Venn diagram currently shown in Figure 3B does not help very much to make this point clear. This point is very clear in Figure 3E, so perhaps the language just needs to be changed.

Overall, the data presented on the global transcription do provide evidence that the transcriptional program that occurs at ZGA is impaired in the mutant mice.

Response: We thank the reviewer for pointing this out for improving the readability of our manuscript. We have reworded the sentence pointed out by the reviewer, and provided a schematic (Fig 3B, right panel) to explain our point that “maternal *Pabpn1l*-deletion disrupts zygotic genome activation”.

Figure 4 begins to address mechanism by exploring the idea that PABPNL1 interacts with BTG4 to target specific transcripts for CCR4-NOT-mediated decay. The authors employ HeLa cells for these biochemical studies. The first set of experiments employs Flag-tagged proteins and the only control is deletion of the PABPNL1 RRM. The authors should really employ another poly(A) binding protein, PABPN1 would be the most obvious one (especially since the authors show PABPN1 does not interact with BTG4 in Figure 4G), as a control. The RRM is a large domain of PABPNL1 so these data provide little evidence for specificity. There are also no immunoblots provided to demonstrate that the ΔRRM protein is expressed at comparable levels in HeLa cells under the conditions employed. There is an immunoblot shown in Figure 5C, but the context is different and the levels of ΔRRM PABPNL1 do appear lower than WT PABPNL1. There is an easy solution to this issues as additional controls that employ the Arg-171->Ala protein are actually presented in Figure S6. These data should be incorporated into the main text to shore up these results.

Response:

1) We thank the reviewer for suggesting this control experiment. In the revised manuscript, we have used the R171A mutant PABPN1L (PABPN1L^{R171A}) as an additional negative

control for the BTG4-RNA immunoprecipitation assays in Fig 4A. Consistent with Fig 6D, mRNAs are not enriched by the R171A mutant PABPN1L in this RIP assay.

2) In our opinion, overexpressing PABPN1 is not a good control, because the HeLa cells abundantly express endogenous PABPN1. In fact, the RIP assay was performed in HeLa cells with the presence of endogenous PABPN1, but we found BTG4 alone cannot interact with representative transcripts. These results actually have already indicated that PABPN1 is invalid in mediating BTG4-mRNA interaction.

The authors then turn to defining the domain of BTG4 that interacts with PABPNL1. They map this interaction to a C-terminal domain and also show specificity because PABPNL1 shows no interaction with the related protein BTG2 (Figure 4E).

Figure 5 provides compelling in vivo rescue data. The only issue with the results presented here is that a control employed, Arg171->Ala variant comes out of nowhere with the statement (an essential RNA-binding residue in the RRM) with no reference or evidence to back up this statement. While a single amino acid change that impairs RNA binding is a far preferable control to a large deletion such as the ΔRRM employed (which the authors show here is expressed at lower levels than WT PABPNL1), there needs to be some basis for the choice of this particular variant. There is language later in the Results and a Supplemental Figure (S6) that argues based, primarily it seems on sequence conservation, that this residue is important for RNA binding. This information needs to be presented at this point in the manuscript. Indeed, there are structures of related poly(A) binding proteins (PDB# 2JWN), which might allow the authors to model the structure of the RRM domain in PABPNL1 and provide a more compelling argument for why this residue is likely to be critical for RNA bidning. Ideally, they could incorporate this PABPNL1 variant into the experiments presented in Figure 4A to provide a far more compelling control for that experiment than the current ΔRRM.

Response: Thank the reviewer to point this out. According to previously published nuclear RRM structure (Domingues et al., 2015), the CsPABPN1 RRM domain existed as a stable homodimer in solution and R136 in CsPABPN1 shows major contribution to Poly(A) binding. By sequence analysis, we found that PABPN1L shows conserved R171 site (Fig EV4B; broken circle and arrowhead indicate), thus we performed experiments on R171 point mutation experiments. Indeed, in PABPN1L, R171 contributes to Poly(A) binding (Fig 6C-D). We also incorporated this PABPN1L variant as a control into the experiments presented in Fig 4A.

