EMBO reports

Manuscript EMBO-2016-42573

The E3 ubiquitin ligase RNF114 and TAB1 degradation are required for maternal-to-zygotic transition

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Review timeline:

Submission Date:	24 April 2016
Editorial Decision:	27 May 2016
Revision Received:	26 October 2016
Editorial Decision:	17 November 2016
Revision Received:	20 November 2016
Accepted:	25 November 2016

Editor: Esther Schnapp

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 27 May 2016

Thank you for the submission of your manuscript to EMBO reports. I apologize for the slight delay in getting back to you; we have only now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that a role for RNF114 in MZT through its effect on TAB1 is potentially interesting. However, they also raise several concerns that would need to be satisfactorily addressed for publication of the study by our journal. Both referees 1 and 2 point out that endogenous TAB1 ubiquitination and degradation by endogenous RNF114 needs to be demonstrated at the 2 cell stage, and referees 2 and 3 pinpoint two inconsistencies that would need to be clarified. All missing controls and quantifications and statistical analyses must further be provided.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. The most important concerns that must be addressed are mentioned above. 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 this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 25,000

characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

REFEREE REPORTS

Referee #1:

The study entitled "RNF114 is required for maternal-to-zygotic transition by the clearance of TAB1" describes a novel E3 ligase, RNF114, and its role in Maternal to Zygotic transition (MZT) by using standard biochemical tools.

The authors observed that RNF114 is highly expressed in oocyte and localised in the cytoplasm during early embryonic developmental stages. To investigate the functional role of RNF114 as an E3 ubiquitin ligase during this stage, the authors suppressed RNF114 expression using siRNA mediated gene knock-down and they could observe defects in MZT progression. Moreover, it was shown that defective degradation of TAB1, a known TAK1 interacting protein which contribute to activation of NF- B signalling, caused such a defect. They confirmed ubiquitination activity of RNF114 toward TAB1 and the effect of TAB1 degradation on downstream NF-kB signalling pathway. By performing these experiments, the authors could suggest a physiological role of RNF114 as a key regulator of MZT, through degradation of TAB1.

This study provides novel insights into the role of newly identified E3 ligase, RNF114, during MZT. Since it is still not fully understood how ubiquitin-mediated protein degradation via the proteasome associate with early embryo development, this work broadens our understanding of the ubiquitin-proteasome system in MZT. For instance, it is clear that targeted degradation of TAB1 by RNF114 is essential for MZT and that this event triggers NF- B signalling downstream; specific experiments have been performed to dissect the role of RNF114 and its substrate degradation.
However, in order that this article meets the standards of EMBO Reports, major revisions are needed and the authors should address the following questions.

Major concerns:

1. One major question is what triggers RNF114 mediated TAB1 degradation at initial stage. Is there any specific upstream signal that induces activation of RNF114?
2. In this study the authors showed functional role of RNF114 as an E3 ligase by only using in-vitro co-overexpression system. It would be appreciable to show endogenous TAB1 ubiquitination and its degradation by endogenous RNF114.
3. Related to point 2, does this endogenous level TAB1 ubiquitination occurs specifically at two cell stage?
4. The authors observed RNF114 protein expression level and localization during MZT. How is the protein level of TAB1 in the course of early embryonic development? Does TAB1 protein level show specific decrease around 2-4 cell stage?
5. What happens to those arrested cell in the end? Are they cleared by certain mechanism such as apoptosis, necrosis, etc.?
6. Which part of TAB1 is crucial for the binding to RNF114?
7. According to Fig EV3 and EV4, TAB1 shows two bands but in the pull-down with RNF114 TAB1 only shows one single band. Are any post-translational modifications on TAB1, such as phosphorylation, required for its recognition by RNF114?

Minor concerns:

1. Page 5, line 2, title "RNF14 is predominately ... embryonic development" should be changed to "RNF114 is predominately ... embryonic development"

2. In figure legend 3C, "Zygotes were were microinjected..." should be changed to "Zygotes were microinjected..."

Referee #2:

This short report concisely demonstrates an important role for RNF114 in mouse preimplantation development. It goes on to identify through a clear set of experiments a set of substrates of this E3 ligase and identifies the likely substrate and mediator of the preimplantation RNF114 phenotype. However the manuscript falls down in the second part and some points mentioned below still need addressing before publication.

Major:

Throughout the manuscript the authors describe a two-cell stage arrest after either RNF114 knockdown or Tab1 expression, although the figures are only 35% or 27% respectively. Although this proportion is likely significant and represents a genuine effect on development, the term 2-cell stage arrest should be changed throughout. The endogenous expression patterns of Tab1 + Cd74, Tnip1, Psat1, Ralgps1 in preimplantation development are not analysed. Are they expressed? Are they downregulated in the zygote? If Tab1 is a critical substrate of RNF114 it should be downregulated in the zygote endogenously at least at the protein level. Tab1 mRNA and protein expression analysis during early preimplantation development (Oocyte to 2-cell stage) should be performed.

- Figure 3D. No apparent change in TAB1 levels after Rnf114 knockdown. Quantification and statistical testing should be performed.
- Figure 3E. Control group 25% embryos arrest at 2-cell stage. This is unusually high and differs from their previous results (Figure 1D and 3C). Statistical testing should be performed to check significance.
- Figure 4A. This result has been previously reported (Nishikimi et al Biol Reprod 1999), which should therefore be cited.
- The authors mention that the NF-KB pathway is crucial for the early stages of development in the mouse embryo (page 9, 1st sentence). The reference provided here refers to the bovine embryo. The reference mentioned above can also be used instead.
- Figure 4B. P-IKBalpha actually appears to increase up to the 2-cell stage. At the 4-cell stage the amount of loading control is clearly lower. This result should either be quantified (relative to loading control across at least 3 independent experiments) or removed.
- Figure 4F. The results that the negative regulator (P-IKBalpha) of the NF-KB pathway is downregulated when RNF114 is downregulated or Tab1 overexpressed does not seem to support the authors conclusion that 'RNF114-mediated ubiquitination and degradation of TAB1 might be essential to the activation of NF- B pathway during MZT'. In this case that in the absence of TAB1 degradation the pathway would not be activated, and therefore one would see higher levels of inhibitor?

