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Mfn2 modulates the UPR and mitochondrial function via repression of PERK

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editors: Isabel Arnold, Anke Sparmann

1st Editorial Decision 24 October 2012

Thank you for submitting your research manuscript (EMBOJ-2012-83337) to our editorial office. It has now been seen by three referees and their comments are provided below.

As you can see below, all referees find the concept of the study interesting, but raise significant experimental concerns. These issues would have to be addressed by a considerable amount of additional work. However, since this appears feasible based on the constructive suggestions made by the reviewers, we would be willing to grant the opportunity to significantly extend and revise the current manuscript. As this will entail time-consuming experimentation, we would understand if you decide to seek potential rapid publication elsewhere.

However, in case you do embark on revisions for our journal, please take the specified demands into careful consideration to avoid disappointments later in the process. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the all raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1

Starting from previous findings that correlate Mfn2 ablation to ER stress, authors aim at demonstrating that Mfn2-deficient cells are less prone to ER stress induced apoptosis and encounter instead paraptosis. They suggest a direct interaction between PERK and Mfn2, that would be responsible for mitochondrial morphology and function defects in Mfn2 loss of function cells. This could represent an outstanding report from that ones on the field of mitochondrial dynamics regulation and connection between ER function and cell death. However, some major issues need to be addressed by the authors.

Major comments:

1. Authors describe Mfn2-/- ER morphology defects during ER stress as "ER vacuolization". In the images they show it appears that distribution of ER is compromised as a consequence of cytosol vacuolization (as shown by pEGFP images in fig2H) and not of ER. Supplementary figure 1 shows expansion, not vacuolization of ER. This must be addressed

2. As mentioned in "minor comments" several experiments on cell death analysis are not clearly interpretable. We suggest to add flow cytometric analysis of cells labeled with Propidium Iodide and Annexin V to show percentage of dying cells. This could also reinforce the hyphotesis of a form of paraptosis (see point 5), since it was shown that paraptotic cells are characterized by PS externalization(Wang Y. et al., 2003). By Annexin V staining upon addition of caspase inhibitor Z-VAD-FMK, authors should be able to discriminate between apoptotic and paraptotic cells. The same analysis should be performed with addition of cycloheximide.

3. Authors show that Mfn2-/- MEFs are resistant to ER stress induced apoptosis. Is this resistance restricted to conditions of ER stress or is manifested in a more general picture of cell death inducing stimuli? This important control must be added.

4. During ER stress Mfn2 ablation results in impaired autophagy. This finding does not fit well with the overall main claims of the manuscript, since the proposed mediator of Mfn2 loss of function phenotype PERK, seems not to play a role in the dysregulated autophagy associated to Mfn2 ablation (Figure 5C). In addition, Mfn2 ablated cells seem to display impaired autophagy also in basal condition (Lc3b II is detected also at time 0 in Fig3A and B, and transcripts' levels of Beclin-1 and Lc3b are decreased also in absence of Tg in fig 3D, when xbp1 shorter transcript is not present (fig4D)), suggesting that the defects retrieved in ER stress conditions are a consequence of an autophagic process that is impaired independently of activation of UPR branches. Moreover several experiments are not convincing on this part, as explained in "minor comments". In conclusion, data on autophagy authors show can generate confusion in the reader and distract him/her from the real message;

5. The authors suggest that under ER stress Mfn2 deficient cells undergo paraptosis. However, this is not adequately supported by biochemical analyses of this mode of cell death. Please expand this part.

6. Mfn2-/- cells show dysregulated PERK, IRE-1 and ATF6 UPR branches in response to ER stress. However, caspase and autophagy are ameliorated only by silencing of PERK or Xbp1, while ATF6 loss of function aggravates the phenotype. This suggests that the three different ER stress pathways play different and specific roles in Mfn2 ablated cells. PERK downregulation can surprisingly rescue mitochondrial defects of Mfn2-/- cells, implying that Mfn2 does not play a key role in mitochondrial fusion, or that maybe Perk controls some inhibitor of Mfn2-mediated fusion. To further substantiate this finding, one wonders if silencing of Xbp1 or ATF6 or simply chemical attenuation of ER stress rescues defects of Mfn2 ablation like PERK.

