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Tetracyclines activate mitoribosome quality control and reduce ER stress to promote cell survival

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **21st Apr 2023**

Dear Pere,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also raise some concerns and have several suggestions for how the study could be improved. I think all suggestions are reasonable and should be addressed. Please let me know in case you disagree, and we can discuss the revisions further, also in a video chat, if you like.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. 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 major 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 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 (22nd Jul 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

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2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) 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: <https://www.embopress.org/page/journal/14693178/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.

4) 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.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/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.

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7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). 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 (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript 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.

9) Our journal also 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/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n Program fragment delivered error ``Can't locate object method "less" via package "than" (perhaps you forgot to load "than"?) at //ejpvfs23/sites23b/embor_www/letters/embor_decision_revise_and_review.txt line 56.' 2, use scatter blots showing the individual data points.

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 also include scale bars in all microscopy images.

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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I look forward to seeing a revised form of your manuscript when it is ready.

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The study presents an analysis of mitochondrial translation inhibition by doxycycline that promotes survival of HEK cells through suppression of the ER stress IRE1α protein. It shows that doxycycline treatment of HEK cells leads to the association of a mitoribosomal large subunit factor MALSU1. The authors interpret these results as stalling of translation and subunit splitting. They further show that doxycycline reverse cell-death signaling in the ER through the attenuation of IRE1α oligomerization and inhibition of effector UPR function. MALSU1 deficient cells exhibit heightened sensitivity toward the activation of ER stress. Together, the data suggest signaling between mitochondria and ER in the context of mitochondrial diseases.

I have several suggestion how to improve the manuscript.

1. Since the study describes effects induced by doxycycline and not other tetracyclines. For consistency, it might be better to replace 'tetracycline' with 'doxycycline' throughout the introduction, discussion, abstract, and title.

2. Throughout this work, the effect of the antibiotic is described as inhibition of translation elongation. However, no evidence is provided for that. Tetracyclines work on elongation and initiation of bacterial ribosomes. So perhaps, better to use a more general term, like 'translation inhibition' or similar?

3. It's unclear why a GTP analog is added to the mitoribosome prep? This would inhibit elongation and rescue factors, leading to ribosome stalling. I couldn't find a reasoning for the procedure.

4. If I understand correctly, it's suggested that MALSU1 is a dissociating factor, however no data is presented or has been ever published showing such an experiment. MALSU1 was shown in the association with the mitoribosomal large subunit assembly intermediate, but no subunit dissociating activity has ever been recorded. Maybe better to use a bit more careful description.

5. An additional possible scenario is that another factor splits the subunits, for example mtIF3 that binds to the mitoribosomal small subunit. However, it seems that the small subunit hasn't been monitored in the experiments. It's a bit risky to rely on the data from the large subunit only, because it's always in access over monosomes, which might bias the results.

6. Figure 1E, unclear what are the shapes associated with MALSU1, looks like labels are missing? The schematic with the membrane is a bit unusual, perhaps it's possible to design a figure that is scientifically more accurate?

7. Line 97, the corre4ct reference is 10.1038/nsmb.3464

Referee #2:

In this work, Ronayne et al have investigated how exposure to tetracyclines promote survival of cells of a mitochondrial disease model. They found that components of a recently identified pathway for resolving stalled mitoribosomes interact genetically with tetracycline antibiotics. One of these components, MALSU1, a factor also playing roles in mitoribosome assembly, was a specifically interesting candidate. In further sets of biochemical experiments, this study found that MALSU1 accumulated to higher levels upon exposure to the antibiotic, which was accompanied by an increased binding of MALSU1 to the mitoribosomes and a concomitant shift of the large subunit to lighter fractions in sucrose gradient analyses. The authors then went on to show that, in addition to these effects on mitoribsome assembly and MALSU1 interaction, exposure to a tetracycline impaired the induction of the UPRer and decreased the accumulation of proteins within the ER. This connection between tetracycline toxicity and an impairment of ER loading was unexpected and could potentially explain how antibiotic administration could promote survival of cells of a mitochondrial disease model. The previously reporter beneficial effect of inhibition of mitochondrial translation to cure mitochondrial defects in cells has generated a substantial interest in understanding how this occurs mechanistically. The current manuscript will therefore be of interest for the community working on mitochondrial proteostasis, aging and diseases.
Overall, the data are of very good quality and the manuscript is well written. The proposed mechanism by which tetracyclines modulate mitoribosome activity and the causality between changes in mitochondrial translation and ER protein loading/UPRer induction should be further elaborated.

Major points:
1. The proposed mechanism suggests that exposure to tetracyclines activates the rescue of stalled mitoribsomes via MALSU1, but not via MTRES1 or mtRF-R. While these conclusions are based primarily on genetic interactions between the drug and these genes and the biochemical characterization of MALSU1-mitoribosome binding, it is important to identify the molecular sequences of events. The increased interactions of MALSU1 with the mitoribosome was detected after 48h of exposure to tetracycline. If indeed tetracyclines induce ribosome splitting, as the authors suggest, this should occur immediately or very shortly after drug exposure. If this does not occur in such short time frames, it is likely that the increased MALSU1-mitoribosome interaction more reflects an adaptive response of the cells with impaired mitochondrial translation. Both outcomes would be interesting but could potentially change the proposed mechanism. Hence the experiments from Fig2 B and D-E should be repeated with cells exposed to tetracyline for much shorter times. Moreover, it would be important to monitor the effects of tetracycline on mitochondrial translation in these experiments, either through metabolic labeling or other methods (PMID: 33586863).

