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The mitochondrial ribosomal protein mRpL4 regulates Notch signaling

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Dear Dr. Zhang,

Thank you for the submission of your research manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, the referees have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your 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 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. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: 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. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) 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: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. See also:
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12) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. See also guide to authors:
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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.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The manuscript by Mo and colleagues describes a "non-conventional" function of the mitochondrial ribosomal protein, mRpL4. They show that disruption of mRpL4 in Notch-receiving cells impairs expression of Notch target genes. They further demonstrate that mRpL4 physically interacts with Wap, a WD40 repeat protein, to regulate Notch target gene expression. Importantly, they find that mRpL4 regulates Notch target genes in zebrafish as well, indicating that the phenotype is conserved in vertebrate systems. In addition, the phenotypes observed when mRpL4 is disrupted cannot be explained by altered ROS formation as disruption of several other mitochondrial ribosomal proteins altered ROS formation without affecting the expression of Notch target genes. Altogether, this is an exciting finding that adds to the growing list of observations demonstrating that some mitochondrial ribosomal proteins may have extra-mitochondrial functions. However, I have suggested two experiments that I think are necessary to help reinforce the conclusions drawn.

1. The authors should quantify the wing margin (notch) defects produced as a result of RNAi-mediated disruption of a Notch signaling component that produces robust wing margin defects, as well as the defects produced as a result of RNAi-mediated disruption of mRpL4 and RNAi-mediated disruption of another mitochondrial ribosomal protein (such as mRpS28 or mRpL24) to provide a better sense of the degree of penetrance observed when mRpL4 is knocked down.
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Minor concerns:

1. Why was E(spl) mβ-LacZ used in some panels while NRE-GFP was used in others to assess Notch signaling?
2. In addition to Su(H), Hrb27 is another nuclear protein that regulates Notch signaling. Can the authors discuss whether they think the phenotype involves Su(H) or Hrb27 or both?
3. Please provide a brief description of the somatic screen described in the first paragraph of the results.

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The manuscript identifies a mitochondrial ribosomal protein mRPL4 as a novel component required for Notch receptor signaling and demonstrates that it's function is by distinct activity from its normal role in protein translation. This conclusion appears robust as it utilises both mutants, RNAi and rescue of the mutan phenotype by cDNA expression. Using methodology which is standard and well proven in the literature the authors show that the protein is required only in the signal receiving cells not the signal sending cells, and that the function is one which is down stream of release of the Notch ICD. This is likely to be at the level of Su(H) recruitment to the Notch target genes as shown by the CHIP assay in Figure 2. It would be nice to have this interesting conclusion backed up by an additional method, for example it is possible to image Su(H) recruitment to the E(spl) locus on the polytene chromosome as I think this would enhance the robustness of this conclusion.

The authors extend these interesting findings further by identifying an interacting partner, wap, which they show binds by two hybrid assay and by ip. Wap expression appears to rescue the effects of mRPL4 mutation providing additional support for a functional link. Finally the authors increase the interest in the work by demonstrating some cross species conservation of function between human, zebrafish and fly proteins.

Overall the manuscript is well written and clear, the methodology is straight forward and the experimental design is sound. The findings are interesting and somewhat unexpected in nature and should be of interest to a wide audience.

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In this work, Mo et al report a novel role for mitochondrial ribosomal protein L4 (mRpL4) beyond synthesizing mtDNA encoded proteins. They show that mRpL4 is localized both in the cytoplasm and the nucleus and it is implicated in Notch signaling activation in Drosophila wing discs. They further show that this role is conserved in zebrafish and mammals.
The authors have performed elegant genetic experiments to show that mRPL4 acts in signal receiving cells to regulate Notch signaling activity in a manner independent of OXPHOS protein synthesis. They reveal a physical interaction of mRPL4 with the WD40 protein wap, an interacting partner of mnb, a Ser/Threonine Kinase. They report the presence of mnb phosphorylation

consensus motif in Su(H), the DNA binding partner of Nicd, and they speculate that mRpl4 and wap recruit mnb to phosphorylate Su(H) and affect Notch target gene activity. Finally, they find that this new role of mRpl4 is conserved in vertebrates. Their work is significant to the field of developmental biology, Notch signaling and mitochondria as it reinforces the idea that there is intense crosstalk among mitochondria and signaling cascades in the cell and this crosstalk affects developmental processes.