7. Along the same line,
Authors suggest that Mfn2 is an upstream regulator of PERK, and that in this way PERK regulate mitochondrial morphology. Are mitochondrial function and morphology compromised in wt cells overexpressing PERK?

8. Mfn2 is known as a mitochondrial pro -fusion protein. How can silencing of PERK induce mitochondrial elongation in absence of Mfn2 (are levels of expression of other mitochondria shaping proteins such as Opa1, Drp1, Fis1 or Mfn1 changed)? Mitochondrial shape changes have to be quantified.

9. Related to the last point, in order to strengthen the claim of a specific and functional interaction between Mfn2 and PERK, authors should address if downregulation of PERK in Mfn1 knock out cells alters morphology and function of mitochondria.

Minor comments
There are a number of missing control experiments. In more detail

1. In Fig 1A, resolution is not good enough to visualize autophagosomes.
2. In Fig2 A it seems that also uncleaved caspase3 is downregulated in Mfn2-/- cells under ER stress condition. The ratio of cleaved/uncleaved caspase3 seems not to be different between wt and ko in tg 12 and 24h. From this WB we can not conclude apoptotic pathway is not activated in Mfn2 ko cells under ER stress conditions. Moreover the measurement of the DEVDase activity (fig 2B) is suitable to artifacts since it is based on a fluorimetric assay. As said above, a flow cytometric analysis of Propidium Iodide and Annexin V stained cells should be added.
3. Fig2 B lacks loading control
4. In Fig2D also in Mfn1-/- cells cleavage of caspase 3 is lower than in wt cells upon treatment with TG. Please add a WB for Mfn1-/- treated with Tm and Bref to confirm that caspase 3 is not less cleaved.
5. In the WB of fig 2F it looks like in Mfn2 kd cells caspase 3 is more cleaved than in the wt controls (see 16h lane). In addition, tubulin is saturated. Please comment and change the loading control with an appropriate one.
6. Supplementary fig2C: PI stains also necrotic cells; please do the same experiments with cicloheximide to show you are staining paraptotic cells.
7. Figure 3A: LC3b signal is saturated. Please change blot. Moreover it looks like the amount of LC3b II over the total amount of Lc3 (I+II) is not changed upon ER stress in wt and mfn2-/- cells. A densitometric analysis could be helpful here
8. The same has to be performed for experiment with bafilomycin on Fig 3B, where the loading control has to be changed since again it looks that tubulin signal is saturated.
9. From experiment in figure 3C authors cannot conclude that autophagosomes-lysosomes fusion is impaired, since they do not show marker for lysosomes. Absence of GFP signal can be a consequence of the decrease in the number of lysosomes available for the formation for autophagolysosomes. In any case, quantification of data is required.
10. Fig4B: tubulin is saturated. Authors should change loading control or use a less exposed WB. The same has to be done for WB in suppl.figure4B.
11. Fig4: PERK phosphorylation is shown until 6 hours of treatment with TG; elF2alpha phosphorylation increases until 9 hours; levels of expression of Chop and GADD34 are shown also at 24 hours after treatment. Authors should show what happens to PERK phosphorylation and Xbp1 splicing at 9, 12 and 24 hours of Tg treatment.
12. Fig 5C: authors should change loading control or exposure time: tubulin immunodetection is saturated.
13. Plot of figure 5B and E lack measurement of DEVDase activity in wt cells. In figure 2 wt cells have a 20 fold increase of DEVDase activity. The level of the DEVDase activity in Mfn2-/- treated with tg and Xbp1 siRNA reaches about 3.5, which is still far from the level of wild type cells. Authors should add Mfn2 KO + Mfn2 as a control in this experiment. In addition authors should also add immunodetection of caspase3 cleavage for both knockdown experiments performed;
14. Figure 5C and F lack immunodetection of LC3b for wt cells or Mfn2 -/- + Mfn2;
15. Figure 6A lacks the immunodetection of not-phosphorylated PERK
16. Figure 6B: same of figure 5C: authors should change loading control or exposure time: tubulin WB is saturated.

17. Figure 7D and H: OCR for wt is about 550pmol/min, while for Mfn2 KO is about 200 in fig7D; in 7H scr Mfn2 KO cells show an increase of two times of OCR (from 200 to 400 pmol/min); authors should add Mfn2 KO + Mfn2 as a control in this experiment. The same has to be performed for figure 7G. In addition, authors should add oligomycin as a control of coupling of mitochondrial electron chain and cyanide to completely block respiration.

Referee #2

This manuscript describes a role for MFN2 in modulation of the ER stress response in a cell. The manuscript is presented in a fragmented manner, jumping from a description of vacuolization and defective apoptosis and autophagy, transitioning to changes in UPR signaling and ending with analysis of mitochondrial function. Within this presentation, data are not internally consistent. In addition, data often directly contradict a recent study reporting that MFN2 is upregulated in response to ER stress and that loss of MFN2 leads to an amplified ER stress and an increase in ER-stress induced apoptosis. The authors do not offer an explanation for these potential discrepancies. The most novel and interesting component of the story is the direct interaction of PERK with MFN2 and the changes in mitochondrial function in Mfn2-/- cells with PERK silencing. however, these observations are underdeveloped.

Comments:

1. The arrows indicating autophagosomes in the thin section, negative stain EM images in Figure 1A do not show characteristics typical of autophagosomes. In addition, the fluorescent images of ER morphology in MEFs are not consistent with previously published data including deBrito 2008 and Ngoh 2012 - there should be some ER tubules visible at the edges of the cell. This could be a problem with the resolution of the images in the file.

2. In contrast to previously published observations (Ngoh 2012), this manuscript describes a decrease in caspase activation and a decrease in cell death, as measured by PI staining following induction of ER stress. The western blot analysis should be quantified and normalized to loading controls to strengthen the argument. Authors must comment on possible explanations for the direct contradiction to published data.

3. Autophagy is examined by western blot analysis of LCB3 cleavage and confocal imaging of GFP-mCherry-LC3. The gel in 3A is overloaded and all western analysis must be quantified.

Authors conclude from these data that both autophagosomal and autophagolysosomal formation are impaired in MFN2 knock out cells. However, from the thin section negative stain EM data, authors conclude that autophagosomes accumulate in MFN2 knock out cells. If formation is impaired, why would they accumulate?

4. Consistent with published data, authors report a sustained activation of PERK, which phosphorylates eIF2, leading to lower levels of cellular translation. In contrast to published data, this report shows increased expression of the eIF2 dephosphorylase, GADD34, and a subsequent decrease in eIF2 phosphorylation. However, they also observe an increased expression of the eIF2 target, CHOP. If eIF2 is dephosphorylated by GADD34, how is CHOP activated? If CHOP is activated, why isn't there increased caspase activation? If ATF6 is promoting cell survival, this must be tested.

Also in contrast to previous reports (Ngoh 2012), Figure 4 suggests that all branches of the ER stress pathways are induced. Please address the discrepancy.

5. Authors reduce PERK expression in MFN2 null cells and examine mitochondrial activity and report that cells lacking both PERK and MFN2 have less severe defects in ROS production, respiration and calcium overload. Is this due to a relief in translational repression? Does silencing of PERK alone alter mitochondrial function?

Minor concerns:
1. There are no sizes indicated for any scale bars. The fluorescent images in Figure 1C would benefit from being displayed in greyscale to improve contrast. Also, the insets should be more clearly separated from the original image and should contain scale bars. Finally, the original images could be slightly larger so that the cell fills the panel.

2. Quantification/normalization of western analysis would greatly strengthen the conclusions.

3. For the PERK silencing experiments, wild type should be included in parallel with scramble RNA controls.

Referee #3

The manuscript by Juan Pablo Muñoz et al entitled "Mfn2 operates upstream of PERK to modulate the UPR and mitochondrial function" described novel findings indicating that Mfn2-deficient cells experiment an impaired response to drugs that induces ER stress. Due to the known role of Mfn2 in the interaction between ER and mitochondrial membrane, this study may have important implications. However, in some experiments the author miss important controls. Data should be confirmed by reconstituting in Mf2 KO cells and by using RNAi. Some Western blot fail to show the phenotype that the authors describe in the text. Due to these problems, quantifications is necessary to strength the conclusions of the study. There are not functional experiments in vivo or in a physiologic condition of ER stress to assess the importance of Mfn2 over the PERK pathway. There is only one inmunoprecipitation assay using a PERK-myc ectopic expression but endogenous immunoprecipitation are absent. This is also a correlative study, no mutagenesis is provided to conclude causality. In summary, I believe the story has great potential but has to be improved.