Minor:

- Page 3, start of 2nd paragraph, 'various oocyte stages' are mentioned - the reference actually describes that the ubiquitination pathway is zygote enriched (9).
- Figure 1C. In the text zygotes were used, in the legend it is specified oocytes. In addition the negative control should be described in the legend.
- Figure 1D. In the legend it is mentioned oocytes were injected, yet in the text it is zygotes. It is a development experiment so presumable it should be zygotes?
- Figure 1E. Scale bars should be included.
- Figure EV1. Scale bars should be included.
- Page 7, 2nd paragraph 'All 10 FLAG-tagged candidate substrates' are mentioned. However in the previous paragraph there were 13, thus the word 'all' cannot be used.
- Figure 3A+B N numbers + quantification
- Page 8, last paragraph, a reference for Tab1 regulation of MAPKKK should be included.
- Page 9, 2nd paragraph 'nuclear localization of p65 at the two-cell stage in normal zygotes' This sentence is confusing e.g. 'at the 2-cell stage in embryos derived from control zygotes.

Referee #3:

The authors present evidence that ubiquitination pathway, specifically ubiquitin ligase Rnf114 is important in maternal to zygotic transition (MZT) during early mouse development. Downregulation of Rnf114 by siRNA results in persistence of TAB1 which in turn presumably interferes with the activation of NF- B pathway. The results presented are convincing for the most part and represent significant addition to our understanding of the important role that degradation of maternal proteins plays in normal development. However, there are several inconsistencies and problems in the manuscript that have to be resolved before acceptance. As the authors failed to number the pages of the manuscript the following comments presume that the title page is page 1, Abstract page 2, Introduction page 3, Results and Discussion page 5, etc.

Specific comments:

1. Page 3, lines8-10, In referencing past work the authors should also include Solter et al., Cold Spring Harbor Symp. Quant. Biol. 69: 11-17 as the first description of the essential role that proteasomes play in early development.
2. There is rather disturbing inconsistency throughout the manuscript regarding the timing of siRNA injection. Page 5, line 13 and page 11, lines 22-23 state that siRNA was injected into zygotes while on page 20, line 7 it is claimed that the oocytes were injected. Similarly, on page 12, line 4 the authors state that siRNA was injected into GV oocytes 24 hours before IVF but on page 21, line 13 it is stated that the injection took place after IVF. Such discrepancies imply that the people doing experiments and people writing the manuscript failed to communicate and diminish the confidence in the described results.
3. Page 8, lines 14-16 and Figure 3E. What does it mean "partially rescued to some extent"? It is hard to understand why oocytes injected with two different siRNAs develop better than un-injected? controls. Results in Fig. 3E are presented as the percentage of embryos arrested at 2-cell stage so what is the meaning of the error bars?
4. Page 9, line 6 and Figure 4B. Western blot does not support the statement in the text. Amount of p-I B is increased and not decreased as stated in the text. The 4-cell lane cannot be interpreted since the loading control is completely off.
5. Page 9, line 15 and figure 4F. The authors should state which embryonic stage was used for this Western blot. The level of p-I B is indeed significantly decreased following Rnf114 knock-down or Tab1 overexpression which may be counterintuitive considering the authors' arguments that these treatments hinder NF- B pathway so why would inhibitor of the pathway be decreased. Maybe if they reinterpret Figure 4B and realize that the amount of p-I B actually increases during normal development, the observed decrease following siRNA treatment may start making sense. Accepting the reality of the results may encourage the authors to reconsider the connection between Rnf114, Tab 1 and NF- B pathway and their role in MZT and early development.

1st Revision - authors' response 26 October 2016

Thank you for considering the revised version of our manuscript “RNF114 is required for maternal-to-zygotic transition by the clearance of TAB1,” by Ye Y. et al. for publication as a Scientific Report in *EMBO reports*. We are very thankful to the referees and the editor for pointing out some important modifications needed in the report. We have thoughtfully taken into account these comments. Our detail responses to each of the comments could be found in the Point-by-point response. We believe that the comments have been highly constructive and very useful to restructure the manuscript. We also believe that the new data included in the revised manuscript really improved the quality of our research. Indeed, we now show the endogenous TAB1 ubiquitination and degradation by endogenous RNF114 at the 2 cell stage.

The major inconsistencies pointed out by referees 2 and 3 should be caused by our inaccurate description on the known regulation mechanisms of NF-B pathway, we are so sorry for our negligence, such mistake has been carefully corrected and the relevant references have been provided. Moreover, the missing quantifications and statistical analyses have been added in the current version, and according to the author checklist, we provided the source data underlying all

graphs (the exact statistical test results could also be found in the source data). We hope that all these changes fulfill the requirements to make the manuscript acceptable for publication in EMBO Reports.

Point-by-Point Response:

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

Referee #1:

The study entitled "RNF114 is required for maternal-to-zygotic transition by the clearance of TAB1" describes a novel E3 ligase, RNF114, and its role in Maternal to Zygotic transition (MZT) by using standard biochemical tools. The authors observed that RNF114 is highly expressed in oocyte and localised in the cytoplasm during early embryonic developmental stages. To investigate the functional role of RNF114 as an E3 ubiquitin ligase during this stage, the authors suppressed RNF114 expression using siRNA mediated gene knock-down and they could observe defects in MZT progression. Moreover, it was shown that defective degradation of TAB1, a known TAK1 interacting protein which contribute to activation of NF-kB signalling, caused such a defect. They confirmed ubiquitination activity of RNF114 toward TAB1 and the effect of TAB1 degradation on downstream NF-kB signalling pathway. By performing these experiments, the authors could suggest a physiological role of RNF114 as a key regulator of MZT, through degradation of TAB1. This study provides novel insights into the role of newly identified E3 ligase, RNF114, during MZT. Since it is still not fully understood how ubiquitin-mediated protein degradation via the proteasome associate with early embryo development, this work broadens our understanding of the ubiquitin-proteasome system in MZT. For instance, it is clear that targeted degradation of TAB1 by RNF114 is essential for MZT and that this event triggers NF-kB signalling downstream; specific experiments have been performed to dissect the role of RNF114 and its substrate degradation. However, in order that this article meets the standards of *EMBO reports*, major revisions are needed and the authors should address the following questions.