Some additional biochemical experiments could consolidate their main message. My main suggestions are the following.

1) Mo et al. nicely show that on one hand loss or knockdown of mRpl4 reduces the expression of Wg, Cut and NRE-GFP (Notch reporter) in wing discs and on the other hand that SuH recruitment at E(spl) locus is reduced. As wg and ct genes were used as readouts for Notch signaling it would be nice to show Su(H) occupancy at those two loci in wt vs mRpl4 compromised wing discs.

2) The authors show that mRpl4 interacts with wap in a Yeast Two Hybrid (YTH) assay. They confirm this interaction in Drosophila wing discs. They also mention that wap is a partner of mnb, a Ser/Thr kinase. They mention a mnb consensus domain in Su(H) and speculate that mRpl4 and wap could bring mnb to phosphorylate Su(H). It would be nice to have a few more evidence to support this mechanistic model. As in the YTH assay no Notch related proteins were found as interactors of mRpl4, the authors could for instance perform co-IPs to show interaction between wap and mnb or Su(H) in wing discs. Furthermore, does the phosphorylation status of Su(H) change upon knockdown of mRpl4 or wap or mnb in wing discs? Finally, a phospho-mimetic or phospho-deficient Su(H) mutant in T426 could be helpful to address whether this modification has consequences in Notch target genes activation and wing phenotypes. However, the latter experiment might be beyond the revision timeline.

3) Finally, the authors propose "a model that mRpL4 interacts with wap to regulate Notch signaling activity, potentially through modifying Su(H) to influence its recruitment on chromatin". However, residue T426, which is a potential phosphorylation target of mnb, and could be affected by mRpl4 and wap interaction, lies in the C-terminal domain (CTD) of Su(H). Crystal structures of Su(H)/CSL have shown that this domain serves as an interacting surface for NICD ankyrin Repeats and mastermind (mam). Thus, one could speculate that this T426 phosphorylation could potentially affect Su(H)/Nicd/mam complex stability/ its turnover in Notch target genes relative to co-repressor complex binding or its capacity to activate Notch target genes but perhaps not its recruitment on chromatin. If the DNA binding capacity of Su(H) is compromised in mRpl4 mutants, this could be achieved through another modification within a less conserved consensus motif of Su(H) deposited by mnb or through a completely different modification deposited in the N-terminal domain (NTD) or the β trefoil domain (BTD) of Su(H) by another wap interacting kinase. NTD and BTD domain of Su(H) have been shown to bind the major and minor DNA groove. The authors should discuss this with more clarity.

Minor comments:
- In the introduction, a more recent review about Notch signaling could be added along with the Bray 2006.
- In Sup Fig 1: also show staining of Notch and Dl in WT clones
- In Figure 1: Does ectopic expression of UAS-mRpl4 in wt MARCM clones induce Notch target genes like wg or ct?
- In Figure 2: A cartoon with the area of the wing disc manipulated with each Gal4 driver would be helpful for the reader.
- In Sup Fig 3: also include wt MARCM clones stained for Nicd
- In Materials and methods please state the origin of Nicd.
- In Figure S6 it is nicely shown that mRPL4 and wap are found both in cytoplasm and nucleus. does mRpl4 have a nuclear localisation signal? If not, does wap have one? HOw to they translocate to the nucleus? Any hypothesis?
- It is stated that wap interacts with the Ser/Thr kinace mnb. And that Su(H) has a residue T426 that looks like the mnb phosphorylation consensus sequence. Do Nicd and mastermind have similar phosphorylation consensus sequences?

Response to reviewers

Ref: Submission EMBOR-2022-55764V2

We thank the editor and three reviewers for critical reading of our manuscript and for their valuable suggestions. In the revised version of manuscript, we have performed experiments to address the reviewer's concerns, improved the quality of data presentation and writing, and enriched the discussions. We are confident that we have addressed the reviewers' concerns in the current form of our manuscript. Our point-by-point response to the reviewers are listed as below and marked in red. Corrections made in the revised manuscript are also marked in red.

Referee #1:

The manuscript by Mo and colleagues describes a "non-conventional" function of the mitochondrial ribosomal protein, mRpL4. They show that disruption of mRpL4 in Notch-receiving cells impairs expression of Notch target genes. They further demonstrate that mRpL4 physically interacts with Wap, a WD40 repeat protein, to regulate Notch target gene expression. Importantly, they find that mRpL4 regulates Notch target genes in zebrafish as well, indicating that the phenotype is conserved in vertebrate systems. In addition, the phenotypes observed when mRpL4 is disrupted cannot be explained by altered ROS formation as disruption of several other mitochondrial ribosomal proteins altered ROS formation without affecting the expression of Notch target genes. Altogether, this is an exciting finding that adds to the growing list of observations demonstrating that some mitochondrial ribosomal proteins may have extra-mitochondrial functions. However, I have suggested two experiments that I think are necessary to help reinforce the conclusions drawn.

1. The authors should quantify the wing margin (notch) defects produced as a result of RNAi-mediated disruption of a Notch signaling component that produces robust wing margin defects, as well as the defects produced as a result of RNAi-mediated disruption of mRpL4 and RNAi-mediated disruption of another mitochondrial ribosomal protein (such as mRpS28 or mRpL24) to provide a better sense of the degree of penetrance observed when mRpL4 is knocked down.
We appreciate the reviewer's suggestion and have quantified the wing margin defects caused by mRpL4, mRpL24 and mRpS28 RNAi (Fig EV2C and D in the revised manuscript). Nearly 1/3 of mRpL4 RNAi wings displayed marginal nicks, while none of the other 2 MRPs affected wing margin formation. We concluded that mRpL4 RNAi could cause moderate wing margin defects.

2. The authors should use RNAi to knock down mRpL4 and another mitochondrial ribosomal protein (mRpS28 or mRpL24) in muscles or brain and use qRT-PCR of Notch target genes to examine the extent of tissue specificity of the phenotype in flies. Alternatively, you could use the NRE-GFP or another reporter of Notch signaling to

address this question, if necessary.

We agree with the reviewer that whether mRpL4 regulates Notch signaling in other tissues should be examined. Using the NRE-GFP reporter, we showed that knock-down of mRpL4 but not mRpL24 led to reduction of Notch activity in larval neuroblasts (Fig EV3A-C) and salivary gland imaginal rings (Fig EV3D-F). In adult midgut, the expression level of *Su(H)*-LacZ was also reduced upon *mRpL4* RNAi (Fig EV3G-I).

Minor concerns:

1. Why was E(spl) mβ-LacZ used in some panels while NRE-GFP was used in others to assess Notch signaling?

The mRpL4 mutant line used in this study is caused by insertional mutagenesis using the P-element construct P{lacW}, which contains the LacZ cDNA sequence. In order to avoid misjudgment about the LacZ expression level, the changes of Notch signal activity in mRpL4 mutant clones were examined by Cut, Wg and the *NRE*-GFP reporter. In other experiments, the choice of reporter lines was made to be convenient for genetic manipulations. We always crossed the reporter line with Gal4 line at the first step, and used the progenies to cross with RNAi or transgenes. Thus, when *hh*-Gal4 was used, we always chose reporter line that has been inserted on the second or X chromosome. Generally speaking, multiple Notch signal targets and/or reporter lines were examined whenever possible to validate the effects on Notch activity.

2. In addition to Su(H), Hrb27 is another nuclear protein that regulates Notch signaling. Can the authors discuss whether they think the phenotype involves Su(H) or Hrb27 or both?

The role and relevance of Hrb27/Hrp48 are included in the revised manuscript. Hrb27 utilizes at least two separate pathways to modulate Notch signaling. In female flies, Hrb27 represses the expression of the sex determination master gene Sex-lethal (Sxl) to ensure a proper amount of Notch during wing development (*Suissa* et al, 2010). Sxl protein binds Notch mRNA and inhibits Notch protein translation in ovary cells (Penn & Schedl, 2007), but whether similar mode of action exists in wing disc cells has not been directly tested yet. In both males and females, Hrb27 interacts with the ubiquitin ligase Deltex (Dx) to attenuate Notch signaling activity in a Sxl-independent manner (Dutta *et al*, 2017; Dutta *et al*, 2020). Epistasis assays demonstrate that Hrb27 functions up-stream of NICD in both pathways (Suissa *et al*, 2010; Dutta *et al*, 2017). In our hands, mRpL4 regulates Notch signaling activity in both sexes and likely functions down-stream of NICD. We believe that Hrb27 and mRpL4 might not directly collaborate with each other to regulate Notch signaling.