Figure 6 addresses an additional function of PABPNL1 in stabilizing BTG4 in oocytes. The data shown in Figure 6A is compelling and 100 oocytes were required to collect the data. It would be preferable to see this result from more than one experiment with quantitation, but

the amount of material required may be prohibitive. Perhaps this result is representative of multiple experiments. Subsequent experiments use transgenic cell lines, so this is really the key result. The authors address the nature of this regulation in Figure S5. They have one statement that the "Btg4 3'UTR was normally activated in maturing Pabpnl1-/- ooctyes." What they mean by "activated" here is not clear. Activation of a 3'UTR is not a common terminology. Probably what the authors mean is that the translational activation that is regulated by the 3'UTR of Btg4 is intact in the Pabpnl1-/- oocytes. Figure 6B requires a control such as expression of PABPN1 to demonstrate that the protection of BTG4 from rapid turnover is specific to PABPNL1. Alternatively, the authors mapped the domain near the C-terminus of PABPNL1 that binds to BTG4 so the prediction would be that this PABPNL1 variant would not alter the decay rate of BTG4. This critique also applies to Figure 6D, to demonstrate that the protection of BTG4 from ubiquitylation is specific to PABPNL1 or the interaction with PABPNL1.

Response:

1) We rephrased the related description in Results (line 283-284) as the reviewer suggested.
2) The Co-IP assay showing BTG4 polyubiquitination in Fig 7D was performed in HeLa cells which endogenously expressed abundant PABPN1, but the results indicated that βTrCP1-mediated BTG4 polyubiquitination cannot be prevented by PABPN1. Similar in Fig 7B, endogenous PABPN1 did not stabilize BTG4, suggesting that the protecting effect of PABPN1L is specific. We agree with the reviewer that it will be ideal to include a control group by overexpressing PABPN1L$^{Cter\Delta}$. However, due to the significant disruption that is being caused by the COVID-19 pandemic, our laboratory has been shut down, and we will have difficulty in meeting the timeline of submitting the revised manuscript if we wait to add this new result. To some extent, we have generated the negative control of BTG2 which showed no interaction with PABPN1L (Fig 4E) and the time-dependent BTG2 degradation was not prevented by PABPN1L overexpression (Fig EV5E), which suggested that BTG4-PABPN1L specific interaction protects BTG4. Because this issue is relatively minor, we ask the reviewer to kindly consider if the manuscript can be accepted without this result.

Finally, the last figure employs thermal stability to explore RNA binding. This is not the most direct assay, but together with all the other data is reasonable. These data might have made more sense before the part of the manuscript about BTG4 stability (because the story moved away from RNA binding), but there is a great deal of data here. Perhaps the BTG4 protein regulation could have been reserved for another study, but this is what the authors present. The RNA binding studies only employ poly(A) RNA. Authors really should have included other sequences to provide evidence for specificity. Most poly(A) binding proteins have binding sites on the order of 10-20 nucleotides so the 20N having no effect may suggest that the change in thermal stability does not represent the direct binding to RNA. This protein has

a single RRM similar to conventional PABPN1. These data are some of the lest convincing for the conclusions that the authors draw, although they are almost certainly correct that PABPNL1 binds polyadenosine, there are far more direct binding assay that could be employed.

Response:

1) We appreciate the reviewer's suggestion. We have rearranged the Figs 6 and 7 in the main text.

2) Please kindly note that in Fig 6A (left panel), the RNA substrate ($20N+A_{10}$) comprising 20 non-poly(A) nucleotides followed by a 10-adenosine poly(A) tail showed no effect on the Tm of PABPN1L. These results suggested that PABPN1L cannot interact with 20-nucleotides or 10-adenosines, which as a negative control for implying that TSA is sensitive.