Major concerns:

1. One major question is what triggers RNF114 mediated TAB1 degradation at initial stage. Is there any specific upstream signal that induces activation of RNF114?

Response: Thanks for pointing out this key question! The maternal-to-zygotic transition is the first major developmental transition that occurs following fertilization. The degradation of RNAs and proteins from the oocyte is supposed to be a prerequisite for this transition. Evidence is accumulating that the ubiquitin-proteasome system (UPS) is one of the main pathways for clearance of the maternal proteins in early embryos, and disruption in the UPS compromises embryo developmental potential [1-3]. Proteasomal degradation by the UPS occurs very fast after sperm entry, and only approximately 50% of the maternal proteins remain in late two-cell embryos[4]. In light on these results, we speculate that the RNF114 mediated TAB1 degradation at initial stage maybe depend on the activation of UPS which occurs shortly after fertilization. However, the mechanisms underlying the UPS activation at the initiation of embryonic development are still unclear. As a newly identified protein, the understanding of the functional role of RNF114 is mainly limited to immune response, but the regulatory pathway is poorly known. Our current study focuses on the role of RNF114 in early embryo, and we hope to address the upstream signals that induce RNF114 activation during early embryonic development in the near future.

2. In this study the authors showed functional role of RNF114 as an E3 ligase by only using in-vitro co-overexpression system. It would be appreciable to show endogenous TAB1 ubiquitination and its degradation by endogenous RNF114.

Response: Thanks a lot for this suggestion. According to your suggestion, lysates of mouse 2-cell stage embryos were immunoprecipitated by anti-TAB1 antibody, followed by western blotting with

antibodies against TAB1 or Ub. The results showed that TAB1 was effectively immunopurified by anti-TAB1 antibody, and Ub chains were detected on those endogenous TAB1 (Figure EV5), thus confirming the ubiquitination of endogenous TAB1 at two cell stage. In addition, we found that the degradation of TAB1 is mediated by RNF114 through Time-lapse microscopy in mouse early embryos. Tab1-GFP mRNA was microinjected into the mouse zygotes at the condition without or with Rnf114 knockdown. The results showed that the GFP fluorescence signal decreased at the two cell stage. However, once Rnf114 was knocked down, TAB1-GFP levels remained approximately constant (Figure 4D), suggesting that endogenous RNF114 is indeed required for TAB1 degradation during mouse early embryo development.

3. Related to point 2, does this endogenous level TAB1 ubiquitination occurs specifically at two cell stage?

Response: We also performed immunoprecipitation in mouse zygotes, the results showed when the TAB1 proteins were adjusted to comparable level, there were more Ub chains on TAB1 in the two cell stage embryos than in the zygotes (Figure 4C), suggesting endogenous TAB1 ubiquitination mainly occurs at two cell stage.

4. The authors observed RNF114 protein expression level and localization during MZT. How is the protein level of TAB1 in the course of early embryonic development? Does TAB1 protein level show specific decrease around 2-4 cell stage?

Response: Thanks for this question. We have detected the protein level of TAB1 from MII oocytes to 4 cell stage embryos by western blotting. Indeed, we found that TAB1 decreased sharply during maternal-to-zygotic transition, and it only weakly expressed at 2- and 4- cell stages (Figure 4B).

5. What happens to those arrested cell in the end? Are they cleared by certain mechanism such as apoptosis, necrosis, etc.?

Response: We found that the arrested embryos still stayed in two-cell stage at E4.5 (shown below). It has been proven that there is a unique mode of embryo demise for the early mammalian embryos at 2- to 4-cell cleavage stage where they enter a permanent cell cycle arrest, like a senescence-like state [5], since at this special stage, they can survive pretty long time, we do not further pursue this issue.

(Data not included in the Peer Review Process File)

6. Which part of TAB1 is crucial for the binding to RNF114?

Response: Thanks for this question. To detect the potential interaction between TAB1 and RNF114, we expressed and purified the GST-RNF114 from E. coli. The purified proteins were used to pulldown either mammalian cell expressed GFP-TAB1 or E. coli expressed FLAG-TAB1, we found no detectable interaction between these two proteins at either of the conditions (shown below). A lot of E3 ligases assemble polyubiquitin chains by "Hit and run" mode, the interaction between the E3 ligase and the substrate is very week which could not be detected by normal biochemical methods, this mechanism might be applied to RNF114 and TAB1, so we could not further address the question "which part of TAB1 is crucial for the binding to RNF114." No detectable interaction between RNF114 and TAB1. GST-RNF114 which was expressed and purified from E.coli was used to pull-down mammalian expressed GFP-TAB1 (A) or E.coli expressed and purified FLAG-TAB1 (B). GST protein was used as a control.

(Data not included in the Peer Review Process File)

7. According to Fig EV3 and EV4, TAB1 shows two bands but in the pull-down with RNF114 TAB1 only shows one single band. Are any post-translational modifications on TAB1, such as phosphorylation, required for its recognition by RNF114?

Response: There is no result of the RNF114 pull-down experiment in this research, while the western blot analysis of TAB1 in mouse early embryos revealed bands corresponding to its predicted position. In our opinion, the extra-bands occurred in the exogenous expression system

(Figure EV3 and EV4) might come from cleavage by some proteases. Usually, if there were post-translational modification, the protein might migrate a bit slowly than the unmodified proteins.

Minor concerns:

1. Page 5, line 2, title "RNF14 is predominately ... embryonic development" should be changed to "RNF114 is predominately ... embryonic development"

Response: Thanks for this reminding, it has been corrected.

2. In figure legend 3C, "Zygotes were were microinjected..." should be changed to "Zygotes were microinjected..."

Response: Thanks, it has been corrected.

Referee #2:

This short report concisely demonstrates an important role for RNF114 in mouse preimplantation development. It goes on to identify through a clear set of experiments a set of substrates of this E3 ligase and identifies the likely substrate and mediator of the preimplantation RNF114 phenotype. However the manuscript falls down in the second part and some points mentioned below still need addressing before publication.

