JCB

Mitochondrial translation and dynamics synergistically extend lifespan in C. elegans through HLH-30

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Review Timeline:

Submission Date:	2019-07-12
Editorial Decision:	2019-08-09
Revision Received:	2020-01-22
Editorial Decision:	2020-02-18
Revision Received:	2020-03-04

Monitoring Editor: Richard Youle

Scientific Editor: Marie Anne O'Donnell

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.)

DOI: https://doi.org/10.1083/jcb.201907067

August 9, 2019

Re: JCB manuscript #201907067

Dr. Riekelt H Houtkooper
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Dear Dr. Houtkooper,

Thank you for submitting your manuscript entitled "Mitochondrial translation and dynamics synergistically extend lifespan in C. elegans through HLH-30". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that all three reviewers are very interested in the link made here between longevity and inhibition of mitochondrial translation and dynamics and are particularly positive about the suitability of these findings for JCB. Reviewer #1 and #2 do emphasize that more insight into the underlying mechanisms that directly connect altered mitochondrial dynamics / ribosome inhibition with HLH-30 expression, and how the resulting lysosomal biogenesis enhances longevity, would be necessary and Reviewer #1 has provided some suggestions for experiments to address this. Reviewer #2 recommends more rigorous analysis of a number of experiments, and all the other specific technical comments noted by the reviewers necessary to substantiate the main claims should be addressed for resubmission.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Richard Youle, Ph.D.
Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Liu and colleagues describe studies to determine mechanistically how mitochondrial dynamics (fission or fusion) interact with reduced mitochondrial translation to prolong worm lifespan. The authors demonstrate that the lysosomal biogenesis transcripton factor TFEB/HLH-30 is required for lifespan extension when mitochondrial ribosomes and mitochondrial fusion are simultaneously inhibited. Surprisingly, the authors demonstrate that despite being activated under these conditions, the UPRmt is not required for the increased longevity. The manuscript is well written and likely of interest to a broad audience. I have several concerns and suggestions below that I hope the authors will find helpful.

My main concern is that the authors conclude that UPRmt is not required for the longevity caused by mito ribosome and mito fusion inhibition. Considering the impact of the author's previous paper demonstrating UPRmt is required for longevity caused by mito ribosome inhibition (Houtkooper et al., Nature 2013), the conclusion here will likely have broad impact. My concern is that conclusion is based on two relatively weak UPRmt inhibitors. 1) Mutations/deletions of haf-1 do not fully suppress UPRmt activation and 2) RNAi of atfs-1 in combination with mrps-5 RNAi may not be a complete loss of ATFS-1 function. A complete loss of function allele of atfs-1 was recently published and would be an ideal test of the author's model (Deng et al, PNAS 2019).

The findings supporting a role for HLH-30/TFEB in promoting longevity upon mito ribosome and fusion inhibition are interesting. The incredible increase in MVB and lysosomal compartments in mrp-5/eat-3 worms is striking (Fig 6C). Does HLH-30 inhibition prevent this?

It should be clearly mentioned that HLH-30 is known to be required for lifespan extension during mitochondrial dysfunction (Lapierre et al., Nat Comm 2013).

As stated by the authors, it remains unclear how HLH-30 is regulated by mito ribosome/fusion inhibition. While I don't think a complete mechanism is required, but a bit more discussion and perhaps experimentation would be appreciated. As inhibition of both mito fusion and fission is required for mrps-5 RNAi lifespan extension, the authors should determine if HLH-30 activation requires mitochondrial dynamics? Or, does inhibition of mito fission and fusion impair HLH-30 activation? Is HLH-30 regulated by mitochondrial dynamics?

Minor concerns.
Fig 1B. Lifespan extension by mrps-5 RNAi is much weaker than previously demonstrated (Houtkooper et al, Nature 2013). The authors should comment on this. Does doxycycline extend worm lifespan? If so, this may be a more reliable assay than using multiple RNAi's.

Page 15. Pink-1 and dct-1 are not transcription factors.

A bit more discussion on the physiologic relevance of impairing mitochondrial protein synthesis and mitochondrial fusion simultaneously would be appreciated.

Reviewer #2 (Comments to the Authors (Required)):

Liu et al. provide insights into the interplay between mitochondrial form and function which govern the rate of aging in an organism through increased lysosomal content. In a comprehensive follow up to Houtkooper et al. 2013 this paper investigates the mechanism of lifespan extension that occurs upon knockdown of both mitochondrial translation, via mrps-5 RNAi, and mitochondrial fusion, via eat-3 and fzo-1. The authors claim that although impairment of mitochondrial fusion and translation activate UPRmt, both independently and collectively, the increased lifespan extension by inhibiting fusion under impaired mitochondrial translation is independent of UPRmt. Through a broad proteomic analysis of animals with impaired mitochondrial networks and translation, the authors determine that the lifespan extension of abrogated mitochondrial fusion and translation is dependent on reduced reproductive capacity. Lastly, the authors link the lifespan increase of the mrps-5/eat-3 animals to the transcription factor HLH-30 and increased lysosomal biogenesis.

This manuscript is founded on the interesting finding that decreasing both mitochondrial translation and mitochondrial network dynamics leads to a synergistic increase in lifespan. The authors then go on to investigate four somewhat connected findings: the effect of combining decreased mitochondrial fission with decreased mitochondrial translation, the effect of UPRmt on the lifespan extension of decreased mitochondrial translation combined with decreased mitochondrial fusion, a not so informative proteomic experiment which implicates decreased reproductive capacity as a link to lifespan extension, and a loosely connected tie to activation of HLH-30 with lifespan.

We find the manuscript to present an interesting phenomenon characterized with thorough experiments. However, acceptance of the manuscript should depend on a more unified narrative and increased mechanistic understanding of the link between decreased mitochondrial translation

and fusion with activation of HLH-30 and lysosomal biogenesis. Without a more direct link, it is unclear how specific this phenomenon is to the added alteration of mitochondrial dynamics versus a metabolic or nutrient shift that may mimic a starved state. Major concerns and questions are outlined below.

MAJOR COMMENTS

1. Figure 3B shows that decreasing both mitochondrial fusion (via fzo-1) and fission (via drp-1) reverses the mrps-5 mediated lifespan extension, which is a very intriguing result but not well explained or characterized. Since it is surprising that inhibition of fission and fusion - seemingly opposing pathways for mitochondria - have the same downstream effect, the authors should better characterize the effect of drp-1 and the drp-1/fzo-1 double mutant on UPRmt (using the hsp-6p::GFP reporter strain) and atfs-1 dependence of the lifespans. Showing the morphology of the mitochondrial network of this double mutant with mrps-5 knockdown looks like would also be informative. As a minor comment, the decrease in size of the worm is not very informative.
2. In the methods, the authors state that only lifespans of key findings were repeated twice. There is also no mention of whether they were blinded. As lifespans can be quite variable and subjective, all lifespans need to be repeated at least twice, and at least once blinded. In addition, key findings should be repeated in triplicate.
3. All lifespans were completed on FUDR. While this can be considered standard practice in the field, it confounds interpretations given Figure 4 in which the authors claim that lifespan extension of this paradigm is also dependent on reduced reproduction. All key lifespans should be completed once without FUDR for clear interpretation of results.
4. The finding of decreased reproduction in this mrps-5/eat-3 double knockdown lifespan extension paradigm is interesting, but Figure 4F does not seem to be the right experiment to explore this point. An experiment that would further validate these claims would be performing a lifespan on adults post reproduction, if these RNAi constructs are only working in the germline than this should not extend lifespan. As an alternative, performing these lifespans in a glp-1 mutant (which is reproductive null) would also demonstrate this point.
5. Figure 5B claims that the eat-3/mrps-5 lifespan is not dependent on atfs-1. This lifespan needs to be repeated using a hypomorph allele of atfs-1(gk3094) or atfs-1(tm4919) in order to make this claim based on both the haf-1 and atfs-1 data.
6. Figure 6 needs many more experiments before making the claim that there is a connection between concurrent decreased mitochondrial translation and fusion and HLH-30 activation
a. qPCR of HLH-30 targets (see Lapierre et al. 2013) is a gold standard for looking at HLH-30 activation and must be included
b. There needs to be a discussion in the paper why there was not an upregulation in lysosomal proteins in the proteomics data, especially since the model is based primarily on upregulation of lysosome biogenesis one would expect to see an increase in lysosomal proteins
c. hlh-30p::hlh-30::GFP localization is known to be variable and needs to be quantified (see Lin et al. 2018)
d. Other reporters of autophagy and lysosomal biogenesis need to be included: lgg-1p::GFP, lmp-1p::GFP, lysotracker, autophagic flux reporter (Chang et al 2017), mitophagy reporter
e. The role of general autophagy and/or mitophagy needs to be addressed
7. The data involving drp-1/mrps-5 and HLH-30 needs to be included somewhere in the manuscript. Does drp-1 alone and in combination with mrps-5 RNAi also cause HLH-30 to go nuclear? Is this lifespan also dependent on hlh-30?
8. Figure 6 is entirely dependent on the lifespans with hlh-30 RNAi however there is a major error in the table listing the median lifespans in Table S1 of the daf-16 RNAi conditions and the representative lifespan shown in Figure S4H. The plot of EV in this lifespan is clearly different from

the 7 other lifespans. The authors cannot make these claims about daf-16 with this error included in the manuscript. daf-16 reporter images should also be included to prove there is no interaction with daf-16, especially since DAF-16 and HLH-30 are known to function as combinatorial transcription factors (Lin et al. 2018).
9. The proposed model in Figure 6E is based on weak data regarding HLH-30 activation and EM images. What about the reverse model where there is reduced fission via drp-1 inhibition? Why is it that knocking down both fission and fusion in combination with reduced mitochondrial translation reverses the beneficial lifespan extension of mrps-5? Is messing with mitochondria just leading to an altered metabolic state that leads to HLH-30 activation? Please address these concerns.