The final paragraph of the Results, which addresses the Arg-171 residue is a bit confusing. The authors refer to another structure and key residues but do not clearly state how those residues relate to Arg-171 (Fig. S6B shows this but not obvious from the text). Regardless, this text should appear earlier in the manuscript when this PABPNL1 variant is first employed.

The model does a nice job of summarizing a great deal of information.

Response: According to previously published nuclear RRM structure (Domingues et al., 2015), the CsPABPN1 RRM domain existed as a stable homodimer in solution and R136 in CsPABPN1 shows major contribution to Poly(A) binding. By sequence analysis, we found that PABPN1L shows conserved R171 site (Fig EV4B), thus we performed experiments on R171 point mutation experiments. We have added more description in the main text. We have rearranged the Figs 6 and 7 in the revised manuscript.

Presentation comments (examples):

Typically results should be stated in the present tense (presumably they are still true). For example, the summary paragraph of the Introduction should make statements in the present tense such as the following suggested change:

"Genetic deletion of Pabpn1l impaired the deadenylation and degradation of a subset of maternal mRNAs during the MZT."

Should really read:

"Genetic deletion of Pabpn1l impairs the deadenylation and degradation of a subset of maternal mRNAs during the MZT."

Even in the Results headers this is an issue:

"Maternal PABPN1L was essential for ZGA"

Presumably this important finding has not changed and was not known before the present study so the statement should be:

"Maternal PABPN1L is essential for ZGA"

The authors use the language on the bottom of page 8- "To confirm our hypothesis in vivo,". Scientists should always TEST hypotheses as they can only be tested, but never confirmed or proven. This is not to detract from the experiments, which are compelling, but a wording change required.

Response: We have corrected the indicated errors. We have also corrected the tense of our results into the present tense. Thanks to the reviewer's positive comments and valuable suggestions.

1st Revision - Editorial Decision 6th May 2020

Dear Dr. Fan

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. As you will see, all referees are very positive about the study and support publication in EMBO reports without further revisions.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- Please provide all figures (main and EV) as individual production quality figure files as .eps, .tif, .jpg (one file per figure). Please ensure a high resolution of the figures. The current resolution is too low for production.

- Data Availability section: Please include a link that resolves to the dataset.

- Author contributions: please specify the contribution of Li Shen.

- Please update the references to the numbered format of EMBO reports. The abbreviation 'et al' should be used if more than 10 authors. You can download the respective EndNote file from our Guide to Authors
https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

- EV tables: Please upload EV tables 1, 3, 4, and 6 as individual Word files including the respective legends (Expanded View option).

- I suggest submitting EV tables 2 and 5 as Datasets. The nomenclature for these is "Dataset EV1" and "Dataset EV2". You can keep the tables in the .xlsx format and please include the legend either in the first column of the respective tabs or in a separate tab labeled "legend". Please update the numbering of the remaining EV tables accordingly.

- Figure 5C: When comparing the image shown in Figure 5C with the Source data you supplied, it appeared that you cut the scan of the Western blot for FLAG to remove the lower MW bands and smear. Please note that such a procedure is not good practice and not in agreement with our journal policies. Please either show the actual data including the background staining or replace the entire experiment with data from a replicate experiment. If you decide for the latter option, please update and supply the source data for this experiment.

- Fig EV1E appears very over-contrasted. If possible, please provide images with less contrast modification.

- Please provide a scale bar for the zoom image in Fig EV3A.

- Source data for Figure 7A: the blots for pERK1/2 and DDB1 in the source data file appear mislabeled. Please correct.
- The same applies to the source data blots for Figure 7D, HA and MYC.

- Finally, I attach to this email a related manuscript file with comments in the figure legends by our

data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.
I have also taken the liberty to make some changes to the Abstract and title. Could you please review it?

We look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Martina Rembold

Martina Rembold, PhD
Editor
EMBO reports

****

Referee #1:

I'm fine with the manuscript as it has been revised.