Major:

1. Throughout the manuscript the authors describe a two-cell stage arrest after either RNF114 knockdown or Tab1 expression, although the figures are only 35% or 27% respectively. Although this proportion is likely significant and represents a genuine effect on development, the term 2-cell stage arrest should be changed throughout.

Response: Thanks for your suggestion. We have carefully checked and revised the inappropriate statements, and changed them to "early embryonic development defect" and similar wordings.

2. The endogenous expression patterns of Tab1 + Cd74, Tnip1, Psat1, Ralgps1 in preimplantation development are not analysed. Are they expressed? Are they downregulated in the zygote? If Tab1 is a critical substrate of RNF114 it should be downregulated in the zygote endogenously at least at the protein level. Tab1 mRNA and protein expression analysis during early preimplantation development (Oocyte to 2-cell stage) should be performed.

Response: Thanks for pointing out this question. We have detected the mRNA expressional level of Tab1, Cd74, Tnip1, Psat1 and Ralgps1 through oocyte to 2-cell stage embryo, the results of RT-PCR (reverse transcription PCR) showed that Tnip1 and Psat1 was nearly undetectable during this period, the expressional level of cd74 was very weak, while Tab1 and Ralgps1 mRNAs were expressed sustainedly (the results were shown below). Additionally, combined with the comments of referee 1, we analyzed the protein level of TAB1 from oocytes to 4-cell stage embryos by western blotting, the results of has been given in Figure 4B, it could be found that the protein level of TAB1 reduced sharply during early preimplantation development, only weak expression could be detected at 2-cell and 4-cell stages. This result further supported that TAB1 is a critical substrate of RNF114 in mouse early embryos. mRNA expressions of the genes corresponding to RNF114 substrates were detected by RT-PCR. Ovary or kidney tissues were used as the positive control, and 18s as the loading control.

(Data not included in the Peer Review Process File)

3. Figure 3D. No apparent change in TAB1 levels after Rnf114 knockdown. Quantification and statistical testing should be performed.

Response: Thanks for this suggestion. We have repeated this experiment for another three times, and the results of total four experiments were quantified. The result was now presented in the revised Figure 4B, the quantitative and statistical result was also shown.

4. Figure 3E. Control group 25% embryos arrest at 2-cell stage. This is unusually high and differs from their previous results (Figure 1D and 3C). Statistical testing should be performed to check significance.

Response: Figure 3E (now presented as Figure 4E in the revised manuscript) showed that the rescue effect of Tab1 knockdown on early embryo development defect which was caused by RNF114 knockdown. Due to the expressional level of TAB1 was very low at two-cell stage (Figure 4B), it is difficult to guarantee the knockdown effect on endogenous TAB1, if the Tab1 siRNA was microinjected into the zygotes same as shown in the previous experiments (Figure 1D and Figure 3C (now presented as Figure 4A). In order to ensure the knockdown efficiency, we microinjected the mixture of Rnf114 and Tab1 siRNAs into the GV oocytes and the injected GV oocytes were arrested with milrinone for 24hr, then the oocytes were transferred into milrinone-free medium for maturation. The mature oocytes were then fertilized by IVF and subsequent development was observed. The details have been described in the Materials and Methods section and the figure legend. Because relatively complex manipulation and long time in vitro culture, the developmental rate of embryos derived from in vitro-matured oocytes was expectedly lower than the in vivo developed embryos, which were used in other experiments. In addition, we have performed statistical analysis for these experiments to show the differences. Thanks for your suggestion.

Figure 4A. This result has been previously reported (Nishikimi et al Biol Reprod 1999), which should therefore be cited.

Response: We have cited their work in the revised manuscript.

The authors mention that the NF-KB pathway is crucial for the early stages of development in the mouse embryo (page 9, 1st sentence). The reference provided here refers to the bovine embryo. The reference mentioned above can also be used instead.

Response: Thanks for this suggestion! And we have cited this paper in the revised manuscript.

Figure 4B. P-IKBalpha actually appears to increase up to the 2-cell stage. At the 4-cell stage the amount of loading control is clearly lower. This result should either be quantified (relative to loading control across at least 3 independent experiments) or removed.

Response: Thanks for this advice. According to your suggestion, we repeated this experiment two more times, the results of three independent experiments were quantified, and in order to reflect the activation of NF-KB pathway more clearly, we calculate the ratio of phosphor- IκBα to total IκBα. The representative figure and quantitative result were shown in the revised Figure 5B. The results showed that from MII oocyte to 4-cell stage, the ratio of p-IκBα to IκBα increased gradually, suggesting the activation of NF-κB pathway during maternal-to-zygotic transition.

Figure 4F. The results that the negative regulator (P-IKBalpha) of the NF-KB pathway is downregulated when RNF114 is downregulated or Tab1 overexpressed does not seem to support the authors conclusion that 'RNF114-mediated ubiquitination and degradation of TAB1 might be essential to the activation of NF-κB pathway during MZT'. In this case that in the absence of TAB1 degradation the pathway would not be activated, and therefore one would see higher levels of inhibitor?

Response: This confusion may be caused by our description in Page 9, lines 6-7 in the previous version, where we described the result as "Western blot confirmed decreased expression of negative regulators of NF-κB pathway, such as IκBα and the IκBα phosphorylated form (p-IκBα), with embryonic development." In fact, this description is not accurate. The NF-κB/Rel transcription factors are present in the cytosol in an inactive state coexisted with the inhibitory IκB proteins, while the phosphorylation and degradation of IκBα can free NF-κB from inhibition, allowing nuclear translocation of active p65 (RelA) [6-8]. The activation and nuclear translocation of NF-κB p65/p50 are key steps for the DNA binding of NF-κB and transcription of downstream target genes [9]. Thus phosphorylated form of IκBα is not also a negative regulator of NF-κB pathway, on the contrary, activation of NF-κB requires phosphorylation of the inhibitory subunit IκBα. The downregulation of p-IκBα when RNF114 was knocked down or Tab1 was overexpressed (Figure 5F) indicated that the activation of NF-kB pathway was impeded. We are so sorry for this improper description, and now it has been modified in the text.