3. Please provide a brief description of the somatic screen described in the first paragraph of the results.

A brief description of the somatic screen has been included in the Methods section, and the relevant references (Ren *et al*, 2018; Mo *et al*, 2022) have been added in the

revised manuscript.

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The manuscript identifies a mitochondrial ribosomal protein mRPL4 as a novel component required for Notch receptor signaling and demonstrates that it's function is by distinct activity from its normal role in protein translation. This conclusion appears robust as it utilises both mutants, RNAi and rescue of the mutant phenotype by cDNA expression. Using methodology which is standard and well proven in the literature the authors show that the protein is required only in the signal receiving cells not the signal sending cells, and that the function is one which is down stream of release of the Notch ICD. This is likely to be at the level of Su(H) recruitment to the Notch target genes as shown by the CHIP assay in Figure 2. It would be nice to have this interesting conclusion backed up by an additional method, for example it is possible to image Su(H) recruitment to the E(spl) locus on the polytene chromosome as I think this would enhance the robustness of this conclusion.

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Overall the manuscript is well written and clear, the methodology is straight forward and the experimental design is sound. The findings are interesting and somewhat unexpected in nature and should be of interest to a wide audience.

We appreciate the reviewer's comments and suggestions. We have tried very hard to image the recruitment of Su(H) to the *E(spl)* locus on the polytene chromosome in larval salivary gland cells, but we were not able to observe a sharp band of Su(H) on the chromosome as shown in previous report (Gomez-Lamarca *et al*, 2018). The expression level of Su(H)-GFP fusion protein in our reporter strain was too weak for direct imaging. We labeled the Su(H)-GFP fusion protein, endogenous Su(H) protein as well as over-expressed HA-Su(H) by immunostaining, and always found a general diffuse distribution of Su(H) even in the presence of over-expressed NICD. We reason that the failure to detect a prominent Su(H) band is likely caused by using of different stocks and imaging systems. Due to the impact of COVID-19, we were not able to acquire the particular Su(H)::GFP strain under the revision timeline. However, we have performed experiments to demonstrate that mRpL4 regulates Notch signaling in multiple tissues (Fig EV3A-I), mRpL4 affects Su(H) occupancy at *Wg* and *Cut* genomic regulatory regions (Fig EV3J), physical interaction exists between mnb and mRpL4 (Fig EV8D), as well as between Su(H) and wap (Fig EV8G). Collectively, we are confident for the main conclusion that mRpL4 interacts with wap-mnb to regulate Notch signaling activity, probably acting on Su(H) to modulate the transcriptional

output.

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We appreciate the reviewer's suggestion and have examined the Su(H) occupancy at the regulatory regions of *Cut* and *Wingless* loci in wing discs. The Su(H) occupancy were decreased when mRpL4 RNAi was introduced (Fig EV3J).

2) The authors show that mRpl4 interacts with wap in a Yeast Two Hybrid (YTH) assay. They confirm this interaction in Drosophila wing discs. They also mention that wap is a partner of mnb, a Ser/Thr kinase. They mention a mnb consensus domain in Su(H) and speculate that mRpl4 and wap could bring mnb to phosphorylate Su(H). It would be nice to have a few more evidence to support this mechanistic model. As in the YTH assay no Notch related proteins were found as interactors of mRpl4, the authors could for instance perform co-IPs to show interaction between wap and mnb or Su(H) in wing discs. Furthermore, does the phosphorylation status of Su(H) change upon knockdown of mRpl4 or wap or mnb in wing discs? Finally, a phospho-mimetic or phospho-deficient Su(H) mutant in T426 could be helpful to address whether this

modification has consequences in Notch target genes activation and wing phenotypes. However, the latter experiment might be beyond the revision timeline.