Referee #2:

my questions have been addressed.

Referee #3:

The authors have addressed the comments from the previous review cycle. Some experiments were not possible due to COVID-19, but these are not experiments required to support the primary conclusions of the study.

Re: EMBOR-2019-49956V2

Responses to editor's comments

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. As you will see, all referees are very positive about the study and support publication in EMBO reports without further revisions.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- Please provide all figures (main and EV) as individual production quality figure files as .eps, .tif, .jpg (one file per figure). Please ensure a high resolution of the figures. The current resolution is too low for production.

Response: We have submitted all figures accordingly.

- Data Availability section: Please include a link that resolves to the dataset.

Response: The link of dataset was added in Data Availability part.

- Author contributions: please specify the contribution of Li Shen.

Response: We have added the contribution of Li Shen in Author Contributions part.

- Please update the references to the numbered format of EMBO reports. The abbreviation 'et al' should be used if more than 10 authors. You can download the respective EndNote file from our Guide to Authors

https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

Response: We have updated the numbered format of our references accordingly.

- EV tables: Please upload EV tables 1, 3, 4, and 6 as individual Word files including the respective legends (Expanded View option).

Response: Tables EV1, EV3, EV4, and EV6 were uploaded as requested.

- I suggest submitting EV tables 2 and 5 as Datasets. The nomenclature for these is "Dataset EV1" and "Dataset EV2". You can keep the tables in the .xlsx format and please include the legend either in the first column of the respective tabs or in a separate tab labeled "legend". Please update the numbering of the remaining EV tables accordingly.

Response: We appreciate the editor's suggestion. Tables EV2 and EV5 were submitted as Datasets EV1 and EV2 with a separated legend. Tables EV1, EV3, EV4, and EV6 were renumbered in order.

- Figure 5C: When comparing the image shown in Figure 5C with the Source data you supplied, it appeared that you cut the scan of the Western blot for FLAG to remove the lower MW bands and smear. Please note that such a procedure is not good practice and not in agreement with our journal policies. Please either show the actual data including the background staining or replace the entire experiment with data from a replicate experiment. If you decide for the latter option, please update and supply the source data for this experiment.
Response: The Western blot for FLAG in Fig 5C was revised in agreement with Source Data.

- Fig EV1E appears very over-contrasted. If possible, please provide images with less contrast modification.
Response: We have replaced Fig EV1E with less contrast modified images.

- Please provide a scale bar for the zoom image in Fig EV3A.
Response: The scale bar was supplemented in the zoom image of Fig EV3A.

- Source data for Figure 7A: the blots for pERK1/2 and DDB1 in the source data file appear mislabeled. Please correct.
- The same applies to the source data blots for Figure 7D, HA and MYC.
Response: We apologize for the wrong labeling. We have corrected the error in the source data blots for Figs 7A and 7D.

- Finally, I attach to this email a related manuscript file with comments in the figure legends by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.
I have also taken the liberty to make some changes to the Abstract and title. Could you please review it?
Response: We appreciate the editors' advice. Statements of replicates and error bars were added to the associated figure legend. Title and Abstract have been revised accordingly. Thank you very much for your help.

2nd Revision - Editorial Decision 14th May 2020

Dr. Heng-Yu Fan
Zhejiang University
Life Sciences Institute
866 Yu Hang Tang Rd
Hangzhou 310058
China

Dear Dr. Fan,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Martina Rembold, PhD
Editor
EMBO reports

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Corresponding Author Name: Heng-Yu Fan
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49956V2

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.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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B- Statistics and general methods

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

Question	Answer
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.
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 all the antibodies information was listed in the Table EV3
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.

* for all hyperlinks, please see the table at the top right of the document

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 provided the "Data Availability" section according to the author guidelines.
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).	We have provided the "Data Availability" section according to the author guidelines.
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

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable.