Peroxisomal support of mitochondrial respiratory efficiency promotes ER stress survival

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers. A key set of issues you must address is 1) test further and discuss your results in the context of other potential cellular defects caused by the loss of peroxisomal function, and 2) quantify their results.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

As peroxisomes are indispensable for mitochondria respiration in yeast, the molecular basis of how these organelles form functional alliance remains unclear. In this manuscript, the authors addressed the role of peroxisomes in mitochondrial respiratory function during ER stress in yeast via an inter-disciplinary approach, including genetics, imaging, oxygen consumption measurement, proteomics, native gel assay, etc. While the ample amount data are present, they did not provide in depth mechanistic insights connecting peroxisomes to mitochondria under ER stress induced by an artificial stressor.
Thus, I do not recommend to publish this manuscript in the Journal of Cell Science. Please see the following for comments.

Comments for the author

Major concerns:
1. The current manuscript seems to be divided into two halves, 1) the role of peroxisomes in mitigate ROS during ER stress and 2) mitochondria response to ROS. Both parts seem solid but I found it difficult to connect these two.
For me the missing link is how peroxisomes are connected to mitochondria. Is it via contact sites? Or via transferring lipids/metabolites? Or other pathways? Addressing any of these questions will provide some mechanisms that compliment well with the current genetic and functional data.
2. All imaging data lack quantification and statistics. This has to be included to strengthen their conclusions. For example, the DHE signal seemed to primarily localized to mitochondria (Figure S4); yet, in the text stated " visualize a low intensity fluorescent staining". Quantification can eliminate this concern.
3. This work is mainly based on the ER stress condition induced by tunicamycin, which is a standard yet artificial. It would be essential to repeat some key experiments in a more physiological-relevant conditions.

Minor point:
1. Please indicate a category for peroxisomal proteins in Table 1 as the authors emphasized in the text.
2. In Figure 1B (Pex11 fluorescence), the induction of peroxisome in rtg KO cells was clear and significant though is partially inhibited as compared to WT cells. And the partial inhibition only occurred in tun, but not DTT condition. However, the authors stated " The efficiency of ...in response to tunicamycin or DRR was partially inhibited....(Fig. 1B). It seems an overstatement to me.
3. There are two panel B in Figure 1.

Reviewer 2

Advance summary and potential significance to field

This manuscript by Hijazi et al. explored the response of peroxisomes and mitochondria during ER stress.
Using tunicamycin to induce ER stress in S. cerevisiae, the authors examined mitochondria metabolic and redox homeostasis state in various strains deficient in peroxisomes or peroxisome function. The authors assert that the role of peroxisomes in the mitochondrial ER stress response is 2-fold. 1) First, the peroxisome b-oxidation is upregulated in response to ER stress to provide fuel for mitochondrial ATP production. The authors report that peroxisome abundance increases in response to ER stress, and genes involved in peroxisome b-oxidation and lipid transport expression were upregulated after 5 hours of tunicamycin treatment. Further, they show that an increase in the mitochondrial ETC complex proteins and enhanced maximal oxygen consumption were

dependent on peroxisomes and peroxisomal b-oxidation. 2) Second, the authors show that while ROS production occurs with ER stress induction (as evidenced by a robust increase in sod1Δ and cta1Δ cells), wt cells can mitigate this increase. The authors show that this ROS mitigation is dependent on a pex34. ER stress is also shown to induce ETC supercomplex formation, to increase the efficiency of electron transfer and reduce ROS production, and show that supercomplex formation is also dependent on pex34.

In addition, they also determine the importance of tsa1 and cta1 in mitigating ER stress-induced ROS production.

Comments for the author

The idea that peroxisomes contribute to mitochondrial function during cellular conditions when energy demands and mitochondrial metabolism are increased, in this case, ER stress, is interesting and novel. This manuscript shows that the loss of peroxisomes or peroxisomal proteins leads to a number of mitochondrial phenotypes following ER stress induction. However, this collection of phenotypes does not lead to a clear mechanism by which peroxisomes contribute to the mitochondrial response to ER stress. Specifically, how do peroxisomes contribute to mitochondrial ROS mitigation through pex34 and supercomplex formation? The authors vaguely allude to this through pex34's role in peroxisome-mitochondria contact, but pex34 facilitated contact has only been shown to be involved in the transport of acetyl-CoA supporting the need for lipid metabolism to fuel the cells. However, it is unclear how the authors propose pex34 is acting to mitigate mitochondrial ROS and influence supercomplex formation. Lipid metabolism increases mitochondrial ROS and does not relieve it. The authors also show that induction of mitochondria ETC complex expression with ER stress depends on peroxisomes, but the mechanism for how peroxisomes, and specifically peroxisomal beta-oxidation, are influencing mitochondrial ETC protein expression is not clear.
There are a number of other possible explanations for the observed phenotype given that there are several other factors that can contribute to mitochondria dysfunction in the absence of peroxisomes. One is the accumulation of lipids in cells in the absence of peroxisomes has been postulated to induce ER and mitochondria stress. Also, peroxisomes have been postulated to be necessary to maintain cellular redox homeostasis during metabolic stress; thus, its loss can result in the redox buffering capacity of the cell. More recently, the accumulation of peroxisomal proteins in peroxisome deficient cells have been shown to induce mitochondria stress. Thus, any or combination of these factors may explain the mitochondria phenotype described in this manuscript. Although the authors do not have to explore all of these factors, they should consider them in the analysis and discussion of their data.

Other concerns.
1) Fig 1: Figures are mislabelled. There are 2 B's
2) Fig 1B(#2): This western blot needs to be quantified. The important measurement here is the change in the protein levels with tunicamycin and not just intensity in +tunicamycin conditions. In this case, it would be helpful to quantify the blot in terms of fold change. Comparing the change or increase in the ETC proteins provide alternative interpretations to the results. More specifically, it seems that a comparable increase is seen in WT/pex34/pox1 for Por1, as well as WT/pex34 for Cox2. The only perceivable phenotype I see is the loss of Cox2 increase with tunicamycin in pox1 cells and not in the pex34. Also is it possible that there is less Cox2 in the pex34 and pox1 cells at the untreated basal state, which would suggest a difference in the mitochondria status of these cells? The pex3delcells should also be assessed as a control for the role of peroxisomes in general.
3) Supp Fig 1: This is not an appropriate assay to do by flow cytometry for PTS1-GFP expressing cells. As PTS1-GFP expression is under an artificial promoter, its expression shouldn't be responsive to endogenous stimuli, thus quantifying GFP intensity should not change with ER stress and is not indicative of peroxisome number. This is evidenced by comparable/ slightly higher baseline signal in pex3Δ cells compared to wt. pex3Δ should not have any peroxisomal structures. Instead, the authors should quantify peroxisomes puncta per cell.
4) Supp Fig 2a: Has peroxisome swelling with impaired export of beta-oxidation products been shown before?
If yes, this should be cited. If not, this phenotype should not be used as evidence for pex34 cells having impaired acetyl-CoA transport to mitochondria. Also, it is not acknowledged that the

peroxisome swelling in pex34del cells seems to be reduced in +tun conditions. If the swelling is indeed due to lipid accumulation shouldn't the same phenotype be seen in the pex34 del +tun?
5) Supp Fig 3: Need to include statistics for CCCP treated pex3Δ between + and – tunicamycin. By eye, it looks like maybe a significant difference. If it is different, how does this fit the authors' model?
6) Fig 2: The rho0 control is not sufficient to determine if the mitochondrial ETC is the major source of ERS-
induced ROS. While the DHE staining does not change with ERS in rho0 cells there is also no change in WT cells, so not seeing an increase in rho0 cells doesn't tell us anything when there was no increase, to begin with. To determine if ROS is mitochondrially produced, the authors should treat sod1Δ and cta1Δ cells with antimycin A, or make a dual sod1Δ rho0, cta1Δ rho0 cells. If ROS is mito produced, we would see no increase however, we would see a comparable increase if ROS is produced elsewhere. It is also possible that the ROS could be peroxisome produced or ER produced. This is especially possible given that ROS increase was highest in cells that did not have an increased oxygen consumption (ie, pex34 tsa1, cta1).
7) Some of the images in Fig 2 have different background levels suggesting a difference in the acquisition setting. Flow cytometry or even fluorometry should be used to quantify the changes in DHE between the different cells and conditions.
8) Fig 3C: The authors state that TMRM fluorescence did not increase in tsa1Δ cells after ERS, but this is not supported by the figure where there is a very obvious increase in TMRM after tun in tsa1 cells.
9) Fig 3D: The TRX2-lacZ activity assay should also be performed in pex3Δ and pex34Δ cells

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First revision

Author response to reviewers' comments

Detailed responses to the reviewers are as follows:

Reviewer 1
Our data suggest that mitochondria and peroxisomes both assist to promote ER stress adaptation and cell survival. Mitochondria increase respiration which helps ameliorate ROS accumulation in response to ERS. We propose that peroxisomes are necessary to supply fuel to drive the TCA cycle and the ETC. In our revision, we provide *new* results to support our model. First, we show that overexpression of Mpc1, the pyruvate carrier that increases mitochondrial substrate for acetyl CoA formation, can suppress ERS-induced ROS accumulation and impaired respiratory response in *pex34Δ* and *pox1Δ* cells (Fig. 3). We also include in this figure a diagram to make more clear the relationships between the three organelles. Second, we show *new* results that the protonophore CCCP, by producing maximal OCR, can reduce ROS accumulation in *pex34Δ* cells, suggesting that the ROS accumulated during ER stress in these cells is mitochondrial in origin (Suppl. Fig. 5). We hope these new results better tie together how the mitochondria and peroxisomes assist during ER stress as the three organelles are physically and metabolically connected.

As requested by the reviewer, we have now quantitated the images in Fig. 2 showing ROS accumulation in wild-type, *pex34Δ* and *tsa1Δ* cells during ER stress. Using Image J software on TIFF images, the results are now presented in Fig. 2C, confirming the differential ROS accumulation in wild- type, *pex34Δ* and *tsa1Δ* in representative fluorescence images.

The most physiologically relevant inducer of ER stress in yeast is expression of the misfolded protein CPY*. In a previous paper, we have shown that the Complex IV subunit Cox2 is increased upon CPY*, suggesting that respiratory remodeling is induced by ER stress. In the current manuscript, we used CPY* as an inducer of ER stress in Fig. 5, showing that supercomplex formation is increased upon constitutive expression of CPY*. In addition to tunicamycin, we also used DTT to induce *TRX2- lacZ* (Fig. 4D) and peroxisomes (Fig. 1B).

As requested by the reviewer, we have now included a peroxisome category in Table 1.

As requested by the reviewer, we have rewritten the conclusion for Fig. 1B to describe better the result that peroxisome induction by DTT is not significantly impaired in *rtg1Δ* cells, but tunicamycin response is somewhat diminished. These results suggest the RTG pathway may at least partially play a role in ERS-induced peroxisome proliferation.

Reviewer 2

We thank the reviewer for noting that our model is novel and interesting.

To account for impaired ER stress response in *pex* mutants and *pox1* cells defective in ß oxidation, the reviewer proposes the mutant cells might have mitochondrial or ER dysfunction. To address the reviewer's hypothesis, we now include assays to examine ERS-induced UPR and OSR in *pex* mutants and *pox1Δ* cells (*new* Suppl. Fig. 2). The results show that UPR and OSR in response to ERS in the mutants are not significantly different from that of wild-type cells. We therefore note that impaired UPR and OSR are unlikely to account for impaired respiratory response to ERS in *pex34Δ* and *pox1Δ* cells.

As described above, to strengthen our model that lipid metabolism via ß oxidation provides fuel to drive the TCA cycle and oxidative phosphorylation during ERS-induced respiration, we now provide a *new* experiment shown in Fig. 3. (Increased fuel is necessary to increase electron transport which can ameliorate ROS production upon increased efficiency of electron transfer and reduced electron leak from the ETC). *pox1Δ* and *pex34Δ* cells cannot respond to ERS with increased respiration, but this impaired response is rescued by overexpression of Mpc1, the mitochondrial pyruvate carrier. Rescue of *pex34* and *pox1* cells by Mpc1 overexpression suggests that an alternative source of acetyl CoA can support ERS-induced respiratory response. These events are illustrated in a new diagram.

As requested by the reviewer, we quantitated the Western blot showing low levels of the respiratory complex protein Cox2 in *pox1Δ* and *pex34Δ* cells. (We removed the results from *acb1Δ* cells because the mutant is not analyzed further in the paper). A poor ERS response in *pex34Δ* and pox1Δ cells, as assayed by Cox2 levels, is consistent with results from OCR assay, DHE assay, adaptation assay and supercomplex formation. The simplest hypothesis is that ß oxidation and peroxisomal tethering play an important role in ER stress adaptation.

The reviewer noted that it is not appropriate to use flow cytometry PTS1-GFP to quantify peroxisome numbers. Because these data were in a supplemental figure (formerly Suppl. Fig. 1) included to support imaging in Fig. 1, we have now removed the figure.

We agree with the reviewer's comment that enlarged peroxisomes do not necessarily reflect a defect in peroxisome-mitochondrial transport in *pex34Δ* cells. However, Pex34 has been suggested independently by Shah et al. to function in transferring ß oxidation intermediates to mitochondria. We have therefore removed the results showing abnormal peroxisome morphology in *pex34Δ* and *pex3Δ* cells.

A supplemental figure (now Suppl. Fig. 1A), shows that tunicamycin treatment increased the maximal O_2 consumption rate of wild-type cells, but insignificantly affected that of *pex34Δ* and *pox1Δ* cells.
Because there were insufficient trials with *pex3Δ* cells to obtain statistics, we have removed the *pex3Δ* results from the figure.

Thanks to the reviewer for suggesting a better control to show that ROS accumulation in mutant cells during ER stress is ETC-derived. We now present a *new* experiment in which CCCP was added to *pex34Δ* and *pox1Δ* cells during tunicamycin treatment, and the cells were then stained with DHE. The results are shown in a new Suppl. Fig. 5. Addition of CCCP, a protonophore that dissipates the MMP and increases OCR to maximal, reduced ERS-induced ROS accumulation in *pex34Δ* and *pox1Δ* cells.

The results strongly suggest that ERS-induced ROS in *pex34Δ* and *pox1Δ* cells is derived from the ETC.

As stated above, the fluorescent images showing ROS staining in Fig. 2 have now been quantitated to confirm the differences shown in the representative images.

In Fig. 4C the description of TMRM fluorescence in *tsa1Δ* cells has been re-written to reflect that although TMRM staining increases slightly after ER stress, mitochondrial membrane potential is still diminished by comparison with that in ERS-stimulated wild-type cells.

New TRX2-lacZ results in *pex3* and *pex34* mutants are now shown in Suppl. Fig. 2B. ER stress-induced OSR in *pex3Δ* cells appears similar to that in WT. *pex34Δ* and *pox1Δ* cells are competent to induce OSR upon ER stress, although the response is somewhat diminished by comparison with wild-type cells. These data suggest that impaired OSR cannot account for impaired respiratory response to ERS in *pex* mutants and *pox1Δ* cells.

Formatting issues: Fig. 1 subsections are now properly labeled. Table 1 is now in table format. The abstract has been shortened to < 180 words. The materials and methods section is now at the end of the discussion section. The manuscript now contains 5 main figures and 1 table with 6 supplemental figures and 1 supplemental Excel file.

Thanks very much to you and the reviewers for your help. We think the manuscript is much improved and we hope it is now suitable for publication.

Second decision letter

MS ID#: JOCES/2021/259254

MS TITLE: Peroxisomal support of mitochondrial respiratory efficiency promotes ER stress survival

AUTHORS: Imaddedin Hijazi, Emily Wang, Michelle Orozco, Sarah Pelton, and Amy Chang
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.