We appreciate the reviewer's suggestions and have performed co-IPs to show the interaction between mnb and mRpL4 (Fig EV8D), as well as between Su(H) and wap (Fig EV8G) in wing disc cells. We have tried to examine the phosphorylation status of Su(H) by western blotting, but no significant changes were observed when the commercial antibody (sc-398453, Santa Cruz) was used. We think that phospo-specific Su(H) antibody might be needed to directly reveal whether mRpl4-wap-mnb could impact the phosphorylation status of Su(H). We are making efforts to construct Su(H) T426A and T426D mutant plasmids and transgenic/knock-in mutant flies. Examination of how T426 mutations impact Su(H) and Notch signal activity *in vitro* and *in vivo* will give clear clues about the functional significance of the T426 phosphorylation, but we agree with the reviewer that these experiments are beyond the revision timeline.

3) Finally, the authors propose "a model that mRpL4 interacts with wap to regulate Notch signaling activity, potentially through modifying Su(H) to influence its recruitment on chromatin". However, residue T426, which is a potential phosphorylation target of mnb, and could be affected by mRpl4 and wap interaction, lies in the C-terminal domain (CTD) of Su(H). Crystal structures of Su(H)/CSL have shown that this domain serves as an interacting surface for NICD ankyrin Repeats and mastermind (mam). Thus, one could speculate that this T426 phosphorylation could potentially affect Su(H)/Nicd/mam complex stability/ its turnover in Notch target genes relative to co-repressor complex binding or its capacity to activate Notch target genes but perhaps not its recruitment on chromatin. If the DNA binding capacity of Su(H) is compromised in mRpl4 mutants, this could be achieved through another modification within a less conserved consensus motif of Su(H) deposited by mnb or through a completely different modification deposited in the N-terminal domain (NTD) or the β trefoil domain (BTD) of Su(H) by another wap interacting kinase. NTD and BTD domain of Su(H) have been shown to bind the major and minor DNA groove. The authors should discuss this with more clarity.

We appreciate the reviewer's insightful suggestions. After going through the relevant literatures, we have to agree with the reviewer that the presumptive mnb target residue (T426) lies in the C-terminal domain (CTD) of Su(H), a region that is not involved in DNA binding (Kovall & Hendrickson, 2004; Wilson & Kovall, 2006). Thus, although mRpL4 and wap might recruit mnb to phosphorylate Su(H), such modification will unlikely alter its affinity with chromosome. The T426 phosphorylation site resides in the conserved β-strand motif that interacts with the ankyrin repeats domain of NICD (Nam et al, 2006; Choi et al, 2012) and the transcription repressor Hairless (Yuan et al, 2016). It is attempting to speculate that T426 phosphorylation could potentially affect the interaction of Su(H) with NICD and Hairless, which in turn may modulate the composition, stability, activity and turnover of Su(H) transcription regulatory complexes. These hypotheses could help to explain the reduced occupation of Su(H) on Notch targets observed in mRpL4 RNAi wing disc cells. Interestingly, the mitogen

activated protein kinase (MAPK) phosphorylates Su(H) at P424 to attenuate Notch signaling (Auer et al, 2015; Fechner et al, 2022). The mnb and MAPK phosphorylation sites are in such close proximity, making it hard to ignore the potential antagonistic effect between them. At present, we could not rule out the possibility that mnb may also target less conserved consensus sites in other domains of Su(H) to modify its activity. Alternatively, unknown kinases that interact with wap could contribute to modification and regulation of Su(H). The vertebrate orthologs of mnb are known to phosphorylate NICD and attenuate Notch signaling (Fernandez-Martinez et al, 2009; Hämmerle et al, 2011; Morrugares et al, 2020), whether mnb could phosphorylate NICD during fly development is still illusive. Further investigations are needed to reveal how mRpL4 and wap-mnb regulates Su(H) and Notch signaling activity.

Minor comments:

- In the introduction, a more recent review about Notch signaling could be added along with the Bray 2006.

Recent review about Notch signaling (Henrique & Schweisgut, 2019) has been added.

- In Sup Fig 1: also show staining of Notch and Dl in WT clones.

Staining of Notch and Dl in wild type clones are included as Fig EV1C and Fig EV1E in the revised manuscript.

