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Local coordination of mRNA storage and degradation near mitochondria modulates C. elegans ageing

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30th Sep 2022

Re: EMBOJ-2022-112446
Local coordination of mRNA storage and degradation near mitochondria modulates C. elegans ageing

Dear Prof. Tavernarakis,

Thank you again for submitting your manuscript proposing a role for coordinated mRNA storage and degradation near mitochondria in aging to The EMBO Journal. We have now received four referee reports on your study, which are copied below. However, given the comments of these experts and further discussion, we have decided that we unfortunately cannot offer to invite a revision for EMBO Journal at this time.

As you will see, all referees clearly acknowledge the interest of the proposed model and the findings to the field. However, at this stage, they are not convinced that the conclusions have been sufficiently supported by experimental data and they raise numerous major issues that relate to both conceptual concerns as well as experimental and technical issues. In particular, the main conclusions regarding the proposed local coordination of mRNA storage and degradation, and the effect of this on aging, would require further support. In our view, the number and breadth of the issues the referees noted would require extensive experimental work and time to resolve, which would be well beyond the scope of a single round of major revision. In addition, this would include experiments with unknown outcome that however affect main messages of the study and could this substantially change the study. Therefore, we unfortunately cannot offer further steps towards publication at the moment.

However, as mentioned, we recognize the interest in the proposed model and we also realize that the number of issues raised also relates to the different fields this work involves. Therefore, we would be open to reconsider a revised version of the study if you are able to resolve the main concerns in the future (or potentially already have preliminary data that could be added to resolve some critical issues). Please however note that in such a case, we again assess novelty and advance at the timepoint of submission and may involve alternative or additional referees if needed. In case you have any questions regarding this procedure or would like to discuss the decision or possibly a transfer to another journal within EMBO Press, please do not hesitate to contact me to set up a call.

I am sorry that I cannot be more positive on this occasion, but hope that you will nonetheless find the comments of our reviewers helpful. Thank you again for giving us the opportunity to consider your manuscript.

Kind regards,

Stefanie Boehm

Stefanie Boehm
Editor
The EMBO Journal

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Referee #1:

Review of "Local coordination of mRNA storage and degradation near mitochondria modulates C. elegans ageing" by Nektarios Tavernarakis and co-workers.
Processing bodies (P-bodies) are ribonucleoprotein (RNP) non-membranous assemblies in the cytoplasm of eukaryotic cells. They contain translationally repressed mRNAs as well as proteins related to mRNA turnover and silencing. P-bodies are thought to play a role in the regulation of post-transcriptional gene expression, particularly in mRNA turnover and storage. In this manuscript, the authors show that in Caenorhabditis elegans components of two different mRNA decay pathways act in the

close proximity of mitochondria to regulate nuclear-encoded, mitochondria-targeted protein transcripts (MTPTs). They report that the decapping and the deadenylation complex form distinct P-body-related granules that are physically and functionally linked to mitochondria. MTPTs are bound and their expression oppositely regulated by decapping and the deadenylation complexes. The authors suggest that "balanced degradation and storage of mitochondria-targeted protein mRNAs is critical for mitochondrial homeostasis, stress resistance and longevity".
The manuscript gives me mixed feelings. On the one hand, the reported observations are certainly very interesting and relevant. On the other hand, I do not agree with the interpretation of some of the experiments.
In general, the manuscript pays too little attention to the molecular functions of the two different degradation complexes. In my view, some observations could be explained "simply" by the enzymatic activities of the decapping vs. the deadenylation complex. When deadenylation is inhibited, mRNAs will retain longer poly(A)-tails, which would explain why RNAi of ntl-2 leads to higher translation rates (Figure 4h). The opposite effect of the dcap-2 RNAi could be explained by the accumulation of deadenylated mRNAs, which are not decapped and degraded and may therefore be inefficiently translated and/or interfere with the translation of other mRNAs. This is just one example where a more molecular/mechanistic/functional and less phenomenological view would lead to deeper and better interpretations of the results. This perspective should be applied to the entire manuscript and also considered in the discussion.
Additional major points
1. Although it is already indicated in the abstract, the authors only reveal in Figure 4 that the ntl-2 and dcap-2 bodies are different entities. Before that, the two bodies are presented in parallel. However, from the very beginning one has the feeling that they are functionally not the same. Wouldn't it be better if the differences were made clear early on?
2. This brings me to the next question - what is the relation between the dcap-2 bodies, the ntl-2 bodies and canonical p-bodies? Are they overlapping? I am missing some counterstainings between different p-body components and dcap-2 and ntl-2 as well as between dcap-2 and ntl-2. In addition, the storage bodies and degradation bodies would need to be characterized in more detail.
Minor points
3. How is the quantification done, e.g. in Figure 1e, 1l, 1m? According to the figure legend three experiments were quantified, so where are the error bars?
4. In figure 1e the different classes are "0 µM", "<=1 µM", "0-1 µM" and ">5 µM". The second and third are the same, so I guess there is some mislabelling here?
5. Why are there no error bars in figure 4h?
6. Wouldn't it be better to show all primers in a table instead of in the text?

Referee #2:

EMBOJ-2022-112446: "Local coordination of mRNA storage and degradation near mitochondria modulates C. elegans ageing"

In this work, the authors show that specific components of the mRNA storage and degradation complexes are localized in close proximity to the mitochondria thereby regulating their content and function in opposite directions. They also showed that the abnormal expression of these components antagonistically impacts their reciprocal expression and is differentially involved in mitochondrial stress response and aging. Moreover, they provide evidence that a specific component of the mRNA storage complex is associated with nuclear-encoded mitochondrial proteins transcripts. These are certainly novel and very interesting findings in the mitochondria and aging biology field, which when substantiated by additional data (see below) will deserve publication in the EMBO Journal.
However, based on the described data, the overall conclusion drawn by the authors that "Local coordination of mRNA storage and degradation near mitochondria modulates C. elegans ageing" seems slightly overstated. Indeed, they clearly showed that some proteins belonging to mRNA-transcripts storage and degradation complexes, are localized near mitochondria and their suppression regulates mitochondrial content and aging. However, to be able to relate these findings to the complex (rather than to the single proteins), data should be provided with additional proteins belonging to the complexes. Moreover, they did not provide any evidence that the "coordination of mRNA storage and degradation", rather than simply the reduced expression of some of their components, is actually the mechanism underlying their effects on stress response and aging. Overall the investigated topic is extremely interesting and of timely importance but in the current state the conclusions are overstated. Moreover, some parts of the manuscript are disconnected and the rationale for carrying out some experiments as well as their description is sometimes clumsy. Different points are discussed below, which I hope will help strengthening and/or revising the main conclusions.

1) The mRNA decapping and the CCR-4/NOT complex physically and functionally associate with mitochondria in an age-dependent manner.
In this first part the authors show the association of mitochondria only with one component of each complexe, NTL-2 and EDC-3. Additional proteins belonging to the complexes (CCF-1, CCR-4, DCAP-2...) should be tested to validate if the complexes (and not simply NTL-2 and EDC-3) actually physically and functionally associate with the mitochondria.
On the other hand, to be able to conclude that alteration of mitochondrial function impact on the expression and association of mRNA-complex-components with mitochondria (figure 1j,n,m), the effect of silencing mitochondria genes not directly related to gene translation should be assessed (mrps-5 is in fact directly affecting ribosomes and thus obviously mRNA regulatory complexes). This is especially in light of their observation that instead atp-3 RNAi (Figure S1) does not impact EDC-3

association with mitochondria.

From Figure S1 it is not clear if the physical association between NTL-2- and EDC-3-containing bodies is actually reduced with aging. In S1a it is very hard to conclude that the association between mitochondria and NTL-2 is lost with aging given that the mitotraker staining (in which tissue?) is completely absent. Given that the structure and dynamics of mitochondria dramatically change during aging (e.g. Regmi et al. 2014; Palikaras et al. 2015), the authors should consider to repeat the experiment using for instance TOMM-20 strain or TMRE used in Figure 1 and possibly at different age and not only in 15 days old animals. Alternatively to be able to use the mtGFP expressing strain, they could cross it with a NTL-2::red reporter. Similarly, to establish that EDC-3-mito association is lost with age, time course experiments to better visualize mitochondria at earlier time points (e.g. 5 and 11 days) might actually help.
It is not clear why the authors, to exclude that altered association during aging is ascribed to abnormal mitochondrial structure/dynamics, choose to silence atp-3 (a subunit of the ATPase which would primarily impact on mitochondrial function) rather than genes directly involved in mitochondrial fusion/fission.
Most importantly, to corroborate the conclusion that mitochondria association with mRNA storage and degradation regulatory proteins is affected during aging, their association should be check in long- and short-lived mutants. Incidentally, the authors used RNAi against atp-3 (Figure S1) and mrps-5 (Figure 1j, m, n), which are known to extend C. elegans lifespan (Dillin et al. 2002; Rea et al. 2017; Houtkooper et al 2013), and showed that the former does not affect mito-ECL-3 association while the latter increase its expression yet seems to reduce its association with the mitochondria. How do the authors reconcile these opposite effects with two pro-longevity interventions? Moreover, if as they suggest, ECD-3-mito association is lost with aging (Figure S1), wouldn't be expected that pro-longevity intervention increase their association? Again, time course experiments during aging in long- and short-lived mutants might help clarifying on this important point of the study.

2) mRNA decapping and CCR-4/NOT complex components oppositely regulate mitochondrial biogenesis and abundance by functioning in discrete foci.
The authors show that dcap-2 and ntl-2 differentially impact on mitochondrial morphology and mass. What is the rationale for switching to dcap-2 instead of also using ecd-3 RNAi? What is the effect of ecd-3 RNAi on the different mitochondrial-related parameters (morphology, ROS and TMRE, SKN-1 and AAK-2 activation)? What is instead the effect of dcap-2 on mitochondrial-complexes physical association? Validating the findings with suppression of different proteins belonging to the same (storage and degradation) complexes, would support the conclusion.
On the same line, the authors conclude (end of first paragraph on pag 6) that perturbation of decapping complex increases the functional mitochondrial population but they actually showed (figure 1 g,h) that dcap-2 RNAi increases mitochondrial ROS and membrane potential. How would be this indicative of "functional mitochondria"? To reach reliable conclusion in this regard, the effect of different decapping regulatory proteins (and not just one) on different mitochondrial functional parameters, e.g. respiration and/or ATP content should be tested.
Given that a primary readout for mitochondrial stress is induction of mtUPR, it would be interesting to know whether reduced expression of mRNA storage/degradation regulatory proteins impacts on hsp-6 expression.

3) Storage bodies constitute local translation coordinators in the vicinity of mitochondria. Data shown in figure 5 are actually not enough to reach this conclusion. The amount of MTPTs associated with NTL-2::GFP (and possibly other storage components) should be addressed upon silencing or overexpression of storage/degradation/translation-inducer proteins, by mitochondrial stress or aging. This would provide further support to the overall conclusion of the work.
Also, if, as they suspect the storage and degradation foci act antagonistically, can they revert alteration in e.g. MTPTs translation by dcap-2 or ntl-2 suppression with akap-1 or tomm-20 depletion?
In the same chapter "to investigate whether storage bodies have a role in local translation of MTPTs..." they perturbed mitochondrial local translation inducers akap-1 and tomm-20. While this is an interesting point to look at, with this experiment they investigate exactly the contrary, that is: if local translation machinery plays a role in storage bodies components association with mitochondria. To actually investigate if storage bodies have a role in local translation of MTPTs it should be assessed (as suggested above) whether modulating the expression of storage bodies components impact on local translation (e.g. by quantifying MTPTs) or on translation inducers expression/activity.
Finally, results shown with atp-3 in Figure S3 seems rather contradictory. Why atp-3RNAi does not affect the percentage of NTL-2 vicinity to mitochondria (S3b) but it increases the amount of NTL-2 associated with mitochondria (S3c)?

4) Balanced mRNA storage and degradation promotes stress resistance and longevity.
In the last chapter the authors investigate the role of dcap-2 and ntl-2 in mitochondrial stress resistance and aging and found that they act in opposite directions. However, to prove that this is due to local imbalance of mRNA storage/degradation/translation the effect of silencing additional components on the same complexes should be tested.
Moreover, altered MTPTs translation in conditions that affect stress response/aging should be assessed, as well as its reversion when suppressing components of the other components of the translation machinery which suppress the lifespan phenotypes.

6) Discussion
Based on the described finding, some of the conclusions seems overstated. On page 10, end of the first chapter of the discussion, the author state that "the "two types of foci form antagonistically to each other and oppositely regulate cytoplasmic translation rate". While they showed that components of the complexes are regulated antagonistically, they provided no evidence on their effect on cytoplasmic translation.

Also, at the beginning of the following chapter they state "Increased global protein synthesis and aberrant translation of target MTPTs triggered by perturbation of storage bodies following ntl-2 genetic inhibition...." yet, they have not actually shown that inhibition of ntl-2 or of other components of the machinery affect translation. Similarly, in the first chapter of page 11, they state, but actually have not shown that "...dcap-2 depletion reduces overall mRNA translation oppositely to ntl-2 genetic inhibition".

Minor concerns

1) Figure 1 and S1
- Figure 1e. The y-axis labels should be changed into something that reflects % protein-mito proximity/distance. The author should provide a clearer description in their methods session on how the distances were calculated.
- Figure 1k-n need clearer self-explanatory graph labelling: is panel k showing the number of ECD-3 foci or protein expression? Similar to panel 1e, in panels l and m it should be clarified what exactly the y-axis indicates (e.g. % mito-ECD-3 proximity?) and if the differences of treatments vs control are significant. Does panel n represent NTL-2 protein content or n of foci associated to mitochondria? What about the quantification upon paraquat?
- Figure S1. The authors should refer to NTL-2 and EDC-3 foci or protein (not bodies) since they have not looked at the expression of other proteins belonging to the complexes.

2) Figure 2 and S2
- In Figure 2 it would help if the different tissues would be specified directly on the figure' panels (and not only in the figure legends). Similarly, panels d and g could specify that mitochondrial mass is being quantified in the intestine at different days after RNAi treatment.
- In Figure S2c it would help if red/lgg-1, green/mitochondria and merge were indicated in the figure (and not only in the figure legend).

3) Figure 3
- Panel 3a. Representative WB is o very poor quality and could be repeated. Also, it is not clear from the quantification what the multiple dots on the bars stands for given that the experiment was only replicated twice.
- Data provided in panels 3b and 3c shows that dcap-2 RNAi further increase the expression of gst-4 in conditions known to already activate skn-1 (genetically or pharmacologically). This suggests dcap-2 RNAi might actually promote gst-4 induction in a snk-1-idependent manner (for instance Detienne et al. 2016). Thus, to clearly establish whether gst-4 is induced by dcap-2 RNAi via skn-1, it should be addressed whether gst-4 expression is increased in the presence of skn-1 RNAi.

4) Figure 4
- Data shown in panels 4e-g should be quantified to include significance.
- The conclusion drawn from data described in panel 4e should be rephrased. Indeed, if as it seems, dcap2 RNAi increase NTL2 expression and viceversa ntl-2 RNAi increases DCAP-2 expression, it means that the they are indeed regulated antagonistically but rather interdependent (in opposite direction but not independently).
- Panels 4f seems in contrast to data shown in Figure S1 where no effects on ECD-3 expression with aging is observed.
- Text describing Figure 4a-e is very succinct. It should be more extensively elaborated to better convey the main message.
- Similarly, to clarify the effect of the complex' components on protein translation, experiment with FRAP (Figure 4h) could be briefly explained. Alternatively, a label on the figure panel could be included to specify what is the strain/fluorescence recovery that is represented. Statistics should be included in the panel.

5) Figure 5 and S3
- Data in panel 5a are not clear, not clearly explained or represented. Does the wild type strain express GFP alone? Although reduced compare to NTL-2::GFP, what is being immunoprecipitated and amplified in the wild-type and in the HIS-72::GFP strains?
- Amplification of other genes translated in the cytosol or nuclei could be used as further controls.
- WB in Panel 5d is not very representative. atp-3 and mrps-5 quantification should be included in panel 5d.

Referee #3:

Overall

Recent advances in our understanding of the mechanisms that underlie mitochondrial homeostasis (function, biogenesis and turnover) have revealed that mitochondrial proteins are synthesised at, or near, mitochondria, thereby facilitating the import of mitochondria destined proteins across the mitochondrial outer membrane. In this manuscript, Daskalaki and colleagues use C. elegans to dissect the molecular cooperation and roles of the CCR-4/NOT and decapping complexes in vivo, and to understand their physiological relevance with regards to ageing and stress resistance. The authors use fluorescently tagged subunits of these complexes (NTL-2::GFP, EDC-3::dsRed, DCAP-2::mCherry, etc) to visualise their localisation in tissues under different conditions and throughout life. Using these tools, the authors show that the CCR-4/NOT and decapping complexes are spatially

and functionally distinct, with one associated with RNA storage and the other associated with RNA degradation. Intriguingly, both complexes localise to mitochondria, suggesting a role in regulating the localised translation of mitochondrial targeted protein transcripts (MTPT). However, these complexes appear to be differentially involved in organismal robustness, with NTL-2 depletion reducing stress resistance and shortening lifespan and DCAP-2 depletion increasing stress resistance. Based on these findings, the authors propose that balancing degradation and storage of MTPTs is crucial for longevity. The work is important and builds nicely on previous work from the Tavernarakis lab and others. In addition, the data provided are convincing (for the most part) and well-presented. Overall, I found this to be an interesting piece of work that is worthy of publication in The EMBO Journal. However, I think that some additional controls and experiments are needed to strengthen some of the claims made, and that some of the conclusions need to be reconsidered before publication. To assist with this, please find my specific comments below:

Specific comments

1. This is a minor point, but the MTCO1 blot in Figure 1f is very poor quality compared to the blot in Figure 5d. Can the authors provide a better representative image here?

2. It is great that the authors attempt to look at the protein levels of SKN-1 in C. elegans (often not a simple task) but the FLAG and tubulin blots in Figure 3a are not of sufficient quality for quantification (The SKN-1::FLAG signal is too feint relative to the uneven background). I suggest the authors load more protein and/or use more sensitive detection reagents to amplify the SKN-1::FLAG signal. Another minor point is that it would be good to have some representative gst-4p::gfp images to accompany the quantification presented in Figure 3b.

3. The opposing effects of ntl-2(RNAi) and dcap-2(RNAi) on the IFE-2::GFP reporter are very interesting. While I fully agree that this reporter is a good indicator of overall translation rates, the authors should strengthen this conclusion by also looking directly at total protein levels in NTL-2 and DCAP-2 depleted animals. This will reveal whether global protein load is being reduced or enhanced when the storage and degradation complexes are perturbed.

4. The authors state that "NTL-2 is required for the increased lifespan of long-lived mutants and that mev-1, nuo-6 and isp-1 mutants, and atp-3(RNAi), suppress the short lifespan of ntl-2(RNAi) worms. However, the data presented in Figure S4 do not appear to support these conclusions. It is true that ntl-2 is required for the lifespan extension observed in akt-1(ok525) animals, and that ntl-2(RNAi) shortens the lifespan of daf-2(RNAi) and age-1(hx546) animals. However, the lifespan of control;ntl-2(RNAi) animals is extended by daf-2(RNAi) and age-1(hx546) to the same extent as in wildtype worms. Similarly, while mev-1(kn1) mutants do ameliorate the short lifespan of ntl-2(RNAi) animals, this does not appear to be the case for nuo-6(qm200) and isp-1(RNAi) worms, which have a similar shortening of lifespan compared to nuo-6 and isp-1 control animals as ntl-2(RNAi) does compared to wildtype. The authors should modify their conclusions accordingly in the text.

5. It is great that the authors included the HIS-72::GFP line as a control for their NTL-2::GFP RIP experiments. This controls well for non-specific pulldowns related to antibody binding and/or beads. BUT, I am not convinced that this is an appropriate control for random interactions with GFP tagged cytosolic proteins. HIS-72::GFP is nuclear; a more appropriate control would be a GFP tagged protein that is cytosolic.

6. In Figure 5d, the authors make the claim that upon akap-1(RNAi) and tomm-20(RNAi), levels of NTL-2 associated with mitochondria decrease. However, these differences are driven by elevated levels of MTCO1 on their western blots, rather than reduced levels of NTL-2. Do akap-1 RNAi and tomm-20 RNAi result in increased levels of MTCO1? The authors should probe this by western blotting. If so, this would suggest that actually, there is no change in NTL-2 mitochondrial association under these conditions.

7. In Figure 6, the authors show that ntl-2(RNAi) sensitizes worms to multiple stresses. Is this also observed using the ntl-2(ok974) mutant used in Figure 7? In addition, does tomm-22 also impact lifespan in a similar way to akap-1(RNAi) and what happens to lifespan in dcap-2(RNAi) worms?

Referee #4:

The study by Daskalaki et al. focuses on the storage and degradation of mRNAs encoding for mitochondrial proteins. They especially focused on the mRNA decapping and the poly-A tail deadenylase CCR-4/NOT complexes, and their association with mitochondria and their influence on mitochondrial abundance and longevity/ageing. Although there might be interesting observations, I am afraid that some experiments are not technically sound and therefore conclusions are questionable. I have several concerns regarding the approaches and analyses as detailed below:

Fig. 1:
GFP is a relatively large tag. Can it be excluded that the tag interferes with the function of the studied proteins?

Fig. 1f:
To get an estimation how much of NTL-2 and EDC-3 co-fractionate with mitochondria it would be better to present a single blot comparing isolated mitochondria, cytoplasm etc. and maybe include a marker for another protein associated with the outer mitochondrial membrane.

Fig. 1i-j vs. Fig. 1a:
The punctuated staining of NTL-2 in the control panels seem to vary.
It is difficult to judge on the effects on NTL-2 in Fig. 1i-j due to the weak signal.

Fig. 1n:
The levels of NTL-2-GFP are normalized to COX1, which is a mitochondrial DNA-encoded protein. Mitochondrial translation should be affected upon downregulation of a mitoribosomal protein (mrps-5 RNAi). Thus, COX1 cannot be used as a loading control.

Fig. 2:
"we conclude that perturbation of the decapping complex increases the functional mitochondrial population,..."
I do not understand how the author can conclude this. The downregulation of dcap-2 disrupts the membrane potential and triggers mitochondrial fragmentation. How can one conclude that dcap-2 ablation increases the functional mitochondrial population? It shows only mitochondrial abundance, but not whether they are functional.

Fig. 3a:
The western blot is not convincing and a-tubulin seems to be overexposed.

Fig. 5a:
This experiment is questionable. If WT and HIS-72:GFP are both negative controls, why do they show different results, especially for spcs-1?

Fig. 5b-5e:
The text is difficult to follow for non-experts. What do the authors mean with "local translation inducers"? TOM20 is a component of the import machinery at the outer membrane. Ablation of TOM20 affects protein import and membrane potential as also shown by TMRE staining (Fig. 5b). Therefore, the conclusion can be misleading.
Again using COX1 as a loading control is not appropriate as the synthesis or stability of mitochondrial DNA-encoded COX1 can be/ is affected in some of the knockdowns.

Comment on RNAi
Efficiency and specificity of downregulation should be shown or include respective reference if RNAi has already been validated elsewhere.
Comment on statistics
In some figures (e.g. Fig. 1n, 2h, 3a, 3g, 5a, 5d, 5e) the authors wrote "n=2", but included p values. How is it possible to perform statistics with n=2?

Response letter

Ms. No.: EMBOJ-2022-112446

A point-by-point response letter to all the comments by the Referees follows below (original comments are quoted in **bold**).

Referee #1:

On the one hand, the reported observations are certainly very interesting and relevant.

We thank the Referee for the encouraging comment.

… I do not agree with the interpretation of some of the experiments. In general, the manuscript pays too little attention to the molecular functions of the two different degradation complexes. In my view, some observations could be explained "simply" by the enzymatic activities of the decapping vs. the deadenylation complex. When deadenylation is inhibited, mRNAs will retain longer poly(A)-tails, which would explain why RNAi of ntl-2 leads to higher translation rates (Figure 4h). The opposite effect of the dcap-2 RNAi could be explained by the accumulation of deadenylated mRNAs, which are not decapped and degraded and may therefore be inefficiently translated and/or interfere with the translation of other mRNAs.

The Referee suggests that a more molecular/mechanistic/functional view will better interpret our results, in the whole manuscript. Towards this direction, the Referee suggests that some results could be linked to the enzymatic activities of NTL-2 and DCAP-2. We fully agree with the Referee's suggestion and we apologize for not having described this efficiently enough.

In fact, to interpret our results we are based on the known enzymatic activities of DCAP-2 and NTL-2. We have shown that *ntl-2* genetic inhibition increases global translation rates. Despite the fact that polyA tail length is transcript-dependent, it is shown that complete deadenylation leads to mRNA destabilization and subsequent decapping and degradation. Nevertheless, incomplete deadenylation triggers an intermediate phase acquisition, where the mildly deadenylated mRNAs are neither translated nor degraded, but stored in a quiescence state, through which they can re-enter translation if needed, or if not, they are finally driven to degradation (Weill et al., 2012). Also, it has been shown that the length of the mRNA polyA tail is positively correlated with its translation efficiency (Xiang and Bartel, 2021). As pointed out by the Referee, *ntl-2* genetic inhibition is expected to decrease the rates of deadenylation. *In vitro* deadenylase assays have shown that *ntl-2* genetic inhibition disrupts the deadenylase activity of the CCR-4/NOT complex, leading to mRNA stabilization and accumulation of proteins most likely due to increased translation rates (Ito et al., 2011a). On the other hand, we have shown that *dcap-2* genetic inhibition leads to decreased global mRNA translation rates. Previous studies have shown that decreased translation initiation is inversely correlated with mRNA decapping and degradation (Schwartz and Parker, 1999). It is worth noting that the molecular underpinnings of protein synthesis regulation and their interface with the mechanisms that influence ageing are challenging and complex and as yet not well understood. A recent study from our lab

showed that genetic inhibition of *edc-3*, which encodes another component of the decapping complex, triggers eIF4E/ IFE-2 sequestration and trapping within P-bodies, thereby reducing global translation rates (Rieckher et al., 2018).

In this study, we provide compelling evidence that the mRNA decapping and the deadenylation (CCR-4/NOT) complexes do not colocalize in P-bodies, as previously thought, but form distinct foci, the degradation and storage bodies, respectively. These two types of foci form independently of each other; they mutually antagonize each other and influence mRNA translation in an opposing manner. Moreover, the two types of foci also oppositely control mitochondrial abundance and alter mitochondrial function. Together, these findings highlight the importance of balanced mRNA storage and degradation mitochondrial homeostasis and organismal ageing. In conclusion, we will modify the text of our revised manuscript accordingly, so that our findings are more clearly interpreted, with regard to the enzymatic activities of the components examined.

Additional major points

1. Although it is already indicated in the abstract, the authors only reveal in Figure 4 that the ntl-2 and dcap-2 bodies are different entities. Before that, the two bodies are presented in parallel. However, from the very beginning one has the feeling that they are functionally not the same. Wouldn't it be better if the differences were made clear early on?

Indeed, we present the diversifying functional properties of the mRNA storage and degradation bodies in order to support the idea of their distinct identity. To this end, we chose to present the two types of bodies in parallel so as to conclude with their identification as different entities. If the approach suggested (i.e. first, introduce the two types of bodies as distinct entities and analyse the properties that support their discrete identity) will better contribute to the reader's understanding, we can re-organize the manuscript accordingly.

This brings me to the next question - what is the relation between the dcap-2 bodies, the ntl-2 bodies and canonical p-bodies? Are they overlapping? I am missing some counterstainings between different p-body components and dcap-2 and ntl-2 as well as between dcap-2 and ntl-2. In addition, the storage bodies and degradation bodies would need to be characterized in more detail.

The prevailing view is that P-bodies are mebraneless formations consisting of translationally silenced mRNAs and RNA binding proteins. It is also known that mRNA degradation mainly through the 5'-3' mRNA decay pathway occurs in P-bodies. In addition, the idea of mRNA storage within P-bodies is now becoming more and more appreciated although there is no direct experimental approach to show mRNA storage. This statement is mainly driven by the known functions of components bound on identified mRNAs.

The main steps of 5'-3' mRNA degradation are deadenylation-decapping and 5'-3' exonucleolytic decay. P-bodies are considered as dynamic formations with variable protein constituents. It is also accepted that components of the decapping and the CCR4/NOT complexes constitute main P-body components (Decker and Parker, 2012). While microscopy and structural analysis show that components of the decapping complex (EDC3/DCAP2/DCAP1/XRN1) colocalize or form a complex accordingly (Eulalio et al.,

2007, Charenton et al., 2016), there is no direct evidence showing that components of the decapping and the CCR4/NOT complexes colocalize within a single formation/granule, the P-body. Further, recent structural analysis has shown that DDX6/CGH-1 (one of the core components of P-bodies) physically associates primarily with the CCR4/NOT complex component CNOT1 to be activated. Also, DDX6 has been found to form mutually exclusive associations with Pat1, Edc3, Lsm14 or 4E-T. Moreover, by *in vitro* analyses, it has been shown that only 4E-T can concomitantly associate with DDX6 and CNOT1. Notably, they show that CNOT1 binding on DDX6 displaces the decapping complex component Edc3 in vitro, implying that in the presence of CNTO1, DDX6 does not associate with Edc3 (the same stands for Pat1 and Lsm14), unless it is in excess. Based on this evidence, the authors discuss the possibility that "different complexes that perform related functions could exist either in isolation or in combination in large mRNP granules" (Ozgur et al., 2015). Moreover, a recent report on P- body purification for the first time from human cell lines and analyses of their protein constituents, identified decapping complex components (DCP1, EDC3) as significantly enriched but it did not reveal a significant enrichment of the CCR4/NOT protein complex components within these (P-body) isolates (Hubstenberger et al., 2017). This again points towards the idea that the decapping and the CCR4/NOT protein complex components do not colocalise within a single formation/granule, the P-body, as was initially thought.

Another point that differentiates our results from already published data is that DDX6/CGH-1 depletion has been shown to lead to defective P-body formation. The same has been shown for CCR4/NOT protein complex components (Ito et al., 2011b, Chen and Shyu, 2013). Despite this, we find that *cgh-1* genetic inhibition cannot disrupt storage or degradation body formation (**Figure 1** and **Figure 2** in this letter) and also *ntl-2* genetic inhibition increases the abundance of DCAP-2(+) and EDC-3(+) foci (Figure 4e in the manuscript and **Figure 3** in this letter). Moreover, *dcap-2* genetic inhibition increases NTL-2(+) and CCF-1(+) foci (Figure 4e in the manuscript and **Figure 4** in this letter.)

Overall, while in the literature there are many references pointing towards the direction that deadenylation is needed for P-body formation and that the CCR4/NOT complex is a structural part of the P-bodies, there is no robust evidence in vivo that supports this. The above, in combination with recent structural analysis and P-body proteome analysis that do not support the physical presence of the decapping and deadenylation complex components within a single formation, the P-bodies, strengthen our findings that the decapping and the CCR4/NOT complex components form distinct foci in vivo. In our study we do not exclude the possibility that these distinct foci merge or associate upon certain conditions. To our knowledge, we show for the first time in vivo that components from the two complexes do form foci, which do not colocalise. For better annotation and based on the enzymatic activities of their constituents we name in our study the foci consisting of decapping protein complex components as "degradation bodies" and the foci that consist of CCR4/NOT protein complex components "storage bodies". Based on the literature described above with regard to DDX6/CGH-1 and our additional/new findings (provided below), we consider CGH-1 and CCR-4/NOT components as storage body-constituents and the decapping complex components together with the exonuclease XRN-1, constituents of the degradation bodies.

To conclude, considering the dynamic nature of P-bodies and the variability of their protein constituents we believe that the mRNA decapping and the deadenylation (CCR-4/NOT) complexes function in two discrete types of bodies (or P-bodies), the degradation and storage bodies accordingly. Despite this, we cannot exclude the possibility that these two types of bodies share components. In addition, we find that

the mRNA decapping and the deadenylation (CCR-4/NOT) complex components do not usually colocalize. Instead, they form distinct foci whose components are differentially expressed. In fact, Fig. 4A (in the manuscript) shows the differential localization pattern of EDC-3::DsRed (EDC-3 is a component of the mRNA degradation pathway, like DCAP-2) and NTL-2::GFP bodies in the hypodermis, which is further supported by the Pearson's correlation coefficient as shown in Fig. 4B (in the manuscript).

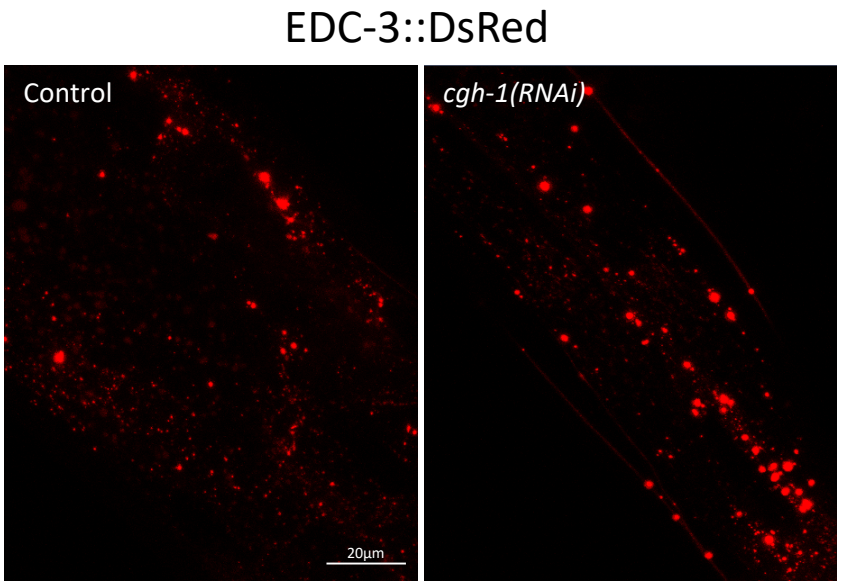

Figure 1. Genetic inhibition of *cgh-1* does not inhibit the formation of EDC-3(+) foci in *C. elegans*.

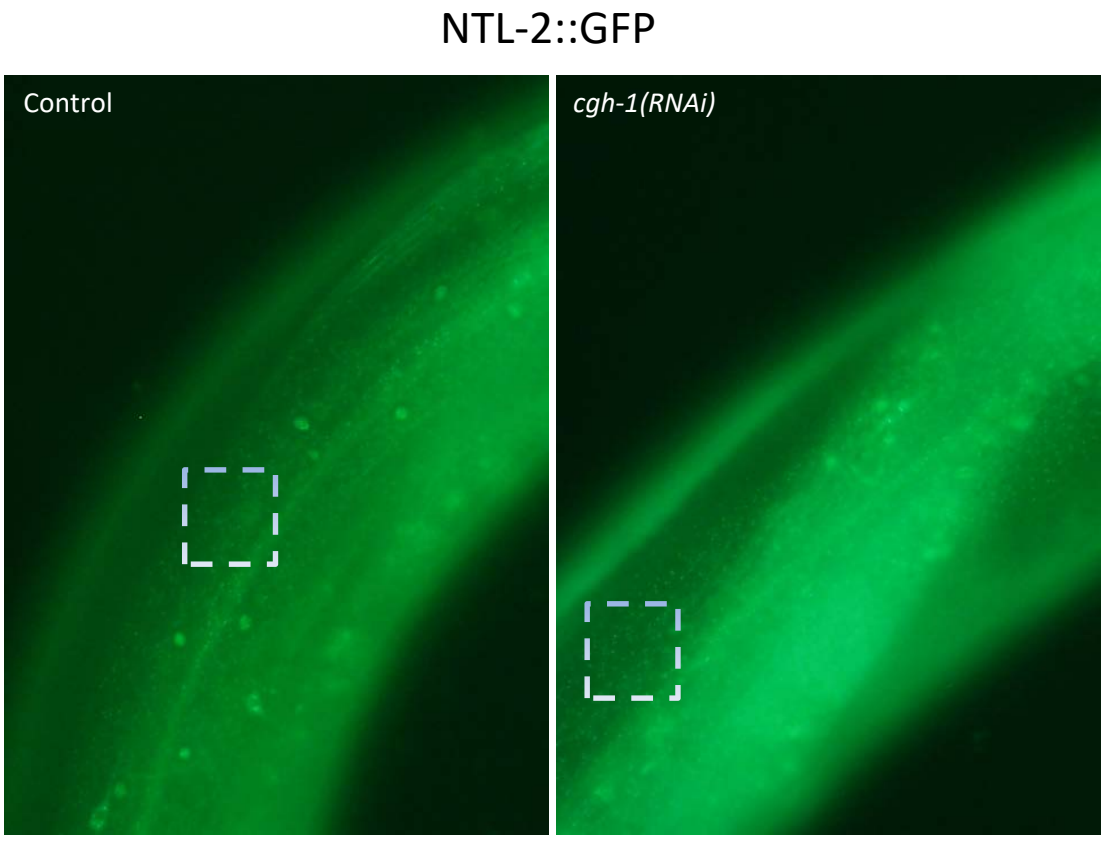

Figure 2. Genetic inhibition of *cgh-1* does not inhibit the formation of NTL-2(+) foci.

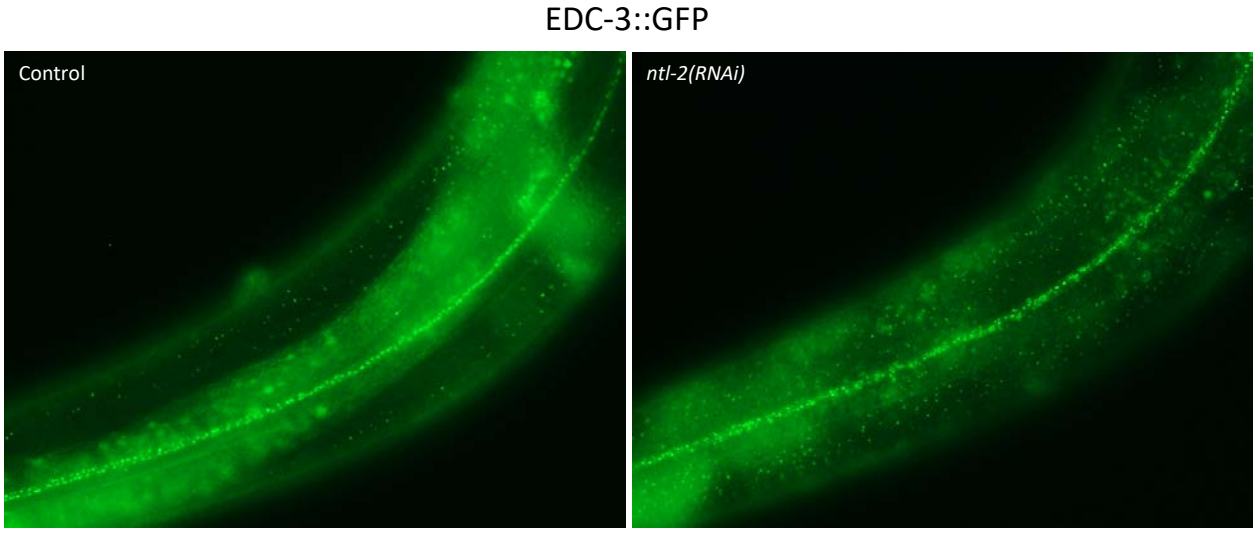

Figure 3. *ntl-2* genetic inhibition triggers an elevation of EDC-3(+) foci.

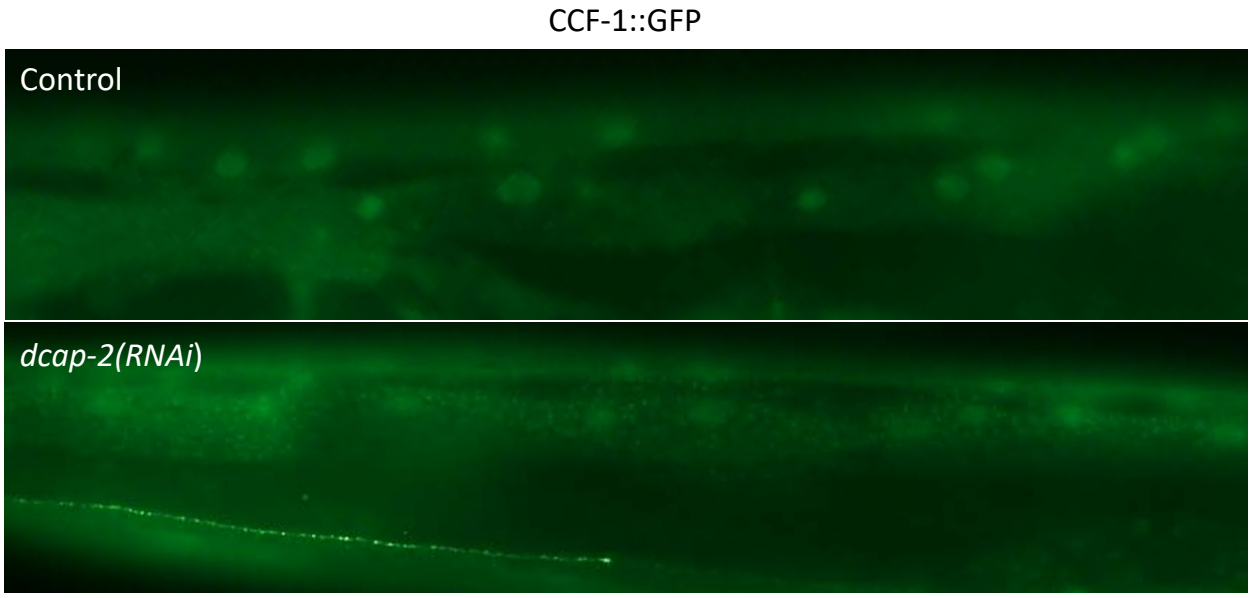

Figure 4. Genetic inhibition of *dcap-2* increases the number of CCF-1(+) foci.

Moreover, we have created the p_{ntl-2}NTL-2::DsRed construct, so we can generate transgenic animals that coexpress NTL-2::DsRed with EDC-3::GFP, NTL-2::GFP with DCAP-2::mCherry, NTL-2::DsRed with CCF-1::GFP and also animals that co-express DCAP-2::mCherry with EDC-3::GFP and with CCF-1::GFP. Furthermore, we can quantify the storage and degradation body abundance upon genetic inhibition of additional components from both formations/foci.

Overall, we believe that co-monitoring of all the above-mentioned combinations of proteins and also monitoring storage and degradation body abundance upon genetic inhibition of several factors belonging to both types of bodies will give a more detailed characterization of storage and degradation bodies, so as to satisfactorily address the Referee's comment.

Minor points

How is the quantification done, e.g. in Figure 1e, 1l, 1m? According to the figure legend three experiments were quantified, so where are the error bars?

We will include error bars in Figure 1e, 1l, 1m. We apologize for any inconvenience this may have caused.

In figure 1e the different classes are "0 µM", "<=1 µM", "0-1 µM" and ">5 µM". The second and third are the same, so I guess there is some mislabelling here?

We thank the Referee for pointing this out. We are sorry for the inconvenience. In the revised manuscript we will correct the mislabelling in the category “0-1 M which should become 1- .

Why are there no error bars in figure 4h?

Figure 4h shows one representative experiment out of 3 biological independent experiments performed with the same results. In the revised manuscript we will merge the experiments and provide the graph with error bars. We apologize for any inconvenience this may have caused.

Wouldn't it be better to show all primers in a table instead of in the text?

We have already provided a detailed table summarizing all primer sequences used in this study up to now at the end of the manuscript. We thank the Referee for pointing this out. In the revised manuscript, we will omit the primer sequences from the text and show them all only in the table.

Referee #2:

…... These are certainly novel and very interesting findings in the mitochondria and aging biology field, which when substantiated by additional data (see below) will deserve publication in the EMBO Journal.

We thank the Referee for the encouraging comments.

However, based on the described data, the overall conclusion drawn by the authors that "Local coordination of mRNA storage and degradation near mitochondria modulates C. elegans ageing" seems slightly overstated. Indeed, they clearly showed that some proteins belonging to mRNA-transcripts storage and degradation complexes, are localized near mitochondria and their suppression regulates mitochondrial content and aging. However, to be able to relate these findings to the complex (rather than to the single proteins), data should be provided with additional proteins belonging to the complexes.

We now have further evidence showing that additional components from the two types of bodies physically associate with mitochondria (Figures 5 and 6 in this response letter). Moreover, we show that their genetic knockdown alters mitochondrial network integrity (Figure 7 in this response letter) and mass in the same (and opposing) manner, consistent with the components already presented in our submitted manuscript (Figure 8 and 9 in this response letter). Also, consistent with the already presented data about *dcap-2* and *ntl-2*, genetic inhibition of additional components also alters mitochondrial function (Figure 10 and 11 in this response letter).

Moreover, we now have additional data from lifespan experiments showing that more components from the two types of bodies oppositely affect longevity (Figure 12 in this response letter). Consequently, we are confident that our new data will robustly support the notion that mRNA degradation and storage complexes oppositely regulate mitochondrial content and whole organism ageing. Hence, the major conceptual point of our manuscript will remain unaffected.

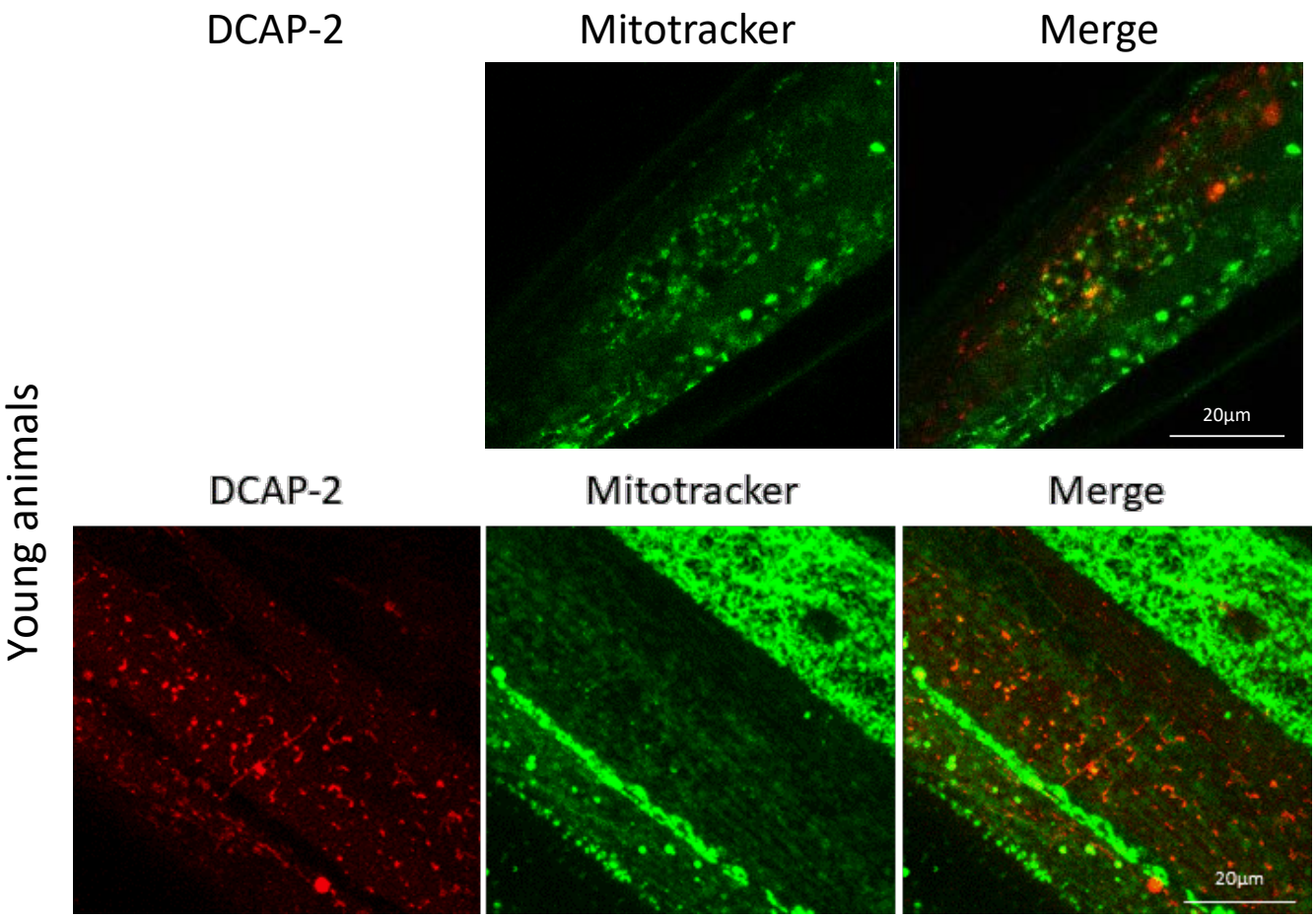

Figure 5. Confocal images showing that majority of the DCAP-2(+) foci localize very close to mitochondria.

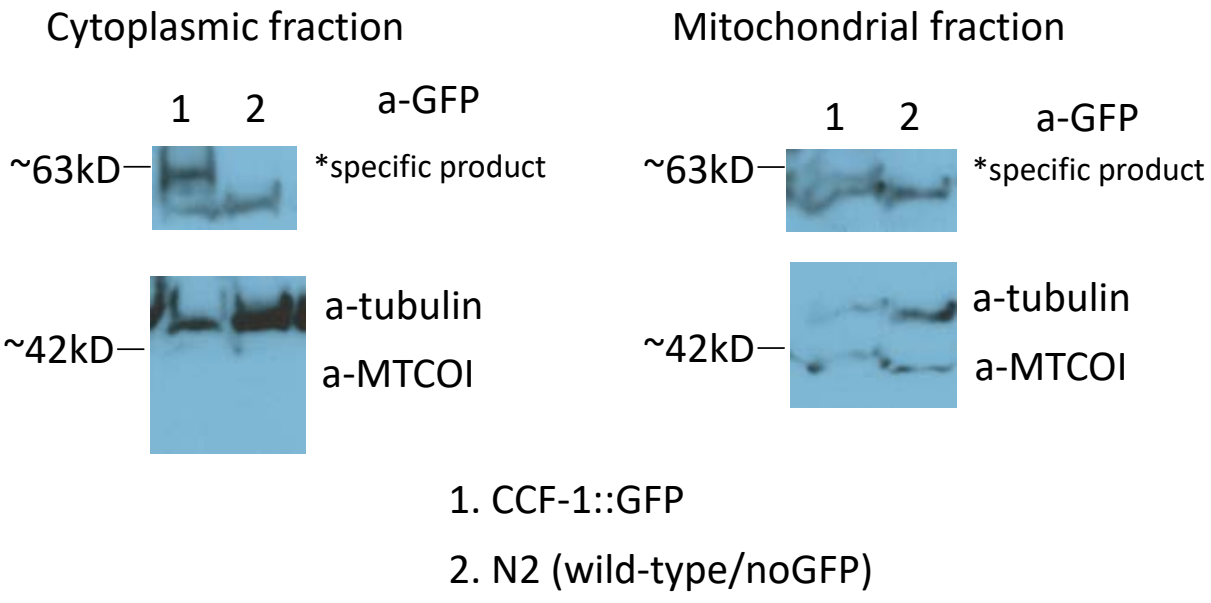

Figure 6. Western blot data showing that CCF-1 (a CCR4/NOT complex component) co-fractionates with mitochondria.

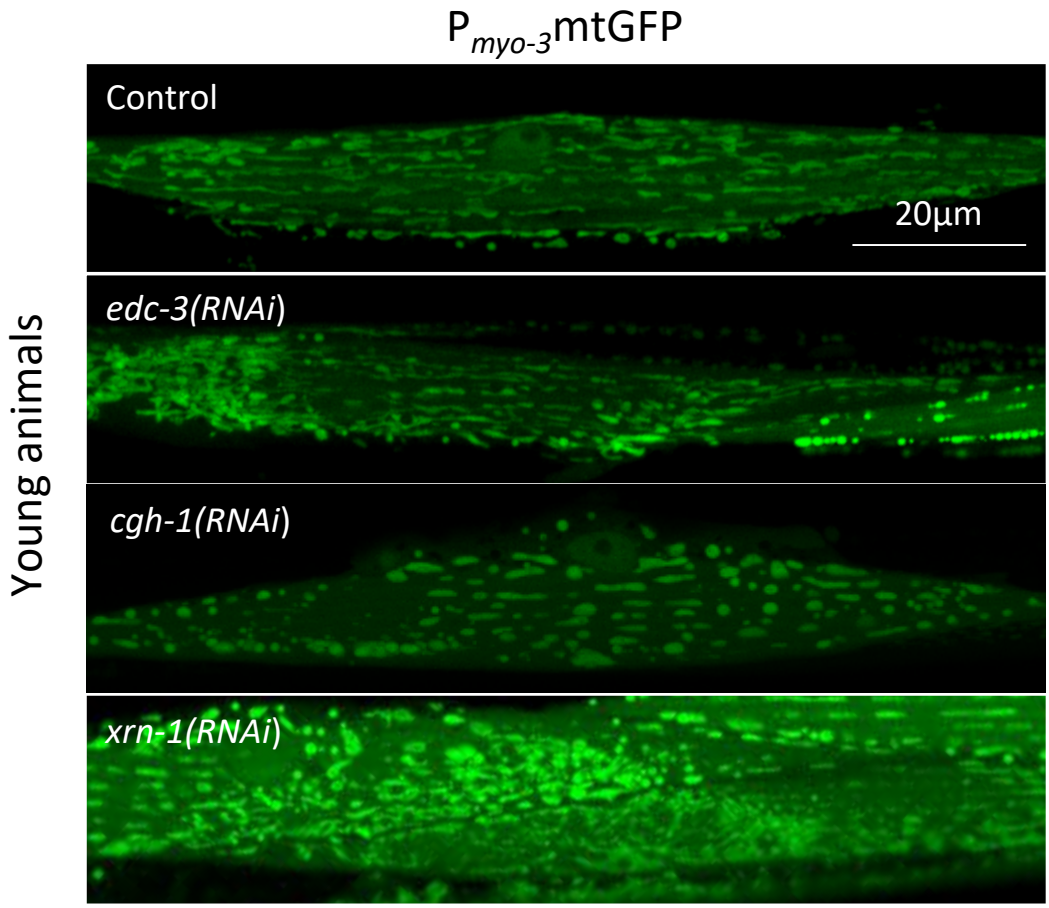

Figure 7. Confocal images showing the effect the genetic inhibition of various storage and degradation body components on the mitochondrial network integrity in *C. elegans* body wall muscle cells.

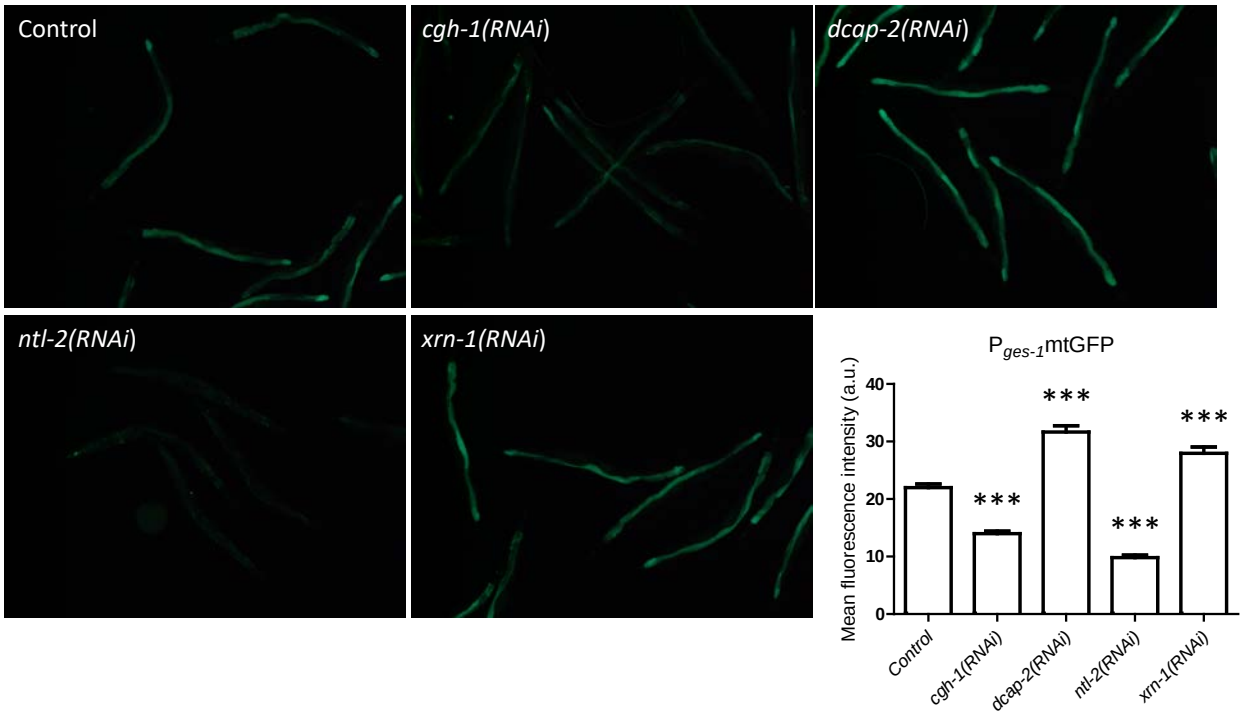

Figure 8. Epifluorescence images showing the effect the genetic inhibition of various storage and degradation body components on the total abundance of mitochondria in the intestine.

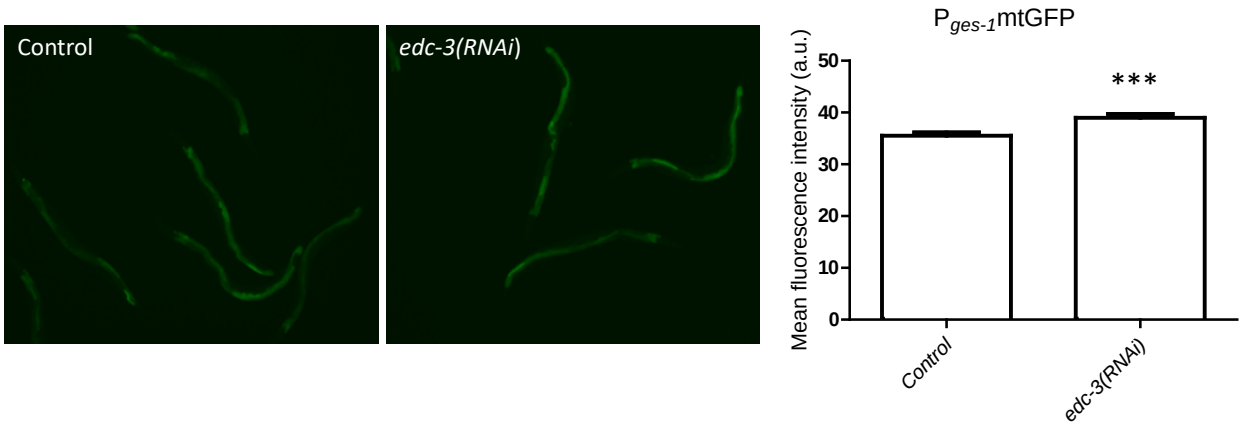

Figure 9. Genetic inhibition *of edc-3* increases total intestinal mitochondrial abundance.

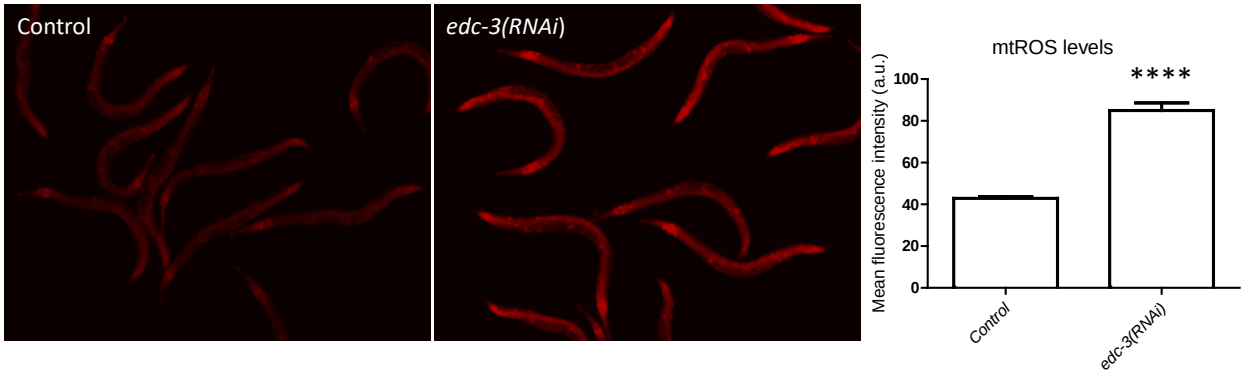

Figure 10. Genetic inhibition of edc-3 increases total mtROS levels.

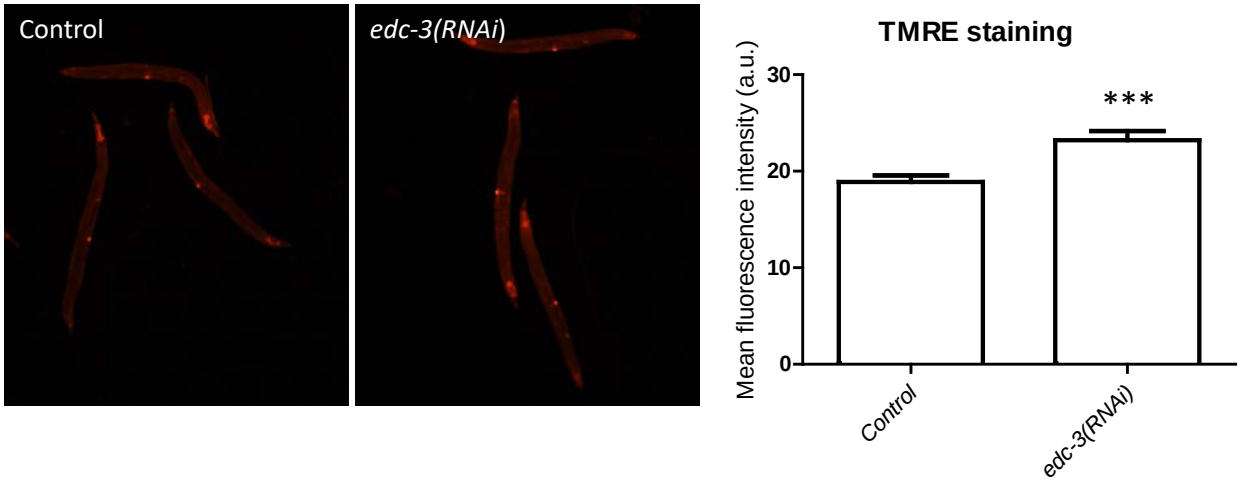

Figure 11. Genetic inhibition of *edc-3* increases mitochondrial membrane potential.

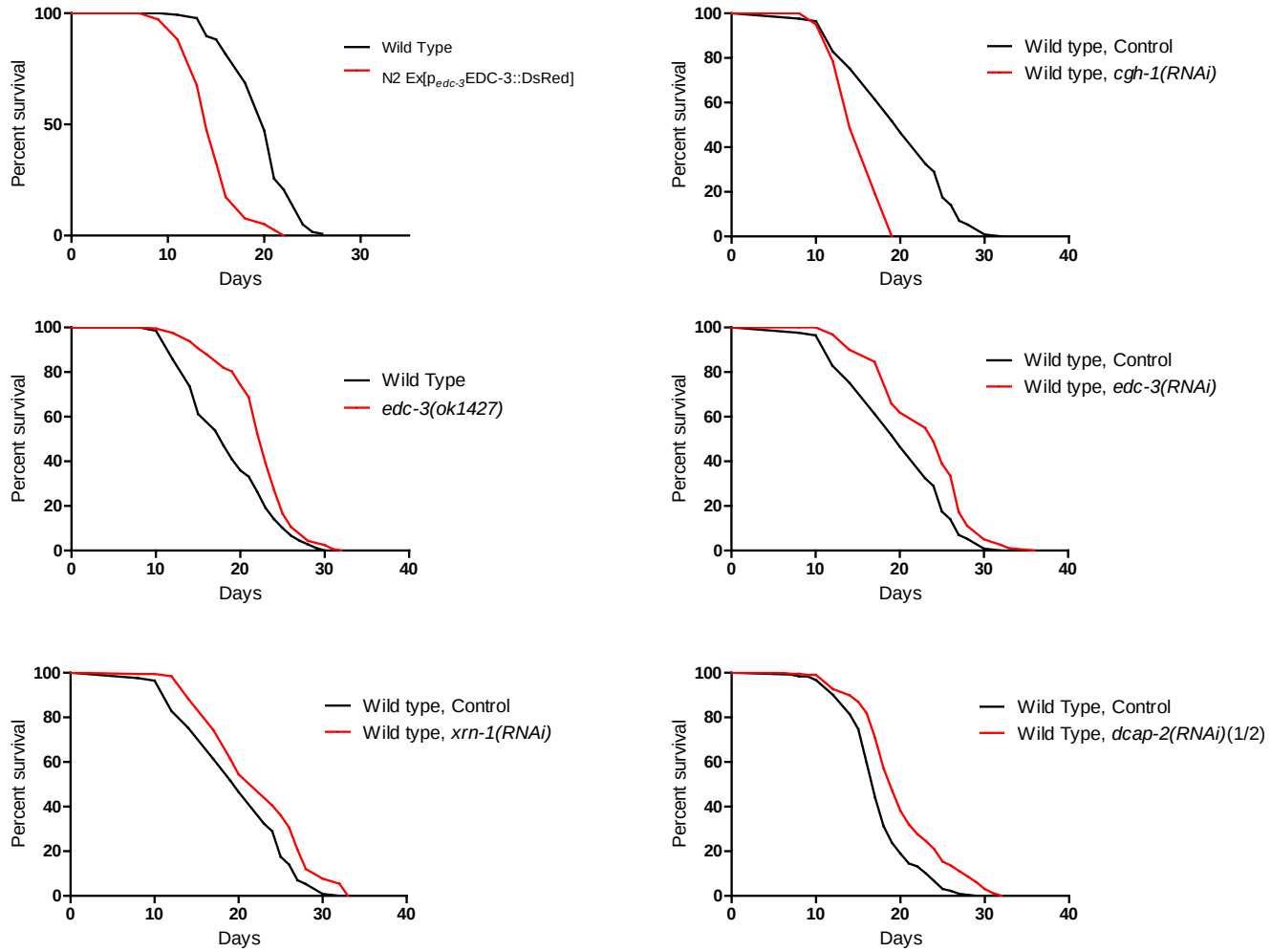

Figure 12. Lifespan assays showing the opposite effects of storage and degradation body components on longevity (in pages 5-6 of the manuscript, we describe the components of storage and degradation bodies in more detail). Please, also see our responses to Referee #3.

Moreover, they did not provide any evidence that the "coordination of mRNA storage and degradation", rather than simply the reduced expression of some of their components, is actually the mechanism underlying their effects on stress response letter and aging.

Our findings indicate that coordination of mRNA storage and degradation modulates ageing. Indeed, in the manuscript, we provide data showing that perturbation of one component (we are now testing additional components; see Figures 3 and 4 of the response letter) from each type of body triggers an increase in the abundance of components from the other type and this impacts ageing.

For example, we show that *dcap-2* genetic inhibition triggers elevation of NTL-2 bodies and *vice versa* (Fig. 4e of the manuscript). Also, we now have additional data showing that, similarly to DCAP-2 bodies, *ntl-2* genetic inhibition triggers an elevation of EDC-3 bodies (Figure 3 response letter). Similarly, *dcap-2* genetic inhibition triggers increased number of CCF-1 foci, similarly to the NTL-2 foci (Figure 4 response letter). Moreover, knockdown *of dcap-2* or of other degradation body genes increases stress resistance compared to control RNAi in wild –type nematodes ((Rieckher et al. 2018, Cell reports and Fig. 6a-c of the manuscript). Furthermore, NTL-2 overexpression (mimicking *dcap-2* genetic inhibition) extends

lifespan, in contrast to *ntl-2* genetic inhibition (Fig. 6e of the manuscript). We now plan to test the effects of combinatory interventions in the two types of foci, on lifespan and stress resistance so as to highlight the crucial role of the coordination of mRNA storage and degradation processes in longevity. For example, we will test whether perturbation of degradation bodies can further extend the lifespan and alter stress resistance of NTL-2 overexpressing animals.

Overall the investigated topic is extremely interesting and of timely importance but in the current state the conclusions are overstated.

We thank the Referee for the appreciation of our work. We have now designed additional experiments to strengthen the conclusions drawn. We will rephrase the text accordingly to avoid overstatements and improve its organization so that every part contributes to the reader's understanding of the central idea.

The mRNA decapping and the CCR-4/NOT complex physically and functionally associate with mitochondria in an age-dependent manner. In this first part the authors show the association of mitochondria only with one component of each complexe, NTL-2 and EDC-3. Additional proteins belonging to the complexes (CCF-1, CCR-4, DCAP-2...) should be tested to validate if the complexes (and not simply NTL-2 and EDC-3) actually physically and functionally associate with the mitochondria.

We are now providing further evidence for the physical and functional association of additional proteins belonging to mRNA degradation and storage complexes with mitochondria (Figures 5 and 6 of the response letter). (These experiments will be repeated so that better image quality is ensured).

On the other hand, to be able to conclude that alteration of mitochondrial function impact on the expression and association of mRNA-complex-components with mitochondria (figure 1j,n,m), the effect of silencing mitochondria genes not directly related to gene translation should be assessed (mrps-5 is in fact directly affecting ribosomes and thus obviously mRNA regulatory complexes). This is especially in light of their observation that instead atp-3 RNAi (Figure S1) does not impact EDC-3 association with mitochondria.

Our data show that alterations in mitochondrial function can dynamically alter the abundance and the associations of storage and degradation bodies with mitochondria. Indeed, our data show that this association is complex and depends on the intervention we perform. This, we believe, is a very interesting finding that further highlights the specificity of the functional associations formed between storage and degradation bodies with mitochondria. To avoid any further confusion, we will explicitly describe this in the text so as to make clear that not each and every perturbation in the mitochondrial function is expected to affect the abundance and associations of storage and degradation bodies with mitochondria in the same manner.

Nevertheless, we show that perturbation of mitochondrial function following paraquat treatment affects the number and association of EDC-3 bodies with mitochondria. In line with this, a previous study showed that knockdown of *eat-3* encoding a mitochondrial dynamin family member homologous to human OPA1, which is essential for resistance of *C. elegans* to free radicals (Kanazawa et al., 2008), caused a substantial increase in DCAP-1:: DsRed bodies during adulthood (Rieckher et al, Cell reports, 2018).

To complement these findings, we could set up experiments to test whether knockdown of other mitochondrial genes not directly related to gene translation (e.g. *drp-1* RNAi) has an effect on the expression and association of mRNA -complex-components with mitochondria.

From Figure S1 it is not clear if the physical association between NTL-2- and EDC-3-containing bodies is actually reduced with aging. In S1a it is very hard to conclude that the association between mitochondria and NTL-2 is lost with aging given that the mitotraker staining (in which tissue?) is completely absent. Given that the structure and dynamics of mitochondria dramatically change during aging (e.g. Regmi et al. 2014; Palikaras et al. 2015), the authors should consider to repeat the experiment using for instance TOMM-20 strain or TMRE used in Figure 1 and possibly at different age and not only in 15 days old animals. Alternatively to be able to use the mtGFP expressing strain, they could cross it with a NTL-2::red reporter. Similarly, to establish that EDC-3-mito association is lost with age, time course experiments to better visualize mitochondria at earlier time points (e.g. 5 and 11 days) might actually help.

In this study, we find that NTL-2(+) (and CCF-1(+) puncta are significantly decreased in aged animals (Figure S1A and Figure 4g in the manuscript). As shown in Figure S1a, there is some remaining signal in the hypodermis of old worms but this is mostly diffused. Based on these observations, we claim that NTL-2(+) foci exhibit decreased associations with mitochondria.

We now provide additional, lower magnification images with increased brightness and contrast. We hope these images provide further support to our claim (Figure 13, response letter).

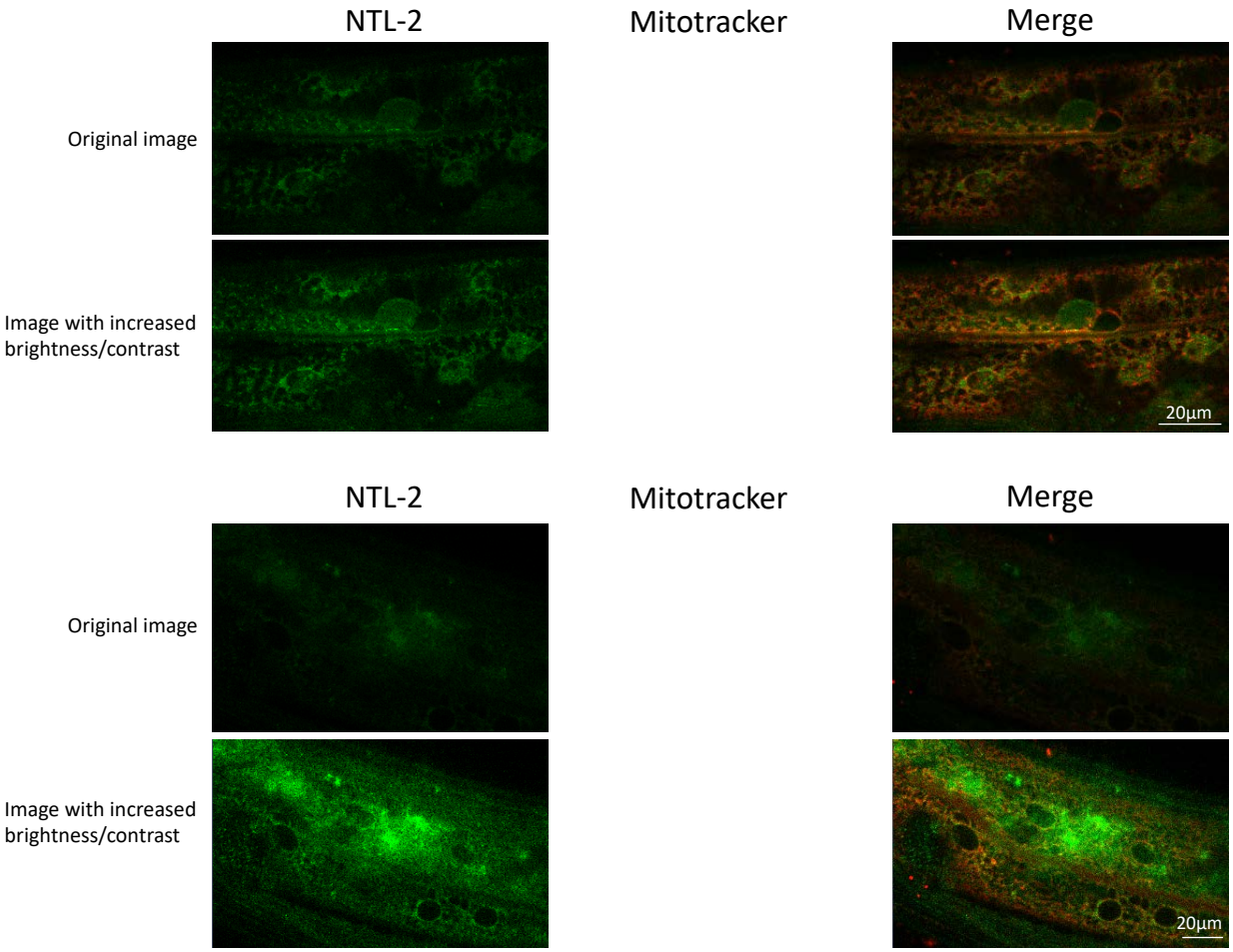

Figure 13. NTL-2 expression relative to Mitotracker fluorescence signal in mitochondria of day 15 animals. Additionally, we plan to perform time course experiments to monitor the expression levels and association of storage and degradation body components with mitochondria at defined time points during life.

It is not clear why the authors, to exclude that altered association during aging is ascribed to abnormal mitochondrial structure/dynamics, choose to silence atp-3 (a subunit of the ATPase which would primarily impact on mitochondrial function) rather than genes directly involved in mitochondrial fusion/fission.

In principle, our aim is to show that loss of contacts between EDC-3- bodies (mRNA degradation bodies) and mitochondria is not simply a corollary of the ageing process, which is accompanied by deterioration of mitochondrial network and function. Indeed, we show that *atp-3* genetic inhibition triggers a severe loss of the mitochondrial network integrity and despite this, the associations of NTL-2(+) foci with mitochondria are not disrupted. This finding further strengthens our results indicating that the associations of storage bodies with mitochondria are local translation-dependent (as unlike *atp-3* genetic inhibition, inhibition of either *tomm-20* or *akap-1* disrupt the associations of storage bodies with mitochondria). Regardless and to further strengthen our results, we plan to test the effects of *drp-1* or *fzo-1/eat-3* RNAi, which are involved in mitochondrial fission and fusion processes, respectively on degradation and storage bodies.

Most importantly, to corroborate the conclusion that mitochondria association with mRNA storage and degradation regulatory proteins is affected during aging, their association should be check in long- and short-lived mutants.

We plan to test the association of the two body types with mitochondria in short and long-lived animals, as suggested by the Referee. We will perform these experiments by RNAi silencing of lifespan-regulatory genes that do not directly affect mitochondrial function. In addition, we will perform time-course experiments in wild type genetic background showing the kinetics of the association of the two types of bodies with mitochondria during aging, in vivo. We believe that these experiments will effectively address the relevant issue raised by the Referees.

We already have data showing that the number of NTL-2(+) foci is increased in animals subjected to *daf-2* RNAi (Figure 14 of the response letter). Also, NTL-2 abundance is increased in animals subjected to *daf-2* RNAi compared to control, as evidenced by the increased GFP signal (Figure 15 of the response letter). By contrast, the abundance of mRNA decapping components such as DCAP-2::mCherry foci and EDC-3::GFP decreases upon *daf-2* knockdown (Fig. 16 and 17 of the response letter).

Together, these findings further support the notion that there is a functional relationship between degradation and storage bodies and this is linked to ageing modulation.

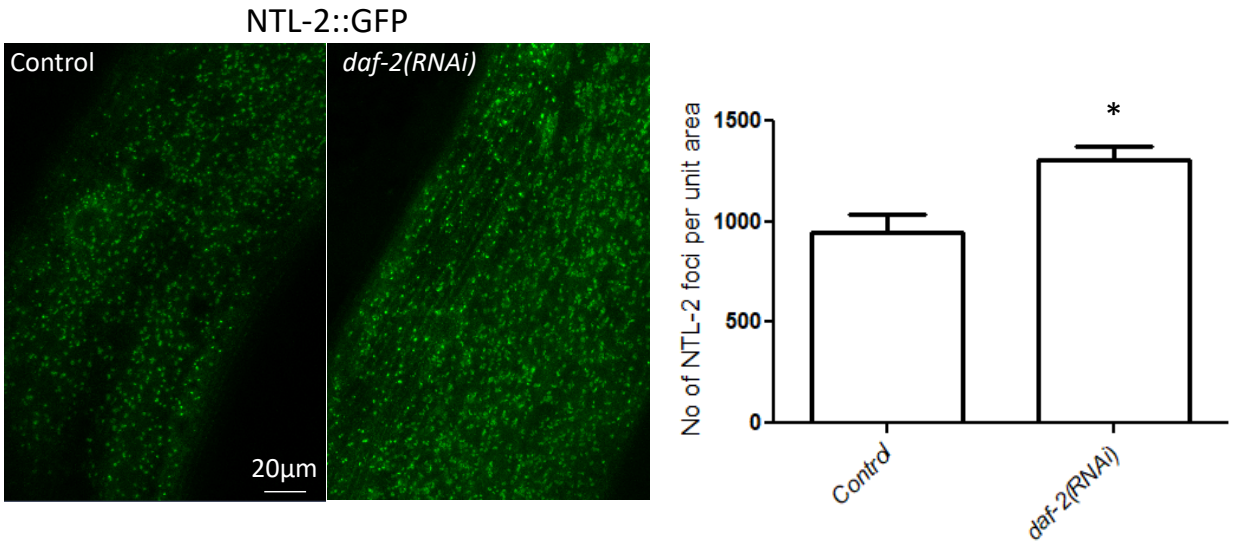

Figure 14. The number of NTL-2(+) foci is increased upon *daf-2* genetic inhibition. Representative confocal images and quantification of NTL-2(+) in animals subjected to *daf-2* RNAi compared to animals fed with control RNAi.

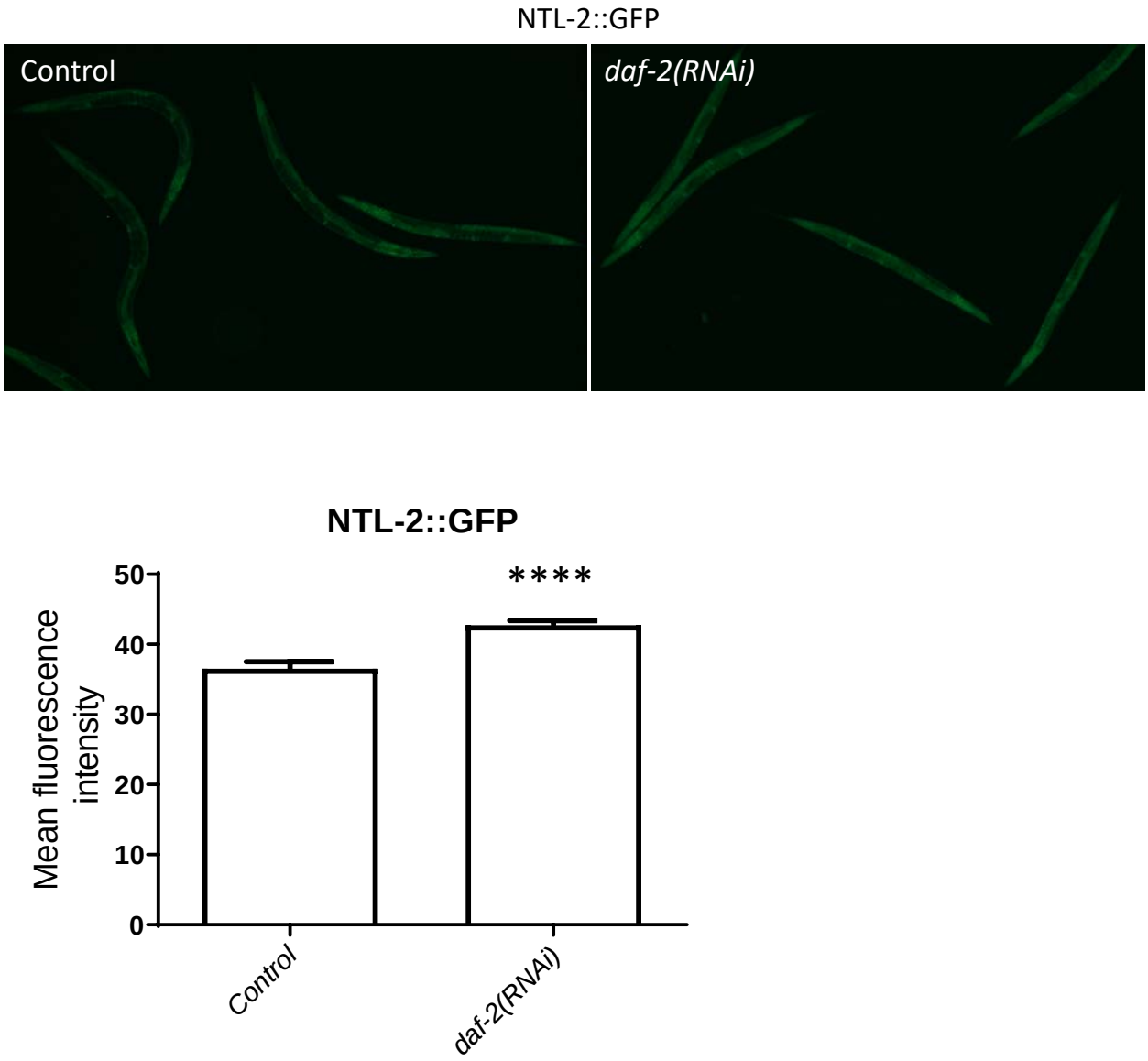

Figure 15. NTL-2 protein levels are increased upon *daf-2* genetic inhibition. Epifluorescence images of transgenic animals expressing the NTL-2::GFP translational reporter upon *daf-2* knockdown and quantification of GFP signal in these animals compared to age-matched controls.

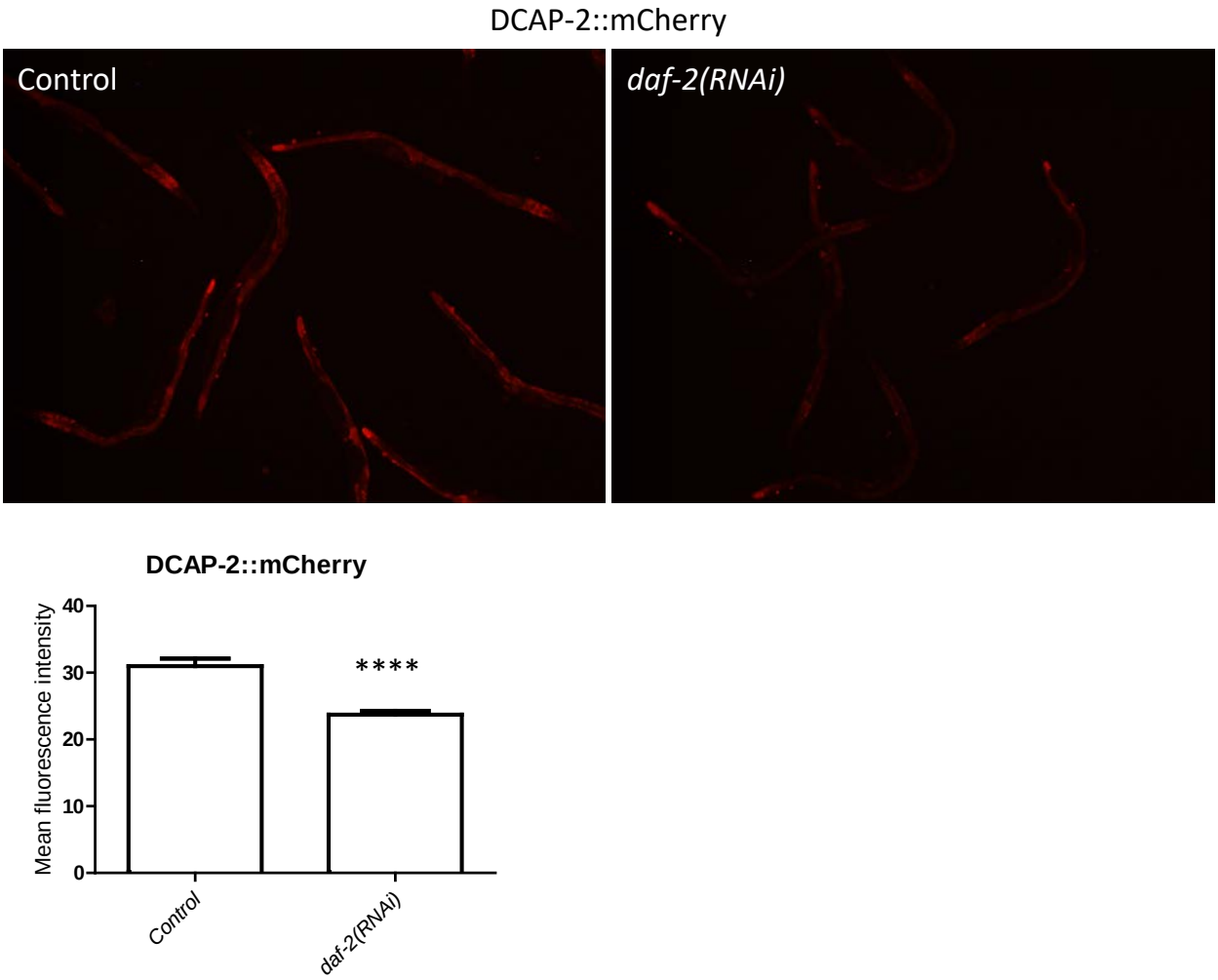

Figure 16. DCAP-2 protein levels are decreased upon *daf-2* genetic inhibition.

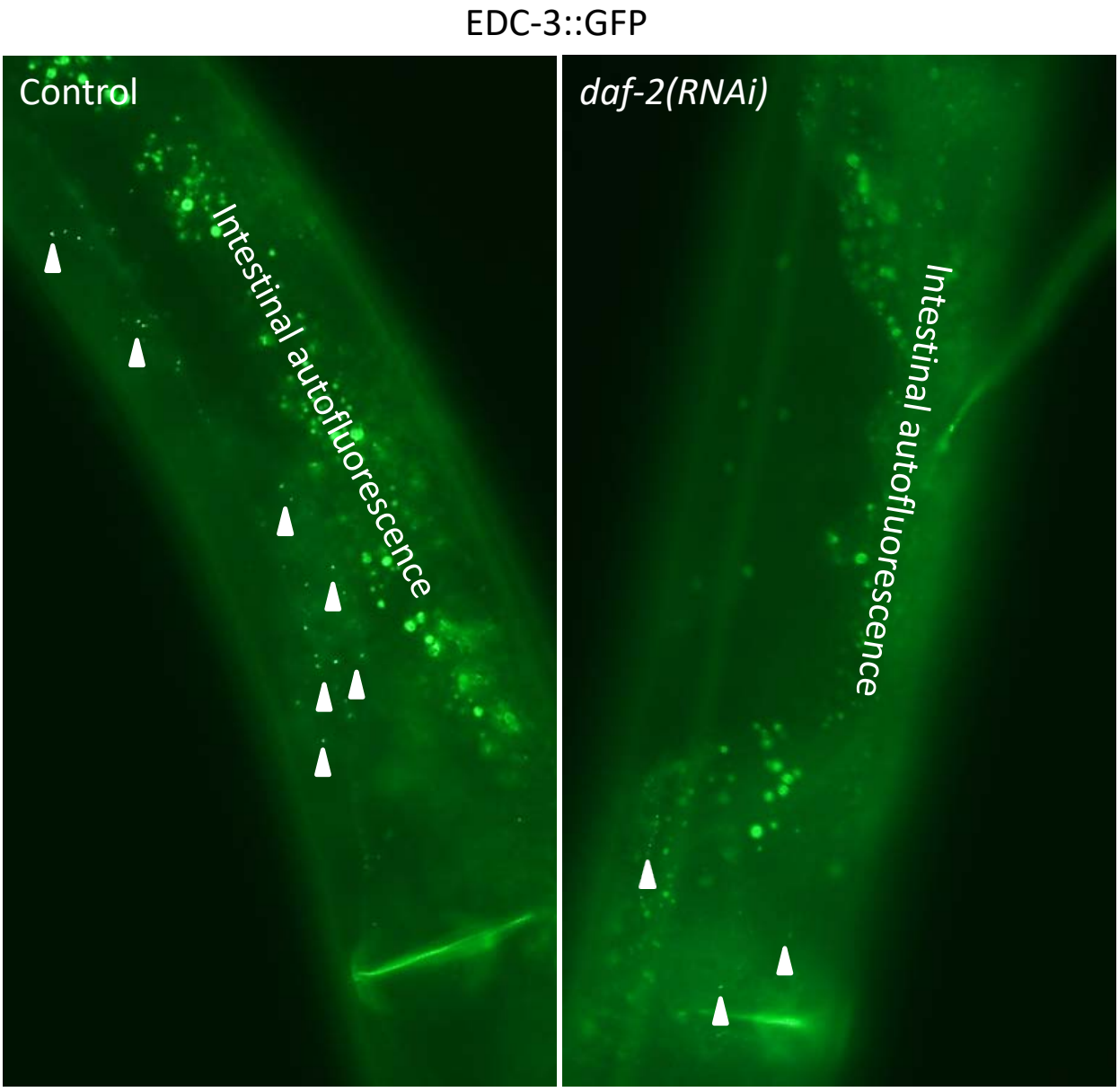

Figure 17. The number of the EDC-3 (+) foci is decreased upon *daf-2* genetic inhibition.

Incidentally, the authors used RNAi against atp-3 (Figure S1) and mrps-5 (Figure 1j, m, n), which are known to extend C. elegans lifespan (Dillin et al. 2002; Rea et al. 2017; Houtkooper et al 2013), and showed that the former does not affect mito-ECL-3 association while the latter increase its expression yet seems to reduce its association with the mitochondria. How do the authors reconcile these opposite effects with two pro-longevity interventions? Moreover, if as they suggest, ECD-3-mito association is lost with aging (Figure S1), wouldn't be expected that pro-longevity intervention increase their association? Again, time course experiments during aging in long- and short-lived mutants might help clarifying on this important point of the study.

We find that EDC-3 bodies are increased in number but their association with mitochondria is reduced upon *mrps-5* RNAi. The finding that the amount of EDC-3 associated with mitochondria, but not total EDC-3, is reduced upon *mrps-5* RNAi suggests that mito-localization of EDC-3 is regulated by *mrps-5* that encodes a ribosomal subunit involved in translation. Following the Referee's suggestion, we plan to perform

time course experiments in long- and short-lived mutants during aging to provide further clarification on this point.

mRNA decapping and CCR-4/NOT complex components oppositely regulate mitochondrial biogenesis and abundance by functioning in discrete foci. The authors show that dcap-2 and ntl-2 differentially impact on mitochondrial morphology and mass. What is the rationale for switching to dcap-2 instead of also using ecd-3 RNAi? What is the effect of ecd-3 RNAi on the different mitochondrial-related parameters (morphology, ROS and TMRE, SKN-1 and AAK-2 activation)? What is instead the effect of dcap-2 on mitochondrial-complexes physical association? Validating the findings with suppression of different proteins belonging to the same (storage and degradation) complexes, would support the conclusion.

The rationale for switching to *dcap-2* RNAi instead of *edc-3* RNAi is just to examine the effects of another degradation body component on mitochondrial-related parameters in the context of the central idea of this study. We now provide additional data showing the effects of *edc-3* knockdown on various mitochondrial-related parameters (Figures 18-23 of the response letter). We hope these results address the Referee's comment.

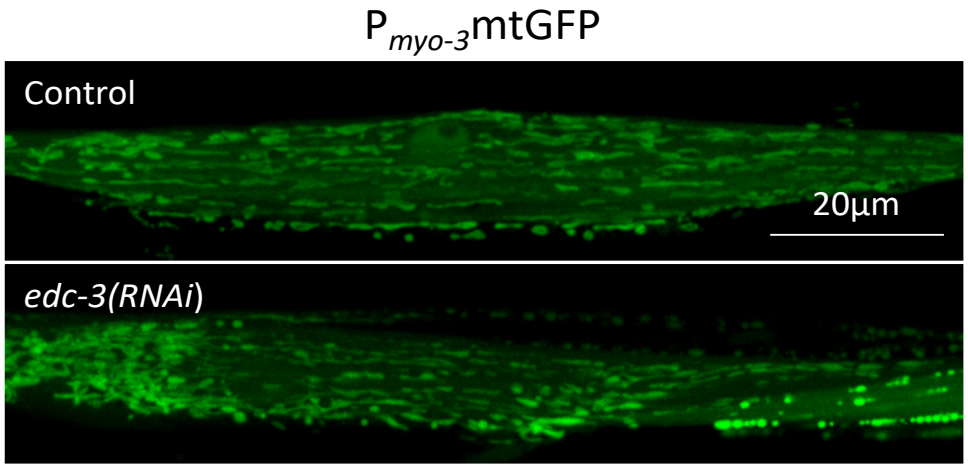

Figure 18. Genetic inhibition of *edc-3* alters mitochondrial network in body wall muscle cells.

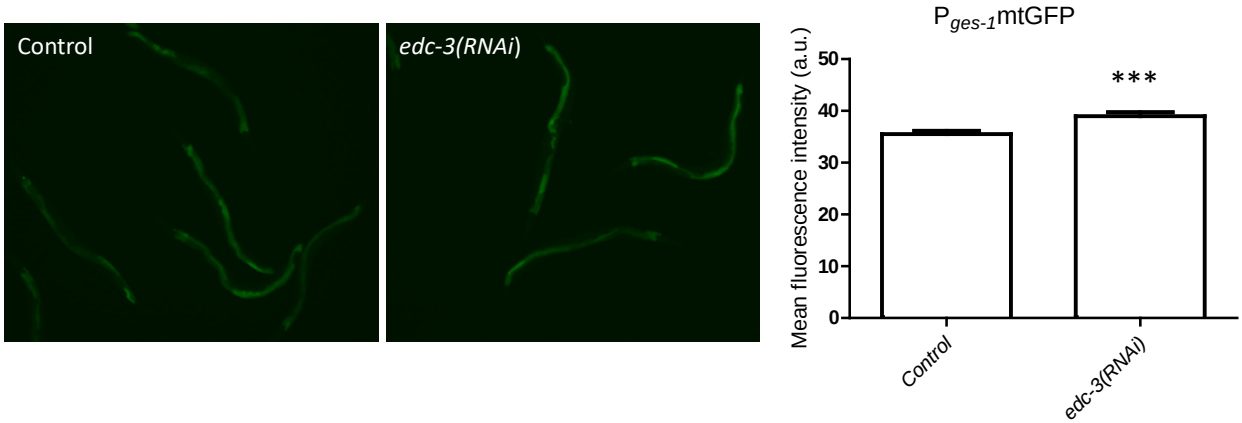

Figure 19. Genetic inhibition of *edc-3* increases total intestinal mitochondrial abundance.

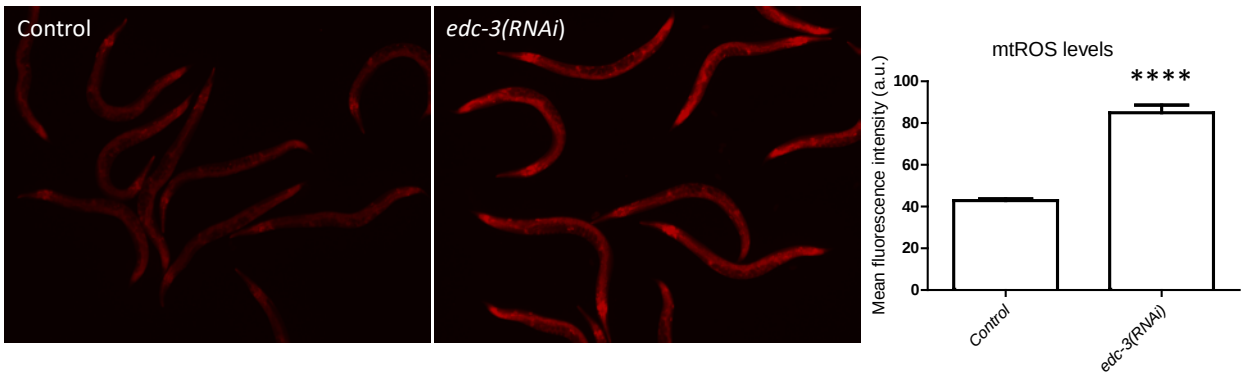

Figure 20. Genetic inhibition of *edc-3* increases total mtROS levels.

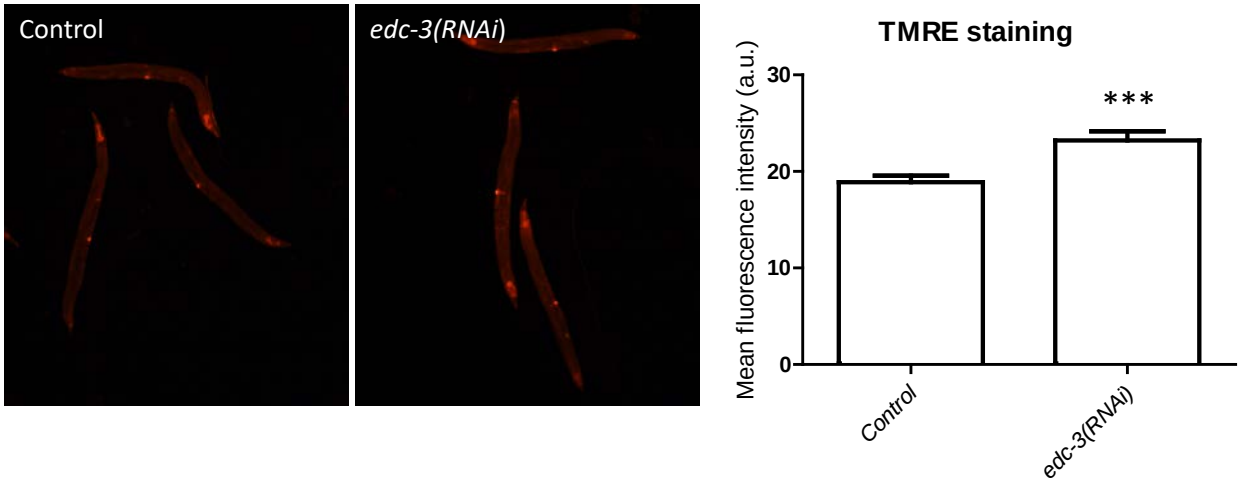

Figure 21. Genetic inhibition of *edc-3* increases mitochondrial membrane potential.

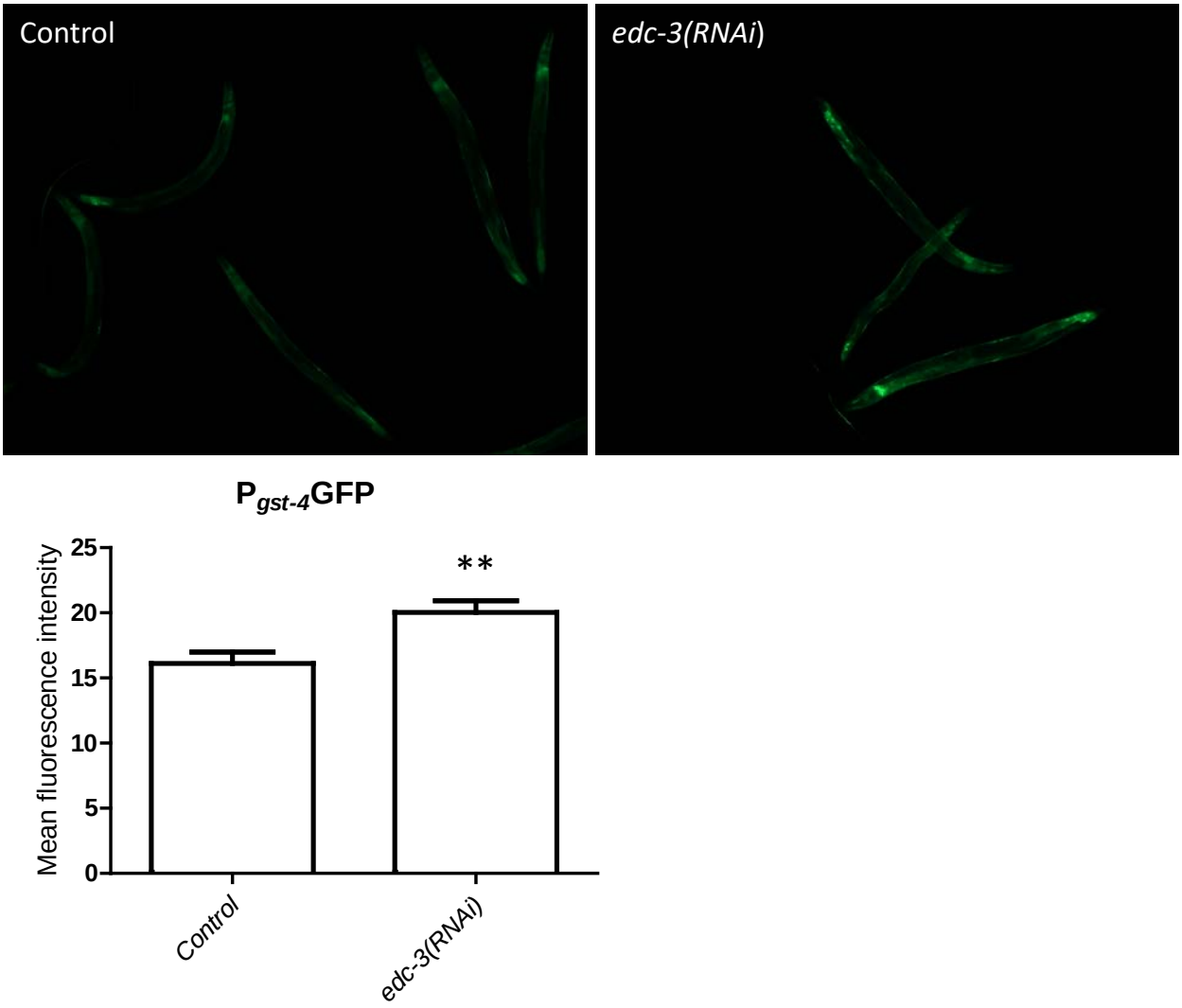

Figure 22. Genetic inhibition of *edc-3* induces the expression of the SKN-1 target gene *gst-4*.

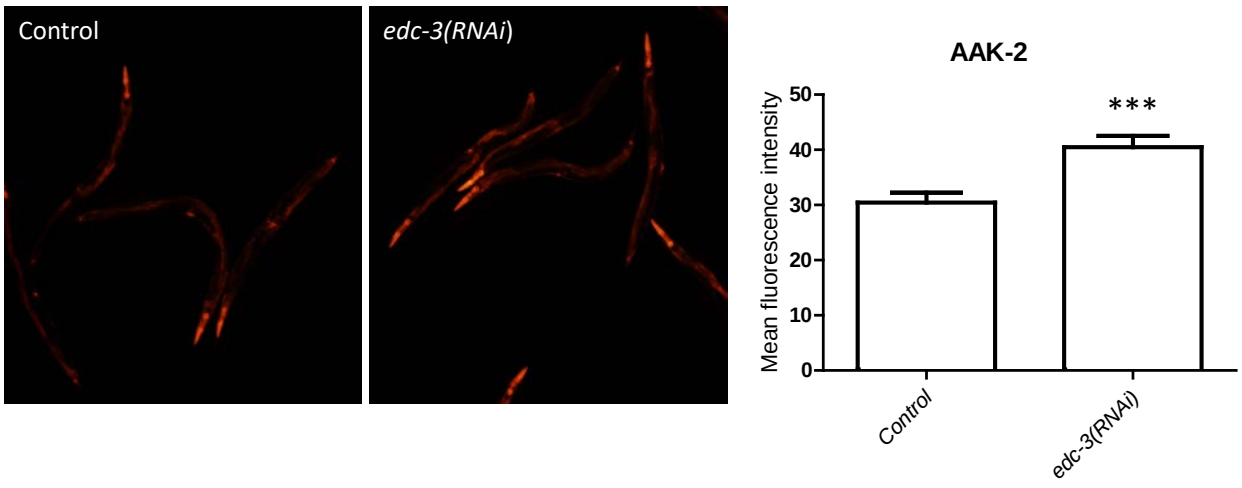

Figure 23. Genetic inhibition of *edc-3* increases AAK-2 total protein levels.

Furthermore, we provide additional preliminary data (below) showing the effect of additional storage and degradation body components on various mitochondrial parameters (Figures 24-28 of the response letter).

