EMBO reports

Reverse electron transfer is activated during aging and contributes to aging and age-related disease

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Referee #1:

This manuscript by Rimal, Lu and colleagues evaluates the role of reverse electron transport (RET) in aging and neurodegeneration, primarily using Drosophila but also in cultured cells and mice. RET is a process by with complex I of the respiratory chain runs backwards to generate NADH and ROS. There is limited understanding of how RET may function in normal physiology and pathology. The authors find that multiple RET inhibitors, and genetic manipulations predicted to inhibit RET, extend lifespan in Drosophila, as well as suppress age-related phenotypes in flies and improve survival in fly and mouse neurodegeneration model.

The results in this paper will be of wide interest, and implicate RET as a novel target in aging and age-associated disease. However, there are significant issues with the the manuscript that need to be address before it is acceptable for publication, in my view:

1. Perhaps I missed it, but have the authors performed epistasis with their primary RET inhibitor CPT together with NMN or anti-oxidants, for example in the context of lifespan? One might predict that there would be little added advantage to combining treatments that hit the same pathway. Such an experiment might provide insight into which consequence of RET is most important in limiting lifespan (NADH or ROS generation).
2. The manuscript relies heavily on the use of CPT. Can the authors verify that it acts as a RET inhibitor in their hands, with the dosage and treatment regimens used in the paper?
3. The mammalian experiments in the manuscript seem underdeveloped. The omission of any learning and memory assays in the mouse AD model are puzzling - one would typically perform these studies standardly in such a model.
4. The statistics in the manuscript require review by a statistician. The lifespan curves appear to have been analyzed point-by-point using a t-test or the like, but a more appropriate analysis would consist of a log-rank rest, with the Wang-Allison test to detect differences in maximum lifespan.
5. In the interest of data transparency, the many "dynamite plunger" bar graphs should be replotted as scatterplots.

Referee #2:

In this manuscript, Rimal et al investigated the impact of reverse electron transfer (RET) interference on aging and lifespan in both fly and mouse models. The authors demonstrated that RET inhibition either by chemicals or by RNA interference, restored both ROS and NAD+ levels, alleviated aging features, and extended lifespan of Drosophila. Similar examination was also done using mouse AD model. Authors claimed that a deregulation of RET is a conserved feature of aging. Overall, this is an interesting study and may help understand the regulation of two important aging regulators, i.e., ROS and NAD+. However, while phenotype observations support the conclusion, mechanistic evidence is rather weak.

Major concerns
1.The key observation that authors wanted to address is aging-related RET activation. However, elevated ROS and decreased NAD+/NADH ratio as supporting evidence is not

enough. For example, does CI (NDUFS3) level increase along with aging? Or Is the activity up-regulated?

2.Both ROS elevation and NAD+ decline might drive aging. It seems that author only applied ROS level as an indicator of RET activation. Is it possible that the effect of RET inhibition on aging and lifespan is in some extent owing to ROS decline? More interestingly, in an earlier study (Cell Metab. 2016 Apr 12;23(4):725-34.), CI was found reduced in aged flies (older than 75 days) and enhanced RET-derived ROS, which is different from general ROS, promotes lifespan and alleviates aging feature. Authors need to distinguish ROS sources in the context of aging regulation.

3.The authors applied mitochondria dysfunction and protein dys-homeostasis as readouts of aging. However, the unanswered questions are how targeting CI affects mitochondria fission/fusion and how mitochondria CI regulates protein homeostasis.

4.In the last part of the manuscript, authors investigated the role of CI inhibition on APP,C99 generation/clearance in both fly and mouse models. It is difficult to understand how RET affect APP,C99 generation/clearance.

Specific points,

1. Figure 1, since the main focus of the study is RET, the key regulator CI level should be examined in aged flies.
2. Figure 4a-e, female flies were used, while in f-h, males were used. What is the rationale?
3. Figure 4d, from the fluorescence images, NDUFS2/S3 RNAi almost eliminated all positive (green) signal, but in the quantification data, only about 50% downregulation, why?
4. Figure 4, is the lifespan extension effect of NMN is NDUFS3/2 dependent? This is important which help answer whether aging-related NAD+ decline is mainly attributable to RET activation. Similarly, what about NMN+CPT combined treatment? Is there any additive effect?
5. Figure 4, many findings are related to the brain, why here only muscle manipulation showed obvious effect? Whether additive effect need to be considered
6. Figure 5, what about NMN? Does the NMN effect on lifespan extension rely on Sirt1/Foxo/ATG1?
7. Figure 6f, how does CPT reduce the aberrant APP.C99 species derived from full-length APP? Is there any independent mechanism?
8. Figure 6k, why RNAi NDUFS3 could remove APP.C99?

Referee #3:

In 'Reverse electron transfer at mitochondrial complex I is activated during aging and contributes to aging and age-related disease phenotypes', Rimal et al. present the role of complex I reverse electron transfer (RET) during ageing. RET is the conversion of NADH from NAD by respiratory complex I (CI) that usually occurs under specific conditions. The

conclusions are not entirely supported by the data and the mechanisms are in general poorly described, involving several effectors which affect several aspects of nuclear, cytosolic, and mitochondrial general functions. It is difficult to determine whether the effects are attributable to RET since no experiments on specific CI activities under different conditions and assembly are performed. Most of the results reflect a correlation but not actual cause. The manuscript, although interesting, should deepen into the biochemical aspects of CI RET in their system.

Major comments:

1. CPT role in preventing RET is not clear. There is no data that comprehensively analyzes complex I specific activities in vitro (forward and reverse) and therefore the effects are not necessarily attributable to RET. IN addition, there is no information on CI assembly under these conditions which leaves many conclusions reliant on assumptions. RET occurs under specific conditions that include i) increased CII activity and therefore changes in NADH:FADH2 equivalents, ii) increased membrane potential (i.e.: excess of protons at the IMS) and iii) CI is dissociated from complexes III2 or III2:IV. Are these conditions applying? To demonstrate that RET is actually happening, the first figures should analyze CI assembly (BN-PAGE, respiratory complexes can be seen directly by Coomassie), test for activity forward and RET using isolated mitochondria and perform deep biochemical analyses. How do other complexes look like, how is their activity? The results can be also interpreted as loss of CI activity (NADH to NAD) attenuates OXPHOS activity and reduces the leak of electrons that may cause ROS. The issue with the inhibitors is that these are not specific to RET activity.
2. CPT acts on NOTCH which controls several cellular processes. Is there any change in global ubiquitination and/or proteasomal activity? This can explain the reduction in proteasomal activity. In addition, mitochondrial labeling in Figure S3C does not suggest mitochondrial fragmentation, it seems more a decrease in mitochondrial density. Is there any de-differentiation issue, a transition to cellular proliferation?
3. It is still unclear the impact of NDUFS2, NDUFV1 and NDUFS3 on CI stability and function. ROS levels should be normalized to total mitochondrial content. This applies to other experiments in the manuscript. CI mutations can result in substantial decreases in cellular mitochondrial mass explaining the decrease in ROS levels.
4. Data on NMN rules out the role of deacetylases. Increases in NAD+ can activate sirtuins that deacetylate their targets. The increase in non-acetylated lysines can favor their ubiquitination and subsequent degradation of proteins. There is a cross-talk between acetylation and ubiquitination that balances activity and degradation of proteins. These hypotheses should be considered. It would be very interesting to understand how Sirt2 (cytosolic) controls CI RET activity.
5. The assessment of CPT on models of neurological diseases is attractive but completely rules out what CPT does, how complex activity is modulated, mitochondrial mass content, glycolysis,....

Minor comments:

1. The entire manuscript lacks controls of gene or protein expression. This is especially

critical for siRNA assays.
2. Graphs need to fit the format of other figures.

1st Editorial Decision **8th Jun 2022**

Dear Bingwei,

Thank you for transferring your manuscript to EMBO Reports, which was previously reviewed at The EMBO Journal.

Having read the manuscript and the referee reports, I would like to invite you to submit a revised manuscript to EMBO Reports as my colleague Daniel mentioned in his previous letter. In particular,

- The effects of CPT treatment in this model and their relevance to the lifespan extension need to be assessed (referee #1 points 1, 2; referee #2 major concerns 1, 2; referee #3 standfirst, points 1, 2)
- Whether aging affects CI/RET activity needs to be investigated (referee #2 major concern 1; specific point 1).

All concerns regarding missing controls, normalization methods and statistical analyses need to be addressed. However, concerns regarding the missing behavioral analyses of mouse AD model (referee #1, point 3), mechanisms explaining the effect of RET inhibition on the mitochondria and protein homeostasis (referee #2, specific concern 3), APP,C99 clearance (referee #2, specific concern 4, specific point 7 and 8) and neurological disease phenotypes (referee #3, major comment 5) are not prerequisite to experimentally address for publication in EMBO Reports.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact the scientific editor handling your manuscript to discuss a revision plan should you need additional time, and also if you see a paper with related content published elsewhere.***

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1. A data availability section providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section should be separate. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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

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

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

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

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

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

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <http://embor.embopress.org/authorguide#datacitation>.

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

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

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. 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:

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

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

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

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

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

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

Kind regards,

Deniz

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Point-by-Point Response to Reviewer Comments

Editor:

We would like to invite a revised manuscript to EMBO Reports where the authors particularly focus on the following concerns:

- The effects of CPT treatment in this model and their relevance to the lifespan extension need to be assessed (referee #1 points 1, 2; referee #2 major concerns 1, 2; referee #3 standfirst, points 1, 2)
We appreciate the editor's suggestion. We have now added the following data to support the mechanism of CPT treatment in relevance to the lifespan extension effect we observed. We show that there is age-dependent increase of RET-ROS and FET-ROS generation in isolated mitochondria (Fig. 1G, H), and that CPT preferentially inhibited RET-ROS generation (Fig. 1I, EV1E) and RET-induced NAD+/NADH decrease (Fig. 1J, EV1F). Moreover, we show that there is age-dependent change of protein-protein interaction involving C-I proteins that participate in RET regulation, and that CPT treatment restored these protein-protein interactions (Fig. EV1G). We also show that there is age-related increase of mitochondrial membrane potential (MMP) and mitochondrial succinate levels (Fig. 1E, F), which are drivers of RET, and that CPT ameliorates these age-related mitochondrial changes.