Minor:

Page 3, start of 2nd paragraph, 'various oocyte stages' are mentioned - the reference actually describes that the ubiquitination pathway is zygote enriched (9).

Response: Yes, as the referee pointed out, Wang et al. compared the protein expressional profile in germinal vesicle (GV), metaphase II (MII) oocytes and zygotes using semi-quantitative mass spectrometry, and found that zygotes were highly enriched in proteins of the ubiquitination pathway, suggesting the ubiquitination pathway may play important roles in degrading the maternal proteins and might be essential for the developmental transition from oocyte to embryo. Now we have modified the description.

Figure 1C. In the text zygotes were used, in the legend it is specified oocytes. In addition the negative control should be described in the legend.

Response: For Figure 1C, it should be the 2-cell stage embryos derived from Rnf114 siRNA or control siRNA injected zygotes, we are sorry for this mistake in writing. Now it has been corrected, and the negative control has been described in the legend.

Figure 1D. In the legend it is mentioned oocytes were injected, yet in the text it is zygotes. It is a development experiment so presumable it should be zygotes?

Response: Yes, It should be "zygotes", we are so sorry for this mistake. It has been corrected now.

Figure 1E. Scale bars should be included.

Response: Thanks. The scale bar has been added.

Figure EV1. Scale bars should be included.

Response: The scale bar has been added.

Page 7, 2nd paragraph 'All 10 FLAG-tagged candidate substrates' are mentioned. However in the previous paragraph there were 13, thus the word 'all' cannot be used.

Response: Thanks for your reminding, we have corrected it.

Figure 3A+B N numbers + quantification ???

Response: The quantification of the results has been added on the Figure.

Page 8, last paragraph, a reference for Tab1 regulation of MAPKKK should be included.

Response: Thanks. The relative references have been added.

Page 9, 2nd paragraph 'nuclear localization of p65 at the two-cell stage in normal zygotes' This sentence is confusing e.g. 'at the 2-cell stage in embryos derived from control zygotes.

Response: It has been modified.

Referee #3:

The authors present evidence that ubiquitination pathway, specifically ubiquitin ligase Rnf114 is important in maternal to zygotic transition (MZT) during early mouse development. Downregulation of Rnf114 by siRNA results in persistence of TAB1 which in turn presumably interferes with the activation of NF-κB pathway. The results presented are convincing for the most part and represent significant addition to our understanding of the important role that degradation of maternal proteins plays in normal development. However, there are several inconsistencies and problems in the manuscript that have to be resolved before acceptance. As the authors failed to number the pages of the manuscript the following comments presume that the title page is page 1, Abstract page 2, Introduction page 3, Results and Discussion page 5 etc.

Specific comments:

1. Page 3, lines8-10, In referencing past work the authors should also include Solter et al., Cold Spring Harbor Symp. Quant. Biol. 69: 11-17 as the first description of the essential role that proteasomes play in early development.

Response: Thanks for this advice. This reference has been added.

2. There is rather disturbing inconsistency throughout the manuscript regarding the timing of siRNA injection. Page 5, line 13 and page 11, lines 22-23 state that siRNA was injected into zygotes while on page 20, line 7 it is claimed that the oocytes were injected. Similarly, on page 12, line 4 the authors state that siRNA was injected into GV oocytes 24 hours before IVF but on page 21, line 13 it is stated that the injection took place after IVF. Such discrepancies imply that the people doing experiments and people writing the manuscript failed to communicate and diminish the confidence in the described results.

Response: We are very sorry for these mistakes. On page 20, line 7 (now page 21, line 10, legend of Figure 1D), the zygotes were injected with Rnf114 siRNAs and collected at 24h after the treatment to assess the knockdown efficiency. On page 21, line 13 (now page 22, line 21-24, legend of Figure 4E), GV oocytes were injected with mixture of Rnf114 and Tab1 siRNAs, then the oocytes matured, and were fertilized by IVF, and the fertilized eggs were transferred into KSOM media, cultured for 48–50 h. Now these discrepancies have been corrected and the full text has been carefully revised.

3. Page 8, lines 14-16 and Figure 3E. What does it mean "partially rescued to some extent"? It is hard to understand why oocytes injected with two different siRNAs develop better than un-injected? controls. Results in Fig. 3E are presented as the percentage of embryos arrested at 2-cell stage so what is the meaning of the error bars?

Response: The logic for that experiment is that: The major function of RNF114 is polyubiquitinating TAB1 and promoting its degradation. Once Rnf114 was knocked down, the TAB1 level will increase, so if we further knock down Tab1 at the condition that Rnf114 has been knocked down, the effect of Rnf114 knock down on MZT will be attenuated to a certain degree because of TAB1 level will be downregulated, and the embryos then could bypass this stage. The experiments have been repeated three times, the error bars mean the variations in the three experiments, in consideration of the comments of referee 2, statistical significance has been added to this figure.

4. Page 9, line 6 and Figure 4B. Western blot does not support the statement in the text. Amount of p-IκBα is increased and not decreased as stated in the text. The 4-cell lane cannot be interpreted since the loading control is completely off.

Response: Here we made a mistake, the description in the text was improper. As shown in Figure 4B (now Figure 5B in the revised version), the protein level of IκBα (the inhibitory subunit of NF-κB) decreased with early embryonic development, accompanied with upregulated phosphorylated form (p-IκBα). The NF-κB/Rel transcription factors are present in the cytosol in an inactive state coexisted with the inhibitory IκB proteins, while the phosphorylation and degradation of IκBα can free NF-κB from inhibition, allowing nuclear translocation of active p65 (RelA) [6-8]. The activation and nuclear translocation of NF-κB p65/p50 are key steps for the DNA binding of NF-κB and transcription of downstream target genes [9]. So there is a positive correlation between the amount of p-IκBα and the activation of NF-κB. Referee 2 also pointed out the similar problem, we are so sorry for our inappropriate description in the text and for the confusion cause by this. Indeed, as you pointed out, the loading control in 4-cell lane is lower than in other lanes. Combing with the advice of referee 2, we repeated this experiment, and quantified the protein levels of total IκBα and p-IκBα, the ratio of p-IκBα/total IκBα was added (Figure 5B) to indicate the activation of NF- κB pathway clearly.