Major points:

Figure 1:
Is necessary to compare WT and KO mfn2 cells. The images show difference in the distribution of ER-RFP, but not an expansion of the ER. This is a problem of the interpretation. To show the effects of Tg, Tm or BrefA in ER expansion, could be made with Brefeldine A_bodipy and FACS analysis as previously reported. The author should consider to do similar experiments with a mfn2 knockdown strategy in WT cells and also reconstitution of mfn2 in KO mfn2 cells. This is very important to discard putative clonal effect between both WT and KO mfn2 cells.

In figure 1C, is necessary to improve the quality of the images to see the dramatic ER vacuolization in mfn2 KO cells, but not in Mfn1. Thus, would be important to see the effect of knocking down of mfn1.

Figure 2:
Nogh G.A et al. (JBC 2012) show before that knock down of Mfn2 increases cell death in MEFs cells exposed to ER stress. However, the authors show that ER stress triggers less cell death in Mfn2 KO cells compared to WT cells. Experiment previously mentioned are then important. Other apoptosis assys should be performed (i.e. Caspase 3 stating).

Figure 3:
A reduced induction of LC3-II in Mfn2 KO cells does not mean that the autophagy is deficient in these cells, because the autophagy flux could be higher than the controls. For this reason it is necessary to perform classical flux assays. The author did flux assay. However bafilomycin addition do not induce LC3-II accumulation. Evaluating if the absent of Mfn2 and not Mfn1 triggers impaired autophagy at basal levels or under ER stress condition could be shown with statistical analysis.

Figure 4
To state that deficient of Mfn2 triggers an enhanced UPR, the best ways to study this is by analyzing basic signaling markers of the pathway: the ER stress sensor activity, the transcriptional factor

regulated by this ER stress sensor and some key UPR gene target. From this perspective, the authors assayed PERK phosphorylation, ATF4 and CHOP induction. However, for IRE1-alpha signaling or XBP-1s induction, the analysis is poor and the XBP1 splicing assay is difficult to interpret. In figure 4D, the smaller band amplified in this assay do not correspond to xbp1 small transcript. This band is smaller because the absent of 26 nucleotides is translated into an smaller amplicon. So, is necessary that the author explain in a better way this assay to avoid misinterpretation. Quantifications of these analyses and the effect of the absent of Mfn2 in the expression of UPR target genes induced by ER stress is necessary to confirm that Mfn2 regulates PERK, IRE1alpha and ATF6 pathways.

Figure 5:
The authors miss to knock down PERK, XBP-1 and ATF6 in WT cells. These controls are important. Another possibility is to evaluate the effect of Mfn1 in PERK, ATF4, IRE1, XBP1 or ATF6 KO cells.

Figure 6: Mfn2 KO cells show and impaired eif2alpha phosphorylation. However, in samples of mice, western blot of eif2alpha phosphorylated show that this is increased. What is the possible explanation?
In 6D, the authors show that ectopic PERK co-inmunoprecipitated with Mfn2. But the authors did not assay the possible interaction with IRE1alpha or ATF6. Endogenous inmunoprecipitation from cells or from tissues should be assayed.

Figure 7: The effect of Knocking down of PERK in Mfn2 KO cells should be compared with WT cells. Surprisingly, PERK KD cells show reduced ROS levels. That is opposite to the data of several papers showing that the PERK-ATF4 branch has an antioxidant role. One possible explanation is that PERK KD was done only in Mfn2 KO cells. An important control here will PERK WT cells.

Minor points (but important):
Molecular weights of Western blots or PCRs are not shown in any figures.

The text is vague and many times "unspecific", especially when referring to mechanisms that have been proposed before. Is necessary to be more rigorous.
Examples:
Line 16: "IRE1alpha activates the xbp-1 transcription factor", must say "IRE1alpha activates expression of xbp-1 transcription factor".
Line 4: "three", must say "at least three"
Line 6: "UPR (unfolding protein response)", must say "UPR (Unfolded Protein Response)".

1st Revision - authors' response 22 February 2013

Referee #1.

Starting from previous findings that correlate Mfn2 ablation to ER stress, authors aim at demonstrating that Mfn2-deficient cells are less prone to ER stress induced apoptosis and encounter instead paraptosis. They suggest a direct interaction between PERK and Mfn2, that would be responsible for mitochondrial morphology and function defects in Mfn2 loss of function cells.
This could represent an outstanding report from that ones on the field of mitochondrial dynamics regulation and connection between ER function and cell death. However, some major issues need to be addressed by the authors.

Major comments:
1. Authors describe Mfn2-/- ER morphology defects during ER stress as "ER vacuolization". In the images they show it appears that distribution of ER is compromised as a consequence of cytosol vacuolization (as shown by pEGFP images in fig2H) and not of ER. Supplementary figure 1 shows expansion, not vacuolization of ER. This must be addressed

Response: We have additionally performed studies using Brefeldin-bodipy FACS analysis in cells upon ER stress induced by thapsigargin. Secretory pathway expansion was quantified by fluorescence-activated cell sorting (FACS) in living cells stained with a red fluorescent version of

brefeldin A as reported (Hetz et al, 2006). ER-Golgi content was similar in WT and Mfn2 KO cells under unstressed conditions. However, increased brefeldin A–BODIPY (boron dipyrromethene difluoride) staining was observed in Mfn2 KO cells undergoing ER stress induced by thapsigargin (Figure 1D). These data together with TEM observations of ER morphology in cells expressing SEC61beta-GFP or or ER-RFP strongly support the view that Mfn2 ablation induces ER expansion.

2. As mentioned in "minor comments" several experiments on cell death analysis are not clearly interpretable. We suggest to add flow cytometric analysis of cells labeled with Propidium Iodide and Annexin V to show percentage of dying cells. This could also reinforce the hypothesis of a form of paraptosis (see point 5), since it was shown that paraptotic cells are characterized by PS externalization(Wang Y. et al., 2003). By Annexin V staining upon addition of caspase inhibitor Z-VAD-FMK, authors should be able to discriminate between apoptotic and paraptotic cells. The same analysis should be performed with addition of cycloheximide.

Response: We have performed flow cytometry analysis of cells labeled with propidium iodide/annexin V. Data indicate that Mfn2 KO cells externalize phosphatidylserine in response to ER stress approximately the same percentage as wild type cells. The z-VAD inhibitor markedly reduced PS externalization in wild type cells but not in Mfn2 KO. Furthermore, cycloheximide decreased propidium iodide uptake in Mfn2 KO cells but not in wild-type cells. In all, these data together with the lack of necrosis induction (Figure 2F) suggest the activation of paraptosis-like cell death in Mfn2-ablated cells after an ER insult. Data are shown in in Figure 2G and Supplementary Figure 3F.

3. Authors show that Mfn2-/- MEFs are resistant to ER stress induced apoptosis. Is this resistance restricted to conditions of ER stress or is manifested in a more general picture of cell death inducing stimuli? This important control must be added

Response: In order to answer the relevant question posed by the reviewer, we have performed studies in which we have induced apoptosis with staurosporine or with the combination TNF-alpha/cycloheximide. Data indicate that apoptosis induced by those agents is normally activated in Mfn2 KO cells. In consequence, we document that Mfn2 KO cells are less sensitive to apoptosis triggered by ER stress. Data are shown in Supplementary Figure 3D.

4. During ER stress Mfn2 ablation results in impaired autophagy. This finding does not fit well with the overall main claims of the manuscript, since the proposed mediator of Mfn2 loss of function phenotype PERK, seems not to play a role in the dysregulated autophagy associated to Mfn2 ablation (Figure 5C). In addition, Mfn2 ablated cells seem to display impaired autophagy also in basal condition (Lc3b II is detected also at time 0 in Fig3A and B, and transcripts' levels of Beclin-1 and Lc3b are decreased also in absence of Tg in fig 3D, when xbp1 shorter transcript is not present (fig4D)), suggesting that the defects retrieved in ER stress conditions are a consequence of an autophagic process that is impaired independently of activation of UPR branches. Moreover several experiments are not convincing on this part, as explained in "minor comments". In conclusion, data on autophagy authors show can generate confusion in the reader and distract him/her from the real message;

Response: We agree with the reviewer that the impact of Mfn2 ablation on autophagy is not the major message of our manuscript, and that basal autophagy may also be dysregulated. However, because of the comments included by the two other reviewers we have performed additional studies, and we have finally decided to maintain this section in the manuscript. Nevertheless, we are open to eliminate it from the manuscript if the editor and reviewers consider it appropriate.
We now provide a quantitative analysis of the estimation of autophagy in Mfn2 KO cells under basal conditions and upon ER stress, we provide better autoradiograms, and we have performed statistics. Data indicate that Mfn2 ablation inhibits basal and ER stress-induced autophagy (shown as Figure 3 and Supplementary Figure 5). This is detected when measuring LC3-II under steady state conditions or under lysosomal blockage induced by Bafilomycin A1.
Mfn2 deficiency causes impaired basal autophagy in MEF cells and in cardiac myocytes (Hailey et al, 2010; Zhao et al, 2012). Our data indicate that Mfn2 ablation causes a deficient ER stress-induced autophagy. Mfn2-ablated cells showed a number of autophagic defects upon ER stress, namely: a) deficient expression of autophagic genes such as beclin-1 or LC3b, b) reduced autophagosomal proliferation, and c) reduced lysosomal abundance, and d) decreased

autophagolysosomal formation. A defective response to ER stress was observed in MEFs as well as in 3T3-L1 pre-adipocytes and in C2C12 myoblasts, which supports the view that these effects are independent of the cell context. We also found that the enhanced IRE-1alpha/XBP-1s activity upon ER stress was responsible for the reduced ER stress-induced autophagy of Mfn2 deficient cells, and silencing of XBP-1 rescued a normal LC3-II formation in Mfn2-ablated cells subjected to ER stress. These effects were not detectable in wild type cells, suggesting specificity of the Mfn2 deficient state, and the effects were not detected under basal conditions of Mfn2 KO cells, suggesting that the role of XBP-1 was limited to ER stress-induced autophagy and not to basal autophagy. These data also support the view that different mechanisms repress basal and ER stress-induced autophagy in Mfn2 KO cells. Based on these ideas, we have modified the discussion section in page 16, last paragraph.

5. The authors suggest that under ER stress Mfn2 deficient cells undergo paraptosis. However, this is not adequately supported by biochemical analyses of this mode of cell death. Please expand this part

Response: We have analyzed the expression of ALIX, a protein that is repressed in paraptosis (Sperandio et al., 2004). Data indicate that wild-type cells undergo a modest induction of Alix protein levels in response to Thapsigargin whereas Mfn2 KO cells do not show any induction and the protein levels are clearly lower than in wild type cells. These data are now shown as Figure 2I. These data together with the observation that Mfn2 KO cells show (i) PS externalization that is not inhibited by z-VAD, (ii) decrease in annexin V+/PI+ cells by cycloheximide, (iii) inhibition of ER expansion with cycloheximide, and (iv) decreased necrosis compared to wild type cells clearly indicate that Mfn2 KO cells undergo a paraptosis-like cell death in response to ER stress.

6. Mfn2-/- cells show dysregulated PERK, IRE-1 and ATF6 UPR branches in response to ER stress. However, caspase and autophagy are ameliorated only by silencing of PERK or Xbp1, while ATF6 loss of function aggravates the phenotype. This suggests that the three different ER stress pathways play different and specific roles in Mfn2 ablated cells. PERK downregulation can surprisingly rescue mitochondrial defects of Mfn2-/- cells, implying that Mfn2 does not play a key role in mitochondrial fusion, or that maybe Perk controls some inhibitor of Mfn2-mediated fusion. To further substantiate this finding, one wonders if silencing of Xbp1 or ATF6 or simply chemical attenuation of ER stress rescues defects of Mfn2 ablation like PERK.

Response: We have analyzed the impact of silencing of XBP-1 or ATF6 on mitochondrial morphology in wild type or in Mfn2 KO cells. Similarly, we incubated Mfn2 KO cells in the presence of chemical chaperones TUDCA and 4-PBA. None of these alterations rescue the effects of Mfn2 deficiency and suggest that is specifically due to downregulation of PERK activity. This is now shown as Figure 7E, and Supplementary Figure 8A.

7. Along the same line, Authors suggest that Mfn2 is an upstream regulator of PERK, and that in this way PERK regulate mitochondrial morphology. Are mitochondrial function and morphology compromised in wt cells overexpressing PERK?

Response: We have overexpressed PERK in the context of wild-type cells. This causes mitochondrial fragmentation and reduced mitochondrial respiration (shown as Figure 8E and 8F).

8. Mfn2 is known as a mitochondrial pro-fusion protein. How can silencing of PERK induce mitochondrial elongation in absence of Mfn2 (are levels of expression of other mitochondria shaping proteins such as Opa1, Drp1, Fis1 or Mfn1 changed)? Mitochondrial shape changes have to be quantified.

Response: We have analyzed the expression of proteins that participate in mitochondrial fusion or fission in cells upon PERK silencing. Data indicate that the effects of PERK silencing on mitochondrial morphology are independent of changes in the expression of mitochondrial fusion or fission proteins (shown as Suppl. Figure 8B). In addition, we have quantified the changes in mitochondrial morphology which are now shown as Figure 7B.

9. Related to the last point, in order to strengthen the claim of a specific and functional interaction between Mfn2 and PERK, authors should address if downregulation of PERK in Mfn1 knock out cells alters morphology and function of mitochondria.

Response: Indeed, the knockdown of PERK in wild type or in Mfn1 KO cells does not rescue mitochondrial morphology or ROS production, although it enhances mitochondrial respiration. This is now shown as Figure 7D, and 7G as well as in Figure 8D.

Minor comments
There are a number of missing control experiments. In more detail

1. In Fig 1A, resolution is not good enough to visualize autophagosomes.

Response: The reviewer is right and we have decided to concentrate into ER morphology. We now include better images in Figure 1A and 1B.

2. In Fig2 A it seems that also uncleaved caspase3 is downregulated in Mfn2-/- cells under ER stress condition. The ratio of cleaved/uncleaved caspase3 seems not to be different between wt and ko in tg 12 and 24h. From this WB we can not conclude apoptotic pathway is not activated in Mfn2 ko cells under ER stress conditions. Moreover the measurement of the DEVDase activity (fig 2B) is suitable to artifacts since it is based on a fluorimetric assay. As said above, a flow cytometric analysis of Propidium Iodide and Annexin V stained cells should be added.

Response: In Mfn2 KO cells, uncleaved caspase 3 shows lower abundance compared to wild type cells. We do not think that this explains the reduced apoptosis in Mfn2 KO cells in response to ER stress because the induction of apoptosis by other agents such as TNFalpha+ cycloheximide or staurosporine is higher in Mfn2 KO cells than in wild type cells.
We now provide quantification of cleaved caspase 3 in Figure 2 and Suppl. Figure 3. In addition, we have performed flow cytometric analyses of Propidium Iodide and Annexin V stained cells. Data indicate that Mfn2 KO cells externalize phosphatidylserine in response to ER stress approximately the same percentage as wild type cells. The z-VAD inhibitor blocked PS externalization in wild type cells but not in Mfn2 KO. Furthermore, cycloheximide decreased propidium iodide uptake in Mfn2 KO cells but not in wild-type cells. In all, these data together with the lack of necrosis induction (Figure 2F) suggest the activation of paraptosis-like cell death in Mfn2-ablated cells after an ER insult. Data are shown in in Figure 2G and Supplementary Figure 3F.

3. Fig2 B lacks loading control

Response: Loading control included (now Figure 2B)

4. In Fig2D also in Mfn1-/- cells cleavage of caspase 3 is lower than in wt cells upon treatment with TG. Please add a WB for Mfn1-/- treated with Tm and Bref to confirm that caspase 3 is not less cleaved.