- In Figure 1: Does ectopic expression of UAS-mRpl4 in wt MARCM clones induce Notch target genes like wg or ct?

Ectopic expression of UAS-mRpl4 in wild type MARCM clones were not sufficient to induce Cut or Wg in our hands (Fig EV1G and H).

- In Figure 2: A cartoon with the area of the wing disc manipulated with each Gal4 driver would be helpful for the reader.

Cartoons showing the area of the wing disc and adult wing affected by the Gal4 drivers are included in the revised manuscript (Fig EV2A and B).

- In Sup Fig 3: also include wt MARCM clones stained for Nicd.

Staining of NICD in WT MARCM clones have been added (Fig EV5D, Fig S3 is now Fig EV5).

- In Materials and methods please state the origin of Nicd.

The origin of UAS-Nicd transgenic stock (Go *et al*, 1998, gift of Dr. Alan Jian Zhu) has been added.

- In Figure S6 it is nicely shown that mRPL4 and wap are found both in cytoplasm and nucleus. does mRpl4 have a nuclear localisation signal? If not, does wap have one? HOw to they translocate to the nucleus? Any hypothesis?

No nuclear localisation signal (NLS) was found in mRpL4, while a small region

similar to pat7-type NLS could be identified in wap using the PSORT predication program (Horton *et al*, 2007). At present, we presume that the translocation of mRpL4 into cell nucleus be assisted by wap.

- It is stated that wap interacts with the Ser/Thr kinace mnb. And that Su(H) has a residue T426 that looks like the mnb phosphorylation consensus sequence. Do Nicd and mastermind have similar phosphorylation consensus sequences?
We identified a potential phosphorylation consensus sequence in NICD but not in mastermind (Figure EV8F), and have included this finding in the discussion.

Dear Dr. Zhang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports.

Before I can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please move the 5 keywords below the abstract.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Please provide a .docx formatted version of the final manuscript text, but without the figures included. Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- You can submit up to 5 images as Expanded View. Presently, there are 9 EV figures. Please select 5 figures to be shown as EV figures (and maybe try to fuse some). Any additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

- I would then suggest to move also 'Expanded View Table 1' (with primer information) to the Appendix. Please name this Appendix Table S1 and use this name for the callouts.

- Please provide a fully completed author checklist, providing information in column D for 'Sample definition and in-laboratory replication' (select responses using the pull down menue).

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

- Please add scale bars of similar style and thickness to all the microscopic images using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- Please make sure that all figure panels are called out separately and sequentially. Presently, it seems separate panel callouts for Fig. EV5 are missing. Please check.

- As the few Western blots shown are significantly cropped, could you please provide the source data for the blots. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the final revised manuscript. Please include size markers for the scans of entire blots, label the scans with figure and panel number, and send one PDF file per figure or one pdf file for all figures shown in the Appendix.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text and comments. Please use the attached file as basis for further revisions and provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have addressed all my concerns.

Referee #2:

The manuscript has been substantially improved in responses to reviewers comments and I believe these interesting findings are now suitable for publication in EMBO reorts.

Referee #3:

This work by Mo et al assigns a novel, conserved and distinct role to mRpL4 in regulating Notch signalling in Drosophila wing discs beyond its normal role in mitochondria protein synthesis. Under this revision round, the authors produced additional evidence to support their model where mRpL4 physically interacts with wap bringing along its interactor, the mnb Ser/Threonine kinase which could phosphorylate Su(H) and affect its recruitment to Notch target genes and their activation.

More specifically, it was nice to show that loss of mRpL4 affects Su(H) recruitment to other Notch target genes, apart from the E(spl) locus, like wg and ct whose expression was affected by genetic knockdown of mRpL4 in the larval wing disc (this addressed my first major comment). Examining also the role of mRpL4 in activating Notch targets in other tissues such as larval brain, salivary glands and gut strengthens the hypothesis that this mechanism is broadly used throughout animal tissues to regulate Notch signalling.
Furthermore, the coIP experiments that the authors produced very elegantly show there is interaction between mRpL4 and mnb as well as between Su(H) and wap. Together with their original observation that mRpL4 interacts with wap, these new data prove that all partners can physically interact thus consolidating the authors' model that mRPL4 regulates Notch target genes activation via affecting Su(H) recruitment to these genomic loci possibly through phosphorylation of Su(H) by mnb. This addressed my second major point to great extent. There is still a lack of data showing that the phosphorylation status of Su(H) is indeed affected in mRpL4 mutants but the authors attempted to resolve this by looking at global Su(H) phosphorylation status which did not change. A more detailed look into various candidate phosphohorylation sites in Su(H) and Nicd will reveal more mechanistic details. However, these experiments were beyond the timeframe of this revision. Hopefully, the authors will pursue this direction in the future.