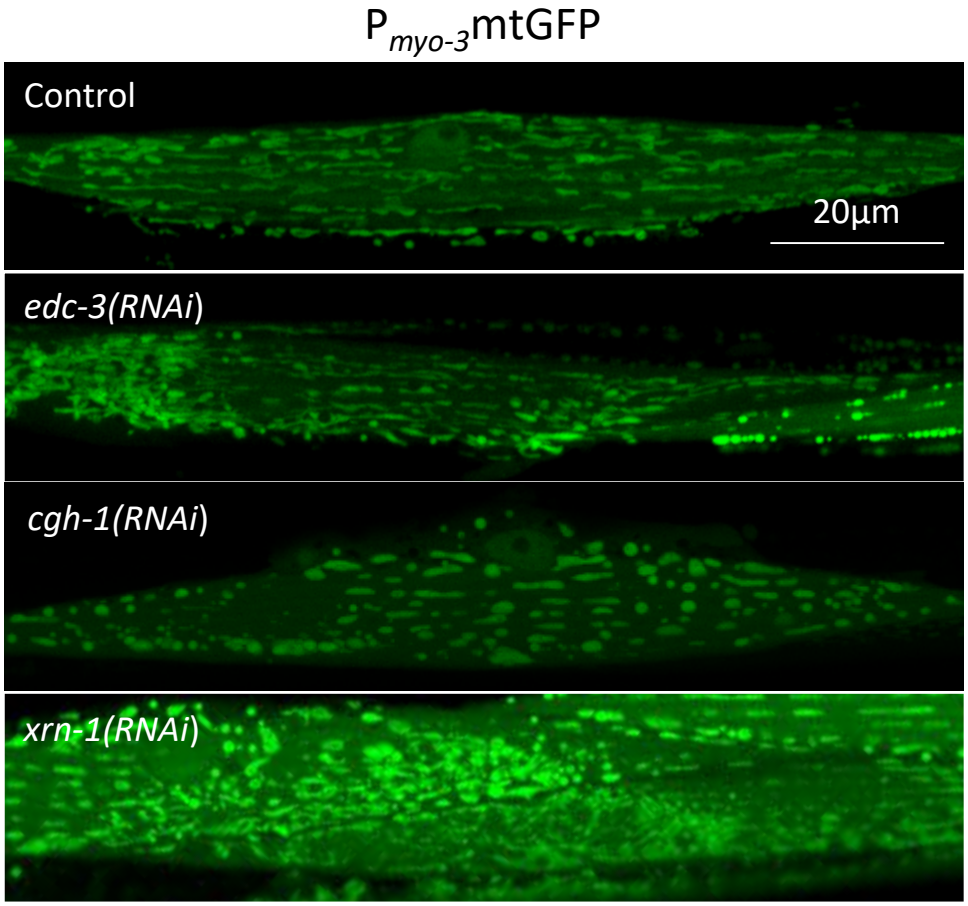

Figure 24. Confocal images showing the effect the genetic inhibition of various storage and degradation body components on the mitochondrial network integrity in *C. elegans* body wall muscle cells.

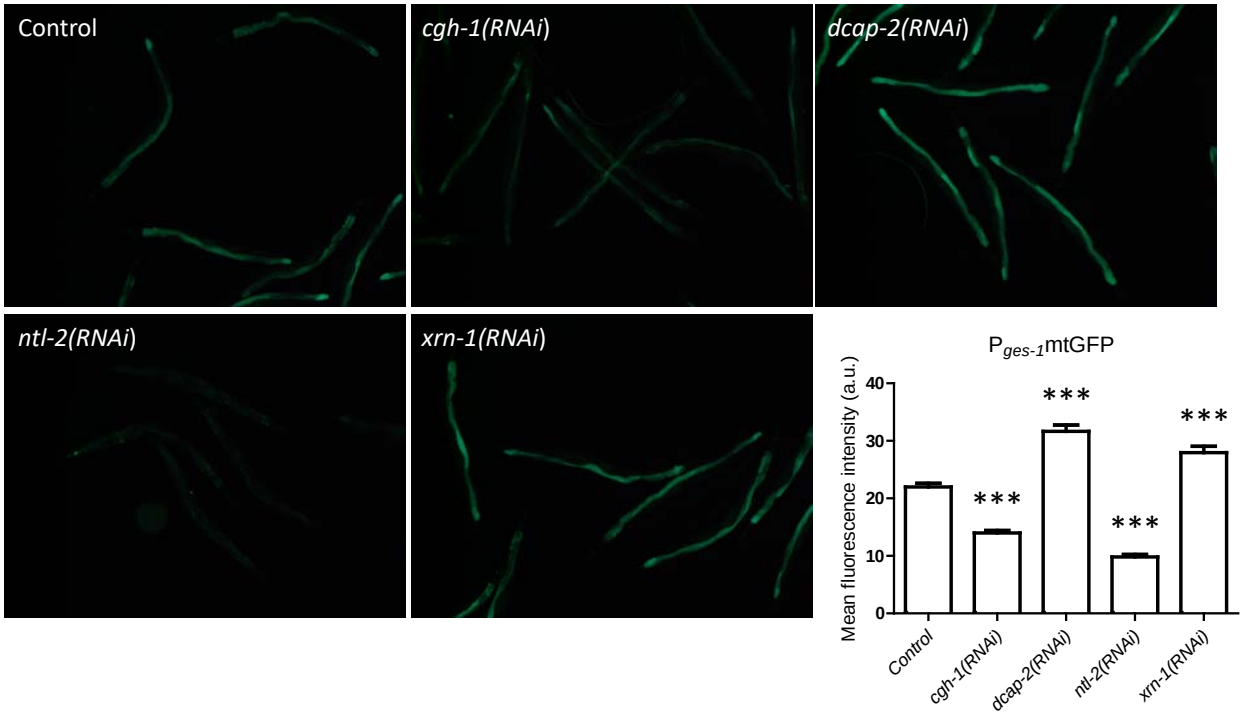

Figure 25. Epifluorescence images showing the effect the genetic inhibition of various storage and degradation body components on the total abundance of mitochondria in the intestine.

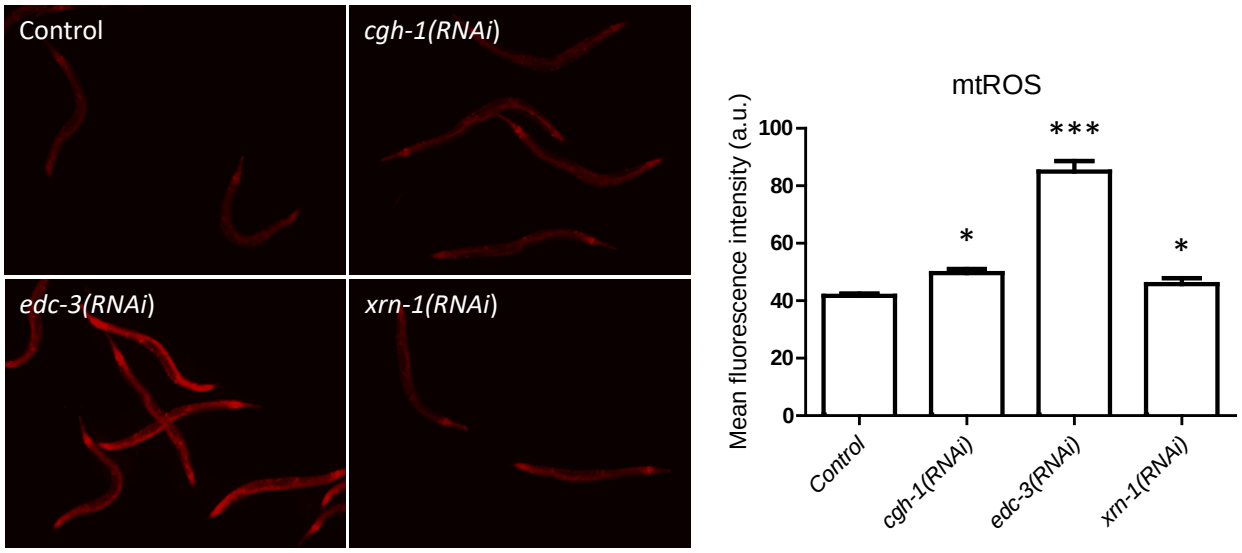

Figure 26. Epifluorescence images showing the effect of the genetic inhibition of various storage and degradation body components on total mtROS levels.

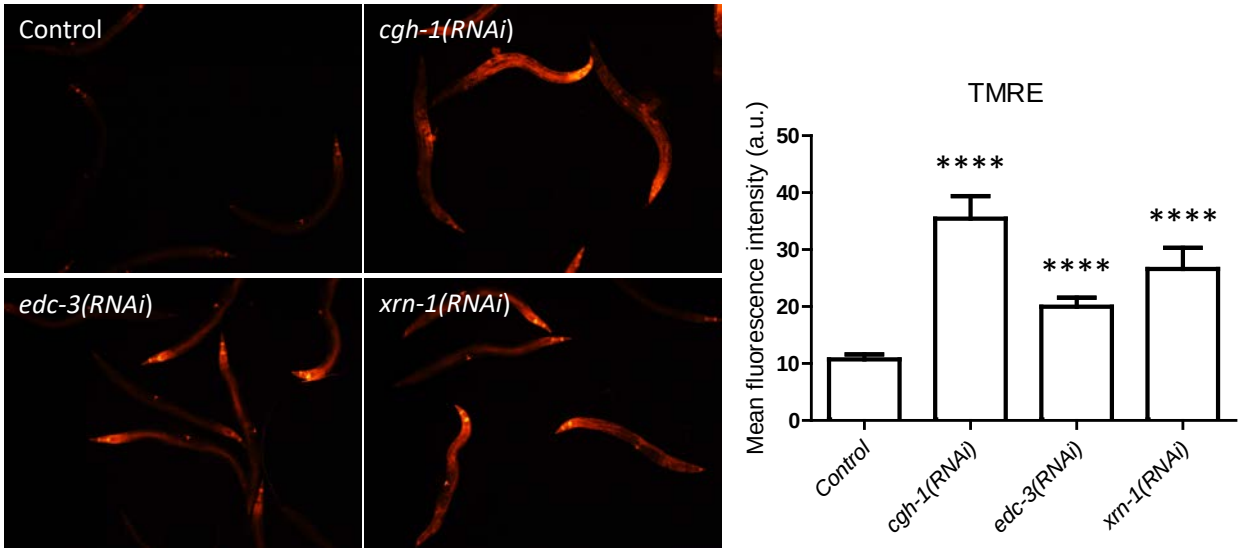

Figure 27. Epifluorescence images showing the effect of the genetic inhibition of various storage and degradation body components on the mitochondrial membrane potential.

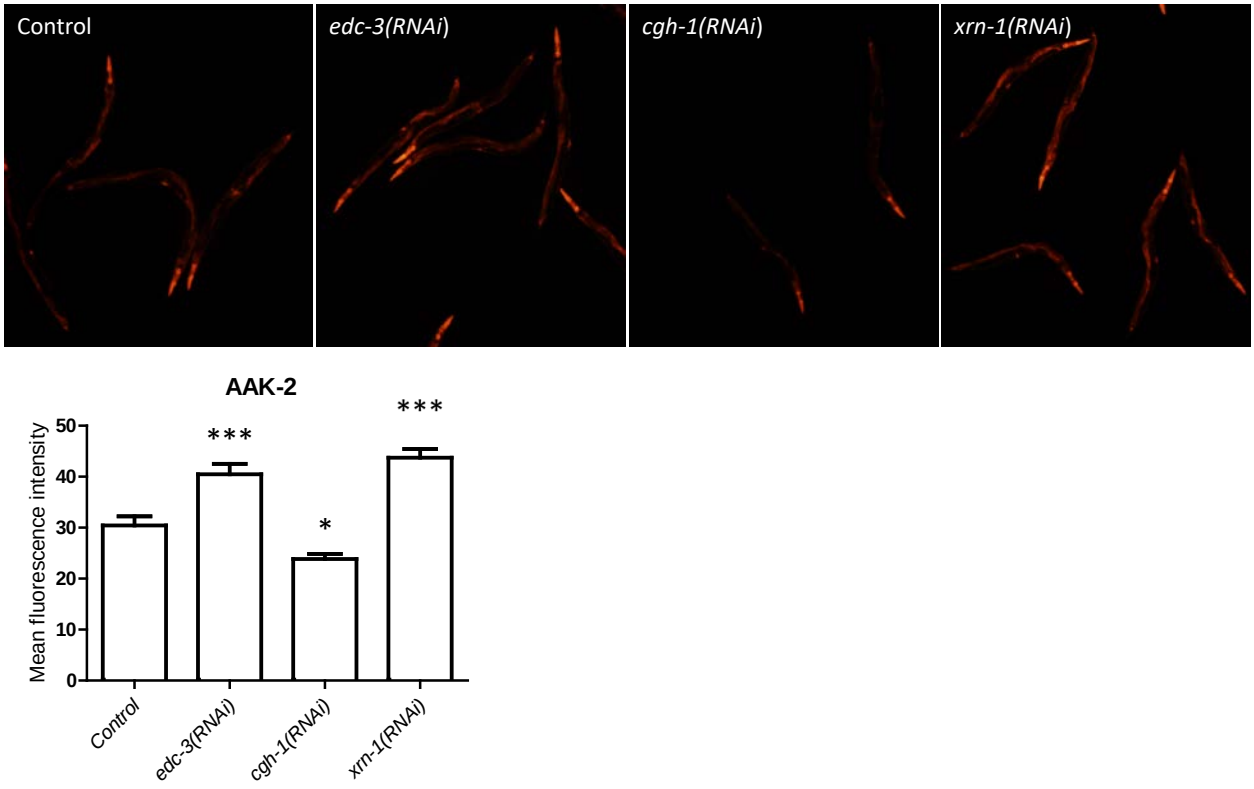

Figure 28. Epifluorescence images showing the effect of the genetic inhibition of various storage and degradation body components on AAK-2 levels.

In addition, we also plan to test the effect of *dcap-2* genetic inhibition on the association of the two types of bodies with mitochondria as suggested by the Referee.

On the same line, the authors conclude (end of first paragraph on pag 6) that perturbation of decapping complex increases the functional mitochondrial population but they actually showed (figure 1 g,h) that dcap-2 RNAi increases mitochondrial ROS and membrane potential. How would be this indicative of "functional mitochondria"? To reach reliable conclusion in this regard, the effect of different decapping regulatory proteins (and not just one) on different mitochondrial functional parameters, e.g. respiration and/or ATP content should be tested.

We now provide additional data showing that *dcap-2* knockdown increases ATP levels, besides ROS production and , compared to control. *dcap-2* genetic inhibition also increases total mitochondrial abundance. These findings invite the speculation that DCAP-2 deficiency triggers elevation of the functional mitochondrial population (as more mitochondria are expected to produce more ATP, ROS and , if functional, compare to a lower number of mitochondria).

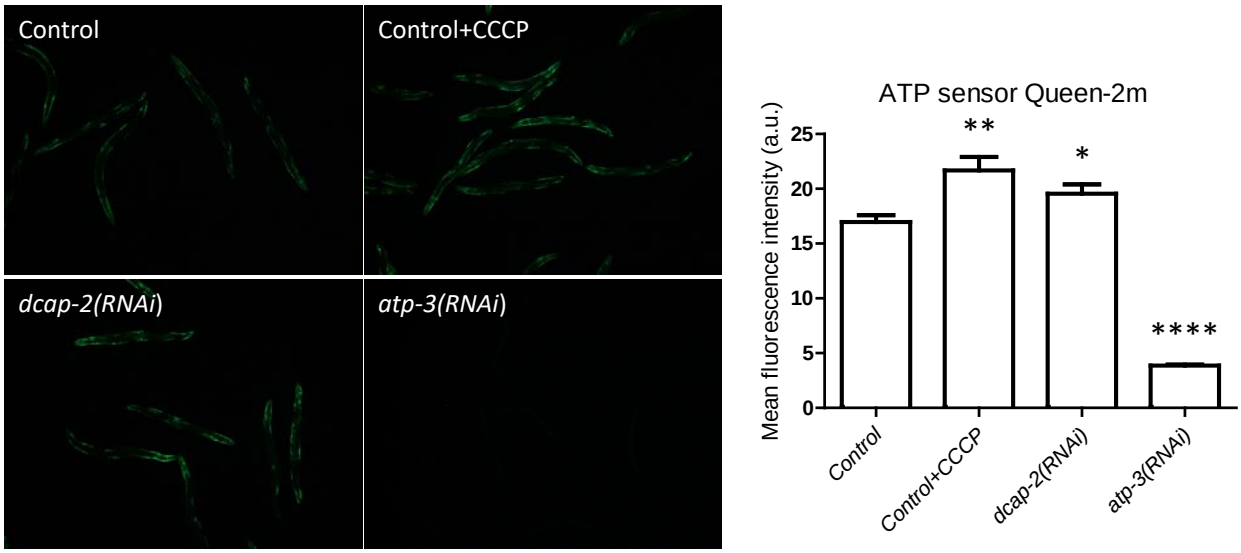

Figure 29. Epifluorescence images showing the effect the genetic inhibition *dcap-2* on total ATP levels.

We also now set up experiments for real-time measurements of oxygen consumption rate using the Agilent Seahorse XF Analyzer, which show that basal oxygen consumption rate is increased upon *dcap-2* knockdown. These results are still very preliminary. In addition, we plan to test additional degradation body components, as suggested by the Referee. Taken together these additional findings will further support our claim and give us confidence that the conceptual context of our study will remain unaltered in the revised manuscript.

Storage bodies constitute local translation coordinators in the vicinity of mitochondria. Data shown in figure 5 are actually not enough to reach this conclusion. The amount of MTPTs associated with NTL-2::GFP (and possibly other storage components) should be addressed upon silencing or overexpression of storage/degradation/translation-inducer proteins, by mitochondrial stress or aging. This would provide further support to the overall conclusion of the work.

In Figure 5 (in the manuscript), our results show that NTL-2, a storage body component binds MTPTs and also that it associates with mitochondria in a local translation-dependent manner. We appreciate the Referee's suggestion, which will strengthen our findings. However, such experiments are quite difficult to perform in large scale/upon many conditions in *C. elegans*. Despite this, we plan to repeat the RNA immunoprecipitation experiment (RIP) to include additional controls and try to detect differences in the amount of MTPTs bound on NTL-2 or an additional storage body component, at least, in one of the conditions that the Referee suggested.

Below, we provide additional evidence (Figure 30, response letter) that the total abundance of MTPTs is increased upon *dcap-2* genetic inhibition. Based on the enzymatic activity of DCAP-2, this could be attributed to decreased decapping and subsequent degradation, leading to accumulation of these MTPTs. For example, knockdown of *dcap-2* increases mRNA levels of *f46b6.6* (ortholog of human mitochondrial translation initiation factor 2, MTIF2) and *t20h4.5* (ortholog of human NADH ubiquinone oxidoreductase core subunit S8, NDUFS8), as shown in Figure 30.

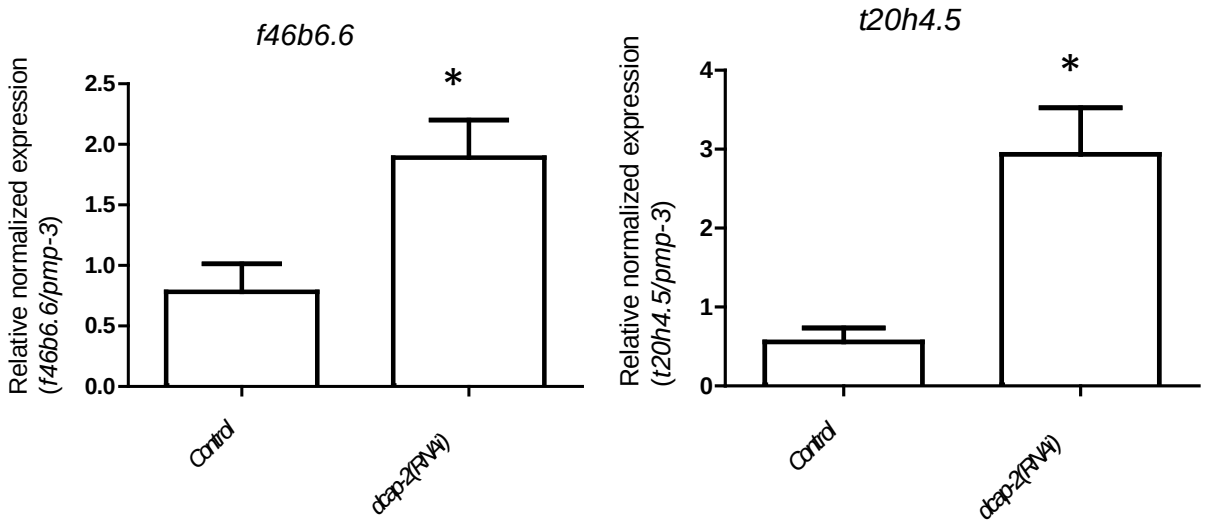

Figure 30. Genetic inhibition of *dcap-2* increases MTPTs' total abundance.

In addition, we provide additional evidence (see below) that *tomm-20* genetic inhibition triggers a drop in the total abundance of DCAP-2 (Figure 31, in the response letter), in contrast to NTL-2, as has been discussed in our manuscript. These results indicate that perturbation of local translation triggers an elevation of storage body components and a concomitant decrease in degradation body components, further supporting an interplay between local translation and coordination of storage and degradation bodies. Considering the effect of *dcap-2* genetic inhibition on storage body components (Figure 4e in the manuscript and Figure 4 in the response letter) and the above-mentioned findings, our claim that the coordinated function of storage and degradation bodies (and not just one component) plays a role in local translation, is further strengthened.

This result also addresses the Referee's comment: "**Moreover, they did not provide any evidence that the "coordination of mRNA storage and degradation", rather than simply the reduced expression**

of some of their components, is actually the mechanism underlying their effects on stress response letter and aging."

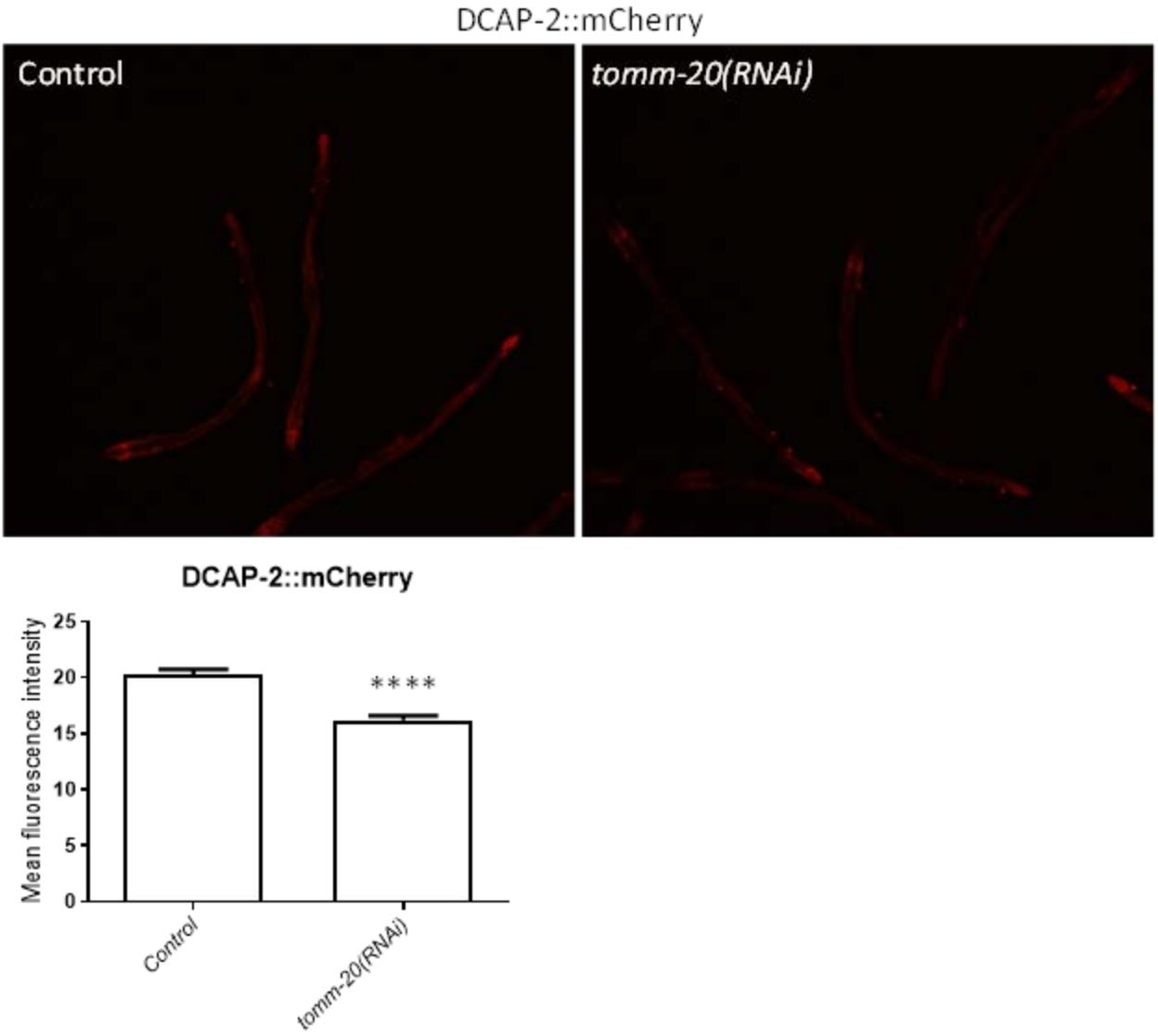

Figure 31. Perturbation of local translation through *tomm-20* genetic inhibition increases total DCAP-2 levels in contrast to NTL-2 levels as described in our manuscript.

Also, if, as they suspect the storage and degradation foci act antagonistically, can they revert alteration in e.g. MTPTs translation by dcap-2 or ntl-2 suppression with akap-1 or tomm-20 depletion?

We plan to set up double RNAi experiments in various combinations e.g. *dcap-2(RNAi);akap-1(RNAi)* and *ntl-2(RNAi); akap-1(RNAi)* to examine their effect on protein levels of select MTPTs depending on antibody availability. We would like to kindly remind you that there is limited availability of antibodies to work with, in *C. elegans*.

In the same chapter "to investigate whether storage bodies have a role in local translation of MTPTs..." they perturbed mitochondrial local translation inducers akap-1 and tomm-20. While this is an interesting point to look at, with this experiment they investigate exactly the contrary, that is: if local translation machinery plays a role in storage bodies components association with mitochondria. To actually investigate if storage bodies have a role in local translation of MTPTs it should be assessed (as suggested above) whether modulating the expression of storage bodies components impact on local translation (e.g. by quantifying MTPTs) or on translation inducers expression/activity.

Indeed, with this experiment we examine whether there is a relationship between local translation regulators and storage body components. We hypothesized that if storage bodies are, by any means, implicated in the regulation of local translation events, then their localization pattern or abundance would be altered in response letter to perturbation in local translation regulators.

We appreciate the Referee's suggestion and we totally agree that these experiments will strengthen our manuscript. For this reason, we will perform such experiments, as suggested (also as mentioned above).

Finally, results shown with atp-3 in Figure S3 seems rather contradictory. Why atp-3RNAi does not affect the percentage of NTL-2 vicinity to mitochondria (S3b) but it increases the amount of NTL-2 associated with mitochondria (S3c)?

We respectfully disagree with the Referee's comment that the results in Figures S3b and S3c are contradictory. In both cases, there is an increase in NTL-2 bodies that closer associate with mitochondria. We agree that the increase as shown in Fig S3c is much more robust. This is expected because in this case, we implement a biochemical approach to monitor the association of NTL-2 with mitochondria, in isolated mitochondria. In Figure S3b, we monitor the associations through imaging and quantification of the distances in the best resolution possible, but still, some differences may be masked. Also, Figure S3b shows quantification from one representative experiment. In the revised manuscript we will merge all our replicates so we expect that the differences will become even more robust in this experimental setup, as well.

4) Balanced mRNA storage and degradation promotes stress resistance and longevity.

In the last chapter the authors investigate the role of dcap-2 and ntl-2 in mitochondrial stress resistance and aging and found that they act in opposite directions. However, to prove that this is due to local imbalance of mRNA storage/degradation/translation the effect of silencing additional components on the same complexes should be tested.

Our findings show that balanced mRNA storage and degradation modulates ageing. In the manuscript, we provide data showing that perturbation of one component (we are now testing additional components, see Figure 32 of the response letter) from each type of body triggers an increase in the abundance of components from the other type and this impacts ageing. For example, we show that *dcap-2* genetic inhibition triggers elevation of NTL-2 (Fig. 1e, in the manuscript). In addition, we find that knockdown of *dcap-2* or of other degradation body genes promotes longevity (Figure 32, response letter). Moreover, in our manuscript we clearly show that NTL-2 overexpression (mimicking *dcap-2* genetic inhibition) also

extends lifespan, in contrast to *ntl-2* genetic inhibition. We now plan to test the effects of combinatory interventions in the two types of foci on lifespan to highlight the crucial role of the coordination of mRNA storage and degradation processes in longevity and stress resistance. For example, we will test whether perturbation of degradation bodies can further extend the lifespan and alter stress resistance of NTL-2 overexpressing animals.

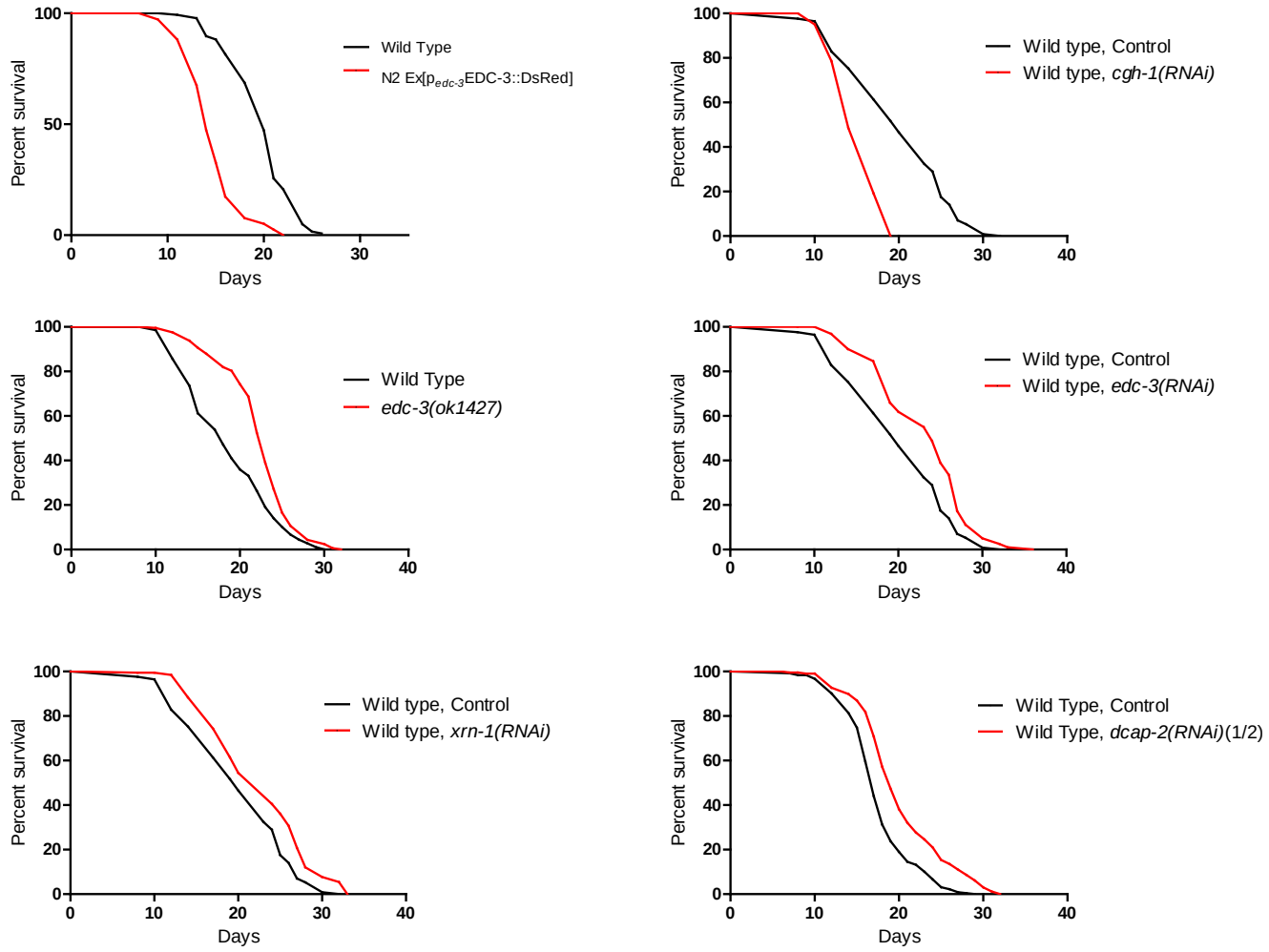

Figure 32. Lifespan assays showing the opposite effects of storage and degradation body components on longevity (in pages 2-4 we describe the components of storage and degradation bodies in more detail).

Moreover, altered MTPTs translation in conditions that affect stress response letter/aging should be assessed, as well as its reversion when suppressing components of the other components of the translation machinery which suppress the lifespan phenotypes.

We are not sure we understand the Referee's comment. It is not clear to us what he/she means by mentioning "components of the other components of the translation machinery which suppress the lifespan phenotypes".

As mentioned previously, we will repeat the RIP experiments trying to identify differences in at least one of the experimental setups as suggested by the Referee**: "The amount of MTPTs associated with**

NTL-2::GFP (and possibly other storage components) should be addressed upon silencing or overexpression of storage/degradation/translation-inducer proteins, by mitochondrial stress or aging."

Furthermore, as mentioned previously, we plan to set up double RNAi experiments in various combinations e.g. *dcap-2(RNAi);akap-1(RNAi)* and *ntl-2(RNAi); akap-1(RNAi)* to examine their effect on protein levels of select MTPTs depending on antibody availability.

Discussion

Based on the described finding, some of the conclusions seems overstated. On page 10, end of the first chapter of the discussion, the author state that "the "two types of foci form antagonistically to each other and oppositely regulate cytoplasmic translation rate". While they showed that components of the complexes are regulated antagonistically, they provided no evidence on their effect on cytoplasmic translation. Also, at the beginning of the following chapter they state "Increased global protein synthesis and aberrant translation of target MTPTs triggered by perturbation of storage bodies following ntl-2 genetic inhibition...." yet, they have not actually shown that inhibition of ntl-2 or of other components of the machinery affect translation. Similarly, in the first chapter of page 11, they state, but actually have not shown that "...dcap-2 depletion reduces overall mRNA translation oppositely to ntl-2 genetic inhibition".

In this study, we assess protein synthesis rates by Fluorescence Recovery after Photobleaching (FRAP) in wild-type animals subjected to RNAi against *dcap-2* or *ntl-2* compared to animals fed with control RNAi (Fig. 4h). This is an established methodology for *in vivo* monitoring of new protein synthesis in cells or tissues of interest (Papandreou et al., 2020, J. Vis. Exp. (163), e61170, doi:10.3791/61170; Kourtis and Tavernarakis, 2017, Bioprotocol, 7(5): e2156; If suggested by the Referee, we are willing to tone down claims that are not validated by an additional advanced biochemical method such as polysome profiling.

Minor points

Fig. 1 and 2 and Fig. S1 and S2

All minor points either textual or graphical are now being addressed in the Revised manuscript. Specifically, corrections have been done with regard to points 1 and 2, per referee suggestions.

Fig. 3

Western blot will be repeated so as to provide a better figure although this is difficult with the strain that expresses the SKN-1::GFP transgene. The other comments are easily addressable and we are in the process of doing so.

Fig.4

Quantification in panels 4e-g will be performed, as referee suggested.

The conclusions drawn will be rephrased.

The abundance of EDC-3 foci increases during ageing. The same stands for DCAP-1 and LSM-3 components of the mRNA decay pathway, as previously reported (Rieckher et al., 2018, Cell reports). In Figure 4f (in the manuscript), we also find that EDC-3 total levels increase during ageing. In Figure S1, we monitor muscle cells specifically. We have not performed quantification of EDC-3 specifically in muscle cells during ageing. We will now measure EDC-3 specifically in body wall muscle cells and if it is increased there as well, Fig. S1 will be substituted with a representative one.

Text describing Figure 4a-e will be more extensively elaborated to better convey the main message.

Similarly, additional details will be provided for the FRAP experiment (Figure 4h) and statistics will be included.

Figure 5 and S3

The results of the RNA immunoprecipitation (RIP) experiment will be presented more clearly.

The wild type strain does not express GFP, this is the reason why we added HIS-72::GFP as an additional control.

In fact, this experiment will be repeated, thus expression levels for additional genes will be quantified and additional controls will be included as suggested by the Referees.

Quantification of ATP-3 and MRPS-5 will be included in Fig. 5e.

Referee #3:

Overall

The work is important and builds nicely on previous work from the Tavernarakis lab and others. In addition, the data provided are convincing (for the most part) and well-presented. Overall, I found this to be an interesting piece of work that is worthy of publication in The EMBO Journal.

We thank the Referee for his/her encouraging comments and for the appreciation of our work.

Specific comments

This is a minor point, but the MTCO1 blot in Figure 1f is very poor quality compared to the blot in Figure 5d. Can the authors provide a better representative image here?