2. While the effect of tetracyline exposure on ERloading and UPRer induction are very interesting, the causality between mitochondrial translation inhibition, these ER changes and the effect on promoting survival of the cells is not firmly established. To this end, the authors should design an experiment where UPRer is artificially induced in their cell model, which should be sufficient to promote survival of these cells independent of tetracycline exposure. Moreover, it would be important to check whether mutants other than MALSU1 ko, which are affecting mitochondrial translation, have similar effects. This would lend further support for a direct role of MALSU1-dependent ribosome recycling in this connectivity between mitochondrial translation and ER proteostasis.

Minor points:
1. actinonin has previously been used to unravel a mitochondrial RNA decay pathways (23453957), however, further experiments suggested that the action of this drug is not on mitochondrial translation or PDF (26504172), but likely inhibits the mitochondrial m-AAA protease to remove faulty proteins. This should be discussed for Fig 1A.

2. The authors argue that MALSU1 ko leads to insensitivity to tetracycline, however in their Fig 1D, two of three clones do show a significant increase in cell growth upon exposure to tetracycline. This experiment should be repeated with more clones and a ratio should be presented of cell numbers obtained with and without DOX addition, both for MALSU1 as well as for MTRES1 and mtRF-R ko cells.

Referee #3:

This manuscript is focused on defining the molecular mechanism by which tetracyclines increase cell survival in mitochondrial mutant disease cells during nutrient deprivation. In previous publications, these authors identified tetracyclines like doxycycline as compounds that could enhance cell survival of cells harboring mutants in mitochondrial proteins (e.g., ND1) in response to respiratory challenge. In other work, the authors indicated that cell toxicity of mitochondrial mutant cells during nutrient stress could be attributed to activation of the UPR sensor IRE1 and p38 signaling. Here, they attempt to connect these two findings to elucidate a molecular mechanism for doxycycline protection. They identify the mitochondrial ribosomal QC factor MALSU1 as a required gene for doxycycline-induced protection of ND1 cells during nutrient stress. This effect appears specific to MALSU1-dependent mito ribosome splitting, as other factors involved in downstream mitoribosome QC are not required for this protection. They go on to try to link MALSU1-dependent mitoribosome regulation to IRE1 by demonstrating that markers of ER stress-dependent IRE1 activation are reduced in doxycycline treated ND1 cells under nutrient stress through an MALSU1-dependent mechanism. Finally, the try to relate these doxycycline-dependent alterations of mitoribosome QC to ER stress-dependent IRE1 activation by attributing this effect to increased ER protein loading. The authors conclude that this represents a unique mechanism of mitochondria-ER regulation mediated through IRE1.

While the identification of MALSU1 as a key component of doxycycline-dependent enhancement of the survival of ND1 mutant cells during nutrient deprivation is an interesting finding, the authors fall short of developing the mechanism of this regulation beyond that first step. There are major gaps in this mechanism that remain, which are critical to address for this current publication. It was already reported that IRE1 activation was potentially involved in reduced viability of ND1 cells during nutrient stress, so the results linking doxycycline to ER stress or IRE1 activation are surprising. Further, the argument that doxycycline-dependent ribosome staling lead to increased ER protein loading is extremely weak for a number of reasons outlined in more detail below. The key questions related to this mechanism remain: 'How does mitoribosome inhibition afforded by doxycycline induce ER stress and subsequent decreases in viability?'. In my opinion, this manuscript does little to advance this beyond identifying MALSU1 as a key component of this mechanism, which should be better developed. Apart from this issue, a number of conclusions drawn from this work are not well supported by the data provided. More on that below, but multiple times, whole cell extracts show one result while isolations that appear to include entire organelles containing the overwhelming majority of proteins being followed (e.g., IRE1) show different results. This doesn't make any sense. I get the arguments that different intra-organellar populations of specific proteins could show different modifications/activations, but this is not supported by the data provided. Quantifications of blots to show reproducibility of major findings are also not included in this manuscript, but are quite

important for the work, especially as what appears to be similar experiments across panels show different results for specific proteins. Ultimately, the minimal conceptual advance combined with the lack of quantification, variability between different replicates of key experiments presented, and numerous issues with the data/interpretations provided significantly limit enthusiasm for this manuscript.

Some specific areas of concern are highlighted below

SPECIFIC COMMENTS.