- Whether aging affects CI/RET activity needs to be investigated (referee #2 major concern 1; specific point 1).
We appreciate the editor's suggestion. We have now added the following data to shed light on how RET/C-I activity is regulated during aging. We show that supercomplex formation by complex-I is reduced with aging, with concomitant increase of holo-complex I, although other complexes are not significantly changed (Fig. 1D). As supercomplexes are devoid of complex II, and are considered not conducive to RET, this offers one explanation of the aging effect on RET. Moreover, we found that succinate and MMP, two driving forces of RET, were increased with aging (Fig. 1E, F). Consistent with these findings, isolated mitochondria from aged animals are more active in RET when provided with the right substrate (Fig. 1H). We also found that there is aging-related increase of NDUFS3, a key C-I subunit involved in RET and a target of CPT (Fig. EV1D), and there are age-dependent changes of protein-protein interactions involving C-I proteins that participate in RET (Fig. EV1G). Thus, multiple changes in mitochondrial C-I that can promote RET are induced during aging. This opens new avenues for future studies of the exact mechanisms involved.

All concerns regarding missing controls, normalization methods and statistical analyses need to be addressed.
Thanks for the suggestion. We have addressed all concerns regarding those issues. Please see our response to the individual concerns when we address each reviewer.

However, concerns regarding the missing behavioral analyses of mouse AD model (referee #1, point 3), mechanisms explaining the effect of RET inhibition on the mitochondria and protein homeostasis (referee #2, specific concern 3), APP,C99 clearance (referee #2, specific concern 4, specific point 7 and 8) and neurological disease phenotypes (referee #3, major comment 5) are not prerequisite to experimentally address for publication in EMBO Reports.
We thank the editor for making exceptions to the list of concerns raised by the reviewers on the manuscript originally submitted for publication in *EMBO J*.

The authors do not need to revise the manuscript prior to transfer. Once the manuscript is transferred, I will then formally invite a revised manuscript as outlined above.'

Referee #1:

This manuscript by Rimal, Lu and colleagues evaluates the role of reverse electron transport (RET) in aging and neurodegeneration, primarily using Drosophila but also in cultured cells and mice. RET is a process by with complex I of the respiratory chain runs backwards to generate NADH and ROS. There is limited understanding of how RET may function in normal physiology and pathology. The authors find that multiple RET inhibitors, and genetic manipulations predicted to inhibit RET, extend lifespan in Drosophila, as well as suppress age-related phenotypes in flies and improve survival in fly and mouse neurodegeneration model.

The results in this paper will be of wide interest, and implicate RET as a novel target in aging and age-associated disease. However, there are significant issues with the manuscript that need to be address before it is acceptable for publication, in my view:
We thank the reviewers for the positive comments. The remaining issues raised by the reviewer have now been adequately addressed.

1. Perhaps I missed it, but have the authors performed epistasis with their primary RET inhibitor CPT together with NMN or anti-oxidants, for example in the context of lifespan? One might predict that there would be little added advantage to combining treatments that hit the same pathway. Such an experiment might provide insight into which consequence of RET is most important in limiting lifespan (NADH or ROS generation).
We appreciate the reviewer's suggestions. As suggested by the reviewer, we have performed the lifespan assay by combining CPT with NMN, NAD^+ synthesis inhibitor, oxidants, and antioxidants. Our results show that although NMN alone can extend lifespan like CPT, its combination with CPT did not offer additional lifespan benefit compared to CPT alone (Fig. EV3E). On the other hand, the NAD^+ synthesis inhibitor FK866 blocked the lifespan benefit of CPT (Fig. 1H), suggesting that the NAD^+/NADH pathway is critically mediating CPT's lifespan effect. Interestingly, treatment with the mitochondrial targeted antioxidant mito-Tempo also extended fly lifespan, but its combination with CPT did not offer additional benefit than CPT alone (Fig. EV3F). Similar results were obtained with the antioxidant melatonin (Fig. EV3G). Moreover, CPT treatment was unable to block the lifespan-shorting effect of the strong oxidant paraquot (Fig. EV3I). Thus, mito-ROS in general appears to be detrimental to fly lifespan as demonstrated by the mito-Tempo effect, but the involvement of ROS generated by specific mitochondrial processes, such as RET-ROS, is complex and will require further investigation, as previous studies suggest RET-ROS may be beneficial to fly lifespan. However, that study was largely based on analyzing the effect of yeast NDI1 expression, which in addition to promoting RET-ROS generation, also increases NAD^+/NADH ratio. This is discussed on page 22.