We will repeat the experiment and try to provide a Western blot of better quality for the MTCO1 detected in the mitochondrial fraction of EDC-3::GFP and NTL-2::GFP transgenic animals.

It is great that the authors attempt to look at the protein levels of SKN-1 in C. elegans (often not a simple task) but the FLAG and tubulin blots in Figure 3a are not of sufficient quality for quantification (The SKN-1::FLAG signal is too feint relative to the uneven background). I suggest the authors load more protein and/or use more sensitive detection reagents to amplify the SKN-1::FLAG signal.

We thank the Referee for acknowledging the difficulties of using biochemistry techniques in *C. elegans*. Nevertheless, we plan to repeat the experiment in order to ensure the best image quality.

Another minor point is that it would be good to have some representative gst-4p::gfp images to accompany the quantification presented in Figure 3b.

We will provide representative images of worms used in quantification of *gst-4::gfp* shown in Fig. 3b, as suggested by the referee (see Figure 33 below).

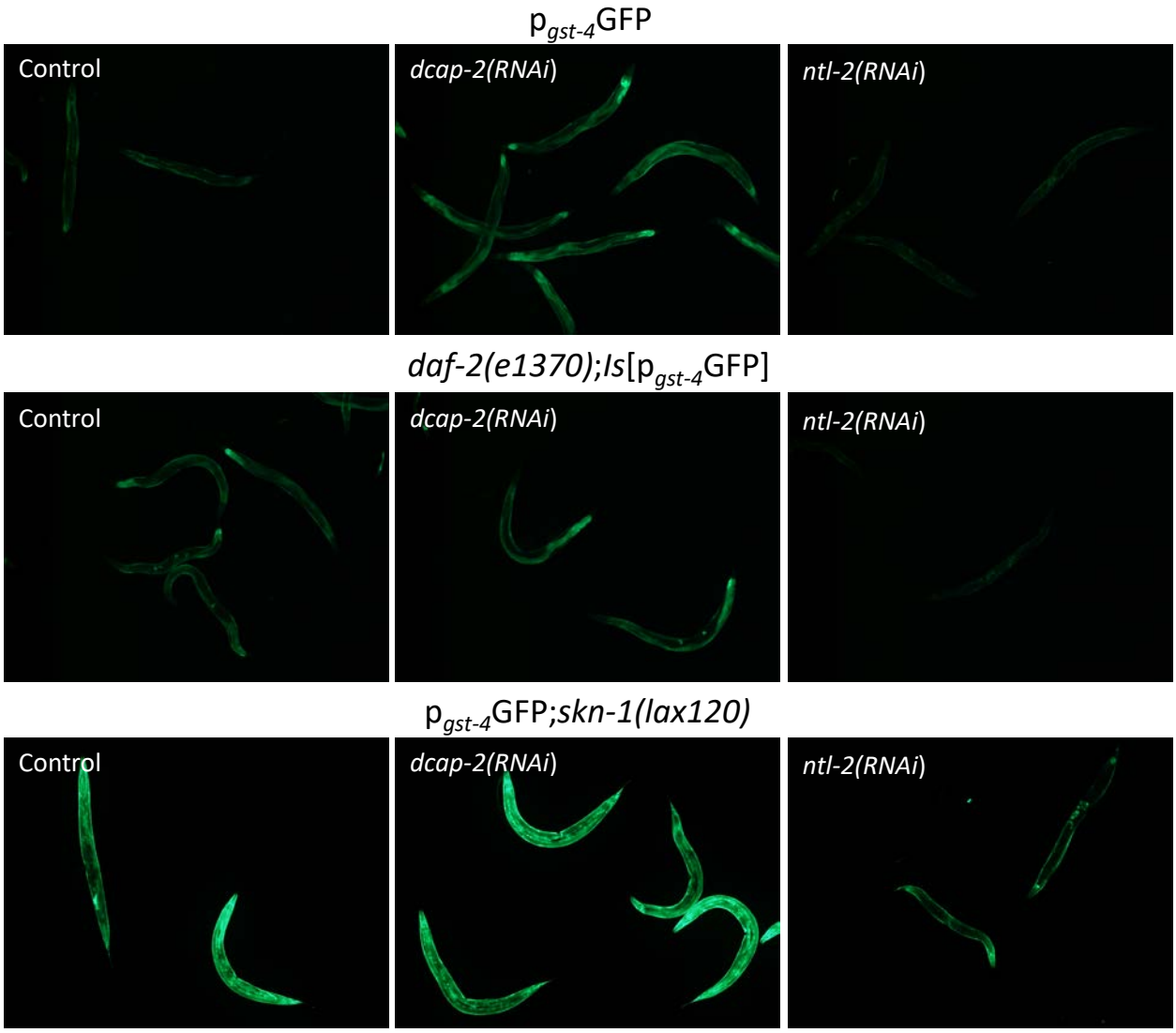

Figure 33. Representative images showing *gst-4* expression (to accompany quantification) in Figure 3b of the manuscript.

The opposing effects of ntl-2(RNAi) and dcap-2(RNAi) on the IFE-2::GFP reporter are very interesting. While I fully agree that this reporter is a good indicator of overall translation rates, the authors should strengthen this conclusion by also looking directly at total protein levels in NTL-2 and DCAP-2 depleted animals. This will reveal whether global protein load is being reduced or enhanced when the storage and degradation complexes are perturbed.

Following the suggestion of the Referee, total protein levels of wild-type animals subjected to *dcap-2* or *ntl-2* RNAi will be determined to strengthen the results obtained with photobleaching and recovery of fluorescence in *vivo* (FRAP) and presented in Fig. 4h (in the manuscript).

The authors state that "NTL-2 is required for the increased lifespan of long-lived mutants and that mev-1, nuo-6 and isp-1 mutants, and atp-3(RNAi), suppress the short lifespan of ntl-2(RNAi) worms. However, the data presented in Figure S4 do not appear to support these conclusions. It is true that ntl-2 is required for the lifespan extension observed in akt-1(ok525) animals, and that ntl-2(RNAi)

shortens the lifespan of daf-2(RNAi) and age-1(hx546) animals. However, the lifespan of control;ntl-2(RNAi) animals is extended by daf-2(RNAi) and age-1(hx546) to the same extent as in wildtype worms. Similarly, while mev-1(kn1) mutants do ameliorate the short lifespan of ntl-2(RNAi) animals, this does not appear to be the case for nuo-6(qm200) and isp-1(RNAi) worms, which have a similar shortening of lifespan compared to nuo-6 and isp-1 control animals as ntl-2(RNAi) does compared to wildtype. The authors should modify their conclusions accordingly in the text.

The Referee's view is another interpretation of lifespan results with mitochondrial mutants subjected to *ntl-2* RNAi. In our view, and consistent with the impact on mitochondrial biogenesis, we find that components of storage and degradation bodies modulate lifespan and stress resistance. Indeed, we show that the detrimental effects of *ntl-2* genetic inhibition on longevity are ameliorated, or rescued, by targeting specific mitochondrial genes.

It is great that the authors included the HIS-72::GFP line as a control for their NTL-2::GFP RIP experiments. This controls well for non-specific pulldowns related to antibody binding and/or beads. BUT, I am not convinced that this is an appropriate control for random interactions with GFP tagged cytosolic proteins. HIS-72::GFP is nuclear; a more appropriate control would be a GFP tagged protein that is cytosolic.

Following the Referee's suggestion, we plan to repeat the experiment by using additional controls.

In Figure 5d, the authors make the claim that upon akap-1(RNAi) and tomm-20(RNAi), levels of NTL-2 associated with mitochondria decrease. However, these differences are driven by elevated levels of MTCO1 on their western blots, rather than reduced levels of NTL-2. Do akap-1 RNAi and tomm-20 RNAi result in increased levels of MTCO1? The authors should probe this by western blotting. If so, this would suggest that actually, there is no change in NTL-2 mitochondrial association under these conditions.

In this experiment we use MTCOI as a loading control, because our loaded sample in this case is isolated mitochondria. We then normalized NTL-2 with MTCOI to ensure that the differences we detect in NTL-2 levels are not due to differences in the sample amount loaded. Nevertheless, we will test whether MTCOI amount changes upon *akap-1* and *tomm-20(RNAi)*. If this is the case, then we will repeat the experiment using another mitochondrial marker as a loading control.

In Figure 6, the authors show that ntl-2(RNAi) sensitizes worms to multiple stresses. Is this also observed using the *ntl-2(ok974)* mutant used in Figure 7?

Given that *ntl-2(ok974)* mutants are homozygous lethal, the strain is balanced. Therefore, it is time consuming to grow a large number of animals to perform stress resistance assays. We believe that owing to

the high efficiency or our RNAi (Figure 34 of the response letter) there is no need to also use the mutant (which is heterozygotes) in this case.

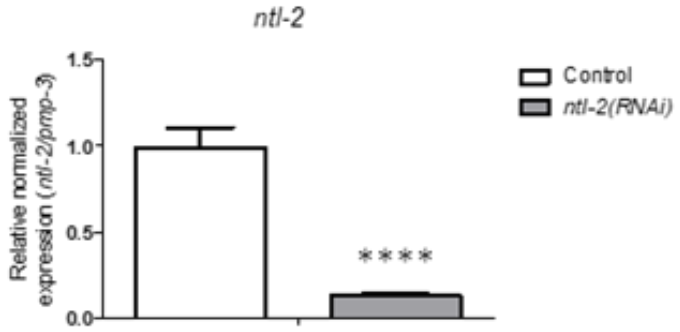

Figure 34. RT analysis verified the efficiency of our RNAi construct.

In addition, does tomm-22 also impact lifespan in a similar way to akap-1(RNAi) and what happens to lifespan in dcap-2(RNAi) worms?

To test the effect of *tomm-22(RNAi)* in lifespan and in comparison to *akap-1(RNAi)* would be out of the scope of the current manuscript. Maybe the Referee's suggestion is to test the effect of *tomm-20* RNAi on lifespan, which is something we plan to do. Knockdown of *dcap-2* increases lifespan (Figure 35).

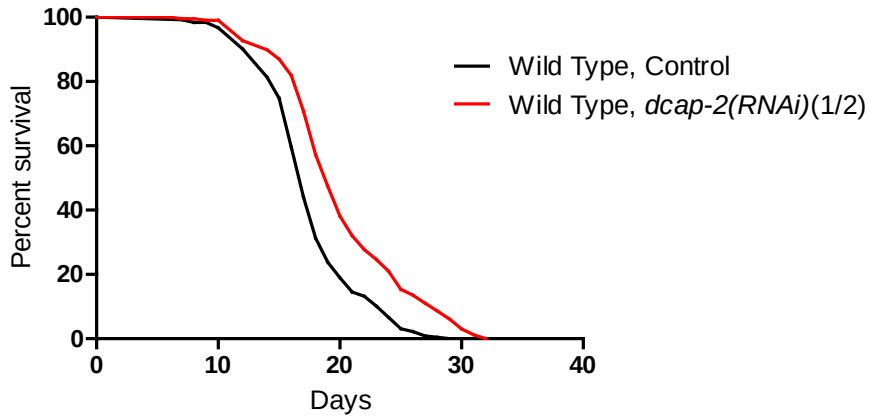

Figure 35. Lifespan assay showing the effect of *dcap-2* genetic inhibition on animal longevity.

Referee #4

Fig. 1

GFP is a relatively large tag. Can it be excluded that the tag interferes with the function of the studied proteins?

The idea to study proteins' function without using a fluorescent tag would be indeed great. However, antibodies for the studied components that work efficiently in *C. elegans* do not exist. Also, the use of antibodies to monitor the formation of the described foci would not allow *in vivo* studies. Therefore, we are afraid that we cannot avoid the use of fluorescent tags in our *in vivo* study.

Based on our data, we are confident enough that GFP does not alter the function of the studied proteins. This notion is supported by the fact that NTL-2::GFP, EDC-3::GFP and CCF-1::GFP acquire the expected subcellular localization pattern (a dotted expression pattern) as seen, for example, in Figures 1a-c and Figure 4g in the manuscript. Also, we have observed that the expression pattern of the studied proteins fused to GFP is dynamic. For example, the expression pattern is dramatically altered upon various treatments (genetic or pharmacological) as well as during ageing (Figures 1i,j, 4e,g and 5b) exhibiting either an increase or a decrease. Notably, in Figure 1j we show that NTL-2::GFP acquires a cytoplasmic, diffused expression pattern upon *mrps-5* genetic inhibition. All these examples prove that NTL-2::GFP expression is upon tight, dynamic and functional regulation. Also, our lifespan data (Figure 6e) are supportive against the notion that GFP interferes with the protein function. This is because 1. we observe a different phenotype compared to control counterparts; NTL-2 overexpressing animals are long-lived (if the protein was not functional we wouldn't except to see a change in the lifespan of the animals) and 2. NTL-2 overexpression has the opposite effect on *C. elegans* lifespan compared to *ntl-2* genetic inhibition. This, to our understanding makes sense and corroborates the notion that GFP does not interfere with the function of the studied protein.

Moreover, the use of fluorescent tags is very common in *C. elegans* research and a well-accepted technical approach. The use of transgenic animals that overexpress certain proteins of interest fused with fluorescent tags or strains that have been generated through the CRISPR/Cas9 technology is ubiquitous in the literature, even in top-rated journals both in *C. elegans* research (Fengxiu Sun, et al., Nature, 2022 (very recent publication-not yet found through EndNote); (Susoy et al., 2021)) and research in other model organisms (Ma et al., 2022) even in related fields or research (Bose et al., 2022).

Overall, while we understand the Referee's concern, based on the wide use of such genetic tools and technical approaches for *in vivo* studies and in combination with our data, which are supportive that our studied molecules are functional, we feel confident for using them. In any case, we are afraid that there are no better tools available to use for such studies *in vivo*.

Fig. 1f:

To get an estimation how much of NTL-2 and EDC-3 co-fractionate with mitochondria it would be better to present a single blot comparing isolated mitochondria, cytoplasm etc. and maybe include a marker for another protein associated with the outer mitochondrial membrane.

We are afraid we don't understand the advance that such an experiment would offer to our manuscript. In the western blot presented in Figure 1f in the manuscript, we have performed mitochondrial isolation in order to test whether NTL-2 and EDC-3 are present in the mitochondrial fraction. Indeed, as shown in this Figure, both NTL-2 and EDC-3 were probed in the cytoplasmic and the mitochondrial fraction. We use this experiment only for qualitative analysis and not for quantitative analysis. We are interested just to show in a biochemical way (as an extra proof to our *in vivo* analysis) the presence or not of the studied proteins in mitochondria.

Due to the known dynamic nature of these formations, we strongly believe that the associations they form with mitochondria would be transient enough and thus it is really hard to extract safe results regarding the real amount of protein that associates with the organelles at the time of animal collection compared to amount of this protein in the cytoplasm. In fact, we can reliably either compare quantifications in samples from mitochondrial fractions or in samples from cytoplasmic fractions. Also, to estimate the abundance of the protein present on mitochondria compared to the cytoplasm, we have to compare samples that have been normalized with two different loading controls, which also may interfere with the real results.

Moreover, the Referee suggests that we "include a marker for another protein associated with the outer mitochondrial membrane" in order to compare with our proteins. It is not clear to us why the Referee suggests that. Each protein that associates with mitochondria may have its own association rates/efficiencies or abundance on mitochondria.

We believe that we will not get a precise measurement of the abundance of the tested proteins present in the cytoplasm versus mitochondria with the suggested experimental approach. For the abovementioned reasons, we feel safe to only use the presented western blot assays just for qualitative analysis and not for precise quantification of the protein amount in each subcellular compartment. We do believe that precise quantification for comparison between the different conditions can only be made within equally handled samples (we can measure protein levels in mitochondrial extracts upon various conditions and make quantitative comparisons among them or in cytoplasmic extracts upon various conditions and make quantitative comparisons among them) that are normalized to the same loading controls.

Fig. 1i-j vs. Fig. 1a:

The punctuated staining of NTL-2 in the control panels seem to vary.

We are sorry but it is not clear to us what the Referee means because he/she does not provide a specific example. In all Control images showing NTL-2::GFP expression, we do observe NTL-2-puncta. The intensity of the signal may vary because of: 1. The different exposure conditions we had to use among the different experimental setups 2. The age of the animals 3. The tissue monitored and 4. The expected variation in the expression levels of the protein from one animal to the other due to mosaicism as the transgene (NTL-2::GFP) is expressed from extrachromosomal arrays and is not integrated.

Fig. 1n:

The levels of NTL-2-GFP are normalized to COX1, which is a mitochondrial DNA-encoded protein. Mitochondrial translation should be affected upon downregulation of a mitoribosomal protein (mrps-5 RNAi). Thus, COX1 cannot be used as a loading control.

We thank the Referee for pointing this out. Indeed, we used MTCOI which is a mitochondrial-encoded protein. We will perform these experiments again using a more appropriate loading control or Ponceau staining. We apologize for any inconvenience this may have caused.

Fig. 2:

"we conclude that perturbation of the decapping complex increases the functional mitochondrial population,..."

I do not understand how the author can conclude this. The downregulation of dcap-2 disrupts the membrane potential and triggers mitochondrial fragmentation. How can one conclude that dcap-2 ablation increases the functional mitochondrial population? It shows only mitochondrial abundance, but not whether they are functional.

We assess ATP levels in animal expressing the ATP sensor Quenn-2m. We find that fluorescence signal is increased in animals subjected to *dcap-2* RNAi compared to controls (Figure 36 of the response letter).

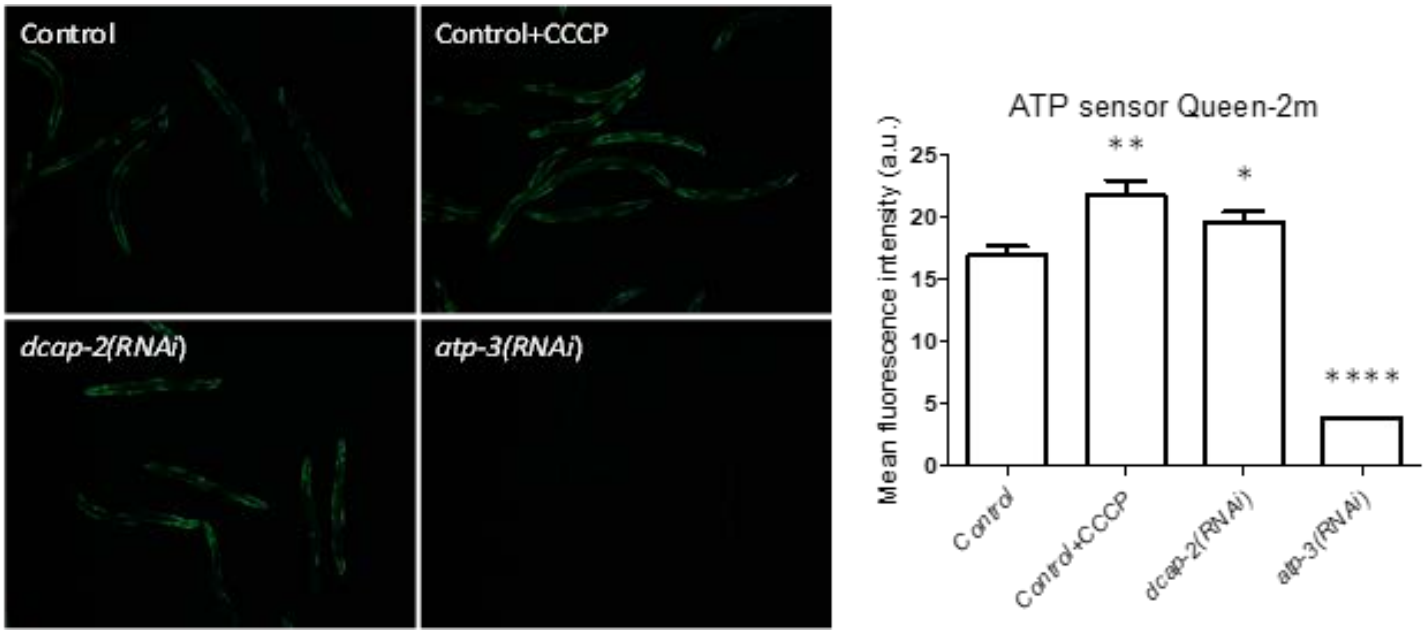

Figure 36. Epifluorescence images showing the effect of the genetic inhibition of *dcap-2* on total ATP levels.

We also have preliminary data from real-time measurements of oxygen consumption rate using the Agilent Seahorse XF Analyzer, which show that basal oxygen consumption rate is increased upon *dcap-2* knockdown.

Fig. 3a:

The western blot is not convincing and a-tubulin seems to be overexposed.

We plan to repeat the experiment and try to provide a Western blot of better quality.

Fig. 5a:

This experiment is questionable. If WT and HIS-72:GFP are both negative controls, why do they show different results, especially for spcs-1?

This is likely due to differences in the genetic background of the two strains. Nevertheless, we plan to repeat the RNA immunoprecipitation (RIP) experiment to include additional controls and try to detect differences in the amount of MTPTs bound on storage body components, at least, in one of the additional conditions as suggested by other Referees (mentioned above in the response letter).

Fig. 5b-5e:

The text is difficult to follow for non-experts. What do the authors mean with "local translation inducers"? TOM20 is a component of the import machinery at the outer membrane. Ablation of TOM20 affects protein import and membrane potential as also shown by TMRE staining (Fig. 5b). Therefore, the conclusion can be misleading.

Again using COX1 as a loading control is not appropriate as the synthesis or stability of mitochondrial DNA-encoded COX1 can be/ is affected in some of the knockdowns.

As described in the Introduction of our manuscript, “following their transcription, MTPTs are exported from the nucleus to the cytoplasm and being in a translationally silenced state, they are transferred to mitochondria where they are anchored on the outer mitochondrial membrane (OMM) by the OMM proteins MDI (AKAP-1 in *C. elegans*), and TOM20 (the nematode TOMM-20) and are locally translated and imported into the organelles (Eliyahu et al, 2010; Gehrke et al, 2015; Zhang et al, 2016). These factors promote local translation of MTPTs, facilitated by OMM bound ribosomes or by free cytoplasmic ribosomes found in the mitochondrial vicinity as revealed by proximity-specific ribosome profiling in yeast.

In fact, TOMM-20 acts as a receptor which binds the presequences of mRNAs to stabilize them locally (Eliyahu et al, 2010). Also the prevailing notion is that mRNAs are cotranslationally imported into the organelle. We find that knockdown of either *tomm-20* or *akap-1* severely affected the associations of NTL-2 (+) foci (storage bodies). The text will be elaborated to better convey the main message.

Regarding MTCOI, we thank the Referee for pointing this out. We will test whether MTCOI levels are affected under the conditions tested and if yes, we will perform these experiments again using a more appropriate loading control or Ponceau staining, as previously mentioned. We apologize for any inconvenience this may have caused.

Comment on RNAi

Efficiency and specificity of downregulation should be shown or include respective reference if RNAi has already been validated elsewhere.

We verified by RT-PCR analysis that our RNAi constructs effectively reduce the expression of our tested genes of interest (Figure 37, response letter).

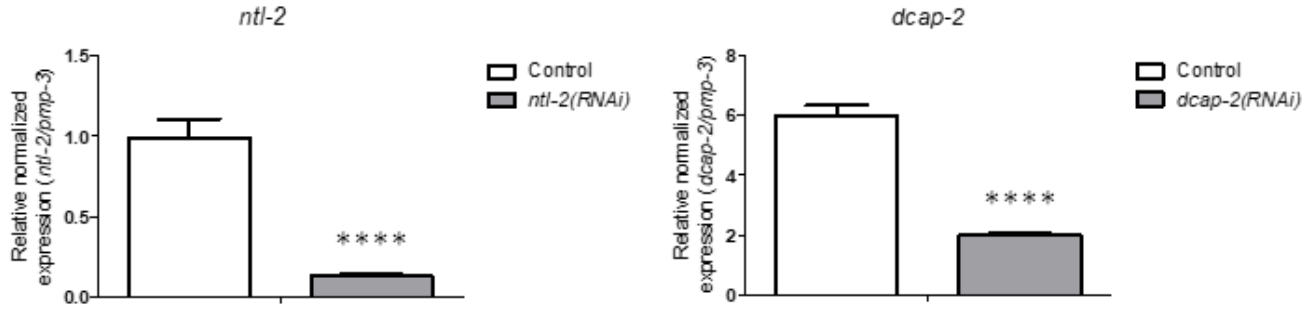

Figure 37. RT analysis verified the efficiency of our RNAi constructs.

Comment on statistics

In some figures (e.g. Fig. 1n, 2h, 3a, 3g, 5a, 5d, 5e) the authors wrote "n=2", but included p values. How is it possible to perform statistics with n=2?

We are not sure we understand the Referee's comment. In these experiments n=2 independent experiments with many animals per experiment. Statistics can be performed normally and the tests used are already described in the Figure legends and the materials and methods section.

Prof. Nektarios Tavernarakis
Foundation for Research and Technology
Institute of Molecular Biology and Biotechnology
Vassilika Vouton
PO Box 1385
Heraklion, Crete 70013
Greece

3rd Nov 2022

Re: EMBOJ-2022-112446R-Q
Local coordination of mRNA storage and degradation near mitochondria modulates C. elegans ageing

Dear Prof. Tavernarakis,
Dear Nektarios,

Thank you again for your comments and further clarifications on our recent decision to reject your manuscript post review (EMBOJ-2022-112446). As we had discussed, I contacted the initial referees and asked for their opinion on your revision plan and preliminary data addressing some of the concerns raised. Both referees appreciate that your planned experiments could address several of the raised concerns and potentially resolve issues. However, they are also still concerned regarding the extent of the revision and if you will indeed be able to provide sufficient experimental data to support a causal link of mRNA storage and degradation to mitochondrial phenotypes and aging. Given the overall interest in the topic and your preliminary response, we have nonetheless decided to invite a revision of the manuscript at this stage. Please however be aware that we will require strong support from all referees in the next round of review and that the crucial points must thus be resolved experimentally or the conclusions revised accordingly in the next submitted version of the manuscript. As discussed, this in particular includes addressing the concerns regarding the proposed local coordination of mRNA storage and degradation and the causal role on mitochondria and aging. Please also remember to provide a detailed response to each of the comments when submitting the revised version and the manuscript must then fulfill all formatting guidelines (please also see below).

Please note that it is our policy to allow only a single round of major revision. Acceptance depends on a positive outcome of a second round of review and therefore on the completeness of your responses included in the next, final version of the manuscript. It is thus important to clarify any questions and concerns as early as possible and I encourage you to contact us if any issues arise during the course of the revision.

At EMBO Press, we ask authors to provide source data for the main and EV figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files

Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

Kind regards,

Stefanie

Stefanie Boehm
Editor
The EMBO Journal

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

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

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

-

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

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

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure).

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

4) A complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author%20Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) All corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database. Please remember to provide a reviewer password if the datasets are not yet public.

Please see the instructions below on how to format the Data Availability section (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

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

[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. ***

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 < https://www.embopress.org/page/journal/14602075/authorguide#referencesformat >.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel).

Please note that source data should be uploaded in the following format:
a) For main figures: please upload the source data as one zip file per figure and label it as source_data_figX.
b) For EV figures please compile individual (zip) files for each figure, then combine these and upload one zip file for all figures. Please label this as source_data_EVfigures.
c) For Appendix figures please also make one file per figure, combine all Appendix files into one final zip file for all figures and label it as source_data_Appendix.

Additional information on source data and instruction on how to label the files are available at < https://www.embopress.org/page/journal/14602075/authorguide#sourcedata >.

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in http://msb.embopress.org/content/11/6/812). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc.. in the text and their respective legends should be included in the main text after the legends of regular figures.

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

https://www.embopress.org/page/journal/14602075/authorguide#expandedview>.

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

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

****
Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (1st Feb 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available
****

Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology

Medical School, University of Crete

N. Plastira 100, Vassilika Vouton, PO Box 1385, Heraklion 70013, Crete, GREECE

March 23, 2023

Dear Reviewers,

We would like to thank you for your time and effort in reviewing our manuscript. We considered each of the points raised very carefully and made every possible effort to address them experimentally. In doing so, we strived to include additional information and data within the text and Figures and also in the Expanded View Figures and Appendix (a total of 35 figures, 4 data tables and 3 video files).

We believe that, with your encouraging and constructive input, we have been able to resubmit a significantly stronger and more comprehensive report. A point-by-point response to all the comments follows below (original comments are quoted in **bold**).

We will be standing by for any further clarifications or information, pertinent to our final version that may be required. Thank you very much again, for your consideration of our manuscript.

Sincerely,

N. Tavernarakis.

Nektarios Tavernarakis,

for the authors

Reviewer #1:

The manuscript gives me mixed feelings. On the one hand, the reported observations are certainly very interesting and relevant. On the other hand, I do not agree with the interpretation of some of the experiments. In general, the manuscript pays too little attention to the molecular functions of the two different degradation complexes. In my view, some observations could be explained "simply" by the enzymatic activities of the decapping vs. the deadenylation complex. When deadenylation is inhibited, mRNAs will retain longer poly(A)-tails, which would explain why RNAi of ntl-2 leads to higher translation rates (Figure 4h). The opposite effect of the dcap-2 RNAi could be explained by the accumulation of deadenylated mRNAs, which are not decapped and degraded and may therefore be inefficiently translated and/or interfere with the translation of other mRNAs.

We thank the Reviewer for appreciating our work. The Reviewer suggests that a more molecular/mechanistic/functional view will better interpret our results, in the whole manuscript. Towards this direction, the Reviewer mentions that some results could be “simply” linked to the enzymatic activities of NTL-2 and DCAP-2 and he/she provides us with a detailed explanation of his/her thought. We fully agree with the Reviewer’s suggestion and we apologize for not having described this view sufficiently enough.

In fact, to interpret our results we are based on the known enzymatic activities of DCAP-2 and NTL-2. On one hand, we have shown that *ntl-2* genetic inhibition increases global protein synthesis rates. As pointed out by the Reviewer, *ntl-2* genetic inhibition is expected to decrease the rates of deadenylation. Indeed, *in vitro* deadenylase assays have shown that *ntl-2* genetic inhibition disrupts the deadenylase activity of the CCR4-NOT complex, leading to mRNA stabilization and accumulation of proteins most likely due to increased translation rates (Ito et al., 2011). Moreover, a recent study has shown that the length of the mRNA poly(A) tail is positively correlated with its translational efficiency in oocytes and early embryos, providing insights into how protein synthesis is controlled by post-transcriptional mechanisms that affect poly(A) tail length (Xiang and Bartel, 2021). On the other hand, we have shown that *dcap-2* genetic inhibition leads to decreased global synthesis rates. In line with this finding, we have previously shown that genetic inhibition of *edc-3*, which encodes the enhancer of mRNA decapping EDC-3 (also component of the decapping complex), triggers eIF4E/ IFE-2 sequestration and trapping within P-bodies, thereby reducing global translation rates (Rieckher et al., 2018). As the Reviewer suggested, the effect of *dcap-2* knockdown on protein synthesis, may be attributed to the accumulation of deadenylated mRNAs, which are not decapped and degraded, and as such, are inefficiently translated or may indirectly affect overall mRNA accumulation and ultimately the rate of bulk protein synthesis (He et al., eLife 2018). These interpretations are now presented in the discussion section of the revised manuscript so that our findings are more clearly linked with the enzymatic activities of the components examined. However, it should also be noted that the molecular underpinnings of protein synthesis regulation and their interface with the mechanisms that influence ageing are complex, and not yet well fully understood.

Additional major points

1. Although it is already indicated in the abstract, the authors only reveal in Figure 4 that the ntl-2 and dcap-2 bodies are different entities. Before that, the two bodies are presented in parallel. However, from the very beginning one has the feeling that they are functionally not the same. Wouldn't it be better if the differences were made clear early on?

Indeed, we first present the diversifying functional properties of the mRNA storage and degradation complexes in order to support the idea of their distinct identity. Therefore, we chose to present the two types of complexes in parallel so as to conclude with their identification as different entities. We tried to change the organization of the manuscript as suggested by the Reviewer, but we felt that the initial structure had a better flow, so we decided to keep it. Nevertheless, we have now made more clear early on that the components of the mRNA degradation complex and the proteins involved in the CCR4-NOT complex display different features. As such, they differentially influence essential cellular processes, including mitochondrial biogenesis.

This brings me to the next question - what is the relation between the dcap-2 bodies, the ntl-2 bodies and canonical p-bodies? Are they overlapping? I am missing some counterstainings between different p-body components and dcap-2 and ntl-2 as well as between dcap-2 and ntl-2. In addition, the storage bodies and degradation bodies would need to be characterized in more detail.

The prevailing view is that P-bodies are mebraneless formations consisting of translationally silenced mRNAs and RNA binding proteins. It is also known that mRNA degradation, mainly through the 5'-3' mRNA decay pathway occurs in P-bodies. Furthermore, the idea of mRNA storage within P-bodies is becoming increasingly appreciated although there is no direct experimental approach to show mRNA storage. This idea is mainly driven by the known functions of P-body components bound to identified mRNAs.

The main steps of 5'-3' mRNA degradation are: deadenylation-decapping and 5'-3' exonucleolytic decay. P-bodies are considered as dynamic formations with variable protein constituents. It is also accepted that components of the decapping and the CCR4-NOT complex constitute main P-body components (Decker and Parker, 2012). While microscopy and structural analyses show that components of the decapping enzyme (DCAP2), decapping activators (DCAP1 and EDC3) and the main cytoplasmic 5′→3′ exoribonuclease (XRN1) colocalize or form a complex accordingly (Eulalio et al., 2007, Charenton et al., 2016), there is not yet direct evidence that components of the degradation and the CCR4-NOT complex colocalize within a single formation/granule, the P-body. Furthermore, recent structural analysis has shown that DDX6/CGH-1 (one of the core components of P-bodies) physically associates primarily with the CCR4-NOT complex component CNOT1 to be activated. Also, DDX6 has been found to form mutually exclusive associations with Pat1, Edc3, Lsm14 or 4E-T. Moreover, by *in vitro* analyses, it has been shown that only 4E-T can concomitantly associate with DDX6 and CNOT1. Notably, CNOT1 binding to DDX6 displaces the decapping complex component Edc3 *in vitro*, implying that in the presence of CNTO1, DDX6 does not associate with Edc3 (the same stands for Pat1 and Lsm14), unless it is in excess. Thus, it is possible that different complexes with related functions could exist either in isolation or in combination in large mRNP granules (Ozgur et al., 2015). Moreover, decapping complex components (DCP1, EDC3) were found to be significantly enriched in P- bodies purified from human cell lines, whereas CCR4-NOT complex components did not show any enrichment within P-body isolates (Hubstenberger et al., 2017). This finding also supports the idea that the decapping and the CCR4-NOT protein complex components do not colocalize within a single formation/granule, the P-body, as was initially thought.

Although there are many reports pointing towards the direction that deadenylation is needed for P-body formation and that the CCR4-NOT complex is a structural part of P-bodies, there is no robust *in vivo* evidence that supports this. The above, in combination with recent structural analysis and P-body proteome analysis, which do not support the physical presence of the decapping and deadenylation complex components within a single formation, the P-body, strengthen our findings, indicating that the mRNA degradation and the CCR4-NOT complex components form distinct foci *in vivo*. It is worth noting, however, that our results do not exclude the possibility that these distinct foci can merge or associate under certain conditions. To our knowledge, we show for the first time *in vivo* that components from the two complexes do form foci, which do not colocalize. For better annotation and based on the enzymatic activities of their constituents, we name the foci that consist of CCR4-NOT protein complex components as "storage bodies" and the foci consisting of decapping protein complex components and the exonuclease XRN-1 as "degradation bodies".

Therefore, considering the dynamic nature of P-bodies and the variability of their protein constituents we believe that the mRNA degradation and the deadenylation (CCR4-NOT) complex function in two discrete types of bodies (or P-bodies), the degradation and storage bodies, respectively. Despite this, we cannot exclude the possibility that these two types of bodies share components. More specifically:

In our study, we find that the mRNA degradation and the deadenylation (CCR-4-NOT) complex components do not usually colocalize. Instead, they form distinct foci whose components are differentially expressed. In fact, Fig 4A shows the differential localization pattern of EDC-3::DsRed and NTL-2::GFP bodies in the hypodermis, which is further supported by the Pearson's correlation coefficient as shown in Fig 4B. Additionally, we have now generated new transgenic animals that co-express the following translational reporters: NTL-2::GFP with DCAP-2::mCherry, DCAP-2::mCherry with CCF-1::GFP and EDC-3::GFP with DCAP-2::mCherry. We found that DCAP-2 foci do not co-localize with NTL-2 or CCF-1 foci, while they extensively co-localize with EDC-3 foci (Appendix Fig S14).