1. In Fig. 1G, the authors claim that doxycycline increases mitochondrial MALSU1, without impacting total levels of cellular MALSU1 or gene expression. Where is this protein coming from? Is there a population of MALSU1 that is not mitochondrial localized that then gets targeted to mitochondria posttranslationally? There is no evidence of this population in any other cellular fractions from the data presented. It would also help if this increase was quantified across multiple experiments, although the mitochondria increase is supported across multiple blots (e.g., Fig. 2A,C, although I don't understand the wildly different increases between replicates in Fig. 2A, right and the huge increase between +/- doxycycline observed in I, as compared to G).
2. With respect to MALSU1, could you get the same protection by overexpressing this mitochondrial QC factor? In other words, is this required or sufficient for the doxycycline-induced protection.
3. The authors claim that IRE1 oligomerization is reduced in crude mito/ER fractions, but not in whole cell extracts (the latter is not shown). However, in Fig. 2G,H when the authors monitor IRE1 across fractions, the vast majority of IRE1 is in these crude mito/ER fractions. A similar distribution is observed for mito proteins such as MALSU1. How is it possible that what appears to be a small population of IRE1 not included in the mito/ER crude fractions have such a big impact on IRE1 oligomerization in whole cell extracts? This doesn't make any sense.
4. Why not show PARP cleavage at 96 h? That appears to be where the biggest differences are observed for viability. The results for PARP cleavage shown in Fig. 3A at 72 h are very minor (although I do see it). This should also be quantified.
5. It appears that doxycycline is globally reducing ER stress and subsequent UPR activation in ND1 cells treated with galactose. This should be tested by monitoring activation of PERK and ATF6 signaling as well. This would support an argument that doxycycline and MALSU1 reduce global ER stress. As written, it is suggested that this is selective for IRE1. I understand the authors focus on IRE1 because of their previous manuscript suggesting IRE1 may be most import, but other pathways should also be considered in these experiments.
6. For Fig. 3E, other markers of UPR activation should be followed apart from IRE1 levels. If the authors are correct, there should be numerous other ER stress-responsive genes/proteins that support their argument. As it is, this is a weak figure attempting to link these findings to a mouse model. More needs to be done to do that in a significant way.
7. The authors indicate that ER stress in ND1 cells subjected to nutrient stress results from increased "ER protein loading". This is followed by levels of cathepsin C in the ER, which are suggested increase in ND1 cells incubated in galactose. However, this is not always the case and should be quantified (e.g., there is no increase at time 0 in the samples from Fig. 4D). Regardless, this does not reflect 'protein loading' as described. This could also simply reflect ER stress-dependent accumulation of cathepsin within the ER (i.e., reduced trafficking). This is not likely a 'cause' of ER stress, but instead more likely a consequence. Along these same lines, the reduction in SRP14 would just reflect reductions in ER stress, as this protein is regulated in response to ER stress. Overall, these data are not convincing in describing a molecular mechanism of ER stress increases in ND1 cells incubated in galactose or in the effects of doxycline in this model (apart from just reducing ER stress).
8. Similar to the above, MALSU1 depletion basally reduces cathepsin-C levels within the ER to levels nearly identical to that observed in doxycycline treated ND1 cells treated with doxycycline (Fig. 1F), while true that doxycycline does not further reduce cathepsin-C in these cells, this is not consistent with a model wherein accumulation of cathepsin C is a mechanism involved in the observed cellular toxicity.

Dear Esther,

We would like to thank the reviewers for their critical and fair assessment of our manuscript titled: “Tetracyclines activate mitoribosome quality control and reduce ER stress to promote cell survival”. We have taken into consideration and addressed the comments raised by all three reviewers and have included below a point-by-point explanation as to how the comments have been incorporated into the revised manuscript. We believe that with the new experiments and further revisions the manuscript is now suitable for publication in EMBO reports.

Thank you again for handling the review process of our manuscript at EMBO reports.

Sincerely,

Pere Puigserver, PhD

Point-by-point response to the Reviewer's critiques

We would like to thank the reviewers for the time devoted to evaluating our manuscript and for their positive critiques and suggestions. We have addressed these critiques and revised the manuscript accordingly, outlined in the point-by-point explanation below. We believe that with these revisions the manuscript has been strengthened and the conclusions are further supported by the data.

Referee #1:

1. Since the study describes effects induced by doxycycline and not other tetracyclines. For consistency, it might be better to replace 'tetracycline' with 'doxycycline' throughout the introduction, discussion, abstract, and title.

We agree with the reviewer in expanding this mechanism beyond doxycycline. To address this, we assessed whether other tetracycline analogs can rescue mitochondrial disease mutant cells depending on MALSU1, the mitoribosome quality control factor found to be required for survival signaling in this manuscript. Here, we included the parental tetracycline, along with synthetic analogs minocycline and 7002, where these analogs, similar to doxycycline, cannot promote survival in MALSU1 KO ND1 cells (Fig. 1E). This indicates that MALSU1 survival dependency is not specific to doxycycline, but is extended to the broad tetracycline class of antibiotics. We appreciate the reviewers understanding in now using 'tetracyclines' throughout the manuscript.

We have included an additional figure (Fig. 1E), and subsequent description in the results page 7, lines 199-209.

2. Throughout this work, the effect of the antibiotic is described as inhibition of translation elongation. However, no evidence is provided for that. Tetracyclines work on elongation and initiation of bacterial ribosomes. So perhaps, better to use a more general term, like 'translation inhibition' or similar?

We agree with this comment, and we have updated the manuscript by changing specific wording of "elongation" to "mitoribosome-targeting" or "mitochondrial translation inhibition" where appropriate.

3. It's unclear why a GTP analog is added to the mitoribosome prep? This would inhibit elongation and rescue factors, leading to ribosome stalling. I couldn't find a reasoning for the procedure.

GMPPCP is a non-hydrolyzable analog of GTP that has been previously used in the isolation of stalled mitoribosomes. Here, we sought to capture mitoribosomes in the presence and absence of tetracyclines. To minimize effects of translation during the isolation procedure, we included GMPPCP to limit the dissociation of GTPases (ex. mtEF-G1 and mtEF-Tu) in any actively translating monosomes. For the reviewer's clarity, GMPPCP was not used during culture, but rather during isolation of the mitoribosomes. This was included both in doxycycline treated and untreated samples, and changes in mitoribosome integrity can be attributed to doxycycline and not GMPPCP.

We have included a sentence in the methods for clarity (page 24, lines 763-765).

4. If I understand correctly, it's suggested that MALSU1 is a dissociating factor, however no data is presented or has been ever published showing such an experiment. MALSU1 was shown in

the association with the mitoribosomal large subunit assembly intermediate, but no subunit dissociating activity has ever been recorded. Maybe better to use a bit more careful description.

We are not claiming that MALSU1 is the dissociating factor – rather tetracycline itself or other unknown factors are responsible for the splitting mechanism. MALSU1 binds to the split mitoribosome subunit, and we illustrate increased association following doxycycline treatment. We have included a more careful description of how MALSU1 affects the translation machinery in response to tetracyclines throughout the manuscript where relevant, and consider other possible scenarios, including the role of mtIF3 in this process (see below).

5. An additional possible scenario is that another factor splits the subunits, for example mtIF3 that binds to the mitoribosomal small subunit. However, it seems that the small subunit hasn't been monitored in the experiments. It's a bit risky to rely on the data from the large subunit only, because it's always in access over monosomes, which might bias the results.

We appreciate the reviewer's suggestion of investigating the dependency of mitoribosome small subunit interacting protein mtIF3, as this protein has been shown to exhibit mitoribosome dissociative activity. In this regard, we have investigated the 1) survival dependency and 2) mitochondrial and mitoribosome accumulation of mtIF3 in response to tetracyclines. We find that ND1 cells deficient in mtIF3 are drastically sensitized under glucose deprivation, and also decrease (to a lesser extent than MALSU1) doxycycline's ability to promote survival (Fig. 2A-B). In this regard, we investigated the doxycycline-dependent regulation of MALSU1 and mtIF3 at the mitochondria and mitoribosome in Expi293 cells at early time points (Fig. 2C-D). Here, as previously illustrated in Fig. 3 (48 hours), we illustrate that MALSU1 is recruited to the mitochondria and mitoribosomes in a doxycycline-dependent fashion. In contrast, mtIF3 mitochondrial localization is unchanged, and mitoribosome interaction was undetectable. We repeated these experiments in mitochondria derived from ND1 cells, where we see a drastic accumulation of MALSU1 at early (1 hr) time points that is maximized from 3-24 hr (Fig. 2E). We also noted an appreciable accumulation of mtIF3, at early time points in these experiments (ND1 cells), suggesting this protein may play a role in potentiating MALSU1-dependent survival signaling at the doxycycline-inhibited mitoribosome in the context of mitochondrial mutations. We interpret that both mtIF3 and MALSU1 are required for doxycycline rescue, but MALSU1 is the primary factor that promotes survival due to its increased magnitude in survival necessity and mitoribosome regulation with tetracyclines.

We have included a new figure (Fig. 2) outlining these results with accompanying section found on page 8 lines 211-266.

6. Figure 1E, unclear what are the shapes associated with MALSU1, looks like labels are missing? The schematic with the membrane is a bit unusual, perhaps it's possible to design a figure that is scientifically more accurate?

We thank the reviewer for pointing out inaccuracies and shortcomings of the current model presented in figure 1E. We have included a revised figure that more accurately reflects the data presented and how it fits with the current literature (Fig. EV4D).

7. Line 97, the correct reference is 10.1038/nsmb.3464

This reference has been updated, and content referenced can be found on page 4 line 98.

Referee #2:

1. The proposed mechanism suggests that exposure to tetracyclines activates the rescue of stalled mitoribsomes via MALSU1, but not via MTRES1 or mtRF-R. While these conclusions are based primarily on genetic interactions between the drug and these genes and the biochemical characterization of MALSU1-mitoribosome binding, it is important to identify the molecular sequences of events. The increased interactions of MALSU1 with the mitoribosome was detected after 48h of exposure to tetracycline. If indeed tetracyclines induce ribosome splitting, as the authors suggest, this should occur immediately or very shortly after drug exposure. If this does not occur in such short time frames, it is likely that the increased MALSU1-mitoribosome interaction more reflects an adaptive response of the cells with impaired mitochondrial translation. Both outcomes would be interesting but could potentially change the proposed mechanism. Hence the experiments from Fig2 B and D-E should be repeated with cells exposed to tetracyline for much shorter times. Moreover, it would be important to monitor the effects of tetracycline on mitochondrial translation in these experiments, either through metabolic labeling or other methods (PMID: 33586863).

Based on the timepoint that doxycycline resolves MAPK and XBP1s signaling in ND1 cybrid cells (Fig. EV2), our initial experiments assessing doxycycline effects on the mitoribosome were performed at 48 hours. We do agree with the reviewer's suggestion to assess the time dependence of MALSU1 recruitment. Here, we performed a time-course doxycycline-treatment experiment in Expi293F cells, followed by mitochondrial and mitoribosome isolations. These experiments revealed that doxycycline-dependent MALSU1 accumulation in mitochondria is apparent at earlier (Fig. 2C-D) time points, with mitoribosome binding initiating as early as 1 hr and increasing to the 12 hr timepoint (Fig. 2D). These results were recapitulated in mitochondria derived from ND1 cells, with MALSU1 recruitment occurring as early as 1 hr, and maximizing from 3-24 hours (Fig. 2E-F). These results suggest the recruitment of MALSU1 in response to doxycycline is an early response to- rather than an adaptive effect of tetracycline treatment. We have included these results in Fig. 2, with accompanying descriptions in the figure legends and results section page 8 lines 211-266.

Regarding monitoring mitochondrial translation, we have previously utilized ^{35}S methionine/cystine incorporation experiments to evaluate tetracyclines ability to inhibit mitochondrial translation. In fact, using chemical synthesis, we previously illustrated that tetracyclines that do not inhibit mitochondrial translation do not promote survival in mitochondrial mutants. Thus, we kindly direct the reviewer to our previous work on mitochondrial translation and its connection to cell survival signaling (Perry *et al.* 2021).

2. While the effect of tetracyline exposure on ER loading and UPRer induction are very interesting, the causality between mitochondrial translation inhibition, these ER changes and the effect on promoting survival of the cells is not firmly established. To this end, the authors should design an experiment where UPRer is artificially induced in their cell model, which should be sufficient to promote survival of these cells independent of tetracycline exposure. Moreover, it would be important to check whether mutants other than MALSU1 ko, which are affecting mitochondrial translation, have similar effects. This would lend further support for a direct role of MALSU1-dependent ribosome recycling in this connectivity between mitochondrial translation and ER proteostasis.

We appreciate the reviewer's suggestion in providing further evidence that the UPR, if activated, can be sufficient to promote survival in mitochondrial mutants under nutrient stress. In fact, we have previously reported that pharmacological activation of PERK is sufficient to promote survival in ND1 mutant cybrid cells. We would like to direct the reviewer to this work as evidence toward UPR activation being protective in the context of nutrient stress and mitochondrial mutations, independent of tetracyclines. We include additional rationale toward the investigation of the UPR in the current work by including this citation (page 3, lines 70-71).

In the second portion of this comment, the reviewer proposes to investigate other factors related to the mitochondrial translation system and their ability to promote survival. We value this feedback and address this comment by depleting ND1 mutant cells of mitochondrial initiation factor mtIF3, along with MALSU1-module protein LOR8F8 (see introduction, page 97 line 99) to investigate the specificity of the MALSU1 dependency. We found that mtIF3 is required for the full rescue of doxycycline survival and is regulated at the mitochondria, albeit to a lesser extent when compared to MALSU1 (Fig. 2). ND1 cells deficient in LOR8F8 are efficiently rescued by tetracyclines, disqualifying this MALSU1-interacting protein as the survival signal (Fig. 2B). This shows that MALSU1 regulation at the mitochondria and mitoribosome, along with its dependency, qualifies it as a likely lead candidate in initiating a survival signal in this context.

These results are presented in a new figure (Fig. 2) and are discussed on page 8 lines 211-266.

Minor points:

1. Actinonin has previously been used to unravel a mitochondrial RNA decay pathway (23453957), however, further experiments suggested that the action of this drug is not on mitochondrial translation or PDF (26504172), but likely inhibits the mitochondrial m-AAA protease to remove faulty proteins. This should be discussed for Fig 1A.

We appreciate the reviewer's insights into additional mechanisms of actinonin. We have included an additional potential interpretation of this drug and its role in attenuating mitochondrial translation as it pertains to the results presented in figure 1A (page 5, lines 155-158). Regardless of the mechanism by which actinonin inhibits mitochondrial translation, it is independent of targeting the mitoribosome directly and is unique to that of tetracyclines (either PDF or m-AAA protease). Figure 1A highlights the necessity of targeting the mitoribosome to promote survival.

2. The authors argue that MALSU1 ko leads to insensitivity to tetracycline, however in their Fig 1D, two of three clones do show a significant increase in cell growth upon exposure to tetracycline. This experiment should be repeated with more clones and a ratio should be presented of cell numbers obtained with and without DOX addition, both for MALSU1 as well as for MTRES1 and mtRF-R ko cells.

The experiments outlined in 1D are not on clones, but rather population-selected cells of CRISPR-KO using different sgRNA targeting different cut-sites on exon 1 of MALSU1. In this regard, we respectfully believe using three independent guides targeting the same gene that recapitulate the same phenotype is sufficient (and standard practice). Small differences on survival between the guides could be in the variable slight differences in complete MALSU1 deletion as shown in the different experiments (Fig. 1D). We also do not believe representing the results as a ratio is the most appropriate when describing survival effects, as any cells that survive under doxycycline-treatment when compared to a completely dead population will be artifactually

amplified and mask a real defect in doxycycline's rescue capacity when compared to controls. We thank the reviewer for their consideration and understanding of our methodology in this regard.

Referee #3:

1. In Fig. 1G, the authors claim that doxycycline increases mitochondrial MALSU1, without impacting total levels of cellular MALSU1 or gene expression. Where is this protein coming from? Is there a population of MALSU1 that is not mitochondrial localized that then gets targeted to mitochondria posttranslationally? There is no evidence of this population in any other cellular fractions from the data presented. It would also help if this increase was quantified across multiple experiments, although the mitochondria increase is supported across multiple blots (e.g., Fig. 2A,C, although I don't understand the wildly different increases between replicates in Fig. 2A, right and the huge increase between +/- doxycycline observed in I, as compared to G).

We appreciate the reviewer's comments and we have addressed the concerns regarding MALSU1 mitochondrial accumulation with additional experiments and rationale. These experiments showed that MALSU1 is specifically (Fig. 3G-H), and dose-dependently (Fig. 3I) enriched at the mitochondria without changes in mitochondrial matrix protein LRPPRC or outer-mitochondrial membrane TOM70 at later time points (48 hr). It has been previously reported that MALSU1 has a short half-life and its expression is lost in rho0 cells, highlighting a tightly regulated and mitochondrial-specific localization. Our new experiments revealed that MALSU1 expression is increased at the whole cell level upon doxycycline-treatment at earlier timepoints (ex. ND1 24 hr and Expi293F 1 and 12 hr, Fig. 2D-F, Fig. EV1G) that may reflect an accumulation of MALSU1 at the mitochondria due to its stabilization at the mitoribosome. Transcriptional regulation of MALSU1 is not substantially altered in doxycycline treated cells at this 24 hr timepoint (Fig. EV1E) as originally observed at the 48 hr timepoint (Fig. EV1D), further supporting an increase in protein stability when compared to controls. MALSU1 expression is apparently unchanged, but not consistently, at the whole cell level when compared to increases in mitochondrial fractions at later time points (48 hr). In these conditions, this may suggest remodeling of the mitochondrial proteome in doxycycline treated cells, that when normalized to mitochondrial protein content reflects higher MALSU1 levels. This is consistent with decreases in mitochondrial markers TOM70 and cytochrome C at the whole cell (Fig. 3G-H). Taken together, we appreciate the reviewer's consideration of increased mitochondrial stability of MALSU1 as a regulatory mechanism upon doxycycline treatment.

We have included the updated figures (Fig. 2D-F, Fig. EV1G) and updated accompanying text for figures 3G-H on pages 8-9 lines 235-266, and page 10 lines 301-314.

2. With respect to MALSU1, could you get the same protection by overexpressing this mitochondrial QC factor? In other words, is this required or sufficient for the doxycycline-induced protection.

We appreciate the reviewers proposed experiment and have addressed this by ectopically overexpressing MALSU1 using a lentiviral vector (Fig. EV1A-C). Here, we show that MALSU1 overexpression is not sufficient to rescue ND1 mutant cells from glucose deprivation (at varying concentrations of glucose and galactose). Further, MALSU1 overexpression does not synergize with doxycycline in promoting survival, indicating that tetracyclines are the limiting pro-survival signal. This experiment suggests that a survival signal must be initiated by targeting the

mitoribosome to inhibit translation that promotes MALSU1 recruitment, and its expression alone is not sufficient. In other-words, MALSU1 is required, but not sufficient to rescue mitochondrial mutants from glucose deprivation.

3. The authors claim that IRE1 oligomerization is reduced in crude mito/ER fractions, but not in whole cell extracts (the latter is not shown). However, in Fig. 2G,H when the authors monitor IRE1 across fractions, the vast majority of IRE1 is in these crude mito/ER fractions. A similar distribution is observed for mito proteins such as MALSU1. How is it possible that what appears to be a small population of IRE1 not included in the mito/ER crude fractions have such a big impact on IRE1 oligomerization in whole cell extracts? This doesn't make any sense.

We thank the reviewer for pointing out this apparent counterintuitive observation. We have modified how we describe our rationale and interpretation of these experiments. When analyzing IRE1α across cellular fractions, we find that it is enriched at the mitochondria/ER. Thus, to further analyze the activation of IRE1α in these samples, we probed for the oligomerization at the ER that is associated with mitochondria. Here, we see a potent decrease in oligomer status using blue-native PAGE (Fig. 4B). We did not see substantial changes in IRE1a oligomerization in whole cell lysates (Fig. EV2A), but we attribute this to a technical aspect of the blue native PAGE in whole cell extracts. To probe for IRE1α activation directly in whole cell extracts, we used a phospho-specific antibody (Fig. 4E). These experiments indicate that IRE1α is activated upon glucose starvation, that is reduced by doxycycline, satisfying the counterintuitive observation raised by the reviewer. The combination of phosphorylation and oligomerization regulates the extent of ER stress IRE1α signaling, where our results indicate a decrease in both with doxycycline treatment. We have also included an additional figure that includes the phosphorylation status of p38 across three independent experiments that shows the doxycycline and MALSU1 dependencies of the response in downstream activation of MAPK (Fig. EV2D).

Revised interpretation of these results has been included on page 11 lines 348-359. We have also included for reference oligomerization state of IRE1α derived from whole cell extracts (Fig. EV2A).

4. Why not show PARP cleavage at 96 h? That appears to be where the biggest differences are observed for viability. The results for PARP cleavage shown in Fig. 3A at 72 h are very minor (although I do see it). This should also be quantified.

At 96 hours glucose deprivation, ND1 cells not treated with doxycycline are late in cell death processes and are not suitable for western blot analysis. We chose 48 and 72 hours as timepoints to capture early cell death processes, in line with time points where doxycycline suppresses ER stress pathway activation (XBP1s).

5. It appears that doxycycline is globally reducing ER stress and subsequent UPR activation in ND1 cells treated with galactose. This should be tested by monitoring activation of PERK and ATF6 signaling as well. This would support an argument that doxycycline and MALSU1 reduce global ER stress. As written, it is suggested that this is selective for IRE1. I understand the authors focus on IRE1 because of their previous manuscript suggesting IRE1 may be most import, but other pathways should also be considered in these experiments.

We agree with the reviewer's interpretation that doxycycline is reducing global ER stress. As suggested, we analyzed the activation of PERK and ATF6 with galactose, along with IRE1, and monitored the ability of doxycycline to suppress this activation. Western blot analysis indicates that galactose strongly activates all three UPR transducers, and doxycycline inhibits the

activation (Fig. 4E). Consistent with our results on XBP1s, ND1 mutation results in an increase in phosphor-IRE1a, BiP, and ATF6 expression levels (and cleavage) when compared to WT cells, where doxycycline cannot reverse this activation in MALSU1 deficient cells. This substantiates the claims that MALSU1 is required for doxycycline's protection of ER stress, and that the resolution of ER stress is not specific, but rather global, on all three branches of the UPR.

We have included an additional figure (Fig. 4E) with accompanying results and discussion pages 13-14 lines 412-441.

6. For Fig. 3E, other markers of UPR activation should be followed apart from IRE1 levels. If the authors are correct, there should be numerous other ER stress-responsive genes/proteins that support their argument. As it is, this is a weak figure attempting to link these findings to a mouse model. More needs to be done to do that in a significant way.

We thank the reviewer in prompting a more substantial investigation into ER stress responses in the context of nutrient stress and tetracyclines rescue capacity. To further expand on UPR activation, we analyzed transcriptional regulation of UPR target genes that are expressed under glucose deprivation (galactose treatment) and suppressed with doxycycline – in line with our hypothesis that doxycycline is reducing global ER stress. Specific activation of genes is linked to respective UPR branches including IRE1α (DNAJB9, SEC24D), ATF6 (HSPA5), and PERK (DDIT3 and PPP1R15A) and we analyzed the expression of these genes by qPCR. Here, we illustrate that galactose stimulates the expression of genes from all three UPR branches, and doxycycline inhibits the expression (Fig. 4F). These results are in line with experiments probing the activation of IRE1α, ATF6, and PERK as described above.

We have included an additional figure (Fig. 4F) and accompanying text on page14 lines 434-441.

7. The authors indicate that ER stress in ND1 cells subjected to nutrient stress results from increased "ER protein loading". This is followed by levels of cathepsin C in the ER, which are suggested increase in ND1 cells incubated in galactose. However, this is not always the case and should be quantified (e.g., there is no increase at time 0 in the samples from Fig. 4D). Regardless, this does not reflect 'protein loading' as described. This could also simply reflect ER stress-dependent accumulation of cathepsin within the ER (i.e., reduced trafficking). This is not likely a 'cause' of ER stress, but instead more likely a consequence. Along these same lines, the reduction in SRP14 would just reflect reductions in ER stress, as this protein is regulated in response to ER stress. Overall, these data are not convincing in describing a molecular mechanism of ER stress increases in ND1 cells incubated in galactose or in the effects of doxycycline in this model (apart from just reducing ER stress).

To kindly clarify for the reviewer, the cathepsin-c blots that are referred to in this comment are from whole cell lysates that are unchanged (Fig. 5H, time=0; ND1 cells). We are not claiming doxycycline promotes a reduction in whole cell levels of cathepsin-c, rather specific reduction at the ER that is represented in numerous blots, including 5H, I, and J. Additionally, the results presented on SRP14 illustrate changes only at the ER (microsome fractions), and not the whole cell (Fig. 5I-J). It is established in the literature that that SRP subunits are transcriptionally regulated by the UPR (Wiseman Mesgarzadeh & Hendershot, 2022). If this were the case in our system, changes would be reflected in the whole cell, where we only observe changes at the ER in a doxycycline and MALSU1 specific manner (Fig. 5I-J). Although we do see regulation of all three branches of the UPR (Fig. 4E-F), SRP seems to be specifically regulated at microsome fractions, consistent with reduced protein loading.