2. The manuscript relies heavily on the use of CPT. Can the authors verify that it acts as a RET inhibitor in their hands, with the dosage and treatment regimens used in the paper?
The reviewer's point is well taken. In the revised manuscript, we have provided the following data to support that CPT acts as a RET inhibitor under the dosage and treatment conditions used in the paper: 1) using purified mitochondria, we show that CPT inhibited RET-ROS formation in a dose-dependent manner, with estimated IC50 at 1-2 uM (Fig. 1I), and it increased NAD^+/NADH ratio at the effective dose (Fig. 1J); 2); Treatment of young flies with the RET inducer DES increased ROS generation and decreased NAD^+/NADH ratio. These effects were inhibited by CPT (Fig. 1K).

3. The mammalian experiments in the manuscript seem underdeveloped. The omission of any learning and memory assays in the mouse AD model are puzzling - one would typically perform these studies standardly in such a model.
We understand the reviewer's point. Learning and memory studies in rodents are substantial on their own and will require significant resources and collaboration to complete. We have now focused our AD model studies in *Drosophila*, which include learning and memory assay. We have removed the 5xFAD mouse data because it does not include aging-related phenotypic study. We keep the PS19 mouse data because CPT extends lifespan in this mouse AD model.

4. The statistics in the manuscript require review by a statistician. The lifespan curves appear to have been analyzed point-by-point using a t-test or the like, but a more appropriate analysis would consist of a log-rank rest, with the Wang-Allison test to detect differences in maximum lifespan.
We thank the reviewer for the constructive suggestion. We have consulted a statistician and have analyzed the lifespan data using long rank test, coupled with Wang Allison test for detection of maximal lifespan difference. We have described these changes in Methods section and figure legends.

5. In the interest of data transparency, the many "dynamite plunger" bar graphs should be replotted as scatterplots.
We thank the reviewer for the constructive suggestion. We have now used scatterplots for data presentation.

Referee #2:

In this manuscript, Rimal et al investigated the impact of reverse electron transfer (RET) interference on aging and lifespan in both fly and mouse models. The authors demonstrated that RET inhibition either by chemicals or by RNA interference, restored both ROS and NAD+ levels, alleviated aging features, and extended lifespan of Drosophila. Similar examination was also done using mouse AD model. Authors claimed that a deregulation of RET is a conserved feature of aging. Overall, this is an interesting study and may help understand the regulation of two important aging regulators, i.e., ROS and NAD+. However, while phenotype observations support the conclusion, mechanistic evidence is rather weak.
We greatly appreciate the reviewer's overall positive comments on our manuscript. We have now provided more mechanistic data supporting aging-related RET changes and interference of these changes by CPT.

Major concerns
1.The key observation that authors wanted to address is aging-related RET activation. However, elevated ROS and decreased NAD+/NADH ratio as supporting evidence is not enough. For example, does CI (NDUFS3) level increase along with aging? Or Is the activity up-regulated?
The reviewer's points are well taken. Please see our response to the general comments highlighted by the editor.

2.Both ROS elevation and NAD+ decline might drive aging. It seems that author only applied ROS level as an indicator of RET activation. Is it possible that the effect of RET inhibition on aging and lifespan is in some extent owing to ROS decline? More interestingly, in an earlier study (Cell Metab. 2016 Apr 12;23(4):725-34.), CI was found reduced in aged flies (older than 75 days) and enhanced RET-derived ROS, which is different from general ROS, promotes lifespan and alleviates aging feature. Authors need to distinguish ROS sources in the context of aging regulation.
The reviewer raised an interesting point. Please see our response to Reviewer Point 1 on the potential role of different sources of mito-ROS in lifespan regulation.

3.The authors applied mitochondria dysfunction and protein dys-homeostasis as readouts of aging. However, the unanswered questions are how targeting CI affects mitochondria fission/fusion and how mitochondria CI regulates protein homeostasis.
The reviewer raised some very important points. We are currently actively investigating these questions.

4.In the last part of the manuscript, authors investigated the role of CI inhibition on APP,C99 generation/clearance in both fly and mouse models. It is difficult to understand how RET affect APP,C99 generation/clearance.
This is an unexpected observation. We are currently actively investigating the mechanisms involved, but it will take some time before we can have a clear answer.

Specific points,

1. Figure 1, since the main focus of the study is RET, the key regulator CI level should be examined in aged flies.
We understand the reviewer's point. However, due to the limited availability of antibodies against *Drosophila* C-I proteins, our tools are limited. But we did find changes in NDUFS3 level with aging (Fig. EV1D). We also show that complex-I supercomplex formation is reduced, whereas holo-complex I is increased with aging (Fig. 1D).

2. Figure 4a-e, female flies were used, while in f-h, males were used. What is the rationale?
We have performed the lifespan studies with both male and female flies and observed similar patterns. For WT fly studies, we mostly used males. For some genotypes due to limitation in the number of male flies we could generate from the crosses, we used females.