MINOR COMMENTS
1. In Figure 1A Mitochondrial dynamics changes can be analyzed to provide more quantitative analysis. For example, determining mitochondrial area:perimeter ratios as done in Weir et al. 2017.
2. In supplemental Figure S2C representative images of hsp-4p::GFP should be included. Additionally, there may be differences in basal hsp-4p::GFP levels that are being masked by only comparing to induction of hsp-4p::GFP with tunicamycin. Tunicamycin treatment should also be done on the RNAi knockdowns if that is what you are going to be comparing to. There should be two separate comparisons done with and without tunicamycin with all of these conditions.
3. The lifespan in Figure 3B includes the fzo-1 mutant in combination with drp-1, however there is no lifespan showing what affect the fzo-1 mutant would have on mrps-5 RNAi alone or on EV.
4. In Figure 3B the lifespan is done with the fzo-1/drp-1 double mutant, however in other experiments eat-3 is used due to the more pronounced phenotype. Is there a reason eat-3 was not used in this experiment?
5. In Figure 4 the decision to do proteomic analysis of these double knockdowns is not clear, nor is much gained from it. Since mrps-5 is knocking down mitochondrial translation and the authors later claim that activation of HLH-30, a transcription factor, is key to their mechanism, it seems that the more obvious and informative experiment would be to perform RNAseq. The authors should explain their decision to choose this experimental method. Additionally, it would have also been informative to include the double mutant of drp-1/fzo-1 to determine why this double mutant in combination with mrps-5 does not have a lifespan extension.
6. Figure 5C should also include data with fzo-1, drp-1, and eat-3 alone to make the claim that hsp-6 is up in all of these conditions. Additionally, there should be imaging data of the hsp-6p::GFP reporter with drp-1 alone, drp-1/mrps-5 and drp-1/fzo-1/mrps-5 RNAi.

Reviewer #3 (Comments to the Authors (Required)):

Summary

Authors reported a synergistic effect of mitochondrial translation and dynamics disruption on lifespan, and found that it was dependent on the transcription factor HLH-30/TFEB but independent on stress responses like the UPRER and UPRmt. The manuscript was overall an easy read, and data generally follows a logical structure. However, some important experiments were missing, and experiments performed were insufficient to delve into the mechanisms of lifespan extension. Additionally, some conclusions made were not justified based on the extent of the data presented. I would recommend it for publication, but with revisions made as recommended below.

Major points

1. Figures 1 and S1: mRNA levels of eat-3 and fzo-1 are reported only in worms treated with double

RNAi with mrps-5, but I feel it's important to additionally assess mRNA levels in worms treated with the respective single RNAi. It's possible that the additional lifespan extension seen with double RNAi may potentially be attributed to stronger knockdown of these genes, also mrps-5 mRNA knockdown was similar with single and double RNAi.

2. Given that the efficiencies of RNAi knockdown as reported for mrps-5, eat-3, and fzo-1 seem pretty low despite lifetime RNAi treatment, I wonder if the lack of abrogation of the extended lifespan in mrps-5/eat-3 animals by most of the transcription factors analysed may be in due to poor knockdown. I would strongly recommend authors to compare mRNA levels of the respective targeted transcription factors and report it in the supplementary alongside the lifespan analyses.

3. The authors should complement the analysis of lysosome structures with functional analyses to support their claims about the roles of lysosomes herein. Here are two recommended experiments to perform:

A. Lysotracker staining of lysosomes to gain insights into lysosomal function
B. Investigating autophagic flux with Bafilomycin A or chloroquine: Does inhibiting lysosomal degradation with these chemicals mimic lifespan reduction as in mrps-5/hlh-30 RNAi-treated eat-3 animals?

4. The logical next step is to assess if mitophagy is upregulated when mitochondrial translation and dynamics are disrupted, since HLH-30 is a master regulator of autophagy. Mitophagy could be a mechanism by which the system employs to cope with the stress/lifespan extension. While it might be beyond the scope of this manuscript, I suggest authors should at least discuss this aspect.

5. Loss of fecundity and GO enrichment of processes associated with reproduction/sexual development in double RNAi knockdown as compared to mrps-5 single knockdown could be a result of lifetime administration of RNAi. I suggest authors to compare results with adult-only RNAi.

6. RNAi knockdown of mrps-5 and mrps-5/eat-3 in germline did not recapitulate lifespan extension seen in whole body knockdown. Does germline-specific knockdown itself affect fecundity/ablate germline? I think it would also be quite interesting and therefore would suggest authors to perform whole body knockdown experiments in germline-less glp-1 worms as a complementary approach to gain further insights into the importance of the germline in mediating the lifespan extension.

7. If possible, it would be interesting to assess effects of daf-16/hlh-30/mrps-5 triple RNAi on lifespan in eat-3 mutants, as DAF-16 and HLH-30 has been shown to interact and co-regulate gene expression (Lin XX et al. 2018 Nat Commun, PMID: 30970250).

Minor points

1. Please indicate the age of worms analysed in all figure legends as this is inconsistently performed.

2. The first sentence of the results section seems incomplete. It sounds like the authors have the intention of reiterating their previous findings that mitochondrial translation through knockdown of mrps-5 extends lifespan but failed to convey this in a succinct manner; please correct this.

3. Figure 2D: Please indicate why sodium azide was applied as this is not immediately clear to readers who are not familiar with mitochondrial research.

4. Figure 3D: Legend not indicated.

5. Figure S5A: Please indicate GFP punctae as was performed in Figure 6B.

6. Figure 6B: Please explain what the difference between images on top and bottom panels is.

7. Authors reported that growth (body length) was compromised with mrps-5 and mrps-5/eat-3 RNAi. Could the authors additionally comment on other aspects of body morphology? I think it would be interesting to see if these treatments altered intestinal morphology/fat content in any way as such morphological changes have been observed with lifespan changes.

8. Can authors please indicate the age of the animals presented in Figure S1C? It seems from Figure 2A that the additional body length reduction from mrps-5/eat-3 knockdown worms is only manifested at the older age of day 7, but not at day 3, but the phrasing used in the text seems to be suggesting that it's the double knockdown per se that caused this effect rather than an inhibition of a further age-dependent growth in body length. I recommend authors to rephrase this so that it's apparent that the double RNAi is inhibiting further body growth with age and that it may be preserving a shorter body length associated with younger age/youthfulness.

Point by point response to the reviewers

Reviewer #1:
Liu and colleagues describe studies to determine mechanistically how mitochondrial dynamics (fission or fusion) interact with reduced mitochondrial translation to prolong worm lifespan. The authors demonstrate that the lysosomal biogenesis transcripton factor TFEB/HLH-30 is required for lifespan extension when mitochondrial ribosomes and mitochondrial fusion are simultaneously inhibited. Surprisingly, the authors demonstrate that despite being activated under these conditions, the UPRmt is not required for the increased longevity. The manuscript is well written and likely of interest to a broad audience. I have several concerns and suggestions below that I hope the authors will find helpful.

We thank the reviewer for the positive evaluation and kind words regarding our work. We are particularly pleased that the reviewer appreciates the scope of our work. Moreover, we highly appreciate the constructive suggestions from the reviewer. In order to address the reviewer's concerns, we have performed additional experiments and revised our Results and Discussion sections to provide more clarity.