Furthermore, we show that *ntl-2* genetic inhibition increases the abundance of DCAP-2 and EDC-3 foci (Fig 4F, G in the manuscript and Figure 1 in the response letter, respectively) and consistent with this, *dcap-2* genetic inhibition increases NTL-2 foci (Fig 4D, E in the manuscript). Furthermore, we have quantified storage and degradation body abundance upon genetic inhibition of additional components from both formations/foci. We found that knockdown of *ccf-1* or *let-711* increases DCAP-2 abundance (Appendix Fig S15A & B). Consistently, knockdown *of edc-3* or *xrn-1* increases NTL-2 abundance (Appendix Fig S15C & D). Moreover, we have generated transgenic animals that co-express EDC-3::GFP and CCF-1::DsRed fusion proteins under the control of *edc-3* operon and *ccf-1* gene promoter, respectively. Co-expression of these proteins severely affected the physiology of the animals and their brood size. As a consequence, we could not have as many progeny per generation as needed to perform experiments with many animals. However, we managed to get some age-synchronized animals and study the relative subcellular localization of EDC-3 and CCF-1 foci. In this case, we also found that the two types of foci do not colocalize. These data are presented only in this response letter (Figure 2, in the response letter).

Overall, co-monitoring the expression of all the above-mentioned combinations of proteins, and also monitoring storage and degradation body abundance upon genetic inhibition of several factors belonging to both types of bodies, provides a more detailed characterization of storage and degradation bodies that hopefully satisfactorily addresses the Reviewer's comment.

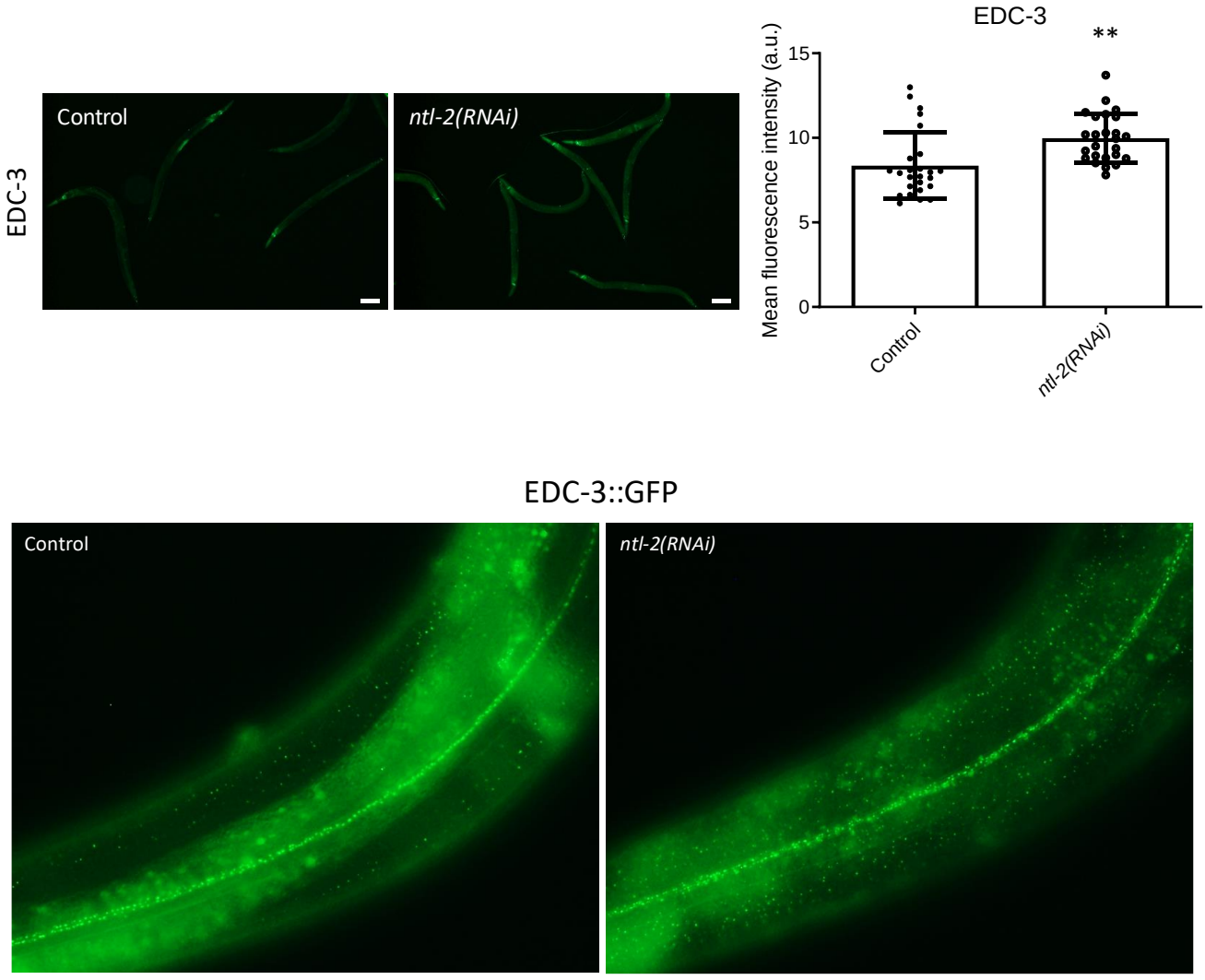

Figure 1. Knockdown of *ntl-2* increases EDC-3 abundance in 1-day-old worms (top) and also increases EDC-3-foci formation (bottom), as shown in animals expressing the p_{edc-3} EDC-3::GFP translational reporter.

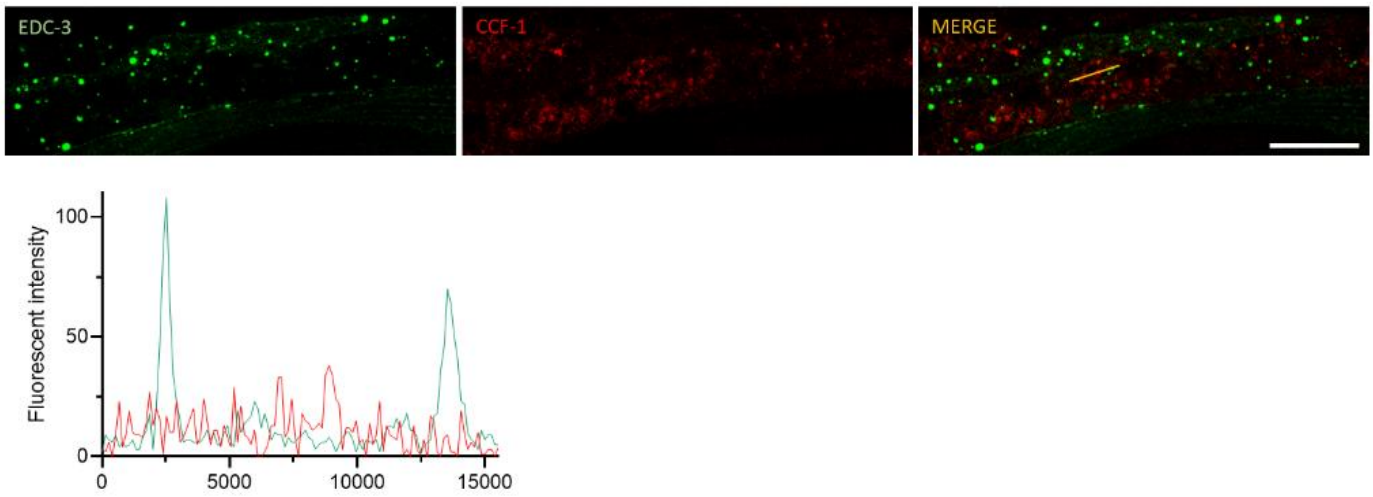

Figure 2. (Upper panel): Representative images showing the localization of EDC-3- (green) and CCF-1- (red) labeled foci and (lower panel): Fluorescent intensity graph of the foci marked with the yellow line (please see, the merged image in upper panel). Images were acquired using the X64 lens, Scale bar, 20µm.

Minor points

How is the quantification done, e.g. in Figure 1e, 1l, 1m? According to the figure legend three experiments were quantified, so where are the error bars?

Error bars have been included in Fig 1E, 1L (old Figure 1L is 1J in the revised manuscript), 1M and graphs have been substituted in the whole manuscript with bar scatter plots containing individual values so that their distribution is clearly shown. We apologize for any inconvenience caused.

In figure 1e the different classes are "0 µM", "<=1 µM", "0-1 µM" and ">5 µM". The second and third are the same, so I guess there is some mislabelling here?

We thank the Reviewer for pointing this out. Indeed, it was a mislabeling and we are sorry for the inconvenience. In the revised manuscript, graphs have been substituted with bar scatter plots containing individual values so that their distribution is clearly shown and labelling has changed.

Why are there no error bars in figure 4h?

Figure 4h showed one representative experiment out of 3 independent experiments performed with the same results. In the revised manuscript, we have merged the experiments and provided the graph with error bars in Fig 4L (figure 4h has become Fig 4L in the revised manuscript). We apologize for any inconvenience this may have caused.

Wouldn't it be better to show all primers in a table instead of in the text?

We have already provided a Table (please, see Reagents and Tools Table) summarizing all primer sequences used in this study at the end of the manuscript. Following the Reviewer’s suggestion, we removed the primer sequences from the text in the revised manuscript. They are now presented only in the Table.

Reviewer #2:

…... These are certainly novel and very interesting findings in the mitochondria and aging biology field, which when substantiated by additional data (see below) will deserve publication in the EMBO Journal.

We thank the Reviewer for the encouraging comments.

However, based on the described data, the overall conclusion drawn by the authors that "Local coordination of mRNA storage and degradation near mitochondria modulates C. elegans ageing" seems slightly overstated. Indeed, they clearly showed that some proteins belonging to mRNA-transcripts storage and degradation complexes, are localized near mitochondria and their suppression regulates mitochondrial content and aging. However, to be able to relate these findings to the complex (rather than to the single proteins), data should be provided with additional proteins belonging to the complexes.

We now provide further evidence indicating that additional components from the two complexes (DCAP-1, DCAP-2 for the degradation complex and CCF-1 for the CCR4-NOT complex) physically associate with mitochondria (Fig 1F in the manuscript and Appendix Fig S1-S3). We have also shown that genetic inhibition of these additional components influences mitochondrial mass in intestinal cells (Appendix Fig S11) in a manner that is consistent with the results already presented in the initially submitted manuscript (Figure 2). In addition, and in line with our previous data about *dcap-2* and *ntl-2*, genetic inhibition of other mRNA degradation (*edc-3*, *xrn-1*) and storage complex components (*ccf-1*, *let-711 (ortholog of human CNOT1*) also alters mitochondrial function in a manner consistent with our previous findings. Specifically, we have shown that knockdown of either *edc-3* or *xrn-1* increases mitochondrial mtROS levels, mitochondrial membrane potential, ATP production and Oxygen consumption rates (Appendix Fig S7-S10). By contrast, RNAi against either *ccf-1* or *let-711* decreases ATP production and increases mtROS levels and mitochondrial membrane potential (Appendix Fig S7-S9).

Moreover, we present additional data from lifespan experiments showing that components from the two types of bodies oppositely influence longevity. Specifically, we show that depletion of storage body components shortens lifespan, while in contrast, knockdown of degradation body components extends lifespan (Fig 6E in the manuscript and Appendix Fig S21 & S22). In addition, we have tested the combined effects of genetic inhibition and/or overexpression from the two types of bodies on lifespan. Our findings further highlight the significance of storage and degradation bodies coordination for lifespan regulation (Appendix Fig S21& S22).

Taken together, our new data robustly support the notion that mRNA degradation and storage complexes coordinately function near mitochondria to oppositely regulate mitochondrial content and function, as well as organismal ageing. Therefore, the major conceptual point of our manuscript is further strengthened by these additional experiments.

Moreover, they did not provide any evidence that the "coordination of mRNA storage and degradation", rather than simply the reduced expression of some of their components, is actually the mechanism underlying their effects on stress response and aging.

In our manuscript, we provide data showing that perturbation of one component (we have now tested additional components; please see Appendix Fig S15) from each type of body triggers an increase in the abundance of components from the other type and this impacts ageing.

For example, we show that *dcap-2* genetic inhibition triggers an elevation of NTL-2 bodies and *vice versa* (Fig 4D & E and 4F & G). Similarly, genetic inhibition of *edc-3* or *xrn-1* increases the abundance of NTL-2 (Appendix Fig S15C & D). Consistent with this finding, genetic inhibition of either *ccf-1* or *let-711* increases DCAP-2 abundance and *ntl-2* genetic inhibition increases total EDC -3 protein levels and foci formation (Appendix Fig S15A & B and Figure 1 in the response letter). Moreover, knockdown *of dcap-2*, *edc-3* or *xrn-1* increases stress resistance compared to control RNAi in wild–type nematodes (Fig 6 A-C and Fig EV4A, C & D). By contrast, genetic inhibition of *ntl-2*, *ccf-1* or *let-711* compromises resistance to diverse stress stimuli (Fig 6A-C and Fig EV4B, C&D).

Moreover, to identify whether the coordinated functions of storage and degradation bodies is needed for organismal stress response we genetically inhibited both storage and degradation body-components using different combinations. In this regard, we found that, *ntl-2* knockdown abrogates the enhanced resistance of EDC-3-or DCAP-2 deficient animals to heat stress and paraquat treatment (Fig EV4 C & D).

We have also tested the effects of storage and degradation body components on longevity. NTL-2 overexpression (mimicking *dcap-2* genetic inhibition) extends lifespan in contrast to its genetic inhibition (Fig 6E). Now, we have tested the effect of RNAi-mediated knockdown of several additional components on longevity, as also described in our answer to the previous comment (Appendix Fig S21). These results showed that genetic inhibition of storage body components is detrimental for longevity in contrast to genetic inhibition of degradation body components.

Further, to determine whether the coordinated functions of storage and degradation bodies are needed for longevity, we have tested the effects of combinatory interventions in the two types of foci on lifespan. We found that knockdown of *edc-3*, *dcap-2* or *xrn-1* further increases the lifespan of both NTL-2 overexpressing and CCF-1 overexpressing animals (Appendix Fig S21 B & C). By contrast, knockdown of *ntl-2*, *ccf-1* or *let-711* decreases the lifespan of EDC-3- or DCAP-2- overexpressing animals (Appendix Fig S21 D & E). Furthermore, knockdown of *ntl-2* or *let-711* abrogates the extended lifespan of *dcap-2(RNAi)*, *edc-3(RNAi)* and *xrn-1(RNAi)* animals (Appendix Fig S22). In total, our findings indicate that a balance between mRNA storage and degradation is critical for modulating ageing and organismal stress responses.

Overall the investigated topic is extremely interesting and of timely importance but in the current state the conclusions are overstated.

We thank the Reviewer for the appreciation of our work. We have now performed additional experiments to strengthen the conclusions drawn. We have rephrased the text accordingly to avoid overstatements and improved its organization so that every part helps the reader perceive our main findings/claims.

1) The mRNA decapping and the CCR-4/NOT complex physically and functionally associate with mitochondria in an age-dependent manner. In this first part the authors show the association of mitochondria only with one component of each complexe, NTL-2 and EDC-3. Additional proteins belonging to the complexes (CCF-1, CCR-4, DCAP-2...) should be tested to validate if the complexes (and not simply NTL-2 and EDC-3) actually physically and functionally associate with the mitochondria.

We now provide further evidence for the physical and functional association of additional proteins belonging to the mRNA degradation and storage complexes with mitochondria during ageing. More specifically, our new data show that DCAP-1 and DCAP-2 foci localize very close to mitochondria stained with DiOC(6)3 (a green fluorescent membrane potential-dependent dye) in 1-day-old animals. Their distance from mitochondria is increased in 7-day-old animals (Appendix Fig S1 & S2A-C). Also, we show that DCAP-2 co-fractionates with mitochondria of 1-day-old animals but not of the 7-day-old counterparts, as evidenced by immunoblot analysis (Appendix Fig S2D & E). Similarly, CCF-1 (a CCR4-NOT complex) localizes very close to mitochondria stained with Mitotracker Deep-Red and also co-fractionates with mitochondria in 1-day old animals (Appendix Fig S3 and Fig 1F in the revised manuscript). CCF-1 interaction with mitochondria is significantly weakened in 7-day-old worms. (Appendix Fig S3). Additionally, complementary to the imaging analysis, we now provide immunoblot analysis data showing that NTL-2 and EDC-3 dissociate from mitochondria in aged animals, while they physically associate with them in young animals (Fig 1F and EV1A-H, in the revised manuscript). Taken together, these results demonstrate that mRNA degradation and CCR4-NOT complex components physically associate with mitochondria in an age-dependent manner.

On the other hand, to be able to conclude that alteration of mitochondrial function impact on the expression and association of mRNA-complex-components with mitochondria (figure 1j,n,m), the effect of silencing mitochondria genes not directly related to gene translation should be assessed (mrps-5 is in fact directly affecting ribosomes and thus obviously mRNA regulatory complexes). This is especially in light of their observation that instead atp-3 RNAi (Figure S1) does not impact EDC-3 association with mitochondria.

Our data show that alterations in mitochondrial function can alter the abundance and association of storage and degradation bodies with mitochondria. Intriguingly, our results show that this association is complex and depends on the intervention performed, further highlighting the specificity of the functional associations of storage and degradation bodies with mitochondria. To avoid any further confusion, we have described this in the text in more detail so as to clarify that not each and every perturbation of mitochondrial function is expected to affect the abundance of storage and degradation bodies and their associations with mitochondria in the same manner.

Nevertheless, we show that perturbation of mitochondrial function following paraquat treatment affects the number and association of EDC-3 bodies with mitochondria (Fig 1I J, K). In line with this, a previous study showed that depletion of EAT-3 (a mitochondrial dynamin family member homologous to human OPA1), which is essential for resistance to free radicals (Kanazawa et al., 2008), caused a substantial increase in DCAP-1:: DsRed bodies during adulthood (Rieckher et al, 2018).

Regarding *mrps-5* genetic inhibition, we would like to add to the Reviewer's comment that the mRNA metabolism regulatory complexes we study in this manuscript are all localized in the cytoplasm and implicated in the regulation of the fate of cytoplasmic mRNAs. By contrast, *mrsp-5* is only implicated in translation of a relatively small set of 13 mRNAs coding for subunits of the oxidative phosphorylation and is strictly localized within mitochondria, as current knowledge suggests. Thus, since these factors are localized and act in different subcellular compartments we would not necessarily expect an effect of *mrps-5* genetic inhibition on the tested mRNA regulatory complexes. However, such a possibility cannot be excluded as a recent study showed that *mrps-5* RNAi-treated worms display a decrease in global translation, as a consequence of an evolutionarily conserved mito-cytosolic balance (Molenaars et al., 2020).

To complement our findings, we have performed additional experiments to test whether knockdown of other mitochondrial genes not directly related to gene translation has an effect on the expression and association of mRNA storage or/and degradation complex components with mitochondria. We found that knockdown of *drp-1* or *fzo-1* required for mitochondrial fission and fusion, respectively decreases the amount of both the EDC-3 and NTL-2 proteins specifically in the mitochondrial fraction isolated from 1-day-old animals compared to control conditions (Figure 3, in the response letter). Of note mitochondrial fission and fusion events are integral parts of mitophagy and mitochondrial biogenesis. By contrast, knockdown of *cyc-1* that encodes a protein predicted to enable electron transfer activity increases EDC-3 and NTL-2 levels in the mitochondrial fraction, indicating enhanced association with mitochondria, which has been further verified by *in vivo* experiments (Figure 3, in response letter and Appendix Fig S5).

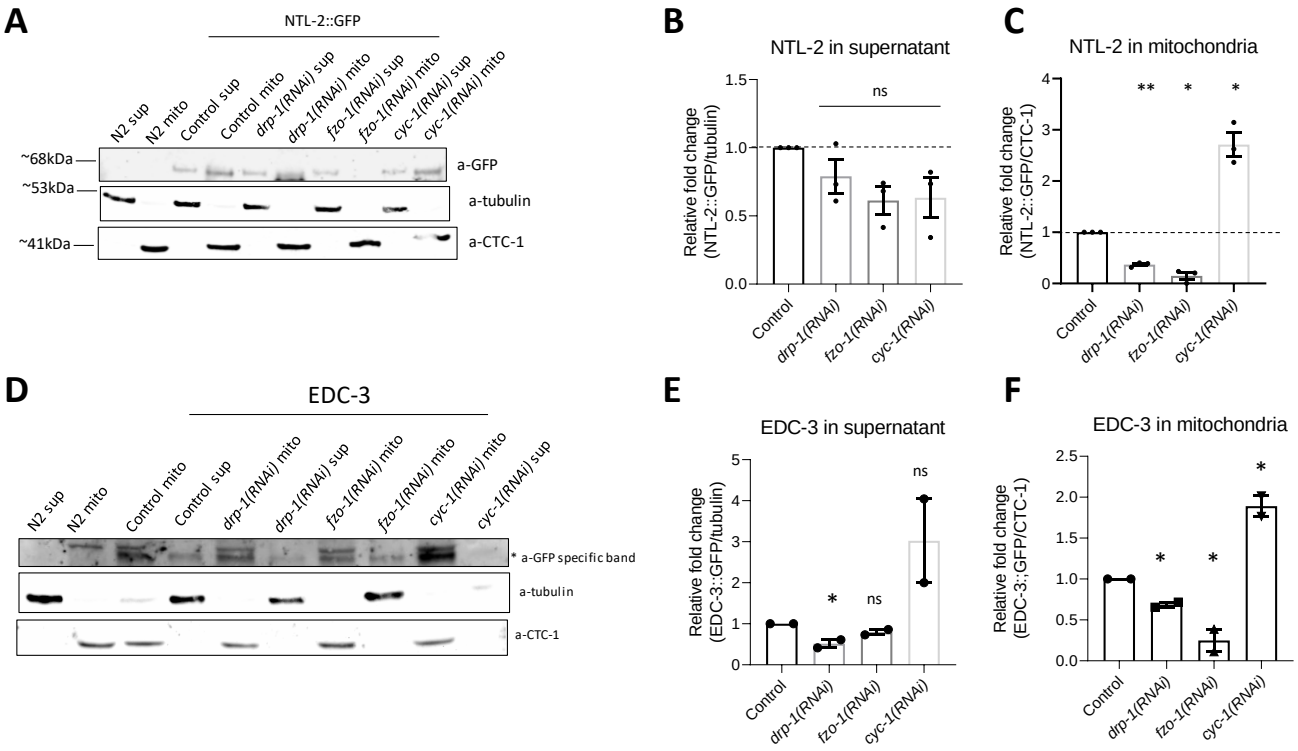

Figure 3: EDC-3- and NTL-2-foci are less associated with mitochondria upon *drp-1* and *fzo-1* genetic inhibition but associate more upon *cyc-1* downregulation. A, Immunoblot analysis of 1-day-old animals showing the protein levels of NTL-2 present in either the supernatant or the mitochondria under control conditions and upon the indicated genetic inhibitions. B, quantification of NTL-2 in the supernatant and C, In

the mitochondrial fraction (n= 3 independent experiments, *P<0.05, **P<0.01; Welch's one-way analysis of variance (ANOVA), followed by Dunnett's T3 multiple comparisons test). D, Immunoblot analysis of 1-day-old animals showing the protein levels of EDC-3 present in either the supernatant or the mitochondria under control conditions and upon the indicated genetic inhibitions. E, quantification of EDC-3 in the supernatant and F, In the mitochondrial fraction (n=2 independent experiments, *P<0.05; Welch's one-way analysis of variance (ANOVA) followed by Dunnett's T3 multiple comparisons test). Error bars denote SEM.

From Figure S1 it is not clear if the physical association between NTL-2- and EDC-3-containing bodies is actually reduced with aging. In S1a it is very hard to conclude that the association between mitochondria and NTL-2 is lost with aging given that the mitotraker staining (in which tissue?) is completely absent. Given that the structure and dynamics of mitochondria dramatically change during aging (e.g. Regmi et al. 2014; Palikaras et al. 2015), the authors should consider to repeat the experiment using for instance TOMM-20 strain or TMRE used in Figure 1 and possibly at different age and not only in 15 days old animals. Alternatively to be able to use the mtGFP expressing strain, they could cross it with a NTL-2::red reporter. Similarly, to establish that EDC-3-mito association is lost with age, time course experiments to better visualize mitochondria at earlier time points (e.g. 5 and 11 days) might actually help.

We thank the Reviewer for pointing this out. We have now repeated these experiments using different specific dyes and different time points (day1 and day5) so that staining of the animals is more efficient through feeding. To further strengthen our findings, we have also tested the presence of storage and degradation body components through immunoblotting in isolated mitochondria of young and aged animals (day 1 and day 7 of adulthood). We hope that our new data clearly show that NTL-2 and CCF-1 foci strongly associate with mitochondria in young animals but their association is significantly decreased in aged animals (Fig 1F, Fig EV1A-D and Appendix Fig S3). We have observed that NTL-2 and CCF-1 foci are markedly reduced in aged animals compared to young worms and consequently they were hardly visible late in life (Fig 4J, K, Fig EV1A and Appendix Fig S3A & B). Despite this, we measured the distance of remaining NTL-2::GFP and CCF-1::GFP foci from mitochondria stained with Mitotracker Deep-Red FM and found that it increases with age, indicating that the association of NTL-2 and CCF-1 foci with mitochondria is significantly decreased in aged animals. (Fig EV1A & B and Appendix Fig S3A-C). We further verified this age-dependent decrease in association of storage components with mitochondria by assessing the amount of NTL-2 and CCF-1 in the mitochondrial fraction at day 1 and day 7 of adulthood (Fig 1F in the manuscript, Fig EV1C & D and Appendix Fig S3D & E).

Similarly, the association of EDC-3, DCAP-1 and DCAP-2 foci with mitochondria was markedly decreased in the hypodermis of 7-day-old animals, as shown by staining mitochondria with DiOC(6)3 (Fig EV1E & F, Appendix Fig S1 and S2A-C). This decreased association was further verified by immunoblot analysis, which showed that the amount of both EDC-3 and DCAP-2 is significantly reduced in the mitochondrial fraction of aged animals compared to 1-day-old animals (Fig EV1G & H and Appendix Fig S2D & E), despite the fact that these components display an increased abundance with age (Fig 4H & I).

It is not clear why the authors, to exclude that altered association during aging is ascribed to abnormal mitochondrial structure/dynamics, choose to silence atp-3 (a subunit of the ATPase which would primarily impact on mitochondrial function) rather than genes directly involved in mitochondrial fusion/fission.

In principle, our aim is to show that alterations in the association of mRNA degradation/storage complexes with mitochondria are not simply a corollary of the ageing process, which is known to be accompanied by deterioration of mitochondrial network and function. Indeed, we show that *atp-3* genetic inhibition triggers a severe loss of the mitochondrial network integrity and despite this, the associations of NTL-2 foci with mitochondria are not disrupted in young animals (Fig EV1K-M). This finding further strengthens our results suggesting that the associations of storage bodies with mitochondria are local translation-dependent (as unlike *atp-3* genetic inhibition, inhibition of either *tomm-20* or *akap-1*, which encode outer mitochondrial membrane proteins promoting protein synthesis on the mitochondrial surface (Zhang et al., 2016) disrupts the associations of storage bodies with mitochondria (Fig 5B, C, E & F in the revised manuscript).

Nevertheless, we have also tested the effects of *drp-1* and *fzo-1* RNAi on degradation and storage bodies, as suggested by the Reviewer. We found that the amount of both EDC-3 and NTL-2 is decreased in the mitochondrial fraction of FZO-1- and DRP-1- depleted young adults compared to controls (Figure 3, in the response letter). It is worth to note, however, that mitochondrial dynamics have been reported to interfere with mitochondrial turnover. Indeed, a recent study showed that DRP-1 deficiency leads to mitochondrial hyperfusion and less mitochondrial turnover, ultimately accelerating cellular senescence or ageing phenotype in mice (Yu et al., 2020). In addition, several studies suggested the existence of a functional relationship between mitochondrial dynamics and mitochondrial biogenesis, but the underlying mechanisms remain largely obscure (for example, Seo et al., 2010). Since the main focus of our manuscript is the regulation of mitochondrial biogenesis by storage and degradation bodies we would expect that interfering with mitochondrial fission and fusion has an impact on the association of the studied components with mitochondria. The implication of mitochondrial dynamics in the associations of storage and degradation body components with mitochondria would need further investigation which falls out of the scope of the current study. For this reason, we have not included these data in the revised manuscript (Figure 3, in the response letter).

Nevertheless, to further verify our findings, we also tested the effect of RNAi-mediated knockdown of *cyc-1*, which encodes a protein predicted to enable electron transfer activity and its genetic inhibition also perturbs mitochondrial network integrity (Appendix Fig S5). We found that the amount of EDC-3 and NTL-2 proteins is increased in the mitochondrial fraction isolated from 1-day-old animals subjected to *cyc-1* RNAi compared to controls, suggesting increased interaction with mitochondria (Figure 3, in the response letter), again despite mitochondrial network disturbance. This increased interaction was further verified by measuring the distance of NTL-2 or EDC-3 foci from mitochondria *in vivo* in 1-day-old animals subjected to *cyc-1* knockdown compared to age-matched controls (Appendix Fig S5). Taken together, these findings indicate that mitochondrial network fragmentation is not sufficient to disturb the associations of storage and degradation bodies with mitochondria.

Most importantly, to corroborate the conclusion that mitochondria association with mRNA storage and degradation regulatory proteins is affected during aging, their association should be check in long- and short-lived mutants.

We have now tested the association of mRNA storage and degradation regulatory proteins with mitochondria in short- and long-lived animals, as suggested by the Reviewer. We performed these experiments by RNAi silencing of longevity regulatory genes that do not encode mitochondrial proteins, such as *daf-2*, and *nhr-49*. We found that the associations of NTL-2 and CCF-1 foci with mitochondria are increased in the long-lived *daf-2(RNAi)* animals, whereas they are decreased in the short-lived *nhr-49*(*RNAi)* animals, even from day 1 of adulthood, as evidenced by both *in vitro* and *in vivo* experiments (EV Fig 2A-F and Appendix Fig S6). By contrast, the associations of mRNA degradation components, such as DCAP-1, DCAP-2 and EDC-3, with mitochondria decrease in both long-lived and short-lived animals (EV Fig 2G-N and Appendix Fig S6). The decreased association of mRNA degradation components with mitochondria coinciding with an increase in the number of storage bodies (Figure 4, in the response letter) and the enhanced association of the latter with the organelles in long-lived animals may represent a state of balance between mRNA storage and degradation which contributes to the maintenance of cellular and organismal homeostasis, as suggested by our data. Accordingly, the reduced association of mRNA degradation and storage components with mitochondria in short-lived animals, reflects a state in which both mRNA storage and degradation near mitochondria are impaired, which may contribute to their decreased survival and compromised stress responses.

As previously mentioned, we have also performed experiments in wild-type animals at defined time points (day 1, day 5 and day 7 of adulthood) and found that the association of mitochondria with mRNA degradation (e.g. EDC-3, DCAP-1 and DCAP-2) and storage (e.g. NTL-2, CCf-1) regulatory proteins decreases during aging, *in vivo.*

We have cross-verified all the above-mentioned results by using a strain that co-expresses the storage body component, NTL-2 and the degradation body component, EDC-3. The results of *in vivo* imaging and *in vitro* biochemical approaches are shown in Appendix Fig S4 and S6, as also mentioned above.

Additionally, we have tested the effect of *daf-2* and *nhr-49* genetic inhibition on the total protein abundance and foci formation. We found that *daf-2* genetic inhibition increases the total abundance of NTL-2 and the number of the NTL-2 foci, but it decreases the abundance of DCAP-2 and EDC-3. Further, the life-shortening *nhr-49* genetic inhibition decreases the number of NTL-2 foci, in contrast to the lifespan-extending *daf-2* genetic inhibition (Figure 4 in response letter).

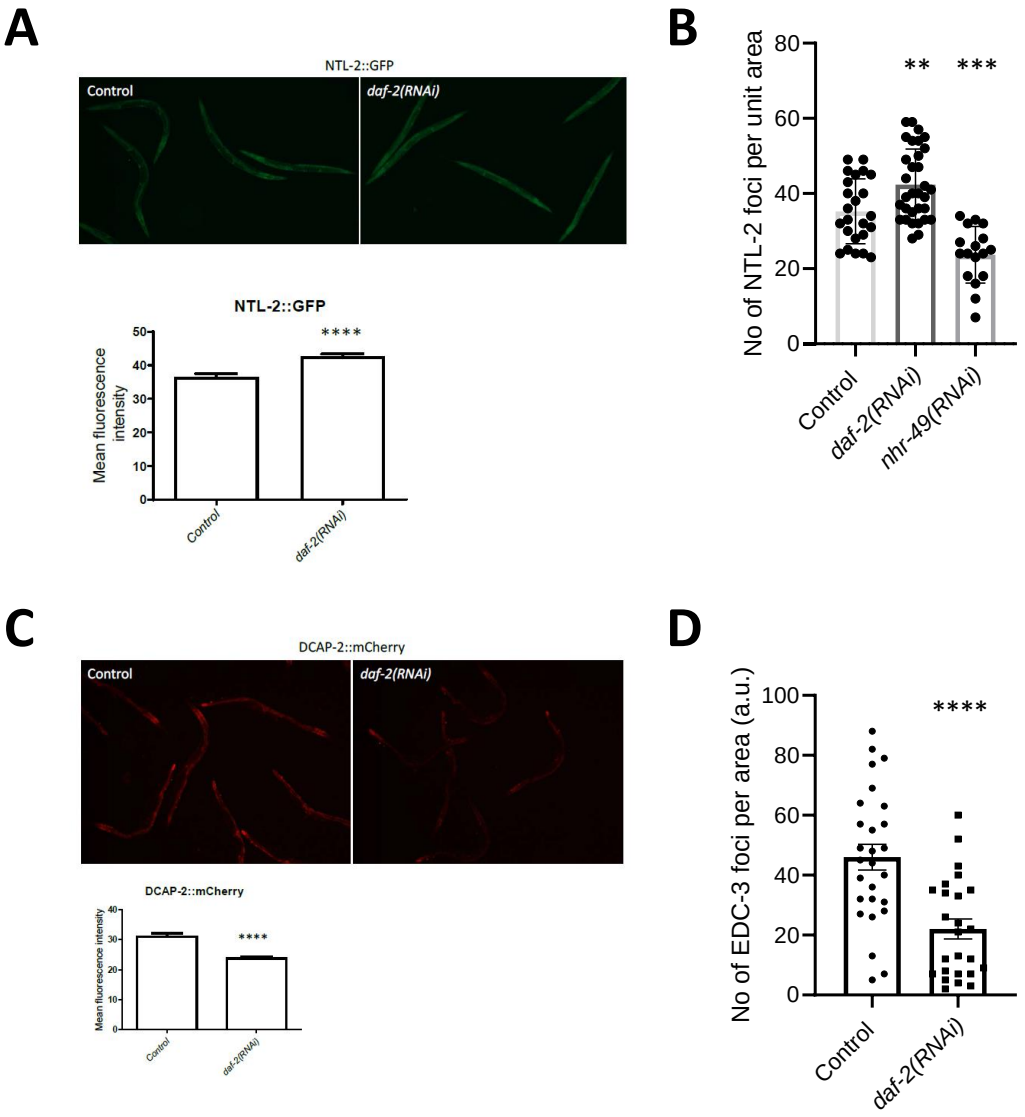

Figure 4. *daf-2* and *nhr-49* genetic inhibition influences total protein abundance and foci formation of storage and degradation body components. A, top: Representative images showing that the total protein levels of NTL-2 increase upon genetic inhibition of *daf-2* and bottom: Respective quantification is shown, ****P<0.0001; two-tailed unpaired t-test. B, The number of NTL-2 foci increases upon genetic inhibition of *daf-2*, but decreases upon *nhr-49* knockdown, **P<0.01, ***P<0.001; one-way analysis of variance (ANOVA). C, top: Representative images showing that the total protein levels of DCAP-2 drop upon genetic inhibition of *daf-2* and bottom: Respective quantification is shown, ****P<0.0001; two-tailed unpaired t-test. D, the number of EDC-3 foci drops upon genetic inhibition of *daf-2*, ****P<0.0001; two-tailed unpaired t-test.