3. Figure 4d, from the fluorescence images, NDUFS2/S3 RNAi almost eliminated all positive (green) signal, but in the quantification data, only about 50% downregulation, why?
The reviewer's point is well taken. We have now replaced the original images with more representative ones.

4. Figure 4, is the lifespan extension effect of NMN is NDUFS3/2 dependent? This is important which help answer whether aging-related NAD+ decline is mainly attributable to RET activation. Similarly, what about NMN+CPT combined treatment? Is there any additive effect?
We understand the reviewer's point. In newly provided data, we show that NMN had no additional effect on lifespan in NDUFS3 RNAi flies (Fig. EV4F), and that NMN treatment did not offer additional lifespan effect when combined with CPT (Fig. EV3E).

5. Figure 4, many findings are related to the brain, why here only muscle manipulation showed obvious effect? Whether additive effect need to be considered
We appreciate the point raised by the reviewer. We observed tissue-specific effect on lifespan with complex I knockdown. Whereas muscle manipulation showed strong effect on lifespan, neuronal manipulation had limited effect. It is possible that there exists additive effect of the tissue-specific manipulation on lifespan. However, multi-tissue knockdown of C-I subunits turns out to be toxic, making it difficult to test the combination effect on lifespan.

6. Figure 5, what about NMN? Does the NMN effect on lifespan extension rely on Sirt1/Foxo/ATG1?
The reviewer's point is well taken. We tested whether the lifespan benefit provided by NMN is dependent on Sirtuin, ATG1, or Foxo. We found that dSirt2-RNAi sufficiently blocked the NMN effect in lifespan extension, whereas ATG1-RNAi and Foxo-RNAi were less effective (Fig EV4E-I). Moreover, NMN did not offer additional lifespan benefit in NDUFS3-RNAi flies (Fig EV4F). Thus, although dSirt2 RNAi was effective in blocking the lifespan effect of CPT and NMN, ATG1-RNAi and Foxo-RNAi were effective in blocking the effect of CPT but not NMN, suggesting that ATG1 and Foxo may mediate other effects of CPT, such as its antioxidant activity, in lifespan regulation. We have discussed this point on page 14-15.

7. Figure 6f, how does CPT reduce the aberrant APP.C99 species derived from full-length APP? Is there any independent mechanism?

This is an unexpected observation. We are currently investigating the mechanisms involved, but it will take some time before we can have a clear answer.

8. Figure 6k, why RNAi NDUFS3 could remove APP.C99?
This is related to Point 7. We are actively investigating the mechanisms involved, but there is no telling when we can have a clear answer.

Referee #3:

In 'Reverse electron transfer at mitochondrial complex I is activated during aging and contributes to aging and age-related disease phenotypes', Rimal et al. present the role of complex I reverse electron transfer (RET) during ageing. RET is the conversion of NADH from NAD by respiratory complex I (CI) that usually occurs under specific conditions. The conclusions are not entirely supported by the data and the mechanisms are in general poorly described, involving several effectors which affect several aspects of nuclear, cytosolic, and mitochondrial general functions. It is difficult to determine whether the effects are attributable to RET since no experiments on specific CI activities under different conditions and assembly are performed. Most of the results reflect a correlation but not actual cause. The manuscript, although interesting, should deepen into the biochemical aspects of CI RET in their system.

Major comments:

1. CPT role in preventing RET is not clear. There is no data that comprehensively analyzes complex I specific activities in vitro (forward and reverse) and therefore the effects are not necessarily attributable to RET. IN addition, there is no information on CI assembly under these conditions which leaves many conclusions reliant on assumptions. RET occurs under specific conditions that include i) increased CII activity and therefore changes in NADH:FADH2 equivalents, ii) increased membrane potential (i.e.: excess of protons at the IMS) and iii) CI is dissociated from complexes III2 or III2:IV. Are these conditions applying? To demonstrate that RET is actually happening, the first figures should analyze CI assembly (BN-PAGE, respiratory complexes can be seen directly by Coomassie), test for activity forward and RET using isolated mitochondria and perform deep biochemical analyses. How do other complexes look like, how is their activity? The results can be also interpreted as loss of CI activity (NADH to NAD) attenuates OXPHOS activity and reduces the leak of electrons that may cause ROS. The issue with the inhibitors is that these are not specific to RET activity.
The reviewer's points are well taken. Please see our response to the general comments heighted by the editor, where have we addressed all the points raised by this reviewer.

Regarding the point of "The results can be also interpreted as loss of CI activity (NADH to NAD) attenuates OXPHOS activity and reduces the leak of electrons that may cause ROS. The issue with the inhibitors is that these are not specific to RET activity". We respectively disagree with this interpretation. Many OxPhos inhibitors would increase rather than decrease ROS production. Also, loss of C-I activity would decrease NAD^+/NADH ratio, whereas CPT increased NAD^+/NADH ratio. Moreover, we found that ATP level is increased after CPT treatment (Fig. 3G), which would be difficult to explain by a loss of C-I activity.