My main concern is that the authors conclude that UPRmt is not required for the longevity caused by mito ribosome and mito fusion inhibition. Considering the impact of the author's previous paper demonstrating UPRmt is required for longevity caused by mito ribosome inhibition (Houtkooper et al., Nature 2013), the conclusion here will likely have broad impact. My concern is that conclusion is based on two relatively weak UPRmt inhibitors. 1) Mutations/deletions of haf-1 do not fully suppress UPRmt activation and 2) RNAi of atfs-1 in combination with mrps-5 RNAi may not be a complete loss of ATFS-1 function. A complete loss of function allele of atfs-1 was recently published and would be an ideal test of the author's model (Deng et al, PNAS 2019).

We agree that both *haf-1* deletion and *atfs-1* RNAi may result in an incomplete block of the UPR^{MT}. Following the reviewer's suggestion, we have performed lifespan experiments in two *atfs-1* deletion mutants. These mutants are *atfs-1(cmh15)* and *atfs-1(gk3094)*, the first of which was reported in Deng et al., PNAS 2019, PMID: 30850535. We found that the combined RNAi depletion of *mrps-5* and *eat-3* was still capable of significantly prolonging lifespan in both these mutants. Together with our previous findings from the lifespan analyses in the *haf-1* mutant and the *atfs-1* RNAi worms, these data suggest an ancillary role of UPR^{MT} in *mrps-5*;*eat-3* RNAi-mediated longevity. These results have been included in our revised figure (Fig. S2, D-E) and the Results section (page 12).

The findings supporting a role for HLH-30/TFEB in promoting longevity upon mito ribosome and fusion inhibition are interesting. The incredible increase in MVB and lysosomal compartments in mrp-5/eat-3 worms is striking (Fig 6C). Does HLH-30 inhibition prevent this?

To examine if HLH-30 inhibition prevents the observed increase of lysosomal-related structures with *mrps-5*;*eat-3* double RNAi, we conducted electron microscopy analysis followed by blind quantification of the structures. In brief, we observed that double RNAi of *mrps-5* and *hlh-30* decreased the number of both lysosome-like structures and multivesicular bodies relative to *mrps-5* RNAi alone in the *eat-3(tm1107)* mutant. These data strengthen our findings that simultaneous inhibition of mitochondrial translation and fusion activates HLH-30-mediated lysosome biogenesis. We have included these finding in our revised figure (Fig. 7, E and F) and Results sections (page 18).

It should be clearly mentioned that HLH-30 is known to be required for lifespan extension during mitochondrial dysfunction (Lapierre et al., Nat Comm 2013).

We apologize for this oversight and we have adapted the manuscript to make clear that there is a requirement of HLH-30 in lifespan extension during mitochondrial dysfunction. This point is clarified in the text of the manuscript on page15 in the Results section.

As stated by the authors, it remains unclear how HLH-30 is regulated by mito ribosome/fusion inhibition. While I don't think a complete mechanism is required, but a bit more discussion and perhaps experimentation would be appreciated. As inhibition of both mito fusion and fission is required for mrps-5 RNAi lifespan extension, the authors should determine if HLH-30 activation requires mitochondrial dynamics? Or, does inhibition of mito fission and fusion impair HLH-30 activation? Is HLH-30 regulated by mitochondrial dynamics?

We thank the reviewer for the suggestions. We have expanded our discussion to elaborate on potential mechanisms underlying regulation of HLH-30 nuclear accumulation by mitochondrial translation and dynamics (page 21 in the Discussion section).

To examine HLH-30 nuclear translocation in the *drp-1(tm1108)* mutant and *drp-1;fzo-1* double mutant we outcrossed a transgenic strain expressing a GFP-tagged HLH-30 with both the *drp-1(tm1108)* mutant and *drp-1;fzo-1* double mutant. We then analyzed the nuclear enrichment of HLH-30::GFP in these mutants with and without *mrps-5* RNAi treatment. These results are now included in Fig. 6, E-F and page 16 in the Results section of the revised manuscript. We observed that mutation of *drp-1* increased the nuclear enrichment of HLH-30::GFP relative to the wild type controls and that the RNAi knockdown of *mrps-5* amplified this effect. In comparison, although an increase in HLH-30::GFP nuclear accumulation also occurred upon double mutation of *drp-1* and *fzo-1* relative to the wild type, no further enhancement of this process was observed upon RNAi of *mrps-5*. In summary, we conclude that double mutation of *drp-1* and *fzo-1* prevents *mrps-5* RNAi-mediated longevity involving a lack of effects of *mrps-5* RNAi on the HLH-30 nuclear enrichment.

Minor concerns.
Fig 1B. Lifespan extension by mrps-5 RNAi is much weaker than previously demonstrated (Houtkooper et al, Nature 2013). The authors should comment on this. Does doxycycline extend worm lifespan? If so, this may be a more reliable assay than using multiple RNAi's.

The difference in the *mrps-5* RNAi-induced lifespans between these two studies is explained by the fact that the worms are exposed to RNAi bacteria at two very different stages of the life cycle. In the previous study (Houtkooper et al., Nature 2013, PMID: 23698443), RNAi exposure began at the parental larval stage 4 and then continued through two generations (Strategy 1). In the present study, we started treating worms with RNAi bacteria from the time of hatching (Strategy 2). The reason we used Strategy 2 in this study is to exclude the influences of transgenerational epigenetics on lifespan outcome, which may potentially complicate the interpretation of our findings. Indeed, the previous study reported that doxycycline increases lifespan via inhibition of mitochondrial translation (Houtkooper et al., Nature 2013). However, we chose RNAi feeding methods over doxycycline treatment for two reasons:

(1) Doxycycline inhibits bacterial translation, which not only confounds the interpretation of results, but also makes it difficult to combine with RNAi feeding experiments in which active bacteria are important for efficient dsRNA expression.
(2) We repeatedly observed reproducible significant lifespan increases when treating worm with RNAi against *mrps-5* using Strategy 2.

Therefore, we are confident in the reliability of our RNAi strategy in this study. We have specified the RNAi feeding methods for each experiment in the Materials and Methods in the revised manuscript (page 24).

Page 15. Pink-1 and dct-1 are not transcription factors.

We have corrected this on page15 in the Results section of the revised manuscript.

A bit more discussion on the physiologic relevance of impairing mitochondrial protein synthesis and mitochondrial fusion simultaneously would be appreciated.

We have expanded the Discussion in the revised manuscript to elaborate on the physiologic relevance of impairing mitochondrial translation and mitochondrial dynamics (page 22).

Reviewer #2:

Liu et al. provide insights into the interplay between mitochondrial form and function which govern the rate of aging in an organism through increased lysosomal content. In a comprehensive follow up to Houtkooper et al. 2013 this paper investigates the mechanism of lifespan extension that occurs upon knockdown of both mitochondrial translation, via mrps-5 RNAi, and mitochondrial fusion, via eat-3 and fzo-1. The authors claim that although impairment of mitochondrial fusion and translation activate UPRmt, both independently and collectively, the increased lifespan extension by inhibiting fusion under impaired mitochondrial translation is independent of UPRmt. Through a broad proteomic analysis of animals with impaired mitochondrial networks and translation, the authors determine that the lifespan extension of abrogated mitochondrial fusion and translation is dependent on reduced reproductive capacity. Lastly, the authors link the lifespan increase of the mrps-5/eat-3 animals to the transcription factor HLH-30 and increased lysosomal biogenesis.
This manuscript is founded on the interesting finding that decreasing both mitochondrial translation and mitochondrial network dynamics leads to a synergistic increase in lifespan. The authors then go on to investigate four somewhat connected findings: the effect of combining decreased mitochondrial fission with decreased mitochondrial translation, the effect of UPRmt on the lifespan extension of decreased mitochondrial translation combined with decreased mitochondrial fusion, a not so informative proteomic experiment which implicates decreased reproductive capacity as a link to lifespan extension, and a loosely connected tie to activation of HLH-30 with lifespan.
We find the manuscript to present an interesting phenomenon characterized with thorough experiments. However, acceptance of the manuscript should depend on a more unified narrative and increased mechanistic understanding of the link between decreased mitochondrial translation and fusion with activation of HLH-30 and lysosomal biogenesis. Without a more direct link, it is unclear how specific this phenomenon is to the added alteration of mitochondrial dynamics versus a metabolic or nutrient shift that may mimic a starved state. Major concerns and questions are outlined below.

We thank the reviewer for their kind words, and we highly appreciate the suggestion regarding a more unified narrative and mechanistic understanding of our findings. To address the points raised by the reviewer, we have performed additional experiments and adapted them in the revised manuscript where appropriate.