We appreciate the reviewer's comment regarding the potential effects of cathepsin-c as a result of reduced trafficking that is rescued by doxycycline. To test this hypothesis, we utilized brefeldin A (BFA), an inhibitor of ER-Golgi trafficking, to analyze the ability of doxycycline to 1) promote survival and 2) inhibit UPR activation when vesical trafficking is inhibited. We also included an inhibitor of ERAD (eeyarestatin I, ESI) in these experiments to assess whether ER protein extraction and degradation are necessary for doxycycline's protective effects. Here, we illustrate that at concentrations where ND1 cells are sensitive to ESI or BFA treatment (Fig. 5B&E), the UPR is hyperactivated as evidenced by BiP and XBP1s under basal conditions, where doxycycline can still reverse this activation. In line with this, doxycycline can still rescue ND1 cell death from glucose deprivation in the presence of BFA or ESI, indicating that doxycycline is working independent of vesical trafficking (secretion) or ERAD processes.

Additional figures (Fig. 5D-G) and accompanying text can be found on pages 14-15 lines 461-470.

Combining these results with our knowledge that doxycycline is suppressing the global activation of UPR transducers, we hypothesized that doxycycline is either regulating 1) global translation or 2) ER co-translational protein loading processes. To examine global translation, we utilized puromycin incorporation experiments, where puromycinylated peptides were analyzed with a puromycin-specific antibody (Fig. EV4A). Here, we illustrate that glucose deprivation reduces global translation rates that are not further affected by doxycycline, providing evidence that doxycycline does not reduce global translation rates to protect cells from ER stress. In this regard and in line with our results on cathepsin-c in the ER, it is likely that doxycycline is reducing ER stress by inhibiting ER protein loading through a currently unknown mechanism.

Additional figure (Fig. EV4A) and accompanying text can be found on page 16 lines 501-506.

8. Similar to the above, MALSU1 depletion basally reduces cathepsin-C levels within the ER to levels nearly identical to that observed in doxycycline treated ND1 cells treated with doxycycline (Fig. 1F), while true that doxycycline does not further reduce cathepsin-C in these cells, this is not consistent with a model wherein accumulation of cathepsin C is a mechanism involved in the observed cellular toxicity.

We appreciate the reviewer's comment, and to address this we have included additional interpretation (page 15, lines 485-593). In this experiment, we observe that along with cathepsin-c, all other probed ER makers (with the exception of SRP14) are reduced in sgMALSU1 microsomes when compared to controls (Fig. 5J). This may be a result of a maladaptive response in these cells that results in a reduced chaperone (ex. BiP, calnexin), and UPR signaling (ex. IRE1α) under nutrient stress. Alternatively, this may reflect reduced total ER in these cells. Nonetheless, the apparent reduced cathepsin-c in sgMALSU1 cells is proportionately similar to the non-target control cells when normalized to other ER proteins (Fig. 5J). Interestingly, SRP14 expression is the same across microsomes derived from NTC and sgMALSU1 cells, which would indicate a potentially higher association of the SRP in MALSU1 deficient cells, when again normalized to the reduced levels of other ER proteins. As the reviewer also appreciated, doxycycline does not reduce cathepsin-C in the ER of MALSU1 depleted cells, which substantiates this mitoribosome quality control protein in mediating associated ER stress responses that is associated with enhanced sensitivity to glucose deprivation.

We have included additional explanation and interpretation of this experiment that can be found on page 15, lines 485-493.

Dear Pere,

Thank you for your patience while your revised manuscript was re-reviewed. Unfortunately, neither referee 3 nor referee 1 were available to re-review your revised study for us, so I asked referee 2 whether s/he can please also assess your response to the other referees' concerns. Referee 2 has only one more minor comment that needs to be addressed, see below, and I am happy to say that we can in principle accept your manuscript now.

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Esther Schnapp, PhD
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Referee #2:

The authors have performed new experiments and provided satisfactory answers to the initial questions. These revisions have further improved the manuscript, which will be very interesting for the community working on mitochondrial stress signaling.

I had a look at the point-by-point response again and think that the authors did a good job in replying to reviewer#3´s concerns as well. They added a few more controls , changed the text accordingly and expanded on the characterization of UPR induction (or here its repression). I would be confident to accept the manuscript in light of how the authors responded to reviewer#3 as well.

I had now time to look carefully at the rebuttal letter and the revised manuscript and I think that the authors did a good job in answering the questions of reviewer #1 as well. They did new experiments and adjusted the text accordingly. Having said this, I agree with this reviewer´s general comment that the manuscript lacks a clear mechanism, specifically the claim that MALSU could be a splitting factor that is recruited to the ribosomes upon tetracycline exposure is not well-aligning with the literature. Here, MALSU is described as a mitoribosome assembly factor. But nevertheless, the current study reports a very interesting

connection of mitochondrial translation inhibition, activation of UPR and cell survival.

The authors might want to adjust the labeling of figure EV4, where the order of IMM, OMM and Matrix is incorrect.

All editorial and formatting issues were resolved by the authors.

Prof. Pere Puigserver
Dana Farber Cancer Institute; Harvard Medical School
Cancer Biology; Cell Biology
360 Longwood Ave
Boston, Massachusetts 02215
United States

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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	NA
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	NA
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	NA

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	NA
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	NA
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	NA
If publicly available data were reused, provide the respective **data citations in the reference list**.	**Yes**	References, Materials and Methods, Figures