5. Page 9, line 15 and figure 4F. The authors should state which embryonic stage was used for this Western blot. The level of p-IκBα is indeed significantly decreased following Rnf114 knock-down or Tab1 overexpression which may be counterintuitive considering the authors' arguments that these treatments hinder NF-κB pathway so why would inhibitor of the pathway be decreased . Maybe if they reinterpret Figure 4B and realize that the amount of p-IκBα actually increases during normal development, the observed decrease following siRNA treatment may start making sense. Accepting the reality of the results may encourage the authors to reconsider the connection between Rnf114, Tab 1 and NF-κB pathway and their role in MZT and early development.

Response: Thanks for this suggestion. In Figure 4F (now Figure 5F), the embryos at 2-cell stage were collected for western blotting, and embryos for each group were collected at the same time after microinjection into the zygotes. The level of p-IκBα is indeed decreased obviously after Rnf114 was knocked down or Tab1 was overexpressed. As we explained above, IκBα is an inhibitory subunit of NF-κB complex, while phosphorylation of IκBα is required for NF-κB activation [6-8]. Thus, the amount of p-IκBα is the positive indicator of activation of NF-κB pathway, instead of the "negative regulator", which we mistakenly wrote in the text (Page 9, line 6-7 in the previous version). Considering both Figure 4B and 4F (now Figure 5B and 5F in the revised version), our results showed NF-κB pathway was quickly activated during early embryonic development, while its activation was impeded following Rnf114 knock-down or Tab1 overexpression. The results showed in Figure 5A, 5D and 5E also supported the same conclusion, since nuclear translocation of p65 also indicating the activation of NF-κB. Now we have revised this sentence and we are so sorry for the mistake.

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2nd Editorial Decision 17 November 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the referee reports, as well as referee cross-comments, which are pasted below.

As you will see, referees 1 and 2 are satisfied with the revised study, and we can therefore in principle accept it. Both referees do not agree with most of the remaining concerns of referee 1, and except for the data in Figure 4C, 4D and EV5, these concerns do therefore not need to be addressed for publication of the study here.

Please explain the data in Figure 4C and EV5. I understand that TAB1 runs at 50 KD (shown in EV5), but this size is not included in the anti-UB blot in Figure 4C. It can therefore not be concluded that TAB1 is more ubiquitinated at the 2 cell stage, as the legend for 4C states. In EV5, only a minor ubiquitination of TAB1 is seen, and it is not compared to the 1 cell stage. If you want to show that TAB1 is ubiquitinated more at the 2 cell stage, these data and figures need to be revised. Please let me know if I misunderstand any of this. There are two misspellings in 4D ("nagative" should be replaced by negative) and 4C ("TAB!" should be replaced by TAB1). Please also make sure that all figures and EV figures are mentioned in the manuscript text.

REFEREE REPORTS

Referee #1:

EMBO rep. reviewer's comments for revised manuscript: The authors of the manuscript have shown the molecular mechanism and the effect of RNF114 mediated TAB1 degradation during Maternal to Zygotic transition (MZT). In the revised manuscript, the authors tried to address the questions raised by the reviewers and succeeded for some of them. However, the unanswered revision questions raise considerable concern weather the quality and reliability of the revised manuscript truly fit to the standard of the EMBO report journal. Below are point-by-point comments to the authors' response.

Major concerns:

1. One major question is what triggers RNF114 mediated TAB1 degradation at initial stage. Is there any specific upstream signal that induces activation of RNF114?

Response: Thanks for pointing out this key question! The maternal-to-zygotic transition is the first major developmental transition that occurs following fertilization. The degradation of RNAs and proteins from the oocyte is supposed to be a prerequisite for this transition. Evidence is accumulating that the ubiquitin-proteasome system (UPS) is one of the main pathways for clearance of the maternal proteins in early embryos, and disruption in the UPS compromises embryo developmental potential [1-3]. Proteasomal degradation by the UPS occurs very fast after sperm entry, and only approximately 50% of the maternal proteins remain in late two-cell embryos [4]. In light on these results, we speculate that the RNF114 mediated TAB1 degradation at initial stage maybe depend on the activation of UPS which occurs shortly after fertilization. However, the mechanisms underlying the UPS activation at the initiation of embryonic development are still unclear. As a newly identified protein, the understanding of the functional role of RNF114 is mainly limited to immune response, but the regulatory pathway is poorly known. Our current study focuses on the role of RNF114 in early embryo, and we hope to address the upstream signals that induce RNF114 activation during early embryonic development in the near future.

As the authors wrote above, it is speculation based on previous works from the literature. It is a reasonable speculation that can be accepted due to the limitation of the duration of the revision

process.

2. In this study the authors showed functional role of RNF114 as an E3 ligase by only using in-vitro co-overexpression system. It would be appreciable to show endogenous TAB1 ubiquitination and its degradation by endogenous RNF114.

Response: Thanks a lot for this suggestion. According to your suggestion, lysates of mouse 2-cell stage embryos were immunoprecipitated by anti-TAB1 antibody, followed by western blotting with antibodies against TAB1 or Ub. The results showed that TAB1 was effectively immunopurified by anti-TAB1 antibody, and Ub chains were detected on those endogenous TAB1 (Figure EV5), thus confirming the ubiquitination of endogenous TAB1 at two cell stage. In addition, we found that the degradation of TAB1 is mediated by RNF114 through Time-lapse microscopy in mouse early embryos. Tab1-GFP mRNA was microinjected into the mouse zygotes at the condition without or with Rnf114 knockdown. The results showed that the GFP fluorescence signal decreased at the two cell stage. However, once Rnf114 was knocked down, TAB1-GFP levels remained approximately constant (Figure 4D), suggesting that endogenous RNF114 is indeed required for TAB1 degradation during mouse early embryo development.

Quality of the ubiquitylation assay in Figure EV5 is poor and therefore not convincing. The authors claimed that the GFP signal from TAB1-GFP is consistent when RNF114 is depleted whereas the GFP signal decreases in the control sample (Figure 4D). However, the GFP signal does not look significantly different between the experimental sets.