MAJOR COMMENTS

1. Figure 3B shows that decreasing both mitochondrial fusion (via fzo-1) and fission (via drp-1) reverses the mrps-5 mediated lifespan extension, which is a very intriguing result but not well explained or characterized. Since it is surprising that inhibition of fission and fusion - seemingly opposing pathways for mitochondria - have the same downstream effect, the authors should better characterize the effect of drp-1 and the drp-1/fzo-1 double mutant on UPRmt (using the hsp-6p::GFP reporter strain) and atfs-1 dependence of the lifespans. Showing the morphology of the mitochondrial network of this double mutant with mrps-5 knockdown looks like would also be informative. As a minor comment, the decrease in size of the worm is not very informative.

We thank the reviewer for their suggestions. For the *mrps-5* RNAi in N2, *drp-1(tm1108)*, and *drp-1;fzo-1* double mutant, we examined UPR^{MT} activation by quantifying the transcript level of the classical UPR^{MT} reporter gene *hsp-6* (Fig. 3D). We found that *mrps-5* RNAi consistently activated the expression of *hsp-6*, regardless of the mitochondrial network conditions.

To clarify the role of the UPR^{MT} in our study, we have performed lifespan analyses in two *atfs-1* null allele mutants treated with double RNAi against *mrps-5* and *eat-3*. In both these

mutants, we still observed the lifespan extension brought about by the double RNAi (Fig. S2, D and E, and page 12 of the Results section). Together with the lifespan data obtained in the *eat-3(tm1107)* mutant treated with RNAi against *mrps-5* and *atfs-1*(Fig. 4B), these results suggest that UPR^{MT} is not the primary driver of the lifespan extension observed upon combined inhibition of mitochondrial translation and dynamics.

To examine the mitochondrial network of the *drp-1;fzo-1* mutant upon *mrps-5* RNAi, we have conducted fluorescence microscopy analyses (Fig. 3, E and F of the revised manuscript). We found that RNAi knockdown of *mrps-5* did not significantly restructure the mitochondrial network of *drp-1;fzo-1* at day 2 of adulthood.

Following the reviewer's suggestion, we have moved the size quantification of *mrps-5* RNAi-treated worms to the supplement in the revised manuscript (Fig. S2, A and B).

2. In the methods, the authors state that only lifespans of key findings were repeated twice. There is also no mention of whether they were blinded. As lifespans can be quite variable and subjective, all lifespans need to be repeated at least twice, and at least once blinded. In addition, key findings should be repeated in triplicate.

In the revised manuscript, we have now included 3 or 4 independent lifespan analyses for our key lifespan results, performed by two independent observers and one-time blinded. For lifespan experiments that are of a more confirmatory nature, we have performed the analyses in duplicate. The exception to this are the lifespans in the small-scale lifespan screen we performed that is shown in Fig S4, B-H and Table S1. These lifespans were performed once, thereafter confirmed several times for our positive *hlh-30* hit. The lifespan of *atfs-1(gk3094)* (Fig. S2E and Table S1) was also performed once, but it should be noted that we have obtained confirmation of this phenotype in a second *atfs-1* mutant strain, *atfs-1(cmh15)* (Figure S2D and Table S1), and using *atfs-1* RNAi (Fig. 4B and Table S1). We show the detailed lifespan statistics in the revised Table S1.

3. All lifespans were completed on FUDR. While this can be considered standard practice in the field, it confounds interpretations given Figure 4 in which the authors claim that lifespan extension of this paradigm is also dependent on reduced reproduction. All key lifespans should be completed once without FUDR for clear interpretation of results.

We understand the reviewer's concern about possible confounding factors due to the usage of FUDR. We have conducted lifespans in the germline deficient mutant *glp-1(e2141)* without FUDR (Fig. 5G and Table S1 in the revised manuscript), as also suggested by the reviewer in point #4. Here we again observed a strong synergistic effect on lifespan between inhibited mitochondrial translation and decreased fusion, suggesting that reproduction loss is not the primary driver of the observed lifespan extension. Moreover, our previous data obtained in the germline-only RNAi strain *rrf-1(pk1417)* (Fig. 5F and Table S1) show that RNAi knock-down of *mrps-5* and *eat-3* exclusively in the germline does not lead to lifespan extension, again indicating that in this context the germline is not involved. In the revised manuscript, we clarify this important point in the Results section (page 14).

4. The finding of decreased reproduction in this mrps-5/eat-3 double knockdown lifespan extension paradigm is interesting, but Figure 4F does not seem to be the right experiment to explore this point. An experiment that would further validate these claims would be performing a lifespan on adults post reproduction, if these RNAi constructs are only working in the germline than this should not extend lifespan. As an alternative, performing these lifespans in a glp-1 mutant (which is reproductive null) would also demonstrate this point.

We thank the reviewer for the suggestion and agree that performing lifespans in a *glp-1* mutant helps to clarify the role of reproduction in *mrps-5;eat-3* double RNAi-mediated

longevity paradigm. We have conducted lifespan experiments in *glp-1(e2141)* treated with RNAi against *mrps-5* and *eat-3*, both individually and in combination (Fig. 5G and Table S1 of the revised manuscript). We found that loss of reproduction in *glp-1(e2141)* did not alter the synergistic effects on longevity between suppressed mitochondrial translation and decreased fusion. These data again suggest that although reproduction is further compromised in the long-lived *mrps-5;eat-3* double RNAi worms, it is not directly involved in their longevity regulation. These results are described on page 14 in the revised manuscript.

5. Figure 5B claims that the eat-3/mrps-5 lifespan is not dependent on atfs-1. This lifespan needs to be repeated using a hypomorph allele of atfs-1(gk3094) or atfs-1(tm4919) in order to make this claim based on both the haf-1 and atfs-1 data.

We have conducted lifespan experiments in two *atfs-1* deletion strains including *atfs-1(cmh15)* and *atfs-1(gk3094)* to further validate its dependence in *mrps-5;eat-3* double RNAi-mediated lifespan extension. We have included the lifespan data in Fig. S2, D and E, Table S1, and described the results on page 12 of our revised manuscript. In summary, we observed a consistent lifespan increase in both the *atfs-1* mutants upon combined RNAi of *mrps-5* and *eat-3*. These data confirm our previous observation and further suggest a secondary role of UPR^{MT} in regulating lifespan upon simultaneous inhibition of mitochondrial translation and fusion.

6. Figure 6 needs many more experiments before making the claim that there is a connection between concurrent decreased mitochondrial translation and fusion and HLH-30 activation.

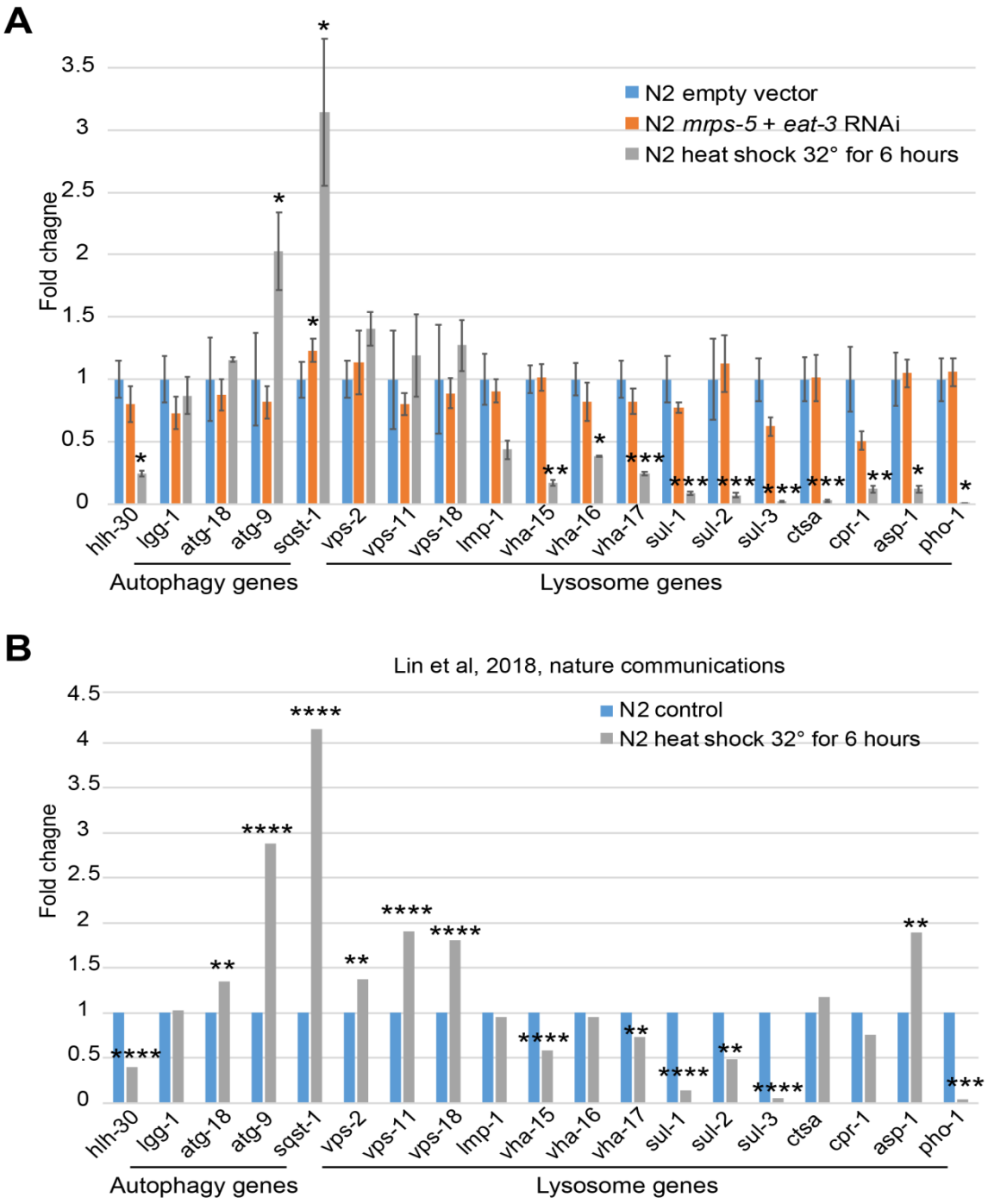
a. qPCR of HLH-30 targets (see Lapierre et al. 2013) is a gold standard for looking at HLH-30 activation and must be included