Together, these findings further support the notion of a functional relationship of degradation and storage bodies with mitochondria and the physiological significance of their coordinated functions in modulation of ageing.

Incidentally, the authors used RNAi against atp-3 (Figure S1) and mrps-5 (Figure 1j, m, n), which are known to extend C. elegans lifespan (Dillin et al. 2002; Rea et al. 2017; Houtkooper et al 2013), and showed that the former does not affect mito-ECL-3 association while the latter increase its expression yet seems to reduce its association with the mitochondria. How do the authors reconcile these opposite effects with two pro-longevity interventions? Moreover, if as they suggest, ECD-3-mito association is lost with aging (Figure S1), wouldn't be expected that pro-longevity intervention increase their association? Again, time course experiments during aging in long- and short-lived mutants might help clarifying on this important point of the study.

We find that EDC-3 bodies are increased in number but their association with mitochondria is reduced in animals subjected to *mrps-5* RNAi (Fig 1L & M). The finding that the amount of EDC-3 associated with mitochondria (but not the total EDC-3 abundance), is reduced upon *mrps-5* RNAi suggests that mito-localization of EDC-3 is influenced by *mrps-5* that functions in mitochondrial translation. *atp-3* encodes the human homologue of nuclear-encoded ATP5O and its downregulation does not influence EDC-3 foci-mitochondria contacts. As the Reviewer mentions, its downregulation during development has been shown to extend lifespan in *C. elegans* (Dillin et al, 2002; Chen at al. 2007; Rea et al, 2007). Nevertheless, not all mitochondrial perturbations are expected to affect the number of storage and degradation bodies and their associations with mitochondria in the same manner, as previously discussed.

Following the Reviewer's suggestion, we have tested the association of mRNA storage and degradation regulatory proteins with mitochondria in short- and long-lived animals, as discussed above (Fig EV2 and Appendix Fig S6). We find that the associations of EDC-3, DCAP-1 and DCAP-2 with mitochondria are reduced in long-lived *daf-2(RNAi)* animals at day 1 and day 5 of adulthood (data about day 5 are not shown). Therefore, it is not surprising that a pro-longevity intervention, like *mrps-5* genetic inhibition, does not increase EDC-3 association with mitochondria in wild-type animals. The associations of the NTL-2 and CCF-1 foci with mitochondria are increased in long-lived *daf-2(RNAi)* animals at day 1 and day 5 of adulthood, as previously mentioned (Fig EV2 and Appendix Fig S6, data about day 5 are not shown). By contrast, short-lived animals display decreased associations of NTL-2 and CCF-1 foci with mitochondria even from day 1 of adulthood (Fig EV2A-F & Appendix Fig S6), as discussed above.

More detailed interpretation of these new results can be found in the discussion section of the revised manuscript.

2) mRNA decapping and CCR-4/NOT complex components oppositely regulate mitochondrial biogenesis and abundance by functioning in discrete foci. The authors show that dcap-2 and ntl-2 differentially impact on mitochondrial morphology and mass. What is the rationale for switching to dcap-2 instead of also using ecd-3 RNAi? What is the effect of ecd-3 RNAi on the different mitochondrial-related parameters (morphology, ROS and TMRE, SKN-1 and AAK-2 activation)? What is instead the effect of dcap-2 on mitochondrial-complexes physical association? Validating the findings with suppression of different proteins belonging to the same (storage and degradation) complexes, would support the conclusion.

The rationale for switching to *dcap-2* RNAi instead of using *edc-3* RNAi was just to examine the effects of another degradation body component on mitochondrial-related parameters in the context of the central idea of this study.

In the revised manuscript, we now provide additional data on the effects of additional genes encoding components involved in mRNA degradation (*edc-3*, *xrn-1, dcap-2*) on mitochondrial abundance and key mitochondrial function parameters (mitochondrial membrane potential, mtROS, oxygen consumption rates, ATP production) and also on SKN-1 and AAK-2 expression and activity. Consistent with our previous findings, mitochondrial abundance and mitochondrial function parameters are increased in 1-day-old animals subjected to RNAi against *edc-3*, *dcap-2* or *xrn-1* (Fig 1G & H, Fig 2 in the revised manuscript and Appendix Fig S7-S11). In addition, both AAK-2 (Fig 3F & G in the revised manuscript) and SKN-1 levels are elevated (Fig 3A & B in the revised manuscript) and SKN-1 is activated in animals subjected to RNAi against degradation complex components (Fig 3C & D and Appendix Fig S12 & S13) compared to controls. By contrast, knockdown of CCR4-NOT complex components decreases the abundance of mitochondria and alters their function in 1-day-old animals (Fig 1G & H and Fig 2 in the revised manuscript and Appendix Fig S7-S9 & S11), decreases SKN-1 (Fig 3A & B in the revised manuscript) and AAK-2 levels (Fig 3F & G in the revised manuscript), as well as SKN-1 activity (Fig 3C & D and Appendix Fig S12 & S13).

Moreover, we have tested the effect of *dcap-2* genetic inhibition on mitochondria-complexes association, as suggested by the Reviewer. We found that the association of NTL-2 foci with mitochondria is significantly increased in 1-day-old animals subjected to *dcap-2* RNAi (Appendix Fig S18A-B and Fig EV 2C & D) in contrast to the associations of EDC-3-foci with the organelles which are significantly impaired (Appendix Figure S18C-F). Since we also show in our revised manuscript that *dcap-2* genetic inhibition increases lifespan (Appendix Fig S21A) and stress resistance (Fig 6A-C and Fig EV4C & D), this result supports previous findings showing that the associations of NTL-2-foci with mitochondria increase in the long-lived DAF-2-depleted animals in contrast to EDC-3-foci which loose association with the organelle under the same treatment.

On the same line, the authors conclude (end of first paragraph on pag 6) that perturbation of decapping complex increases the functional mitochondrial population but they actually showed (figure 1 g,h) that dcap-2 RNAi increases mitochondrial ROS and membrane potential. How would be this indicative of "functional mitochondria"? To reach reliable conclusion in this regard, the effect of different decapping regulatory proteins (and not just one) on different mitochondrial functional parameters, e.g. respiration and/or ATP content should be tested.

We now provide additional data showing that knockdown of genes encoding degradation body components (*dcap-2*, *edc-3*, *xrn-1*) increases mitochondrial mass (Appendix Fig S11), membrane potential (indicative of functional mitochondria) (Fig 1H in the revised manuscript and Appendix Fig S8A-B), mtROS levels and mitochondrial ATP levels compared to control (Appendix Fig S7 &S9A&B). We have also performed real time measurements of oxygen consumption rates using the Agilent Seahorse XF Analyzer. We found that the basal and maximal oxygen consumption rates are increased upon knockdown of *dcap-2, edc-3* or *xrn-1* (Appendix Fig S10), indicative of a healthier bioenergetics profile compared to control (Chacko et al, 2014, Clinical Science). These findings invite the speculation that genetic perturbations in mRNA

degradation components increase the number of functional mitochondria -a high number of healthy mitochondria is expected to produce more ATP, ROS and Δψ, if functional, compared to a lower number of mitochondria-. By contrast, genetic inhibition of genes encoding mRNA storage components decreases mitochondrial mass (Fig 2 & Appendix Fig S11) and alters mitochondrial function-related parameters (Fig 1G & H in the revised manuscript and Appendix Fig S7 & S8C&D and S9C&D)).

Taken together, our findings provide further support to our claim and strengthen the conceptual context of our study.

Given that a primary readout for mitochondrial stress is induction of mtUPR, it would be interesting to know whether reduced expression of mRNA storage/degradation regulatory proteins impacts on hsp-6 expression.

We thank the Reviewer for this suggestion. We have tested whether depletion of storage or degradation regulatory proteins impact on *hsp-60* expression (another reporter of mtUPR) and found that knockdown of *xrn-1* slightly decreases *hsp-60* expression, while knockdown of *dcap-2* does not. By contrast, knockdown of either *ntl-2* or *ccf-1* induces *hsp-60* expression (Figure 5, in the response letter). The possible contribution of mtUPR to healthspan and lifespan changes caused by perturbations in mRNA storage and degradation regulatory proteins is worth considering in the future, so we will not incorporate these data in our manuscript.

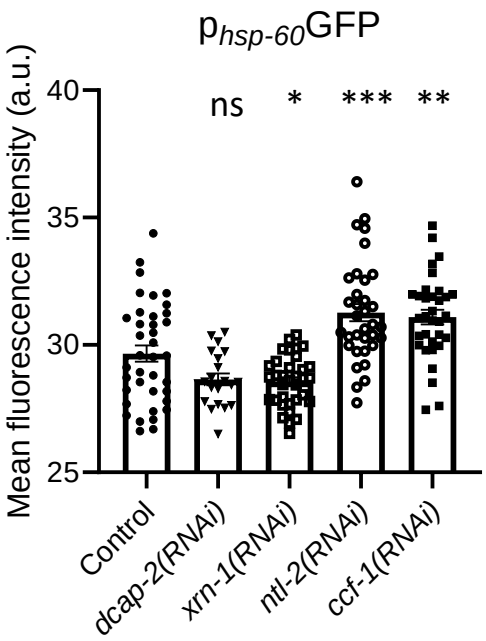

Figure 5. Quantification of *hsp-60p*::GFP reporter upon genetic inhibition of *dcap-2*, *xrn-1*, *ntl*-2 or *ccf-1* in 1-day-old animals. (n=3 independent experiments with at least 160 animals per experiment; *P< 0.05, **P< 0.01, ***P< 0.001; one-way analysis of variance (ANOVA)).

3) Storage bodies constitute local translation coordinators in the vicinity of mitochondria. Data shown in figure 5 are actually not enough to reach this conclusion. The amount of MTPTs associated

with NTL-2::GFP (and possibly other storage components) should be addressed upon silencing or overexpression of storage/degradation/translation-inducer proteins, by mitochondrial stress or aging. This would provide further support to the overall conclusion of the work.

Our results show that NTL-2 binds specifically MTPTs (Fig 5A in the revised manuscript). More specifically, the mRNA levels of *atp-5, atp-1, f46b6.6, mrpl-13, nuo-5, t20h4.5 (*ortholog of human NADH ubiquinone oxidoreductase core subunit S8, NDUFS8)*, as well as skn-1*, which is known to regulate mitochondrial biogenesis (Gureev, 2019; Palikaras et al. 2015,), are all enriched in the immunoprecipitated sample of 1-day-old NTL-2::GFP animals compared to animals expressing the *gst-4p*::GFP reporter and the non-GFP expressing *unc-119(ed3)III* mutants used as controls. Selective association of these transcripts with the NTL-2::GFP protein extract is specific, since mRNAs encoding nuclear proteins (FIB-1*,* NPP-22), endoplasmic reticulum (SPCS-1) or cytoplasmic (RHI-1) proteins have not been detected (Appendix Fig S17A). Detection of *rhi-1* mRNA in the protein extract of *gst-4p*::GFP reporter animals was unspecific.

In addition, we provide further evidence suggesting that NTL-2 associates with mitochondria in a local translation-dependent manner, given that its association with the organelles (Fig 5B & C in the revised manuscript) as well as its abundance in the mitochondrial fraction (Fig 5E and F in the revised manuscript) are decreased upon knockdown of either *tomm-20* or *akap-1*, which are known to promote protein synthesis on the mitochondrial surface (Zhang et al., 2016), as mentioned above.

Moreover, we have now examined MTPTs for their association with NTL-2::GFP bodies upon silencing of *dcap-2*, as well as under mitochondrial stress (CCCP treatment) and ageing, as the Reviewer suggested. We find that genetic inhibition of *dcap-2* increases MTPT binding by NTL-2 (Appendix Fig S17B). Based on the enzymatic activity of DCAP-2, this could be attributed to decreased mRNA decapping and degradation, leading to accumulation of these MTPTs. Moreover, this result is consistent with our findings showing that the number of NTL-2 foci increases upon *dcap-2* genetic inhibition and their association with mitochondria is also strengthened (Fig 4D & E in the revised manuscript and Appendix Fig S18A & B). In contrast, we have found that upon CCCP treatment and during ageing NTL-2 foci formation is significantly decreased (Figure 6 in the response letter and Fig 4J in the revised manuscript) and the selective NTL-2-mitochondria interaction is also impaired (Fig EV1A-D). Consistent with these results we now find that MTPT binding on NTL-2 is abolished in both cases (Appendix Fig S17B). Taken together, our results provide further evidence for the selective binding of MTPTs to NTL-2 bodies.

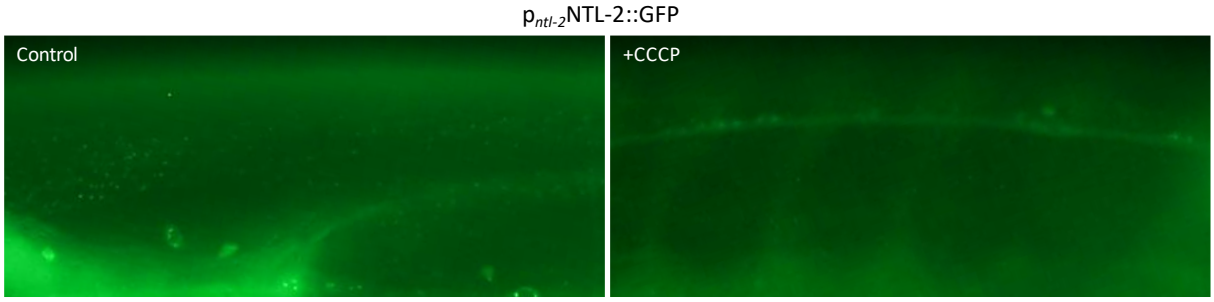

Figure 6. CCCP treatment inhibits the formation of NTL-2-foci.

In addition, we provide evidence that *tomm-20* genetic inhibition reduces DCAP-2 levels (Figure 7 in response letter), whereas it increases NTL-2 levels, as shown in our manuscript (Fig 5D). These results indicate that perturbation of local translation triggers an elevation of storage body components and a concomitant decrease in degradation body components, further supporting an interplay between local translation and coordination of storage and degradation bodies.

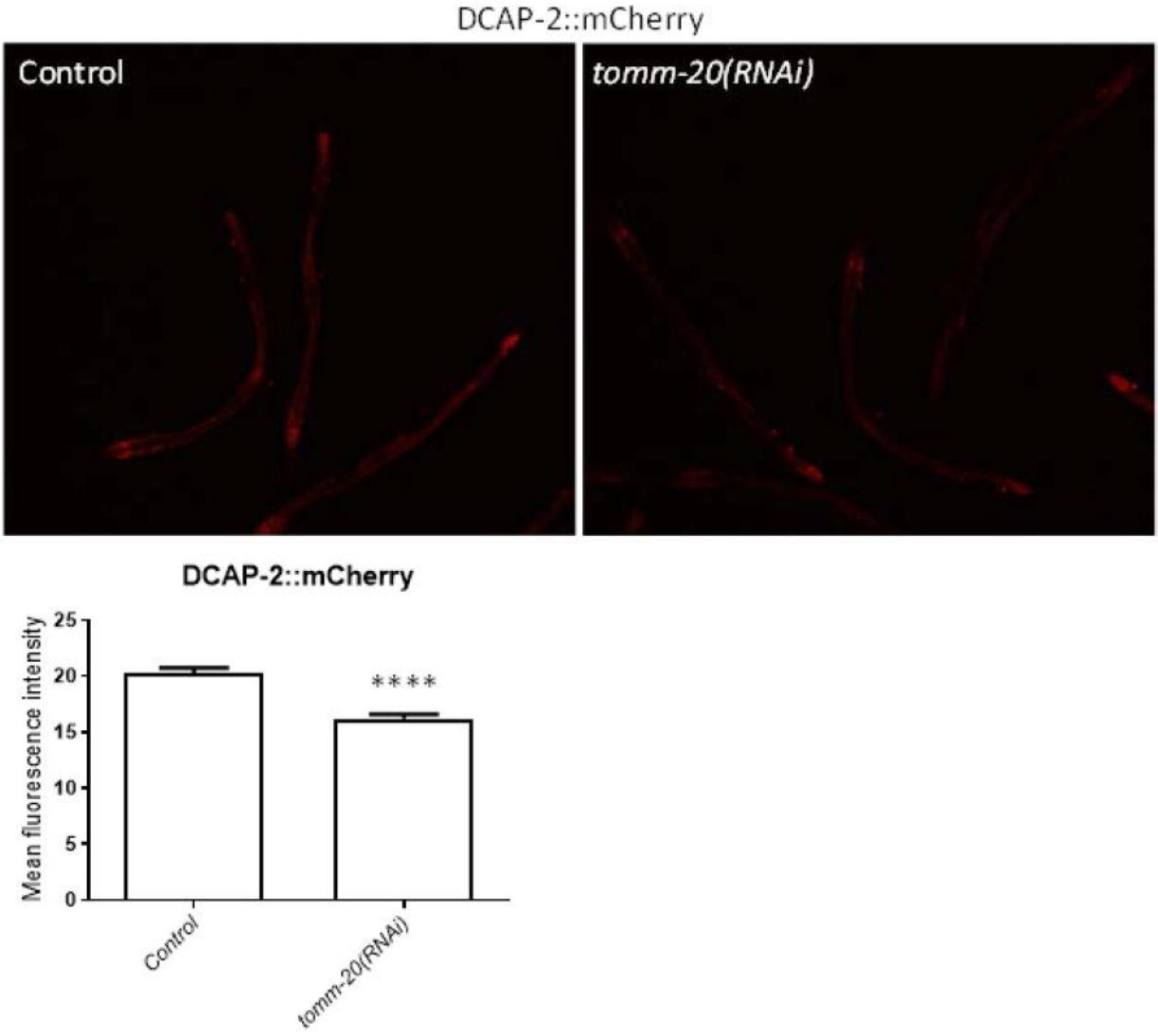

Figure 7. Perturbation of local translation through *tomm-20* genetic inhibition decreases total DCAP-2 levels.

We believe that these new data provide significant insight into the associations of NTL-2/storage bodies with MTPTs and further support the biological significance of NTL-2 binding on MTPTs.

Also, if, as they suspect the storage and degradation foci act antagonistically, can they revert alteration in e.g. MTPTs translation by dcap-2 or ntl-2 suppression with akap-1 or tomm-20 depletion?

We thank the Reviewer for his/her comment. We performed double RNAi experiments in the combinations: *ntl-2(RNAi); akap-1(RNAi) and ntl-2(RNAi);tomm-20(RNAi)* to examine their effects on protein levels of select MTPTs (based on antibody availability; we would like to kindly remind you that there is limited availability of antibodies to work with, in *C. elegans*). We were happy to find that the protein levels of ATP-1, F46B6.6, MRPL-13 and T20h4.5 increase upon *ntl-2* knockdown. This increase was abrogated upon knockdown of either *tomm-20* or *akap-1* (Appendix Fig S20). These findings suggest that MTPT translation is regulated by NTL-2 and this is reversed when we concomitantly genetically inhibit the characterized positive regulators of local translation near mitochondria, AKAP-1 or TOMM-20. These results support the rest of our findings and strengthen the conceptual framework of our work.

In the same chapter "to investigate whether storage bodies have a role in local translation of MTPTs..." they perturbed mitochondrial local translation inducers akap-1 and tomm-20. While this is an interesting point to look at, with this experiment they investigate exactly the contrary, that is: if local translation machinery plays a role in storage bodies components association with mitochondria. To actually investigate if storage bodies have a role in local translation of MTPTs it should be assessed (as suggested above) whether modulating the expression of storage bodies components impact on local translation (e.g. by quantifying MTPTs) or on translation inducers expression/activity.

Indeed, with this experiment, we examine whether there is a relationship between local translation regulators and storage body components. We hypothesized that if storage bodies are, by any means, implicated in the regulation of local translation events, then their localization pattern and/or abundance would be altered in response to perturbations in local translation regulators.

We would like to thank the Reviewer for the suggested experiments, which we totally agree can strengthen our manuscript. Therefore, we have now tested whether knockdown of storage body components affects the expression of *tomm-20* and *akap-1* local translational inducers. We find that knockdown of either *ntl-2* or *let-711* increases the expression of TOMM-20::mKate2 (ubiquitous expression) and TOMM-20::mRFP (expression in body wall muscle cells) transgenes (Appendix Fig S19). We would also like to mention that we tried to detect AKAP-1 through immunoblotting. For this reason, we purchased a commercially available antibody for AKAP-1 which did not work for *C. elegans* samples even when we tried it at very high concentrations.

Finally, results shown with atp-3 in Figure S3 seems rather contradictory. Why atp-3RNAi does not affect the percentage of NTL-2 vicinity to mitochondria (S3b) but it increases the amount of NTL-2 associated with mitochondria (S3c)?

We respectfully disagree with the Reviewer's comment that the results in Figures S3b and S3c (in the original manuscript) are contradictory. In both cases, there is an increase in NTL-2 bodies that closer

associate with mitochondria. We agree that the increase shown in Figure S3c in our original manuscript is much more robust. This is expected because in this case, we implement a biochemical approach to assess the association of NTL-2 with mitochondria, in mitochondrial isolates. In Figure S3b, we monitor the associations through imaging and quantification of the distances in the best resolution possible, but still, some differences may be masked.

Nevertheless, in our revised manuscript the graph has been substituted with bar scatter plot containing individual values so that their distribution is clearly shown (Fig EV1L). In the revised manuscript, we have merged all our replicates, and thus the differences became more pronounced in this experimental setup, as well (please, see Fig EV1L & M).

4) Balanced mRNA storage and degradation promotes stress resistance and longevity. In the last chapter the authors investigate the role of dcap-2 and ntl-2 in mitochondrial stress resistance and aging and found that they act in opposite directions. However, to prove that this is due to local imbalance of mRNA storage/degradation/translation the effect of silencing additional components on the same complexes should be tested.

Our data show that perturbation of one component (we have now tested additional components from each type of body) triggers an increase in the abundance of components from the other complex and this impacts ageing. For example, we find that *dcap-2* genetic inhibition increases NTL-2 levels (Fig 4D & E). Similarly, *edc-3* or *xrn-1* knockdown increases NTL-2 levels (Appendix Fig S15C & D). In addition, we find that knockdown of *dcap-2* or of other degradation body genes promotes longevity (Appendix Fig S21A) and ameliorates lifespan shortening caused by *ntl-2* or *let-711* knockdown (Appendix Fig S22A, C). Furthermore, we clearly show that NTL-2 overexpression (mimicking *dcap-2* genetic inhibition) extends lifespan, in contrast to *ntl-2* genetic inhibition that decreases it (Fig 6E in the revised manuscript and Appendix Fig S21A & C). Genetic inhibition of mRNA degradation components (*edc-3*, *dcap-2*, *xrn-1*) further increases the lifespan of NTL-2- and CCF-1- overexpressing animals (Appendix Fig S21B & C). By contrast, genetic inhibition of *ntl-2*, *ccf-1* and *let-711* decreases the lifespan of EDC-3- and DCAP-2- overexpressing worms (Appendix Fig S21D & E). Moreover, we found that genetic inhibition of *edc-3* or *dcap-2* ameliorates the sensitivity of *ntl-2(RNAi)* animals to heat stress and paraquat treatment (Fig EV4C & D). Taken together, our findings indicate that balanced mRNA storage and degradation processes coordinately modulate ageing and stress resistance.

Moreover, altered MTPTs translation in conditions that affect stress response/aging should be assessed, as well as its reversion when suppressing components of the other components of the translation machinery which suppress the lifespan phenotypes.

We are not sure we understand the Reviewer's comment. It is not clear to us what the Reviewer means by mentioning "components of the other components of the translation machinery which suppress the lifespan phenotypes".

Nevertheless, we performed double RNAi experiments in the following combinations *ntl-2;akap-1(RNAi)* and *ntl-2;tomm-20(RNAi)* to examine their effect on protein levels of select MTPTs, where antibodies were available, as discussed above.

Discussion

Based on the described finding, some of the conclusions seems overstated. On page 10, end of the first chapter of the discussion, the author state that "the "two types of foci form antagonistically to each other and oppositely regulate cytoplasmic translation rate". While they showed that components of the complexes are regulated antagonistically, they provided no evidence on their effect on cytoplasmic translation. Also, at the beginning of the following chapter they state "Increased global protein synthesis and aberrant translation of target MTPTs triggered by perturbation of storage bodies following ntl-2 genetic inhibition...." yet, they have not actually shown that inhibition of ntl-2 or of other components of the machinery affect translation. Similarly, in the first chapter of page 11, they state, but actually have not shown that "...dcap-2 depletion reduces overall mRNA translation oppositely to ntl-2 genetic inhibition".

In this study, we assess protein synthesis rates by Fluorescence Recovery after Photobleaching (FRAP) in wild-type animals subjected to RNAi against *dcap-2* or *ntl-2* compared to controls (Fig 4L in the revised manuscript). This is an established methodology for *in vivo* monitoring of new protein synthesis in cells or tissues of interest (Papandreou et al., 2020, J. Vis. Exp. 163, e61170, doi:10.3791/61170; Kourtis and Tavernarakis, 2017, Bioprotocol, 7(5): e2156). In addition, we have now determined total protein content in DCAP-2- and NTL-2-depleted animals and found that it is substantially reduced in DCAP-2 –depleted worms, but increased in NTL-2 depleted worms compared to controls, indicating that storage and degradation body components influence bulk protein synthesis (Appendix Fig S16). Nevertheless, we tried to tone down claims that are not validated by an additional approach such as polysome profiling, which is not a simple task in *C. elegans*.

Minor points

1) Figure 1 and S1

- Figure 1e. The y-axis labels should be changed into something that reflects % protein-mito proximity/distance. The author should provide a clearer description in their methods session on how the distances were calculated.

The graph in Fig 1E has been substituted with bar scatter plot containing individual values so that their distribution is clearly shown and y-axis labelling has changed.

In addition, we have now provided a more detailed description in the materials and methods section on how the distances were calculated (see “Measurement of the distances between foci and mitochondria” in the Materials and methods section)

- **Figure 1k-n need clearer self-explanatory graph labelling: is panel k showing the number of ECD-3 foci or protein expression? Similar to panel 1e, in panels l and m it should be clarified what exactly the y-axis indicates (e.g. % mito-ECD-3 proximity?) and if the differences of treatments vs control are significant. Does panel n represent NTL-2 protein content or n of foci associated to mitochondria? What about the quantification upon paraquat**?

The graphs have been substituted with bar scatter plots containing individual values so that their distribution is clearly shown and y-axis labelling has changed.

- **Figure S1. The authors should refer to NTL-2 and EDC-3 foci or protein (not bodies) since they have not looked at the expression of other proteins belonging to the complexes.**

Correction have been made, per Reviewer suggestions. It should be noted however, that additional components of both complexes (degradation and storage) have been now tested and their effects are consistent with those reported in the initially submitted manuscript.

2) Figure 2 and S2

- In Figure 2 it would help if the different tissues would be specified directly on the figure' panels (and not only in the figure legends). Similarly, panels d and g could specify that mitochondrial mass is being quantified in the intestine at different days after RNAi treatment.

We prefer to not include tissue specification in Figure panels in order to avoid information overload in images. All information is contained in the Figure legends and the main text of our revised manuscript.

- In Figure S2c it would help if red/lgg-1, green/mitochondria and merge were indicated in the figure (and not only in the figure legend).

This information exists already at the top of the Figure panel (Figure EV3C, top). Thus, we believe that it would be better to avoid further information overload in images.

Fig. 3

- Panel 3a. Representative WB is o very poor quality and could be repeated. Also, it is not clear from the quantification what the multiple dots on the bars stands for given that the experiment was only replicated twice.

Western blot (WB) has been repeated so as to provide a better figure (Fig 3A in the revised manuscript). It should be noted, however, that it is difficult to obtain a high quality WB image with the strain that expresses the SKN-1::GFP transgene. In the new Figure legends, we report that the experiment has been repeated at least 3 times, because for a subset of samples the experiment has been repeated more than 3 times; this is why there are more than 3 dots on some of the bars of the graph (Fig 3B in the revised manuscript).

- **Data provided in panels 3b and 3c shows that dcap-2 RNAi further increase the expression of gst-4 in conditions known to already activate skn-1 (genetically or pharmacologically). This suggests dcap-2 RNAi might actually promote gst-4 induction in a snk-1-idependent manner (for instance Detienne et al. 2016). Thus, to clearly establish whether gst-4 is induced by dcap-2 RNAi via skn-1, it should be addressed whether gst-4 expression is increased in the presence of skn-1 RNAi**.

Following Reviewer's suggestion, we tested whether the effect we observed on *gst-4* expression upon *ntl-2* and *dcap-2* genetic inhibition is SKN-1-depenent. To this end, we performed double RNAi experiments using the following dilutions: control;*dcap-2(RNAi)*, control;*ntl-2(RNAi)*, control;*skn-1(RNAi)*, *dcap-2;skn-1(RNAi)*, *ntl-2;skn-1(RNAi)* diluted 1:1 and found that indeed, the changes observed in *gst-4* expression upon *dcap-2* and *ntl-2* RNAi are SKN-1-dependent (Appendix Fig S12B & C).

Fig.4

- Data shown in panels 4e-g should be quantified to include significance.

Quantification in panels 4e has been performed, as Reviewer suggested (Fig 4E & G) in the revised manuscript. For images in panel 4g of the original manuscript (Fig 4K & L in the revised manuscript) we did not provide additional quantification because NTL-2 and CCF-1 foci are barely detectable in day 10, as mentioned also in the text.

- The conclusion drawn from data described in panel 4e should be rephrased. Indeed, if as it seems, dcap2 RNAi increase NTL2 expression and viceversa ntl-2 RNAi increases DCAP-2 expression, it means that the they are indeed regulated antagonistically but rather interdependent (in opposite direction but not independently).

We thank the Reviewer for pointing this out. The conclusions drawn have been rephrased.

- Panels 4f seems in contrast to data shown in Figure S1 where no effects on ECD-3 expression with aging is observed.

The abundance of EDC-3 foci increases during ageing. The same stands for DCAP-1 and LSM-3 components of the mRNA degradation pathway, as previously reported (Rieckher et al., 2018, Cell reports). In Fig 4H&I, we also show that total levels of EDC-3 increase during ageing. In Figure S1 (initially submitted manuscript), we monitor EDC-3 foci specifically in muscle cells in order to assess their localization relative to mitochondria. We have not performed quantification of EDC-3 specifically in muscle cells during ageing. Nevertheless, we have repeated these experiments and images have been substituted with ones reflecting the increase in EDC-3 protein levels in aged animals (Fig EV1E in the revised manuscript).

- Text describing Figure 4a-e is very succinct. It should be more extensively elaborated to better convey the main message.

The text has been more extensively elaborated to better convey the main message, as Reviewer suggested.

- Similarly, to clarify the effect of the complex' components on protein translation, experiment with FRAP (Figure 4h) could be briefly explained. Alternatively, a label on the figure panel could be included to specify what is the strain/fluorescence recovery that is represented. Statistics should be included in the panel.

Details have been provided for the FRAP experiment in the Materials and methods section together with corresponding references. Also statistics have been included (Fig 4L in the revised manuscript).

Figure 5 and S3

- Data in panel 5a are not clear, not clearly explained or represented. Does the wild type strain express GFP alone? Although reduced compare to NTL-2::GFP, what is being immunoprecipitated and amplified in the wild-type and in the HIS-72::GFP strains?

The RNA immunoprecipitation (RIP) experiment has been repeated, thus expression levels for additional genes have been quantified and different controls have been included as suggested by the Reviewers. Following the Reviewer's suggestion, we repeated the experiment by using the *gst-4p*::GFP reporter strain (GFP driven by the *gst-4* gene promoter which is expressed in the cytosol) and the *unc-119(ed)III* strain (it has the same genetic background with NTL-2::GFP expressing animals but does not contain NTL-2::GFP/GFP) as controls. Please, see our detailed answer above.

Amplification of other genes translated in the cytosol or nuclei could be used as further controls.

Amplification of other genes such as *rhi-1* (cytoplasmic), *spcs-1* (endoplasmic reticulum) and *npp-22* and *fib-1* (nuclear) has also been tested, as suggested by the Reviewer. These mRNAs are not bound by NTL-2::GFP bodies (Appendix Fig S17A).

- WB in Panel 5d is not very representative. atp-3 and mrps-5 quantification should be included in panel 5d.

WB image has been replaced. Quantification upon *atp-3* RNAi and *mrps-5* RNAi treatments has been included in Fig 5F in the revised manuscript.

Reviewer #3:

Overall

The work is important and builds nicely on previous work from the Tavernarakis lab and others. In addition, the data provided are convincing (for the most part) and well-presented. Overall, I found this to be an interesting piece of work that is worthy of publication in The EMBO Journal.

We thank the Reviewer for the encouraging comments and for the appreciation of our work.

Specific comments

1.This is a minor point, but the MTCO1 blot in Figure 1f is very poor quality compared to the blot in Figure 5d. Can the authors provide a better representative image here?

We have repeated this experiment using additional samples. Now we provide an improved Western blot image (Fig 1F of the revised manuscript).

2. It is great that the authors attempt to look at the protein levels of SKN-1 in C. elegans (often not a simple task) but the FLAG and tubulin blots in Figure 3a are not of sufficient quality for quantification (The SKN-1::FLAG signal is too feint relative to the uneven background). I suggest the authors load more protein and/or use more sensitive detection reagents to amplify the SKN-1::FLAG signal.

We thank the Reviewer for acknowledging the difficulties of using biochemistry techniques in *C. elegans*. Nevertheless, we tried different experimental conditions and finally managed to better probe SKN-1 by using higher protein concentrations and the anti-GFP antibody instead of a-FLAG, as these transgenic animals contain both GFP and FLAG tagged to SKN-1 (see material and methods section). Also, we have performed the experiment using additional samples as suggested by the Reviewers. The new results are presented in Fig 3A of the revised manuscript.

Another minor point is that it would be good to have some representative gst-4p::gfp images to accompany the quantification presented in Figure 3b.

We have now provided representative images of worms used in quantification of *gst-4p::GFP* shown in Fig 3C of our revised manuscript, as suggested by the Reviewer. These representative images are presented in Appendix Fig S12A**.**

In addition to *dcap-2* and *ntl-2*, we have also tested other genes involved in mRNA metabolism and found that knockdown of genes encoding components of the mRNA degradation pathway (*edc-3*, *dcap-2, xrn-1*) induces the transcriptional activity of *gst-4* gene promoter, whereas knockdown of genes encoding components implicated in mRNA storage (*ntl-2*, *ccf-1*, *let-711*) reduces *gst-4* transcriptional activity (Appendix Fig S13). It is also worth to note that transcriptional activation upon knockdown of mRNA degradation genes depends on the activity of SKN-1 as it is lost in animals subjected to dsRNA against SKN-1 (Appendix Fig S12A).

3.The opposing effects of ntl-2(RNAi) and dcap-2(RNAi) on the IFE-2::GFP reporter are very interesting. While I fully agree that this reporter is a good indicator of overall translation rates, the authors should strengthen this conclusion by also looking directly at total protein levels in NTL-2 and DCAP-2 depleted animals. This will reveal whether global protein load is being reduced or enhanced when the storage and degradation complexes are perturbed.

Following the Reviewer's suggestion, total protein levels of wild-type animals subjected to *dcap-2* or *ntl-2* RNAi have been determined to strengthen the results obtained with photobleaching and recovery of fluorescence in *vivo* (FRAP). Knockdown of *dcap-2* was found to decrease total protein levels, in contrast to *ntl-2* knockdown (Appendix Fig S16). Knockdown of *let-363*, which is known to inhibit mRNA translation, was used as an additional control. These results provide further support for the impact of *dcap-2* and *ntl-2* knockdown on protein synthesis.