3. Related to point 2, does the endogenous level of TAB1 ubiquitination occur specifically at the two cell stage?

Response: We also performed immunoprecipitation in mouse zygotes, the results showed when the TAB1 proteins were adjusted to comparable level, there were more Ub chains on TAB1 in the two cell stage embryos than in the zygotes (Figure 4C), suggesting endogenous TAB1 ubiquitination mainly occurs at two cell stage.

The data represented in the Figure 4C is sufficient to support the authors' comment.

4. The authors observed RNF114 protein expression level and localization during MZT. How is the protein level of TAB1 in the course of early embryonic development? Does TAB1 protein level show specific decrease around 2-4 cell stage?

Response: Thanks for this question. We have detected the protein level of TAB1 from MII oocytes to 4 cell stage embryos by western blotting. Indeed, we found that TAB1 decreased sharply during maternal-to-zygotic transition, and it only weakly expressed at 2- and 4- cell stages (Figure 4B).

The authors have addressed this point by with their explanation and Figure 4B.

5. What happens to those arrested cell in the end? Are they cleared by certain mechanism such as apoptosis, necrosis, etc.?

Response: We found that the arrested embryos still stayed in two-cell stage at E4.5 (shown below). It has been proven that there is a unique mode of embryo demise for the early mammalian embryos at 2- to 4-cell cleavage stage where they enter a permanent cell cycle arrest, like a senescence-like state [5], since at this special stage, they can survive pretty long time, we do not further pursue this issue.
Knockdown of Rnf114 causes defects in preimplantation embryonic development. (bar=50um)
The Pictures added by the authors are clear enough to address this point.

6. Which part of TAB1 is crucial for the binding to RNF114?

Response: Thanks for this question. To detect the potential interaction between TAB1 and RNF114, we expressed and purified the GST-RNF114 from E. coli. The purified proteins were used to pulldown either mammalian cell expressed GFP-TAB1 or E. coli expressed FLAG-TAB1, we found no detectable interaction between these two proteins at either of the conditions (shown below). A lot

of E3 ligases assemble polyubiquitin chains by "Hit and run" mode, the interaction between the E3 ligase and the substrate is very week which could not be detected by normal biochemical methods, this mechanism might be applied to RNF114 and TAB1, so we could not further address the question "which part of TAB1 is crucial for the binding to RNF114." No detectable interaction between RNF114 and TAB1. GST-RNF114 which was expressed and purified from E.coli was used to pull-down mammalian expressed GFP-TAB1 (A) or E.coli expressed and purified FLAG-TAB1 (B). GST protein was used as a control.

The authors could not detect the interaction between RNF114 and TAB1 and explained that this might be due to the weak interaction between the E3 ligase and its substrate. However, IP is a commonly performed biochemical approach used for the validation of substrates of certain E3 ligase. The authors even co-overexpressed, not at endogenous level, both the E3 ligase and the substrate, so it is very difficult to imagine that such a weak interaction has any meaning in physiology.

7. According to Fig EV3 and EV4, TAB1 show two bands but in the pull-down with RNF114 TAB1 only shows one single band. Are any post-translational modifications on TAB1, such as phosphorylation, required for its recognition by RNF114?

Response: There is no result of the RNF114 pull-down experiment in this research, while the western blot analysis of TAB1 in mouse early embryos revealed bands corresponding to its predicted position. In our opinion, the extra-bands occurred in the exogenous expression system (Figure EV3 and EV4) might come from cleavage by some proteases. Usually, if there were post-translational modification, the protein might migrate a bit slowly than the unmodified proteins.

The explanation provided by the authors is sufficient.

Minor concerns:

1. Page 5, line 2, title "RNF14 is predominately ... embryonic development" should be changed to "RNF114 is predominately ... embryonic development"

Response: Thanks for this reminding, it has been corrected. (Confirmed correction.)

2. In figure legend 3C, "Zygotes were were microinjected..." should be changed to "Zygotes were microinjected..."

Response: Thanks, it has been corrected. (Confirmed correction.)

Referee #2:

I am satisfied that the comments have been fully addressed and firmly believe the revised version of the manuscript is significantly stronger and deserves publication in EMBO Reports.

Referee #3:

The authors addressed reviewers' comments and performed additional experiments. They also corrected several mistakes present in the original version. As mentioned previously the subject is important and the manuscript as presented contributes significantly to our understanding of biochemical processes regulating early mouse development. The manuscript is substantially improved and should be accepted.

Cross-comments from referee 2:

I have looked again at the manuscript and I believe the concern of referee 1 regarding Figure 4D is valid. The GFP signal in the images does not appear to reflect the quantification shown below. The authors should correct this. Have all the images in Figure 4D been manipulated in the same way? I do not think the other concerns are justified. While I am not an expert in ubiquitination assays, Figure EV5 appears to be just a duplication of Figure 4C, and as far as I can tell is not mentioned in the text. Therefore I believe this Figure can be removed from the manuscript without affected the

conclusions. Regarding the interaction, the authors report that they cannot detect the interaction between the two proteins. However the authors are careful in their interpretation of the data, and only suggest that TAB1 might be a bona fida substrate of RNF114, as they do not have enough data to support this. Their main conclusion that RNF114 is involved in the elimination of TAB1 in the embryo is supported by their data.

Cross-comments from referee 3:

Regarding point 1 (upstream regulation/activation of ubiquitynation pathway) I think reviewer 1 is asking too much. It is always possible to ask for more but this is way beyond the scope of this paper and the reviewer in my opinion agrees. The quality of the ubiquitylation assay (Figure EV5) is indeed not perfect but considering the material it was done on (2-cell stage mouse embryo) I think it is sufficient. Considering the number of embryos used to detect the decrease of GFP signal (Figure 4), the observed decrease in injected embryos is small but consistent in comparison with the controls. Overall I think the authors responded well to the critique and provided additional data to make their case quite convincing. I would accept it as it is.