We thank the reviewer for this suggestion. We have performed qPCR for HLH-30 target genes described in Lapierre et al., Nat Commun 2013, PMID: 23925298 in our study, using heat-shocked worms as the positive controls (Reviewer Figure 1). For heat-shock, we used the same method as described in Lin et al., Nat Commun 2018, PMID: 30353013, i.e. 6 hours at 32ºC.

Except for a small but significant increase in *sqst-1* expression, we did not find noticeable changes for any of the tested genes upon double RNAi of *mrps-5* and *eat-3* (Response to Reviewers Figure 1A). Strikingly, heat shock, which evoked substantial HLH-30 nuclear localization both in our study (Fig. S5A) and by Lin et al., Nat Commun 2018, significantly reduced the expression of *hlh-30* as well as 10 of the 14 lysosome genes examined (Reviewer Figure 1A). In fact, only two of the examined autophagy genes, *atg-9* and *sqst-1*, were upregulated in the heat shock condition (Response to Reviewers Figure 1A). To confirm these findings, we carefully compared our results to the gene expression data upon heat shock obtained from the RNAseq experiments in Lin et al., Nat Commun 2018. In line with our findings, they also found decreased gene expressions of *hlh-30* in 6 of the 14 lysosome genes from the HLH-30 target set (shown in Response to Reviewers Figure 1B).

Because we initially expected upregulation of the HLH-30 targets from the Lapierre paper, we explored this apparent discrepancy further. One important note is that the main set of HLH-30 target genes from the Lapierre paper is from long-lived germline deficient worms, i.e. the *glp-1* mutant. At the same time, in *daf-2(e1370)* mutant worms—which show increased HLH-30 nuclear translocation (Lin et al., Nat Commun 2018) and require HLH-30 for their long lifespan—there was upregulation of only 3 genes (*hlh-30*, *atg-9*, and *ctsa*) as shown in Lapierre et al., Nat Commun 2013. Similarly, only 3 genes including *hlh-30*, *atg-9,* and *sqst-1* are upregulated in the *clk-1* mutant, which depends on HLH-30 for its lifespan extension

(Lapierre et al., Nat Commun 2013). With this in mind, we conclude that HLH-30 activates specific gene sets depending on the type of stress.
Finally, it is worth mentioning the electron microscopy we performed in *eat-3(tm1107)* mutants with double RNAi against *mrps-5* and *hlh-30*. We showed that co-silencing *mrps-5* and *hlh-30* markedly reduced the number of lysosome-like structures and multivesicular bodies compared to *mrps-5* RNAi alone. This confirms that *hlh-30* is functionally involved in driving lysosome biogenesis in the context of impaired mitochondrial translation and dynamics.



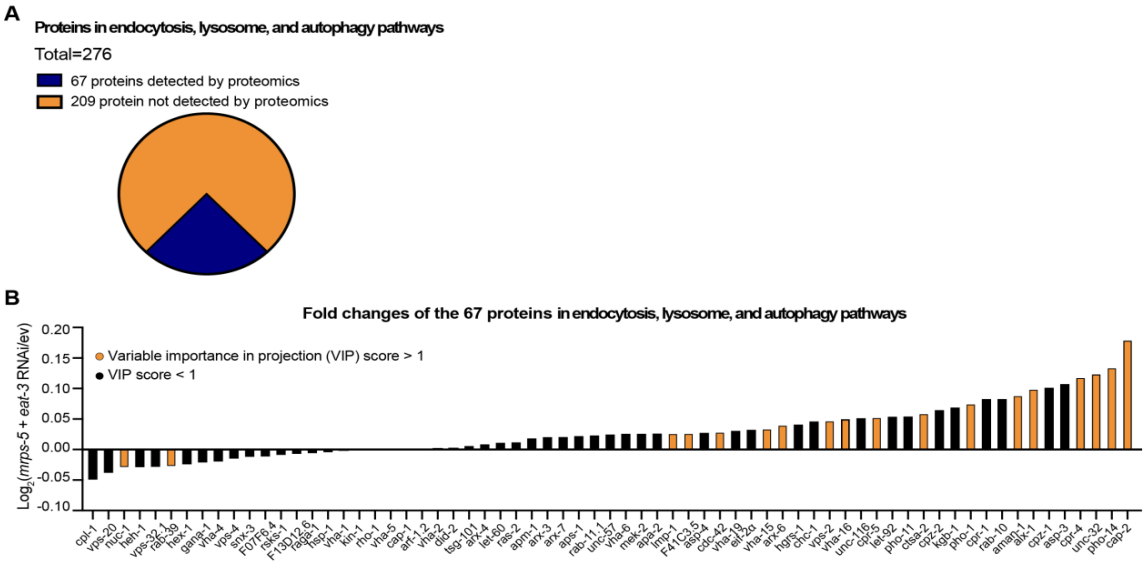
Reviewer Figure 1. Expression of HLH-30/TFEB target genes in worms upon double RNAi of *mrsp-5 and eat-3* or upon heat stress

(A) qPCR analysis of autophagy- and lysosome-related gene expression in worms at larval stage 4 upon double RNAi of *mrps-5* and *eat-3* or upon heat shock at 32° for 6 hours. The expression levels of genes were normalized to reference genes *cdc-42* and *f35g12.2.* The statistical comparisons were performed using Student's t-tests, where statistical significance was calculated by comparing the expression of genes in RNAi treated condition or heat-shock condition to the mean value of control condition, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mean ± SD of n = 6 biological replicates.

(B) Previously published RNA-seq data (Lin et al., Nat Commun 2018) showing expression profiles of HLH-30 target genes upon heat shock.

b. There needs to be a discussion in the paper why there was not an upregulation in lysosomal proteins in the proteomics data, especially since the model is based primarily on upregulation of lysosome biogenesis one would expect to see an increase in lysosomal proteins

To address this notion, we have revisited our proteomics data and specifically checked the levels of proteins in lysosome-related biological processes including endocytosis, lysosome, and autophagy pathways. We detected only about a quarter of the proteins in those lysosome-related pathways in our proteomics data (Reviewer Figure 2A). We have therefore profiled the fold changes of those proteins when comparing *mrps-5;eat-3* double RNAi-treated condition to empty vector-treated controls. As a result, we found that 16 proteins were upregulated while only 2 proteins were downregulated, determined by a variable importance in projection (VIP) score > 1 (Reviewer Figure 2B). These data suggest indeed that a moderate upregulation in lysosome-related proteins is occurring. To reveal the changes of more lysosome-related proteins, we believe that an improved resolution of proteomics is required and suggest it as a focus in our future study. We have added this point to the Discussion of the revised manuscript (pages 20-21).