2. CPT acts on NOTCH which controls several cellular processes. Is there any change in global ubiquitination and/or proteasomal activity? This can explain the reduction in proteasomal activity. In addition, mitochondrial labeling in Figure S3C does not suggest mitochondrial fragmentation, it seems more a decrease in mitochondrial density. Is there any de-differentiation issue, a transition to cellular proliferation?
The reviewer's points are well taken. We did not observe obvious change in global ubiquitination (Appendix Fig. S1C), suggesting that the effect of CPT on proteostasis may be through specific substrates such as APP.C99, or through other mechanisms not involving ubiquitination of proteasome. We have added a new image to illustrate CPT effect on mitochondrial morphology. Since we are studying muscle tissues, which are polyploid, it would be difficult to image that dedifferentiation is involved in the phenotypes we observed. Nevertheless, this is an issue we will visit in the future.

3. It is still unclear the impact of NDUFS2, NDUFV1 and NDUFS3 on CI stability and function. ROS levels should be normalized to total mitochondrial content. This applies to other experiments in the manuscript. CI mutations can result in substantial decreases in cellular mitochondrial mass explaining the decrease in ROS levels.
The reviewer's point is well taken. We have measured mitochondrial mass by probing C-V subunit ATP5a but we did not observe an obvious effect of NDUFS2, S3, V1 knockdown (Appendix Fig. S1E) on its level. Thus, although further studies are needed, this result is inconsistent with these manipulations decrease ROS level by reducing mitochondrial mass.

4. Data on NMN rules out the role of deacetylases. Increases in NAD+ can activate sirtuins that deacetylate their

targets. The increase in non-acetylated lysines can favor their ubiquitination and subsequent degradation of proteins. There is a cross-talk between acetylation and ubiquitination that balances activity and degradation of proteins. These hypotheses should be considered. It would be very interesting to understand how Sirt2 (cytosolic) controls CI RET activity.
These are interesting points. However, our data suggest that RET regulates Sirt2 activity through NAD^+/NADH regulation as shown by the dependence of CPT effect on dSirt2, but not the other way around. Future studies will test if dSirt2 would also feedback regulate C-I/RET.

5. The assessment of CPT on models of neurological diseases is attractive but completely rules out what CPT does, how complex activity is modulated, mitochondrial mass content, glycolysis,....
These studies in mouse models will be the focus of future studies but are outside the scope of current study.

Minor comments:

1. The entire manuscript lacks controls of gene or protein expression. This is especially critical for siRNA assays.
We appreciate the reviewer's point. We have now added controls to show RNAi efficiency (Appendix Fig. S1F).

2. Graphs need to fit the format of other figures.
Thanks for the suggestion. We have made the formats of the graphs consistent throughout the figures.

Dear Dr. Lu

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the delay in handling your manuscript, but we have meanwhile received the reports from former referee #2 (now #1) and referee #3 (now #2). As you will see, while the referees acknowledge that you have addressed a number of concerns, they also point out several points that still need your attention, such as missing controls, unclear BN-PAGE results and oversaturated gels and I would like to give you the opportunity to address these remaining concerns in a second round of revisions.

In addition to these experimental issues, there are also a number of things from the editorial side that need your attention:

- Please reduce the number of keywords to 5.

- Regarding the Author Contributions, we now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section, which therefore needs to be removed from the manuscript text. You can use the free text box in our system if you wish to provide more detailed descriptions. See also guide to authors https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.

- The Data availability section is meant to refer readers to data deposited in external repositories. If you have no data that requires deposition, please state this in this section instead of the text you currently have.

- Please add a callout to Fig 2H in the text.

- Appendix: Please provide page numbers in the table of content and correct the figure names to 'Appendix Figure S#'. Please move the Supplementary Materials & methods to the Materials and Methods section in the main manuscript.
- Figure S1A: please provide scale bars and define their size in the legend.
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- Please use "Data information" if descriptions in the figure legend describe something that applies to several panels. For S1: 'Data information: Data are representative of at least three repeats. The error bars represent SEMs...". [Please define here which data this refers to, also to the quantification? Did you e.g., quantify 5 flies in S1B but repeat this 3 times? Please also define the statistical test used].
- Figure S2A, B: Please provide scale bars and define their size in the legend.
- S2B: please define the treatment conditions 'vehicle' and 'CPT' in the legend.
- S2C: please provide the graph in better resolution
- S2C, D, E: please indicate the number of experiments this is based on and whether these are biological or technical replicates and please state the statistical test used.

- A a general note regarding quantifications: if 'n' is smaller than 5, we recommend showing the individual datapoints in addition to the mean.

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I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The authors have addressed most of the concerns by referee#1 and #2. The original blot of NDUFS3 in Fig. EV1D, which was overexposed making it difficult to tell the difference, need to be replaced with a low-exposure one.