Finally, all my minor comments were addressed and the discussion was greatly improved by addressing my third major comments and a) incorporating literature around the solved crystal structure of Su(H) and its interaction with NICD, b) looking at potential mnb phosphorylation sites in Su(H) and Nicd and c) speculating how the mnb or other kinases brought by mRPL4 and wap could affect Su(H) recruitment to Notch target genes.

All in all, this work is now substantially improved and I would recommend its publication in EMBO reports.

Dear Dr. Breiling,

We would like to thank you for handling our manuscript and for the valuable suggestions. We have modified the manuscript accordingly and our responses are listed as below and marked in red. If you find anything that needs to be corrected or provided, please just let me know.

- Please move the 5 keywords below the abstract.
The keywords have been moved.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file.
The author contributions section has been removed.

- Please provide a .docx formatted version of the final manuscript text, but without the figures included. Please order the manuscript sections like this, using these names: Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends
The manuscript has been revised to meet these requests.

- You can submit up to 5 images as Expanded View. Presently, there are 9 EV figures. Please select 5 figures to be shown as EV figures (and maybe try to fuse some). Any additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.
Five EV figures are kept in the revised manuscript, along with two Appendix figures. The Appendix file is prepared with the required information.

- I would then suggest to move also 'Expanded View Table 1' (with primer information) to the Appendix. Please name this Appendix Table S1 and use this name for the callouts.
The table has been moved to the Appendix file and is cited as Appendix Table S1.

- Please provide a fully completed author checklist, providing information in column D for 'Sample definition and in-laboratory replication' (select responses using the pull down menue).
The fully completed author checklist has been uploaded.

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit

to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics.
See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis. If n<5, please show single datapoints for diagrams.
The nature of *n* has been included in figure legends, as well as the statistical testing method.

- Please add scale bars of similar style and thickness to all the microscopic images using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.
Scale bars have been added for all microscopic images.

- Please make sure that all figure panels are called out separately and sequentially. Presently, it seems separate panel callouts for Fig. EV5 are missing. Please check.
We have checked the manuscript and made corrections to cite all figure panels.

- As the few Western blots shown are significantly cropped, could you please provide the source data for the blots. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the final revised manuscript. Please include size markers for the scans of entire blots, label the scans with figure and panel number, and send one PDF file per figure or one pdf file for all figures shown in the Appendix.
The source data of all western blots are included in the Appendix file.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text and comments. Please use the attached file as basis for further revisions and provide your final manuscript file with track changes, in order that we can see any modifications done.
We have uploaded the word file with track changes.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
The mitochondria ribosome component mRpL4 regulates Notch signaling activity, which is likely independent from its role in mitochondrial protein synthesis. The regulatory role of mRpL4 in Notch signaling is conserved during *Drosophila* and zebrafish development.

- two to four short bullet points highlighting the key findings of your study (two lines each).
  Bullet points:
- mRpL4 positively regulates Notch signaling activity during *Drosophila* development.
- The role of mRpL4 in Notch signaling regulation is likely independent from mitochondrial protein synthesis.

- mRpL4 interacts with wap to modulate the occupancy of Su(H) at the enhancer regions of Notch target genes.
- mRpL4 plays a conserved role in the regulation of Notch signaling during zebrafish development.

- a schematic summary figure that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.
The summary figure/Synopsis Image has been uploaded.

Best wishes!
Junzheng Zhang

Junzheng Zhang
China Agricultural University
China

Dear Dr. Zhang,

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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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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Please note that a copy of this checklist will be published alongside your article.

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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods, Appendix Table S1

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Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

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If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods, Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods, Figure legends

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In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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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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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	