Reviewer Figure 2. Fold changes of lysosome-related proteins upon double RNAi of *mrps-5* and *eat-3*.
A. Pie chart showing the proportion of lysosome-related proteins detected by proteomics.
B. Fold changes of the 67 lysosome-related proteins detected by proteomics upon double RNAi of *mrps-5* and *eat-3*.

c. hlh-30p::hlh-30::GFP localization is known to be variable and needs to be quantified (see Lin et al. 2018)

We thank the reviewer for the suggestion and have now quantified the HLH-30::GFP localization using the same method as described in Lin et al., Nat Commun 2018. The results are now included in Fig. 6, D and F of the revised manuscript.

d. Other reporters of autophagy and lysosomal biogenesis need to be included: lgg-1p::GFP, lmp-1p::GFP, lysotracker, autophagic flux reporter (Chang et al 2017), mitophagy reporter.

To confirm our data on lysosome biogenesis and to calculate the number and morphology of lysosomes, we performed confocal microscopy on *lmp-1p*::GFP transgenic worms in our revised manuscript (Fig. 7, A and B, and page 17 of the Results section). We found that double RNAi of *mrps*-5 and *eat-3* not only increased the number of lysosomes, (as indicated by integrated GFP fluorescence intensity normalized to the area of coelomocyte cells), but also decreased the fraction of enlarged lysosomes with a diameter $\geq$ 2 μm observed in coelomocytes where lysosome-related features are often studied (Fig. 7, A and B, and page 17). We have also examined autophagy flux using a tandem reporter strain named mCherry::GFP::LGG-1 previously described in Chang et al., eLIFE 2017, PMID: 28675140, as suggested by the reviewer. These data are included in Fig. 8, A-D and described on pages 18-19 in the Results section.

In summary: we observed that combined RNAi against *mrps-5* and *eat-3* provokes a significant increase in the number of autolysosomes and the sum of autophagosomes and autolysosomes in both the pharynx and the hypodermal seam cells in day 2 adult worms (Fig. 8, A-D, pages 18-19). Together, these findings are consistent with our previous conclusions from the electron microscopy experiments, namely that *mrps-5;eat-3* double RNAi causes a rise in the number of lysosome-related compartments.

e. The role of general autophagy and/or mitophagy needs to be addressed

As mentioned in our response to the previous question, the results of confocal microscopy experiment performed in mCherry::GFP::LGG-1 reporter strain have shown a significant increase in the number of autolysosomes and the sum of autophagosomes and autolysosomes in both the pharynx and the hypodermal seam cells (Fig. 8, A-D, pages 18-19). These data indicate enhanced autophagy flux in the *mrps-5;eat-3* double RNAi worms, which likely contributes to the lifespan extension (PMID: 21906946; PMID: 30006559; PMID: 31340141; PMID: 31827090).

For the role of mitophagy, we found that neither the knocking down of *pink-1* nor of *dct-1*, the two key mitophagy genes, prevented *mrps-5* RNAi from prolonging the lifespan of *eat-3(tm1107)* (Fig. S4, F and G). However, we do not believe that this rules out the possibility of an important role of mitophagy in *mrps-5;eat-3* double RNAi-mediated longevity outcome, as RNAi knockdown of a single mitophagy gene cannot fully block the mitophagy process. We have revised our Discussion section to provide more clarity on this point (page 20).

7. The data involving drp-1/mrps-5 and HLH-30 needs to be included somewhere in the manuscript. Does drp-1 alone and in combination with mrps-5 RNAi also cause HLH-30 to go nuclear? Is this lifespan also dependent on hlh-30?

To examine HLH-30::GFP nuclear translocation in *drp-1(tm1108)*, we outcrossed a transgenic strain expressing GFP::tagged HLH-30 to *drp-1(tm1108)*. We observed that mutation of *drp-1* increased the HLH-30::GFP nuclear translocation compared to wild type and this increase was further enhanced upon *mrps-5* RNAi. We have included these results in Fig. 6, E and F and page 16 in the Results section of the revised manuscript.

To test if HLH-30 is required for the lifespan extension mediated by combined inhibition of mitochondrial translation and fission, we have performed lifespan analyses on the mitochondrial fission mutant *drp-1(tm1108)* (Fig. 6B and Table S1 in the revised figure section). Interestingly, *hlh-30* RNAi abrogated the lifespan increase mediated by *mrps-5* RNAi in *drp-1(tm1108)*. These results strongly suggest that HLH-30 is a primary driver of the longevity observed upon suppressing mitochondrial translation and mitochondrial fission or

fusion. We have integrated these findings into the Results section of our revised manuscript (pages 15-16).

8. Figure 6 is entirely dependent on the lifespans with hlh-30 RNAi however there is a major error in the table listing the median lifespans in Table S1 of the daf-16 RNAi conditions and the representative lifespan shown in Figure S4H. The plot of EV in this lifespan is clearly different from the 7 other lifespans. The authors cannot make these claims about daf-16 with this error included in the manuscript. daf-16 reporter images should also be included to prove there is no interaction with daf-16, especially since DAF-16 and HLH-30 are known to function as combinatorial transcription factors (Lin et al. 2018).

We apologize to the reviewer for the confusion concerning the lifespans of *daf-16* RNAi conditions. Instead of providing cumulative statistics, we have now reported the statistics for all representative lifespan curves and for their replicates in the revised lifespan table to improve the clarity of our lifespan results (Table S1). According to our results, *daf-16* RNAi showed moderate lifespan reduction in *eat-3(tm1107)* mutant worms, both with and without *mrps-5* RNAi treatment (Fig. S4I and Table S1). This indicates a non-specific role of *daf-16* in this lifespan regulation. While it would be interesting to investigate the interaction between DAF-16 and HLH-30 further, we believe this to be beyond the scope of our manuscript at this moment.

9. The proposed model in Figure 6E is based on weak data regarding HLH-30 activation and EM images. What about the reverse model where there is reduced fission via drp-1 inhibition?

As outlined in our response to point #7 of this reviewer, we have conducted lifespan assays in *drp-1(tm1108)*, where we showed that HLH-30 was essential for *mrps-5* RNAi-mediated lifespan extension (Fig. 6B and Table S1). Moreover, we examined HLH-30::GFP nuclear translocation in *drp-1(tm1108)* with and without *mrps-5* RNAi treatment (Fig. 6, E and F). We found that *mrps-5* RNAi increased the HLH-30::GFP nuclear enrichment in the *drp-1(tm1108)* mutant (Fig. 6, E and F). These data suggest that simultaneously reducing fission and mitochondrial translation also prolongs lifespan by increasing the HLH-30 nuclear enrichment.

Why is it that knocking down both fission and fusion in combination with reduced mitochondrial translation reverses the beneficial lifespan extension of mrps-5?

To explore the underlying mechanism for the loss of the longevity effect of knocking down *mrps-5* by RNAi in the *drp-1;fzo-1* double mutant, we analyzed both the mitochondrial network and HLH-30 nuclear translocation. These data are included in our revised figure (Fig. 3, E and F; Fig. 6. E and F) and in the Results sections (page 11 and page 16). We found that although RNAi knockdown of *mrps-5* significantly reduced the connectivity of the mitochondrial network in wild type worms (Fig. 1, A and B), this effect was absent in the *drp-1;fzo-1* double mutant (Fig. 3, E and F). Moreover, in the *drp-1*;*fzo-1* double mutant, the *mrps-5* RNAi lost its effects on the HLH-30 nuclear translocation (Fig. 6, E and F). In summary, these data suggest that the double mutation of *drp-1* and *fzo-1* abrogates *mrps-5* RNAi-mediated longevity by counteracting its effects on the HLH-30 nuclear enrichment perhaps through a lack of change in mitochondrial network. We have expanded our discussion to elaborate on these points on page 20 of the revised manuscript.

Is messing with mitochondria just leading to an altered metabolic state that leads to HLH-30 activation? Please address these concerns.

We appreciate the insightful points with regard to the relation between metabolic states and HLH-30 activation. To clarify in detail the energy status upon simultaneous suppression of mitochondrial translation and fission or fusion, we have conducted semi-targeted

metabolomics and looked into the “energy charge”. This is an index based on the following calculation ([ATP]+½[ADP])/([ATP]+[ADP]+[AMP]) (Fig. S5B and page 16 of our revised manuscript). We did not detect significant changes in the energy charge between worms with suppressed mitochondrial translation and mitochondrial fission or fusion, either individually or in combination. These data suggest that HLH-30 activation is not merely an outcome of an altered metabolic state. This, at least to some extent, can provide insight into the overall metabolic state.

Although we cannot rule out the possibility that changes in other (specific) metabolites may be associated with the HLH-30 activation, we believe that this is beyond the scope of our current work and better examined in a future study.

MINOR COMMENTS

1. In Figure 1A Mitochondrial dynamics changes can be analyzed to provide more quantitative analysis. For example, determining mitochondrial area:perimeter ratios as done in Weir et al. 2017.