Referee #2:

The study is focused on the study of reverse electron transport (RET).It is mainly based on the capabilities of the CPT compound to restore many different phenotypes in different biological systems. The phenotype appears also to be affected by antioxidant treatment that can also ameliorate the phenotypes shown similar to CPT. Authors should emphasize more the similarities with the antioxidant treatment.

Controls of CI activities are necessary across the most of the key experiments. This is important because the information on CI activities and their phenotypes will strength their conclusions. Also the Knock-down experiments should provide qPCR, WB or both to proof actual downregulation of targeted genes.

It seems that a CI/CII imbalance could be responsible for the increase in ROS. It would be necessary to test CII activity under different experimental conditions and also mildly inhibit it to prevent H+ overflow. Is thus rescuing the phenotype?

In Fig 1D, it is difficult to interpret the BN-PAGE gel since the whole length of the gel is not displayed. WBs showing CI, CIII2, CIV and CV should be necessary to attribute labels to those bands. It seems that the band labeled as CV could instead be CI and the upper bands different supercomplex assemblies, I:III2 and I:III2:IV. CI in-gel activity assays would be also helpful to locate CI and test its activity. WB in EV1D is oversaturated and differences are not distinguishable.

In Fig EV1G, authors show apparent interactions within CI subunits by IP. These subunits are essential for FET and RET. This arises two questions, is there any other electron leak being missed? and, the dissociation of constitutive subunits of CI indicates disassembly?

Fig 3D suggests reduced mitochondrial mass in aged flies and does not represent the graph provided. Loss of mitochondrial mass is critical and may explain most of the phenotype without recurring to RET. The buffering capacity for ROS provided by mitochondria will be compromised and any antioxidant treatment will be functional.

The argument of protein ubiquitination does not consider the fact that specific subpopulations of proteins are being affected resulting in the observed phenotype. This would require an entire deep analysis which may be not the scope of the work. Please, revise these sections accordingly. However, the connection between Sirt2 manipulations and RET and FET activities should be provided.

Point-by-Point Response to Reviewer Comments to EMBOR-2022-55548V2

Referee #1:

The authors have addressed most of the concerns by referee#1 and #2. The original blot of NDUFS3 in Fig. EV1D, which was overexposed making it difficult to tell the difference, need to be replaced with a low-exposure one.

We appreciate the reviewer's comment. We have now replaced the original blot with a low exposure one in **Fig EV1D**.

Referee #2:

The study is focused on the study of reverse electron transport (RET). It is mainly based on the capabilities of the CPT compound to restore many different phenotypes in different biological systems. The phenotype appears also to be affected by antioxidant treatment that can also ameliorate the phenotypes shown similar to CPT. Authors should emphasize more the similarities with the antioxidant treatment.

The reviewer's point is well taken. As we discussed in the text, CPT has a -SH group. So, part of its effects might come from its antioxidant activity. However, the role of antioxidants in aging and lifespan regulation is complex and controversial, mainly because ROS can be beneficial or detrimental depending on the source, intensity, and duration. Particularly relevant to this study, a previous study concluded that RET-generated ROS is beneficial to fly lifespan, whereas our results indicate that a mitochondrial-targeted antioxidant (mito-TEMPO) is also beneficial to fly lifespan, further highlighting the complexity of ROS signaling in lifespan regulation. On the other hand, as a regulator of RET, CPT also increases NAD^+/NADH ratio, and its effect on lifespan is similar as NAD^+ supplementation. Moreover, the facts that co-treatment with CPT and NAD^+ supplementation did not offer further lifespan benefit than individual treatment, and that CPT or NAD+ supplementation provided stronger lifespan effect than antioxidants, strongly supported our conclusion that the effect on NAD^+/NADH ratio is a major contributor to the lifespan benefit of CPT. This is consistent with findings in cancer settings, where CPT exerted anti-cancer effects that antioxidants were not able to provide (Ojha et al., Dev Cell 57(2):260-276). We have further discussed this topic on **page 22-23**.

Controls of CI activities are necessary across the most of the key experiments. This is important because the information on CI activities and their phenotypes will strength their conclusions. Also the Knock-down experiments should provide qPCR, WB or both to proof actual downregulation of targeted genes.

We thank the reviewer for the suggestion. At the beginning of the study, we measured C-I activities using two approaches: 1) *In vitro* C-I activity assay using malate and glutamate as substrates; 2) In-gel C-I activity assay. In both assays, we did not find significant change in C-I activity during fly aging. Therefore, we concluded that RET activity change rather than C-I activity change is primarily associated with fly aging. We have now provided these data in **Fig EV1L, M**.

For the knock-down experiments, in the original Appendix Figure S1F we provided qPCR result for all the RNAi lines used in the study. Due to the unavailability of some of the antibodies, we could not

test protein knock-down for all RNAi line. We have now provided WB results for the RNAi lines for which antibodies are available to us. This is provided in **Appendix Fig S1G**.

It seems that a CI/CII imbalance could be responsible for the increase in ROS. It would be necessary to test CII activity under different experimental conditions and also mildly inhibit it to prevent H+ overflow. Is thus rescuing the phenotype?