4.The authors state that "NTL-2 is required for the increased lifespan of long-lived mutants and that mev-1, nuo-6 and isp-1 mutants, and atp-3(RNAi), suppress the short lifespan of ntl-2(RNAi) worms. However, the data presented in Figure S4 do not appear to support these conclusions. It is true that ntl-2 is required for the lifespan extension observed in akt-1(ok525) animals, and that ntl-2(RNAi) shortens the lifespan of daf-2(RNAi) and age-1(hx546) animals. However, the lifespan of control;ntl-2(RNAi) animals is extended by daf-2(RNAi) and age-1(hx546) to the same extent as in wildtype worms. Similarly, while mev-1(kn1) mutants do ameliorate the short lifespan of ntl-2(RNAi) animals, this does not appear to be the case for nuo-6(qm200) and isp-1(RNAi) worms, which have a similar shortening of lifespan compared to nuo-6 and isp-1 control animals as ntl-2(RNAi) does compared to wildtype. The authors should modify their conclusions accordingly in the text.

The Reviewer's view is another interpretation of lifespan results with mitochondrial mutants subjected to *ntl-2* RNAi. In our view, and consistent with the impact on mitochondrial biogenesis, our findings indicate that the detrimental effects of *ntl-2* genetic inhibition on longevity are ameliorated, or rescued, by targeting specific mitochondrial genes.

5. It is great that the authors included the HIS-72::GFP line as a control for their NTL-2::GFP RIP experiments. This controls well for non-specific pulldowns related to antibody binding and/or beads. BUT, I am not convinced that this is an appropriate control for random interactions with GFP tagged cytosolic proteins. HIS-72::GFP is nuclear; a more appropriate control would be a GFP tagged protein that is cytosolic.

Following the Reviewer's suggestion, we repeated the experiment by using the *gst-4p*::GFP reporter strain (GFP driven by the *gst-4* gene promoter which is expressed in the cytosol) and the *unc-119(ed3)III* strain (it has the same genetic background with NTL-2::GFP expressing animals but does not contain NTL-2::GFP/GFP) as controls. As discussed above, our results show that NTL-2 binds specifically MTPTs (Fig 5A in the revised manuscript). In addition, NTL-2 associates with mitochondria in a local translation-dependent manner as the abundance of NTL-2 foci associated with mitochondria is decreased upon knockdown of *tomm-20* or *akap-1* (Fig 5B-F in the revised manuscript), which are known to promote protein synthesis on the mitochondrial surface (Zhang et al.,2016), as mentioned above. More specifically, the mRNAs of *atp-5,*

*atp-1, f46b6.6, mrpl-13, nuo-5, t20h4.5 (*ortholog of human NADH ubiquinone oxidoreductase core subunit S8, NDUFS8)*, as well as skn-1*, which is known to regulate mitochondrial biogenesis (Gureev, 2019, Palikaras, Nature, 2015), are all enriched in the immunoprecipitated sample of 1-day-old NTL-2::GFP animals compared to animals expressing *gst-4p*::GFP reporter and the non-GFP expressing *unc-119(ed3)III* strain used as controls. Selective association of these transcripts with the NTL-2::GFP protein extract is specific, since mRNAs encoding nuclear (FIB-1*,* NPP-22), endoplasmic reticulum (SPCS-1) or cytoplasmic (RHI-1) proteins have not been detected.(Appendix Fig S17A). Detection of *rhi-1* mRNA in the protein extract of *gst-4p*::GFP reporter animals was unspecific.

Moreover, we have now examined MTPTs for their association with NTL-2::GFP bodies upon silencing of *dcap-2*, as well as under mitochondrial stress (CCCP treatment) and ageing, as the Reviewer suggested. We find that genetic inhibition of *dcap-2* increases MTPT binding by NTL-2. Based on the enzymatic activity of DCAP-2, this could be attributed to decreased mRNA decapping and degradation, leading to accumulation of these MTPTs. Moreover, this result is consistent with our findings showing that the number of NTL-2 foci increases upon *dcap-2* genetic inhibition and their association with mitochondria is also strengthened (Fig 4D & E in the revised manuscript and Appendix Fig S18A & B). In contrast, we have found that upon CCCP treatment and during ageing, NTL-2 foci formation is significantly decreased (Figure 6 in response letter and Fig 4J in the revised manuscript) and the selective NTL-2-mitochondria interaction is also impaired (Fig EV1A-C). Consistent with these results we now find that MTPT binding on NTL-2 is abolished in both cases (Appendix Fig S17B). Taken together, our results provide further evidence for the selective binding of MTPTs to NTL-2 bodies.

6.In Figure 5d, the authors make the claim that upon akap-1(RNAi) and tomm-20(RNAi), levels of NTL-2 associated with mitochondria decrease. However, these differences are driven by elevated levels of MTCO1 on their western blots, rather than reduced levels of NTL-2. Do akap-1 RNAi and tomm-20 RNAi result in increased levels of MTCO1? The authors should probe this by western blotting. If so, this would suggest that actually, there is no change in NTL-2 mitochondrial association under these conditions.

In this experiment, we use a-CTC-1/MTCOI as a loading control, because our sample in this case is isolated mitochondria. We then normalized NTL-2 with CTC-1/MTCOI to ensure that the differences in NTL-2 levels are not due to differences in the sample amount loaded. Nevertheless, we tested whether MTCOI levels change upon *akap-1* RNAi and *tomm-20* RNAi and found that CTC-1/MTCOI levels were not affected by *tomm-20* RNAi, while they were only slightly decreased by *akap-1* RNAi, further supporting our results (Figure 8, in this response letter).

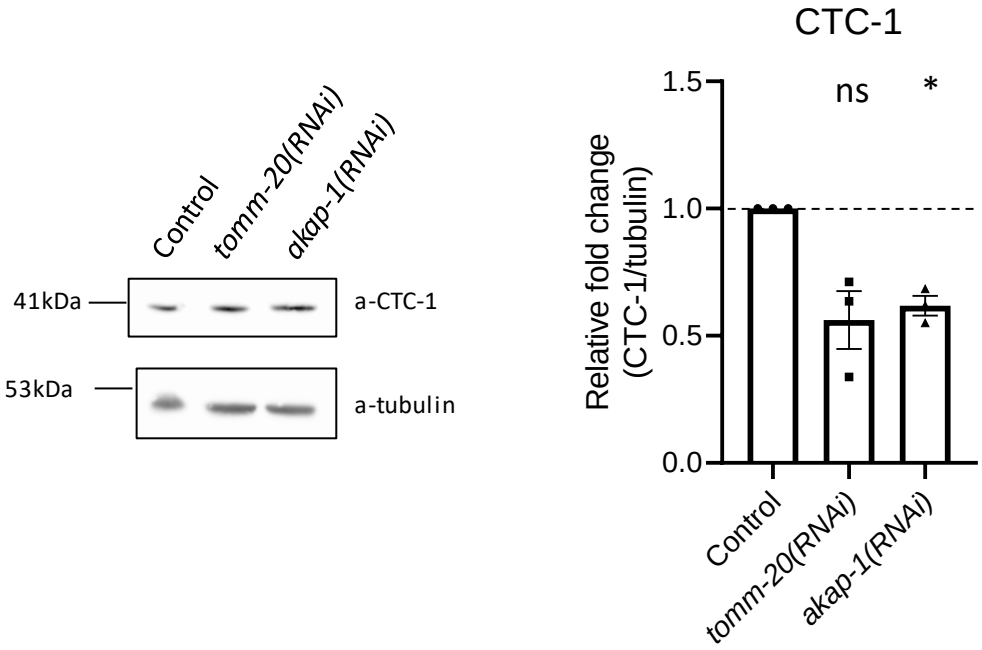

Figure 8. Immunoblot analysis in whole animal extracts showing the protein levels of CTC-1 in control conditions and upon the indicated genetic inhibitions (n= at least 3 independent experiments), *P<0.05; one-way analysis of variance (ANOVA) followed by Dunnett's T3 multiple comparisons test. Error bars denote SEM.

7.In Figure 6, the authors show that ntl-2(RNAi) sensitizes worms to multiple stresses. Is this also observed using the *ntl-2(ok974)* mutant used in Figure 7?

Given that *ntl-2(ok974)* mutants are homozygous lethal, the strain is balanced. Therefore, it is really time consuming to grow a large number of animals to perform stress resistance assays. We believe that owing to the high efficiency of our RNAi (Figure 11, in the response letter), there is no need to also use the heterozygous mutant in this case. It is also worth to note that RNAi-mediated knockdown allows comparison of stress responses in animals subjected to silencing against various genes in an otherwise wild-type background.

In addition, does tomm-22 also impact lifespan in a similar way to akap-1(RNAi) and what happens to lifespan in dcap-2(RNAi) worms?

To test the effect of *tomm-22(RNAi)* on lifespan and in comparison to *akap-1(RNAi)* would be out of the scope of the current manuscript. Maybe the Reviewer's suggestion is to test the effect of *tomm-20* RNAi on lifespan, which is an experiment we performed. We find that knockdown of *tomm-20* extends lifespan in wild-type animals (Appendix Fig S23).

Similarly, *dcap-2* knockdown results in lifespan extension as shown in Appendix Fig S21.

Reviewer #4

Fig. 1

GFP is a relatively large tag. Can it be excluded that the tag interferes with the function of the studied proteins?

The idea to study proteins' function without using a fluorescent tag would be indeed great. However, we are not aware of existing antibodies for the studied components that work efficiently in *C. elegans*. In addition, the use of antibodies to monitor the formation of foci of interest would not allow *in vivo* studies. Therefore, we are afraid that we cannot avoid the use of fluorescent tags in this *in vivo* study.

Based on our data, we are confident enough that GFP does not alter the function of the proteins studied herein. This notion is supported by the fact that NTL-2::GFP, CCF-1::GFP and EDC-3::GFP, DCAP-2::mCherry and DCAP-1::DsRed acquire the expected subcellular localization pattern (a dotted expression pattern) as seen, for example, in Fig 1A-C and Appendix Fig S1-S3) . In addition, we have observed that the expression pattern of these proteins fused to GFP is dynamic. For example, the expression pattern is dramatically altered upon various treatments (genetic or pharmacological), as well as during ageing (see for example Fig 1 and Fig EV1 & 2) exhibiting either an increase or a decrease. Notably, in Fig 1L bottom we show that NTL-2::GFP acquires a cytoplasmic, diffused expression pattern upon *mrps-5* genetic inhibition. All these examples prove that NTL-2::GFP expression is upon tight, dynamic and functional regulation. Furthermore, our lifespan data (Fig 6E, EV Fig 5 and Appendix Fig S21 & S22) suggest that GFP does not interfere with the protein function. More specifically: 1. we observe a different phenotype compared to control counterparts; NTL-2 overexpressing animals are long-lived (if the protein was not functional we wouldn't except to see any change in the lifespan of the animals) and 2. NTL-2 overexpression has the opposite effect on *C. elegans* lifespan compared to *ntl-2* genetic inhibition. These findings corroborate the notion that GFP does not interfere with the function of the studied protein.

Moreover, the use of fluorescent tags is common practice in *C. elegans* research and a well-accepted experimental approach. A wealth of studies published in top-tier journals (for example, Fengxiu Sun, et al., , 2022; Susoy et al., 2021; Feng et al, 2021.) involve the use of transgenic animals overexpressing certain proteins of interest fused with fluorescent tags.

Overall, while we understand the Reviewer's concern, the wide use of such genetic tools and technical approaches for *in vivo* studies as well as our data, which support that the fluorescent fusion proteins used in this study are functional, give us confident for using them. In any case, we are afraid that there are no better tools available to use for such studies *in vivo*.

Fig. 1f:

To get an estimation how much of NTL-2 and EDC-3 co-fractionate with mitochondria it would be better to present a single blot comparing isolated mitochondria, cytoplasm etc. and maybe include a marker for another protein associated with the outer mitochondrial membrane.

We are afraid we don't understand the advance that such an experiment would offer to our manuscript. In the Western blot presented in Fig 1F in the revised manuscript, we have performed

mitochondrial isolation in order to test whether NTL-2, EDC-3 and now additionally CCF-1 are present in the mitochondrial fraction. Indeed, as shown in this Figure, all proteins were probed in the cytoplasmic as well as the mitochondrial fraction. We use this experiment only for qualitative analysis and not for a quantitative assessment. We are interested just to show in a biochemical way (as an extra verification of results obtained from *in vivo* imaging analysis) the presence or not of these proteins in mitochondria.

Due to the known dynamic nature of degradation and storage foci, we strongly believe that the associations they form with mitochondria would be transient, and thus it is really hard to extract safe results regarding the exact amount of protein that associates with the organelles at the time of animal collection compared to amount of this protein in the cytoplasm. In fact, we can reliably either compare quantifications in samples from mitochondrial fractions or in samples from cytoplasmic fractions. Furthermore, to estimate the abundance of the protein present on mitochondria compared to the cytoplasm, we have to compare samples that have been normalized with two different loading controls, which also may interfere with the real results.

Moreover, the Reviewer suggests that we “include a marker for another protein associated with the outer mitochondrial membrane” in order to compare with our proteins. The reason behind this suggestion is not clear since each protein that associates with mitochondria may have its own association rates/efficiencies or abundance on mitochondria.

We believe that we cannot have a precise measurement of the abundance of the tested proteins present in the cytoplasm versus mitochondria with the suggested experimental approach. For the abovementioned reasons, we feel safe to use the presented western blot assay just for qualitative analysis and not for precise quantification of the protein amount in each subcellular compartment.

Nevertheless, we have tried Reviewer’s suggestion. Towards this direction we isolated mitochondria from DCT-1::GFP expressing animals and tried to detect DCT-1 in the mitochondrial isolate together with NTL-2, CCF-1 and EDC- (Figure 9 in the response letter).

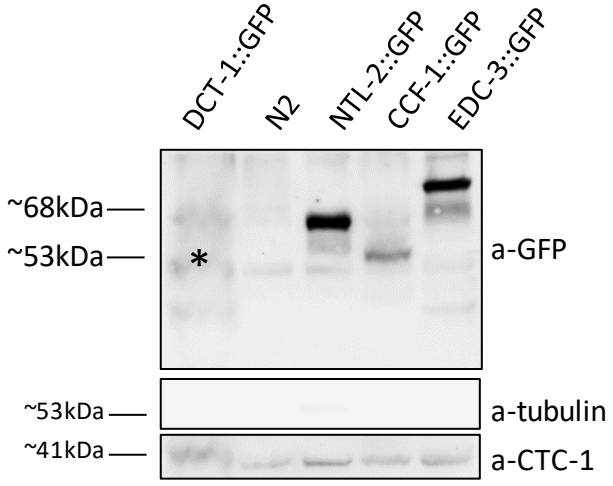

Figure 9: Immunoblot detection of DCT-1::GFP, NTL-2::GFP, CCF-1::GFP and EDC-3::GFP in isolated mitochondria.

Fig. 1i-j vs. Fig. 1a:

The punctuated staining of NTL-2 in the control panels seem to vary.

We are not sure we fully understand what the Reviewer means because he/she does not provide a specific example. In fact, we do observe NTL-2-puncta in all images showing NTL-2::GFP expression under control conditions. The intensity of the signal may vary because of: 1. The different exposure conditions used among the different experimental setups 2. The age of the animals 3. The tissue monitored and 4. The expected variation in the expression levels of the protein from one animal to the other due to mosaicism as the transgene (NTL-2::GFP) is expressed from extrachromosomal arrays and is not integrated.

Fig. 1n:

The levels of NTL-2-GFP are normalized to COX1, which is a mitochondrial DNA-encoded protein. Mitochondrial translation should be affected upon downregulation of a mitoribosomal protein (mrps-5 RNAi). Thus, COX1 cannot be used as a loading control.

We thank the Reviewer for pointing this out. Indeed, we used MTCOI which is a mitochondrial-encoded protein. We have now repeated quantification of this experiment using Ponceau staining to normalize NTL-2 protein levels present in the mitochondrial fraction (Figure 10, in this response letter). Normalization of NTL-2 with Ponceau gave us exactly the same results as NTL-2 quantification following normalization with CTC-1/MTCOI. Therefore, we felt there was no need to change the experiment in our manuscript. However, we have substituted images with ones of better quality (Fig 5E&F in the revised manuscript). We apologize for any inconvenience this may have caused.

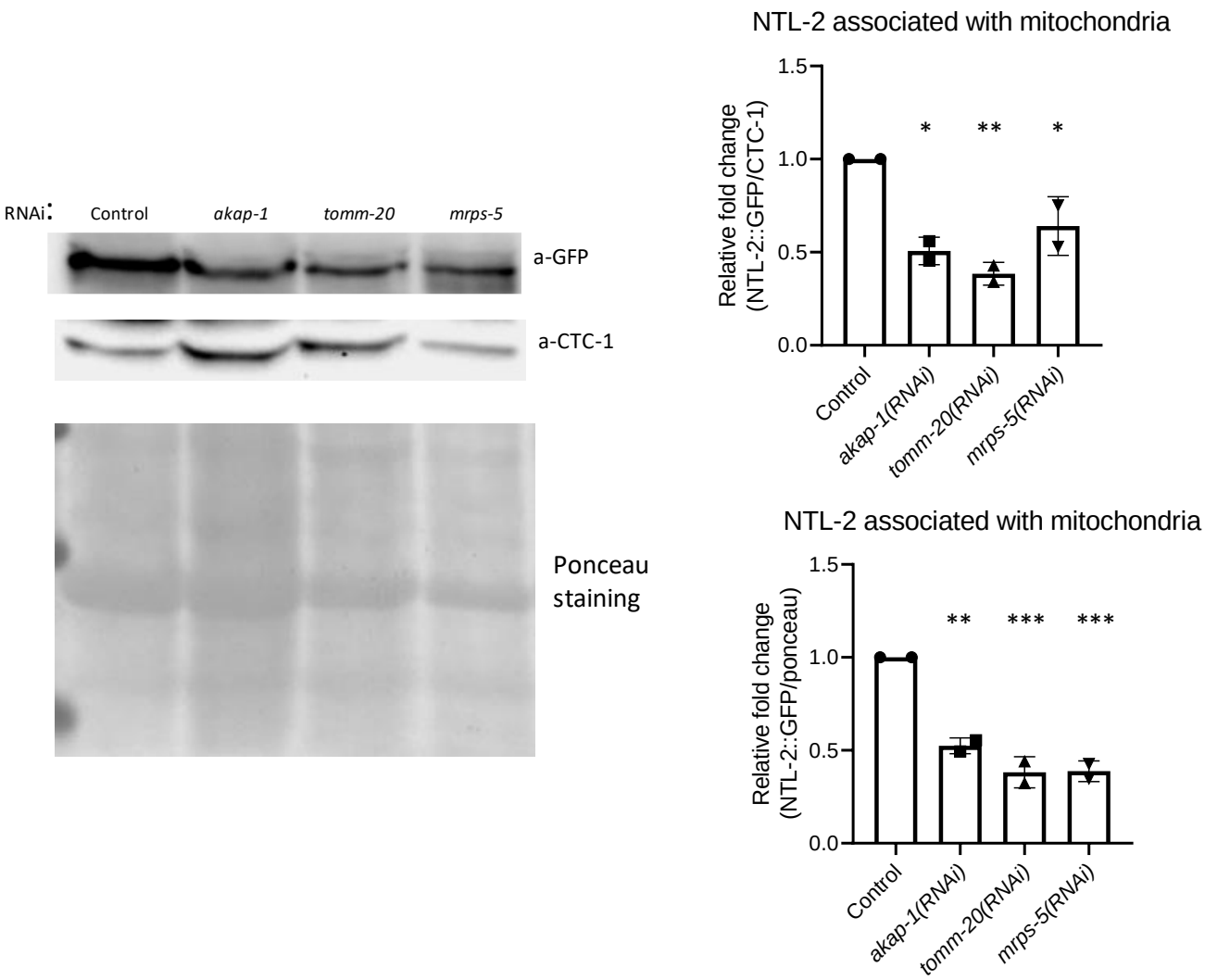

Figure 10. Genetic inhibition of *akap-1*, *tomm-20* or *mrps-5* reduces the association of NTL-2 with mitochondria. The same results were obtained either when CTC-1/ MTCOI or Ponceau staining was used for normalization.

Fig. 2:

"we conclude that perturbation of the decapping complex increases the functional mitochondrial population,..." I do not understand how the author can conclude this. The downregulation of dcap-2 disrupts the membrane potential and triggers mitochondrial fragmentation. How can one conclude that dcap-2 ablation increases the functional mitochondrial population? It shows only mitochondrial abundance, but not whether they are functional.

We now provide additional data showing that knockdown of genes encoding degradation body components (*dcap-2*, *edc-3*, *xrn-1*) increases mitochondrial mass (Appendix Fig S11), membrane potential (indicative of functional mitochondria) (Fig 1H in the revised manuscript and Appendix Fig S8A-B), mtROS levels and mitochondrial ATP levels compared to control (Appendix Fig S7 & S9A & B). We have also performed real time measurements of oxygen consumption rates using the Agilent Seahorse XF Analyzer. We found that the basal and maximal oxygen consumption rates are increased upon knockdown of *dcap-2, edc-3* or *xrn-1* (Appendix Fig S10), indicative of a healthier bioenergetics profile compared to control (Chacko

et al, 2014,). These findings invite the speculation that genetic perturbations in mRNA degradation components increase the number of functional mitochondria -a high number of healthy mitochondria is expected to produce more ATP, ROS and Δψ, if functional, compared to a lower number of mitochondria-. By contrast, genetic inhibition of genes encoding mRNA storage components decreases mitochondrial mass (Appendix Fig S11) and alter mitochondrial function-related parameters (Fig 1H in the revised manuscript and Appendix Fig S7, S8C&D and S9C&D).

Fig. 3a:

The western blot is not convincing and a-tubulin seems to be overexposed.

We have repeated the experiment and provide a Western blot of better quality. However, we would like to kindly note that Western blot analysis, especially with this transgenic strain, is a difficult task in *C. elegans* (Fig 3A in the revised manuscript).

Fig. 5a:

This experiment is questionable. If WT and HIS-72:GFP are both negative controls, why do they show different results, especially for spcs-1?

This is likely due to differences in the genetic background of the two strains. Nevertheless, we repeated the RNA immunoprecipitation (RIP) experiment to include more appropriate controls and detect differences in the amount of MTPTs bound on storage body components, at least, in one of the additional conditions as suggested by Reviewers.

In the new experimental setup we used the *gst-4p*::GFP reporter strain (GFP driven by the *gst-4* gene promoter which is expressed in the cytosol) and the *unc-119(ed3)III* strain (it has the same genetic background with NTL-2::GFP expressing animals but does not contain NTL-2::GFP/GFP) as controls. As discussed above, our results show that NTL-2 binds specifically MTPTs (Fig 5A in the revised manuscript). In addition, NTL-2 associates with mitochondria in a local translation-dependent manner as the abundance of NTL-2 foci associated with mitochondria is decreased upon knockdown of *tomm-20* or *akap-1* (Fig 5B-F in the revised manuscript), which are known to promote protein synthesis on the mitochondrial surface (Zhang et al., EMBO J. Vol 35 | No 10 | 2016 1045), as mentioned above. More specifically, the mRNAs of *atp-5, atp-1, f46b6.6, mrpl-13, nuo-5, t20h4.5 (*ortholog of human NADH ubiquinone oxidoreductase core subunit S8, NDUFS8)*, as well as skn-1*, which is known to regulate mitochondrial biogenesis (Gureev, 2019, Palikaras, Nature, 2015), are all enriched in the immunoprecipitated sample of 1-day-old NTL-2::GFP animals compared to animals expressing *gst-4p*::GFP reporter and the non-GFP expressing *unc-119(ed3)III* strain used as controls. Selective association of these transcripts with the NTL-2::GFP protein extract is specific, since mRNAs encoding nuclear (FIB-1, NPP-22), endoplasmic reticulum (SPCS-1) or cytoplasmic (RHI-1) proteins have not been detected.(Appendix Fig S17A). Detection of *rhi-1* mRNA in the protein extract of *gst-4p*::GFP reporter animals was unspecific.

Moreover, we have now examined MTPTs for their association with NTL-2::GFP bodies upon silencing of *dcap-2*, as well as under mitochondrial stress (CCCP treatment) and ageing, as the Reviewer suggested. We find that genetic inhibition of *dcap-2* increases MTPT binding by NTL-2. Based on the

enzymatic activity of DCAP-2, this could be attributed to decreased mRNA decapping and degradation, leading to accumulation of these MTPTs. Moreover, this result is consistent with our findings showing that the number of NTL-2 foci increases upon *dcap-2* genetic inhibition and their association with mitochondria is also strengthened (Fig 4D&E in the revised manuscript and Appendix Fig S18A&B). In contrast, we have found that upon CCCP treatment and during ageing NTL-2 foci formation is significantly decreased (Figure 6 in response letter and Fig 4K in the revised manuscript) and the selective NTL-2-mitochondria interaction is also impaired (Fig EV1A-C). Consistent with these results we now find that MTPT binding on NTL-2 is abolished in both cases (Appendix Fig S17B). Taken together, our results provide further evidence for the selective binding of MTPTs to NTL-2 bodies.

Fig. 5b-5e:

The text is difficult to follow for non-experts. What do the authors mean with "local translation inducers"? TOM20 is a component of the import machinery at the outer membrane. Ablation of TOM20 affects protein import and membrane potential as also shown by TMRE staining (Fig. 5b). Therefore, the conclusion can be misleading. Again using COX1 as a loading control is not appropriate as the synthesis or stability of mitochondrial DNA-encoded COX1 can be/ is affected in some of the knockdowns.

As described in the Introduction of our manuscript, “following their transcription, MTPTs are exported from the nucleus to the cytoplasm, and being in a translationally silenced state they are transferred to mitochondria where they are anchored on the outer mitochondrial membrane (OMM) by the OMM proteins MDI (AKAP-1 in *C. elegans*), and TOM20 (the nematode TOMM-20) and are locally translated and imported into the organelles (Eliyahu et al, 2010; Gehrke et al, 2015; Zhang et al, 2016). Thus, AKAP-1 and TOMM-20 promote local translation of MTPTs, facilitated by OMM bound ribosomes or by free cytoplasmic ribosomes found in the vicinity of mitochondria, as revealed by proximity-specific ribosome profiling in yeast.

In fact, TOM20 acts as a receptor that binds the presequences of mRNAs to stabilize them to the vicinity of mitochondria (Eliyahu et al, 2010). This interaction seems to be conserved in yeast, flies and humans (Eliyahu et al, 2010; Gehrke et al, 2015). In turn, TOM20 has been shown to interact with PINK1, promoting localized translation of select nuclear encoded mitochondrial targeted protein transcripts (Lesnik et al., 2015). Furthermore, the prevailing view is that mRNAs are co-translationally imported into the organelle. We find that knockdown of either *tomm-20* or *akap-1* severely affects the associations of NTL-2 foci (storage bodies) with mitochondria. This result was verified by two methods; *in vivo* monitoring of NTL-2/storage bodies localization in relation to mitochondria and by mitochondrial isolation and immunoblot detection in this isolate (Fig 5B & C and 5E & F in the revised manuscript). Also, the text has been elaborated to better convey the main message.

Regarding COX1, we thank the Reviewer for pointing this out. We examined whether MTCOI levels are affected under the conditions tested and found that knockdown of *tomm-20* does not affect MTCOI levels whereas *akap-1* knockdown slightly decreases them, further supporting our results (Figure 8, in the response letter).

In addition, we performed Ponceau staining to confirm that our results are not affected when we normalized the amount of NTL-2 present in the mitochondrial extract with CTC-1/MTCOI. Given that the

results of the two quantification methods are in agreement (Figure 10, in this response letter), we believe that CTC-1/MTCOI can be used as a reliable indicator of mitochondria abundance/loading. Since MTCOI is a component encoded by the mitochondrial DNA, we believe that it is the best available choice for normalizing mitochondrial samples.

Comment on RNAi

Efficiency and specificity of downregulation should be shown or include respective reference if RNAi has already been validated elsewhere.

We verified by RT-PCR analysis that our RNAi constructs effectively reduce the expression of the indicated genes of interest (Figure 11, in response letter).

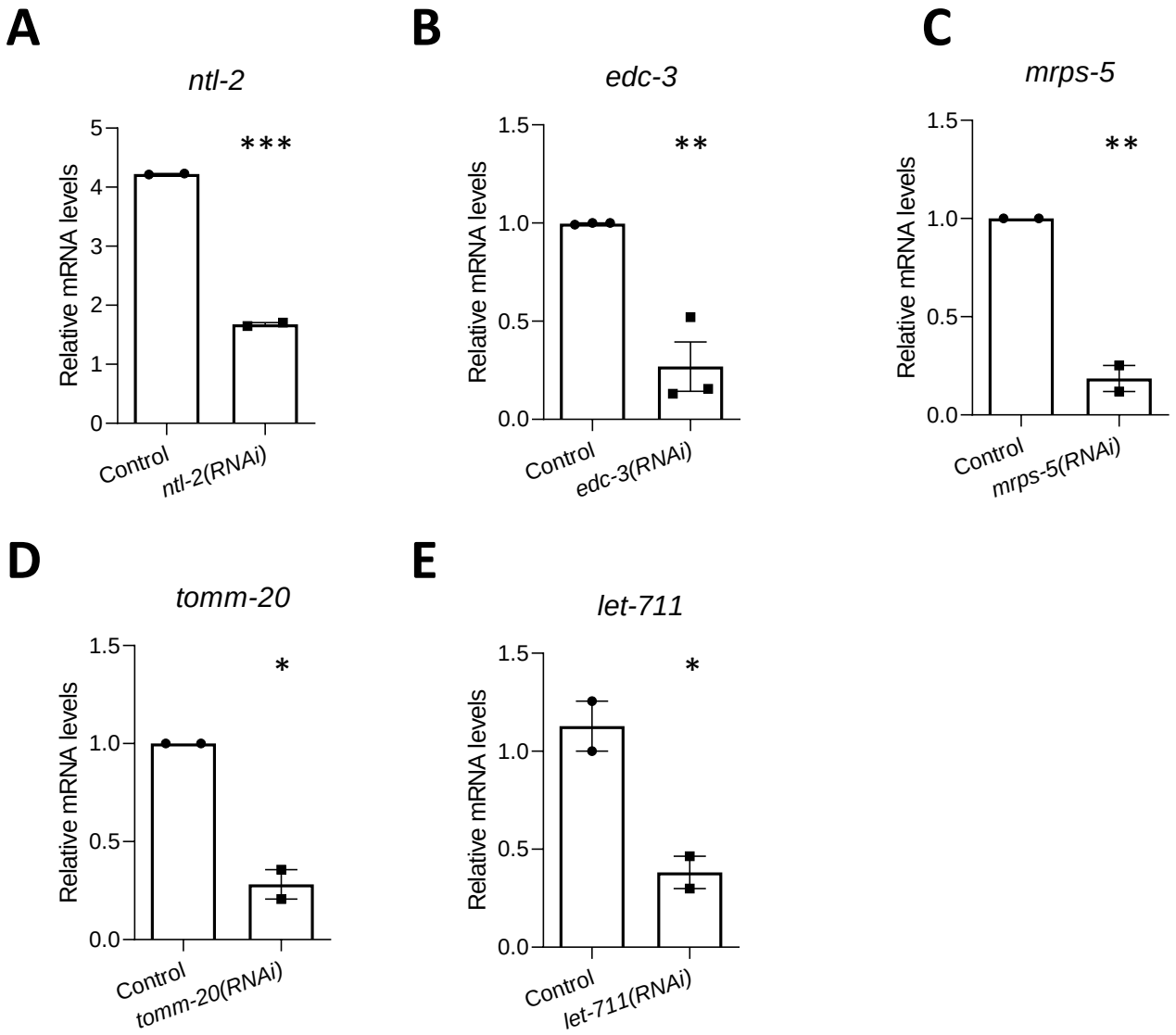

Figure 11. Efficient silencing of the indicated genes by our RNAi constructs as verified by qRT-PCR analysis.

Comment on statistics

In some figures (e.g. Fig. 1n, 2h, 3a, 3g, 5a, 5d, 5e) the authors wrote "n=2", but included p values. How is it possible to perform statistics with n=2?

In these experiments, n=2 independent repetitions were conducted, with a large number of animals per assay (please, see Figure legends for specifics). Statistics are performed as described in the literature, and the statistical tests used are described in the Figure legends and the materials and methods section.

In closing this rather long response letter, we would like to, again, thank all the Reviewers for the constructive and positive input that has enabled us to significantly improve our paper. We do hope that you will find our revisions adequate for publication of our study in the *EMBO Journal*.

With best wishes,

Nektarios Tavernarakis

Dear Dr Nektarios Tavernarakis,

Thank you for submitting your revised manuscript (EMBOJ-2022-112446R) to The EMBO Journal, as well as for your patience with our response at this time of the year. Your amended study was sent back to three referees for their re-evaluation, and we have received comments from all of them, which I enclose below. As you will see, the experts stated that the work has been substantially improved by the revisions and they are now in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

We still need you to take care of a number of minor issues related to formatting and data annotation as detailed below, which should be addressed at re-submission. I will send you an according summary list in a separate message during the next few days.

Please contact me at any time if you have additional questions related to below points.

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Kind regards,

Daniel Klimmeck

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Referee #1:

The revised version of the manuscript now includes new experiments and additions/extensions to previously included experiments. I am pleased to note that all my minor points have been fully addressed. These revisions have undoubtedly strengthened the scientific rigor and reliability of the study.

The authors have made a commendable attempt to shed light on the role of mRNA metabolism in mitochondrial biogenesis and ageing and to explore the effects of different decay pathways, proteins and complexes in this context. However, despite the significant improvements, I still have reservations regarding the elucidation of the underlying mechanism. E.g. I am not fully convinced that all of the observed relationships are causal rather than mere correlations. Despite my doubts, I believe that the findings presented in this manuscript are valuable contributions that will stimulate further discussions and investigations in the field. Therefore, I support publication of the manuscript without further revisions.

Referee #3:

In their revised manuscript, Daskalaki et al. provide evidence that mRNA storage and degradation complexes operate in distinct cellular locations to regulate mitochondrial biogenesis. The authors propose that this is achieved through direct interactions between these complexes and mitochondria, which regulates the local translation of mRNAs encoding mitochondrial proteins. Furthermore, the authors suggest that balancing this mechanism is crucial for longevity.

The authors have made substantial efforts to carefully consider and respond to all of my initial comments. This includes (i) improving the quality of CTC-1/MTCOI and SKN-1 western blots, (ii) including representative images of gst-4p::gfp worms, (iii) conducting further molecular analysis of total and MTCOI protein levels in different treatment groups and (iv) repeating pull-down experiments with a more appropriate control.

While I appreciate the authors including the gst-4p::GFP strain as a negative control for their pulldown experiments, I am not convinced that the levels of GFP present in this strain are comparable to the levels of NTL-2::GFP (the best control would be an ntl-2p::gfp line). Nevertheless, I fully acknowledge that finding a "perfect" control for this experiment is very difficult and that this new set of experiments support the authors original conclusions. In addition, while I still disagree with the authors interpretation of their isp-1/nuo-6/ntl-2 RNAi lifespan data, I also acknowledge that this is not a major focus of the paper.

Overall, I consider the manuscript to be considerably improved and a strong candidate for publication in The EMBO Journal.

Referee #4:

The authors have addressed most of my concerns and provide several additional experiments. Thus, the revised manuscript might be now suitable for publication in the EMBO Journal.

Dear Dr Nektarios Tavernarakis,

Further to above message, please find enclosed a list of remaining minor formatting points to be addressed in your final revision. I also enclose additional comments from our production team for your consideration and integration.

Please let me know anytime should there be any questions related.

Looking forward to your final manuscript version.

with

Best wishes,

Daniel Klimmeck

Daniel Klimmeck PhD

Senior Editor

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Formatting changes required for the revised version of the manuscript:

> Adjust the title of the 'Competing Interests' section to 'Disclosure and Competing Interests Statement'.

> Funding information: please complement our online manuscript system with the complete funding details; currently missing: NIH Office of Research Infrastructure Programs (P40 OD010440), the European Research Council, under grant agreement BIOIMAGING-GR (MIS5002755), Operational Program "Competitiveness, Entrepreneurship and Innovation" (NSRF 2014-2020).

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> The reagent table should be uploaded separately.

> Consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

The authors have addressed all minor editorial requests.

Dear Dr Nektarios Tavernarakis,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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Kind regards,

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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- 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

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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  - definition of error bars as s.d. or s.e.m.

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Please detail **housing and husbandry conditions**.	Not Applicable	

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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	
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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.	**Yes**	Materials and Methods section
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In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	All Figure legends

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Adherence to community standards	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.	Not Applicable	
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	

Data Availability

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)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	