In the revised manuscript, we have quantified mitochondrial morphology for Figure 1A using the same method as in Weir et al., Cell Metabolism 2017, PMID: 29107506. The quantification of these data is now included in Fig. 1B of the revised manuscript.

2. In supplemental Figure S2C representative images of hsp-4p::GFP should be included. Additionally, there may be differences in basal hsp-4p::GFP levels that are being masked by only comparing to induction of hsp-4p::GFP with tunicamycin. Tunicamycin treatment should also be done on the RNAi knockdowns if that is what you are going to be comparing to. There should be two separate comparisons done with and without tunicamycin with all of these conditions.

We appreciate the reviewer’s concerns about the basal *hsp-4*::GFP being masked by only comparing to *hsp-4*::GFP induction in tunicamycin-treated condition. We have now compared each single and double RNAi-treated conditions to their empty vector control and to every other condition (except for DMSO- and tunicamycin-treated conditions) (Fig S1G). In doing so, we still did not find significant differences in the induction of *hsp-4*::GFP among those RNAi-treated groups. In addition, we have included the representative images of *hsp-4*::GFP in Fig. S1F of the revised manuscript.

3. The lifespan in Figure 3B includes the fzo-1 mutant in combination with drp-1, however there is no lifespan showing what affect the fzo-1 mutant would have on mrps-5 RNAi alone or on EV.

We have performed lifespan analyses on the *fzo-1(tm1133)* mutant, where we found a robust lifespan extension induced by *mrps-5* RNAi. We have now included these data and lifespan statistics in Fig. S2C and Table S1 of the revised manuscript.

4. In Figure 3B the lifespan is done with the fzo-1/drp-1 double mutant, however in other experiments eat-3 is used due to the more pronounced phenotype. Is there a reason eat-3 was not used in this experiment?

The reviewer is right that we focus most of our RNAi experiments on *eat-3* RNAi. The reason for this is that double RNAi of *eat-3* and *mrps-5* repeatedly has a more prominent synergistic effect on *C. elegans* lifespan relative to double RNAi of *fzo-1* and *mrps-5* (Fig. 1C and Table S1). We believe this is primarily due to the fact that RNAi knockdown efficiency of *eat-3* in both single and double RNAi conditions is higher than that of *fzo-1* (~66.3% knockdown of *eat-3* versus ~43.7% knockdown of *fzo-1,* Fig. S1, A and B). We then used the *fzo-1;drp-1*

mutant later in the study [which was a kind gift from Dr. William Mair and his team (see Weir et al., Cell Metabolism 2017, PMID: 29107506)], and is therefore the only fission/fusion double mutant available to us during the study period.

5. In Figure 4 the decision to do proteomic analysis of these double knockdowns is not clear, nor is much gained from it. Since mrps-5 is knocking down mitochondrial translation and the authors later claim that activation of HLH-30, a transcription factor, is key to their mechanism, it seems that the more obvious and informative experiment would be to perform RNAseq. The authors should explain their decision to choose this experimental method. Additionally, it would have also been informative to include the double mutant of drp-1/fzo-1 to determine why this Double mutant in combination with mrps-5 does not have a lifespan extension.

We performed proteomics relatively early on in our project and considered this over RNAseq since biological changes captured at the protein level generally provide information closer to the end phenotype in question. Only later when we identified the link with *hlh-30* did we decide to focus on targeted experiments on *hlh-30* downstream effects rather than RNAseq. The same is true for the *drp-1;fzo-1* double mutant. Rather than performing more -OMICs, we have made great efforts to obtain functional data from fluorescent microscopy experiments for both mitochondrial network (Fig. 3, E and F) and HLH-30 nuclear localization (Fig. 6, E and F) in the *drp-1*;*fzo-1* double mutant upon *mrps-5* RNAi. We believe this is more specific to the reviewer's questions and more insightful. As explained in our response to the second sub-question in main point #9, we found that double mutation of *drp-1* and *fzo-1* not only prevented *mrps-5* RNAi from altering the mitochondrial network but also hampered its effect on the HLH-30 nuclear enrichment. Therefore, on the basis of these results, we propose that the double mutation of *drp-1* and *fzo-1* blocks *mrps-5* RNAi-mediated lifespan extension by preventing HLH-30 nuclear enrichment. We go on to discuss the possibility that this results from an immobilized mitochondrial network in *drp-1;fzo-1* double mutant.

6. Figure 5C should also include data with fzo-1, drp-1, and eat-3 alone to make the claim that hsp-6 is up in all of these conditions. Additionally, there should be imaging data of the hsp-6p::GFP reporter with drp-1 alone, drp-1/mrps-5 and drp-1/fzo-1/mrps-5 RNAi.

In our revised paper, we have included *hsp-6*::GFP imaging data with RNAi against *fzo-1* and *eat-3*, individually or in combination with *mrps-5* RNAi (Fig. 2, A and B; and Fig. S1, D and E). We found that single RNAi of *eat-3* or *fzo-1* activated *hsp-6*::GFP expression, with *eat-3* RNAi showing a more prominent increase. For the *hsp-6* expression in *drp-1(tm1108)* and *drp-1;fzo-1* mutants, we provided gene expression data examined by qPCR (Fig. 3D). These data show that RNAi knockdown of *mrps-5* increases *hsp-6* expression in three types of mitochondrial network contexts including N2, *drp-1(tm1108)*, and the *drp-1;fzo-1* mutant, while mutations of *drp-1* alone or of *drp-1* and *fzo-1* in combination do not show any significant effect on *hsp-6* gene expression (Fig. 3D).

Reviewer #3:

Summary
Authors reported a synergistic effect of mitochondrial translation and dynamics disruption on lifespan,and found that it was dependent on the transcription factor HLH-30/TFEB but independent on stress responses like the UPRER and UPRmt. The manuscript was overall an easy read, and data generally follows a logical structure. However, some important experiments were missing, and experiments performed were insufficient to delve into the mechanisms of lifespan extension. Additionally, some conclusions made were not justified based on the extent of the data presented. I would recommend it for publication, but with revisions made as recommended below.

We thank the reviewer for these very kind words, and we are particularly pleased that they found the manuscript clear and suited for publication. To address the points made, we have performed additional experiments, explained in detail below.

Major points
1. Figures 1 and S1: mRNA levels of eat-3 and fzo-1 are reported only in worms treated with double RNAi with mrps-5, but I feel it's important to additionally assess mRNA levels in worms treated with the respective single RNAi. It's possible that the additional lifespan extension seen with double RNAi may potentially be attributed to stronger knockdown of these genes, also mrps-5 mRNA knockdown was similar with single and double RNAi.

In the revised manuscript, we have included the transcript levels of *eat-3* and *fzo-1* in their respective single and double RNAi conditions (Fig. S1, A and B) and described the data on page 7 in the Results section. We found that the knockdown efficiencies of these genes upon single RNAi did not significantly differ compared to those upon double RNAi. Therefore, we conclude that the synergistic effects on longevity of *mrps-5;eat-3* or *mrps-5;fzo-1* double RNAi is not attibutable to a stronger knockdown of *eat-3* or *fzo-1* in double RNAi conditions.

2. Given that the efficiencies of RNAi knockdown as reported for mrps-5, eat-3, and fzo-1 seem pretty low despite lifetime RNAi treatment, I wonder if the lack of abrogation of the extended lifespan in mrps-5/eat-3 animals by most of the transcription factors analyzed may be in due to poor knockdown**.** I would strongly recommend authors to compare mRNA levels of the respective targeted transcription factors and report it in the supplementary alongside the lifespan analyses.

We have measured the transcript levels of all the genes in the lifespan screen and have added these data in Fig. S4 A. We found some variation in the RNAi knockdown efficiencies among those genes (Fig. S4 A). We cannot formally exclude that the lack of lifespan shortening effects on *mrps-5;eat-3* animals is possibly due to insufficient knockdown in some of these cases. However, as our screen clearly (and reproducibly) identified *hlh-30* as critical to the lifespan extension seen with *mrps-5;eat-3* worms, we focused our functional follow-up experiments on this transcription factor.

3. The authors should complement the analysis of lysosome structures with functional analyses to support their claims about the roles of lysosomes herein. Here are two recommended experiments to perform:
A. Lysotracker staining of lysosomes to gain insights into lysosomal function

We have performed additional lysosome imaging as suggestion by the reviewer. We used the *lmp-1*::GFP reporter strain rather than Lysotracker. The reason for this is that Lysotracker stains all acidic compartments and is found mainly enriches in worm intestine, a tissue abundant in acidic and auto-fluorescent materials that do not represent classic lysosomal structures (PMID: 23967012, PMID: 23935448, PMID: 27070172). Therefore, we

used the *lmp-1*::GFP reporter strain as it allows us to more specifically observe lysosomes in worm cells that are active in the endosome-lysosome pathways such as those present in coelomocytes. In the *lmp-1*::GFP reporter strain we performed rigorous quantifications of lysosome number and size. We found that double RNAi of *mrps-5* and *eat-3* not only increased the number of lysosomes in coelomocytes [as indicated by integrated GFP fluorescence intensity normalized to the area of coelomocyte cells (Fig. 7, A and B)], but also prevented lysosome swelling (Fig. 7, A and B). Our results with the *lmp-1*::GFP reporter strain have been included in our Results section (page 17).