2nd Revision - authors' response 20 November 2016

We are very pleased to receive the decision letter of our revised manuscript (EMBOR-2016-42573V2)! We have made further revisions according to your and the referees' suggestions. Our detail responses could be found in the point-by-point response. We agree with your suggestion about the title and abstract, and we are very appreciative of it. The typos in Fig 4C and 4D have been corrected, and the EV tables have been replaced by black and white tables. We also carefully checked the full text to ensure that all figures and EV figures have been mentioned. We hope that the modified manuscript addresses your concerns satisfactorily and is suitable for publication in EMBO Reports now.

Point-by-Point Response:

We thank you and all the referees for the time that you have taken to assess the acceptability of this manuscript and for your helpful comments and suggestions. Followings are our point-by-point responses to the outstanding concerns.

Concerns about Figure 4C and EV5:

Please explain the data in Figure 4C and EV5. I understand that TAB1 runs at 50 KD (shown in EV5), but this size is not included in the anti-UB blot in Figure 4C. It can therefore not be concluded that TAB1 is more ubiquitinated at the 2 cell stage, as the legend for 4C states. In EV5, only a minor ubiquitination of TAB1 is seen, and it is not compared to the 1 cell stage. If you want to show that TAB1 is ubiquitinated more at the 2 cell stage, these data and figures need to be revised.

Response: The data in Figure EV5 firstly proved that TAB1 was ubiquitinated at the 2 cell stage and confirmed the specificity of the immunoprecipitation experiment, there were no bands detected in the IgG control using either anti-TAB1 or anti-Ub antibody. Due to the material limitation of 2-cell stage mouse embryos, the Ub signal was weak, but it still could be found there is clear difference between the IgG control and the anti-TAB1 group. Based on this result, we increased the number of embryos used in Figure 4C, and got more obvious ubiquitination signal. Furthermore, in Figure 4C we also performed immunoprecipitation in mouse zygotes. Thus these two results are not redundant. We are so sorry for the missed mention of Figure EV5 in the text, and now we have added the descriptions of Figure 4C and EV5.

As shown below, there are bands at the 50KD position, and these bands are not ubiquitinated TAB1 because once ubiquitinated it will shift up around 9KD. These kinds of results could be found in a lot of other labs, presumably due to too much protein there, and could be recognized by the antibody nonspecifically. To avoid distracting the readers, we cut them off from the original results.

As shown in Figure 4C, the ubiquitin signal above of TAB1 at two cell stage was clear stronger than that at the zygote stage, when the TAB1 proteins were at comparable level. So our results support that there are more Ub modified TAB1 in the two cell stage embryos than that in the zygote stage.

Concerns about Figure 4D:

Referee #1: The authors claimed that the GFP signal from TAB1-GFP is consistent when RNF114 is depleted whereas the GFP signal decreases in the control sample (Figure 4D). However, the GFP signal does not look significantly different between the experimental sets.

(Data not included in the Peer Review Process File)

Cross-comments from Referee #2: I have looked again at the manuscript and I believe the concern of referee 1 regarding Figure 4D is valid. The GFP signal in the images does not appear to reflect the quantification shown below. The authors should correct this. Have all the images in Figure 4D been manipulated in the same way?Cross-comments from Referee #3: Considering the number of embryos used to detect the decrease of GFP signal (Figure 4), the observed decrease in injected embryos is small but consistent in comparison with the controls.

Response: In Figure 4D, all the time lapse images were taken under the same parameters and manipulated in the same way. The representive images were overlays of DIC image and the GFP fluorescence signal, the grey background maybe make the fluorescent signal seem less clear. Now we replaced the original results with the pure fluorescent images, and sound they are much better than the previous one.

Concerns about Figure 5F:

In Figure 5F, it looks like the bottom right two bands might be spliced together. Can you please explain and provide source data for these bands?

Response: When carrying out this experiment, we also injected the exogenous Optn mRNA into the zygotes because OPTN has also been reported to be involved in regulation of NF-κB activity. Thus the source data of this experiment includes three lanes, from left to right, injected with ddH_2O, Optn mRNA and Tab1 mRNA respectively. However, due to OPTN was excluded from the substrates of RNF114 by the in vitro ubiquitination assays (Fig. 3B), the Optn mRNA injected groups lane was cut off, we are deeply sorry for this non-rigorous treatment! Here are the source data for this result, and following the example in the published paper (Nat Commun. 2015 Apr 13;6:6625.), we have now added a dotted line between the ddH_2O and Tab1 mRNA lanes to indicate there is a cropped lane and supplemented the full western data as Figure EV6. We have also double-checked all the other source data, and ensure there were no more situations of this kind anywhere.

(Data not included in the Peer Review Process File)

Source data of Figure 5G. Western blotting of total-IKBαand TUBULIN were performed with the same run, while western blotting of p-IKBαwas performed with a parallel run with the equal amount of same samples because the size of p-IKBαcould not be distiguished from total-IKBα in SDS-PAGE.

3rd Editorial Decision 25 November 2016

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.

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Corresponding Author Name: Ran Huo.and.Wei.Li
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42573V3

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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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)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen accordingly to studies using similar methods and is comparable to what is generally employed in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable
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.	Before microinjection, the zygotes or oocytes were collected and mixed together, then randomly allocated to different treatment group.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
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.	As mentioned above, the zygotes or oocytes were collected and mixed together, then randomly allocated to different treatment group. In addition, the embryos at different developmental states or with different p65 distribution were counted under microscope blindly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable
5. For every figure, are statistical tests justified as appropriate?	Yes, we use t tests for the ratio data and chi-square tests for categorical data.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We specified the tests used to calculate p-values in the respective figuer legends.
Is there an estimate of variation within each group of data?	The standard deviation (s.d.) or standard error of mean (s.e.m.) were used to estimate the variatuon within groups where no less than three prelicates were performed.
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).	All these information is included in the materials and methods section. And the details of antibodies used for western blotting and immunofluorescence staining are provided in Table EV2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The HEK293T cells used in this study were originally obtained from the ATCC. We have performed a karyotyping and are confident that these are HEK293 cells. They were alsoo tested for mycoplasma and were mycoplasma-negative.

* 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.	Not applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable
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.	Not applicable

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
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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
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	Not applicable
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
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21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Not applicable
22. 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

23. 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