We understand the reviewer's point. However, our data do not support the notion that CI/CII imbalance could be responsible for the increase in ROS. As mentioned earlier, we did not find change in CI activity with aging. We also performed in-gel activity assay for CII and we also did not find significant change in C-II activities with age. This data is provided in **Fig EV1M**.

In Fig 1D, it is difficult to interpret the BN-PAGE gel since the whole length of the gel is not displayed. WBs showing CI, CIII2, CIV and CV should be necessary to attribute labels to those bands. It seems that the band labeled as CV could instead be CI and the upper bands different supercomplex assemblies, I:III2 and I:III2:IV. CI in-gel activity assays would be also helpful to locate CI and test its activity. WB in EV1D is oversaturated and differences are not distinguishable.

We thank the reviewer for the comments. As mentioned in our response to Reviewer 1, we have now replaced the oversaturated WB in **Fig EV1D** with a low-exposure one.

Regarding the BN-PAGE gel, we have now provided the full-length gel to show the various complexes in **Fig 1D**. Our previous image only showed the top of the gel to highlight C-I related changes with age. We are confident about the identities we assigned to the various bands on the BN-PAGE gel. This is based on WBs and in-gel activity assays. Also, the identities we assigned to the various band are consistent with published work of others (Murari et al., J Cell Biol 219(10):e202001071; see below)[Figure for referees not shown.] . It is worth pointing out the BN-PAGE gel mitochondrial respiratory complexes is slightly different from mammals, particularly in the supercomplex assemblies. We have further elaborated this point on **page 7**.

In Fig EV1G, authors show apparent interactions within CI subunits by IP. These subunits are essential for FET and RET. This arises two questions, is there any other electron leak being missed? and, the dissociation of constitutive subunits of CI indicates disassembly?

We appreciate the reviewer's comment. Our results show that there are subtle age-related changes in protein-protein interactions among certain C-I subunits, and that CPT treatment restored these changes, supporting the notion that CPT modulates RET by changing protein-protein interactions within C-I. Since our data showed that CI activities are not changed in aged flies (**Fig EV1L, M**), the simplest explanation is that the detected age-associated protein-protein interactions specifically affect RET. It is unlikely that they indicate C-I disassembly. Otherwise, we would have detected C-I activity changes. Extensive further studies will be needed to understand in more details how these protein-protein interaction change specifically affects RET. We have discussed this point on **page 25**.

Fig 3D suggests reduced mitochondrial mass in aged flies and does not represent the graph provided. Loss of mitochondrial mass is critical and may explain most of the phenotype without recurring to RET. The buffering capacity for ROS provided by mitochondria will be compromised and any antioxidant treatment will be functional.

We understand the reviewer's point. The reviewer might have interpreted the weak mito-GFP signal in one of the two dopaminergic neurons shown in aged brain sample as reduced mitochondrial mass. That cell was simply out of focus. We have now provided new images in **Fig 3D** showing only representative neurons in focus, and we have also quantified mito-GFP signals in **Appendix Fig S1H**. Our data show that there is no significant difference in dopaminergic neuron mitochondrial mass between young and aged flies.

The argument of protein ubiquitination does not consider the fact that specific subpopulations of proteins are being affected resulting in the observed phenotype. This would require an entire deep analysis which may be not the scope of the work. Please, revise these sections accordingly. However, the connection between Sirt2 manipulations and RET and FET activities should be provided.

The reviewer's point is well taken. We cannot exclude the possibility that small subset of proteins might be affected. However, protein ubiquitination is only nebulously related to this study, and deep analysis of protein ubiquitination is out of the scope of the current work as the reviewer appropriately pointed out. We have further discussed this point on **page 11**. Regarding the role of Sirt2, our data support the model that by changing NAD+/NADH ratio, CPT activates the NAD+-containing Sirt2 deacetylase, which mediates the effect of CPT on lifespan, since the lifespan effect of CPT is blocked by Sirt2 knockdown. While we cannot exclude the possibility that Sirt2 may feedback regulate C-I to influence RET or FET, this feedback regulation is unlikely to be the main mechanism of Sirt2 action; Otherwise, CPT would be able to rescue the shortened lifespan of Sirt2 RNAi flies. Instead, we observed that Sirt2 RNAi blocked CPT effect on lifespan. We have now discussed this on **page 24**.

Dr. Bingwei Lu
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United States

Dear Bingwei,

I have already informed you about the outcome of the re-review process and copy the comments from referee 2 again below my signature. Thank you for incorporating the last minor changes into the manuscript and Appendix. I have uploaded the modified files and am now very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #2

The authors improved the paper and data is now more convincing. It is interesting to say that overtime, they see increases in CI abundance in aged flies but not its activity. This indicates that specific CI activity declines upon aging, and it might be of interest to comment on this.

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The data shown in figures should satisfy the following conditions:

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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
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  - are tests one-sided or two-sided?
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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	