B. Investigating autophagic flux with Bafilomycin A or chloroquine: Does inhibiting lysosomal degradation with these chemicals mimic lifespan reduction as in mrps-5/hlh-30 RNAi-treated eat-3 animals?

We appreciate this comment and suggestion. However, we would suggest that performing lifespan testing with bafilomycin A or chloroquine treatments in *C. elegans* is not feasible for two main reasons:

(1) Bafilomycin A1 and chloroquine have only been successfully used in very high concentrations and for short-term analysis (i.e., a few days) in *C. elegans* to block autophagy (PMID: 28237114, PMID: 25569839, PMID: 28696216). For example, the current two available methods, namely feeding and injection, administer bafilomycin A1 to *C. elegans* at 100 μg/ml (160 μM) and 50 μM, respectively (PMID: 28237114, PMID: 25569839). These concentrations are much higher than that used in cell culture (a concentration that is usually lower than 1 μM). Therefore, we expect that the consequence of using this method would be generally catastrophic to the animals' longevity.
(2) Optimization of the feeding/injection concentrations of bafilomycin A1 and chloroquine for lifespan assays is an incredibly time-consuming effort, one that is not feasible within the timeframe of this revision.

Thus, we have not included this experiment in the current manuscript. However, to further investigate the autophagy flux in these RNAi-treated worms in our study, we used a transgenic strain expressing a tandem autophagy reporter, with GFP and mCherry tagged to LGG-1 (Fig. 8, A-D, pages 18-19 in the Results section). In this reporter strain, both GFP and mCherry fluoresce in autophagosomes visualized as yellow punctae. Upon the maturation of the autophagosomes to autolysosomes, GFP fluorescence is quenched, resulting in only mCherry fluorescence signals in autolysosomes (PMID: 28675140). With this tandem reporter, we found that double RNAi of *mrps-5;eat-3* significantly increased the number of autolysosomes in both the pharynx and seam cells in day 2 adult worms. These data again provide additional support to our finding that combined inhibition of *mrps-5;eat-3* promotes lysosome-associated processes (autophagy flux towards autolysosomes), possibly due to the increased lysosome biogenesis.

4. The logical next step is to assess if mitophagy is upregulated when mitochondrial translation and dynamics are disrupted, since HLH-30 is a master regulator of autophagy. Mitophagy could be a mechanism by which the system employs to cope with the stress/lifespan extension. While it might be beyond the scope of this manuscript, I suggest authors should at least discuss this aspect.

We thank the reviewer for this point. We agree it is beyond the scope of our manuscript, but have expanded our discussion to better clarify this notion on page 20.

5. Loss of fecundity and GO enrichment of processes associated with reproduction/sexual development in double RNAi knockdown as compared to mrps-5 single knockdown could be

a result of lifetime administration of RNAi. I suggest authors to compare results with adult-only RNAi.

In our previous study (Houtkooper et al., 2013, Nature, PMID: 23698443), we have shown that the timeframe of development (from embryo phase to larval stage 4) proves crucial for *mrps-5* RNAi-mediated lifespan extension, whereas *mrps-5* RNAi only during adulthood is not sufficient to promote longevity.

6. RNAi knockdown of mrps-5 and mrps-5/eat-3 in germline did not recapitulate lifespan extension seen in whole body knockdown. Does germline-specific knockdown itself affect fecundity/ablate germline? I think it would also be quite interesting and therefore would suggest authors to perform whole body knockdown experiments in germline-less glp-1 worms as a complementary approach to gain further insights into the importance of the germline in mediating the lifespan extension.

We have performed lifespan analysis on *glp-1(e2141)* mutant treated with RNAi against *mrps-5* and *eat-3*, individually and in combination. These lifespan data have now been included in the revised manuscript (Fig. 5G and page 14 in the Results section). In brief, we again observed the synergistic lifespan effects between *mrps-5* RNAi and *eat-3* RNAi in *glp-1(e2141)* mutant worms that are sterile when raised at the restrictive temperature. These data offer additional supports to the finding that reproduction is not essentially required for the long lifespan of *mrps-5;eat-3* RNAi-treated worms.

7. If possible, it would be interesting to assess effects of daf-16/hlh-30/mrps-5 triple RNAi on lifespan in eat-3 mutants, as DAF-16 and HLH-30 has been shown to interact and co-regulate gene expression (Lin XX et al. 2018 Nat Commun, PMID: 30970250).

We appreciate this point. Although it would be interesting to investigate the interactions between HLH-30 and DAF-16 in *mrps-5;eat-3* double RNAi-mediated longevity, we believe that triple RNAi significantly compromises the RNAi efficiency for each gene, thereby hindering the interpretation of the results. Alternatively, developing a double mutant would take much more time than allowed in this revision process. Thus, as this proposed experiment is not vital to support our conclusions, we suggest it as a focus of future study.

Minor points
1. Please indicate the age of worms analysed in all figure legends as this is inconsistently performed.

We have now included the age of worms in all figure legends of the revised manuscript.

2. The first sentence of the results section seems incomplete. It sounds like the authors have the intention of reiterating their previous findings that mitochondrial translation through knockdown of mrps-5 extends lifespan but failed to convey this in a succinct manner; please correct this.

To describe the initial conception of this study better, we have modified the first paragraph in the result section of the revised manuscript on page 6.

3. Figure 2D: Please indicate why sodium azide was applied as this is not immediately clear to readers who are not familiar with mitochondrial research.

We apologize for this oversight. We have now indicated the function of sodium azide in worm respiration assays in the legend of Figure 2 of our revised manuscript (pages 44-45).

4. Figure 3D: Legend not indicated.

We thank the reviewer for identifying this error. Following the suggestion of reviewer 2 (point #1), we have now moved Fig. 3D to Fig. S2B and included the legend in the revised manuscript.

5. Figure S5A: Please indicate GFP punctae as was performed in Figure 6B.

Instead of showing the representative images in the supplement figure, we have quantified HLH-30 nuclear localization as suggested by reviewer 2 and included the data in the revised manuscript (Fig. 6D).

6. Figure 6B: Please explain what the difference between images on top and bottom panels is.

We have explained the differences between the top and bottom panels in the legend of Fig. 6C in the revised manuscript.

7. Authors reported that growth (body length) was compromised with mrps-5 and mrps-5/eat-3 RNAi. Could the authors additionally comment on other aspects of body morphology? I think it would be interesting to see if these treatments altered intestinal morphology/fat content in any way as such morphological changes have been observed with lifespan changes.

We thank the reviewer for this comment. In this study, we did not observe obvious changes in intestinal morphology in worms treated with *mrps-5;eat-3* double RNAi.

8. Can authors please indicate the age of the animals presented in Figure S1C? It seems from Figure 2A that the additional body length reduction from mrps-5/eat-3 knockdown worms is only manifested at the older age of day 7, but not at day 3, but the phrasing used in the text seems to be suggesting that it's the double knockdown per se that caused this effect rather than an inhibition of a further age-dependent growth in body length. I recommend authors to rephrase this so that it's apparent that the double RNAi is inhibiting further body growth with age and that it may be preserving a shorter body length associated with younger age/youthfulness.

We have indicated the age of the animals in Fig. S1C and modified the text to better clarify the notion of the growth inhibition with age by the *mrps-5;eat-3* double RNAi and its implication in youthfulness on pages 7-8 in the Results section.

February 26, 2020

RE: JCB Manuscript #201907067R

Dr. Riekelt H Houtkooper
Amsterdam UMC, University of Amsterdam
Laboratory Genetic Metabolic Diseases Room F0-116 Meibergdreef 9
Amsterdam 1105 AZ
Netherlands

Dear Dr. Houtkooper:

Thank you for submitting your revised manuscript entitled "Mitochondrial translation and dynamics synergistically extend lifespan in C. elegans through HLH-30". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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- Add scale bars to Fig 6E, inset in Fig 7C, 7E
- Provide tables as excel files

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Sincerely,

Richard Youle, Ph.D.
Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have addressed all of my concerns.

Reviewer #3 (Comments to the Authors (Required)):

The authors have adequately addressed my concerns.