Response: We have performed studies in which Mfn1 KO cells were treated with Tm and Bref and we have assayed caspase 3 cleavage. Data indicate that Mfn1 KO cells show an intermediate activation of apoptosis as measured by caspase 3 cleavage or caspase activity between wild type cells (maximal activation) and Mfn2 KO cells (minimal activation). These data are now shown as Suppl. Figure 3B and 3C). The effect of Mfn1 ablation on apoptosis intermediate between wild type and Mfn2 KO cells may be explained by the interaction between Mfn1 and Mfn2 at the mitochondrial-ER contact sites.

5. In the WB of fig 2F it looks like in Mfn2 kd cells caspase 3 is more cleaved than in the wt controls (see 16h lane). In addition, tubulin is saturated. Please comment and change the loading control with an appropriate one.

Response: We have repeated these studies in C2C12 and in 3T3-L1 cells and data are shown in Figure 2E and in Suppl. Figure 2E. Data indicate that Mfn2 deficiency in 3T3-L1 and in C2C12 reduces the activation of caspase 3 and thus in apoptosis in response to thapsigargin. The reviewer is

right in the sense that there is less uncleaved caspase-3 in Mfn2 KO or KD cells in basal condition, which might be due to the lower expression or higher turnover. There is also less uncleaved caspase-3 after Tg treatment, which can also be due to the higher degradation, however, we have not performed any experiments to confirm this point.

6. Supplementary fig2C: PI stains also necrotic cells; please do the same experiments with cicloheximide to show you are staining paraptotic cells.

Response: We have performed flow cytometry studies with cells stained with Annexin V and Propidium Iodide upon treatment with thapsigargin. Data indicate phosphatidylserine externalization both in wild type and in Mfn2 KO cells but Mfn2 KO cells were insensitive to z-VAD (Figure 2F). On the other hand, Mfn2 KO cells, but not wt, were sensitive to CHX, which decreased the percentage of annexin V+/PI+ cells upon Tg treatment (Suppl. Figure 3F).

7. Figure 3A: LC3b signal is saturated. Please change blot. Moreover it looks like the amount of LC3b II over the total amount of Lc3 (I+II) is not changed upon ER stress in wt and mfn2-/- cells. A densitometric analysis could be helpful here

Response: Done. We have quantitated the data as the abundance of LC3b relative to tubulin. We think that this is the best way to present the data because Mfn2 KO cells seems to have a defective expression of total LC3. Thus, under conditions in which LC3-II is enhanced, LC3-I becomes limiting and therefore, the expression as ratio LC3-II/LC3-I or LC3b-II/LC3-I+II is inappropriate (Klionsky et al., 2012).) Our data indicate that Mfn2 deficient cells do not fully activate autophagy under ER stress conditions compared to wild type cells (Figure 3A-3D, Suppl. Figure 5A, and 5B).

8. The same has to be performed for experiment with bafilomycin on Fig 3B, where the loading control has to be changed since again it looks that tubulin signal is saturated.

Response: Autoradiogram has been changed and data have been quantitated (now shown as Figure 3B).

9. From experiment in figure 3C authors cannot conclude that autophagosomes-lysosomes fusion is impaired, since they do not show marker for lysosomes. Absence of GFP signal can be a consequence of the decrease in the number of lysosomes available for the formation for autophagolysosomes. In any case, quantification of data is required.

Response: We agree with the reviewer. We have quantified the immunofluorescence analysis of puncta in control and Mfn2 KO cells under basal or ER stress conditions. In addition, we have analyzed the abundance of lysosomes by immunodetecting LAMP1, and by Lysotracker staining. Data indicate that Mfn2 KO cells show less Lysotracker staining, and reduced LAMP1 expression already under basal conditions, and reduced number of acidic autophagolysosomes upon ER stress (Figure 3C, 3D, 3E and 3F).

10. Fig4B: tubulin is saturated. Authors should change loading control or use a less exposed WB. The same has to be done for WB in suppl.figure4B.

Response: Done. We have also quantified data from UPR branches now shown in figure 4A.

11. Fig4: PERK phosphorylation is shown until 6 hours of treatment with TG; elF2alpha phosphorylation increases until 9 hours; levels of expression of Chop and GADD34 are shown also at 24 hours after treatment. Authors should show what happens to PERK phosphorylation and Xbp1 splicing at 9, 12 and 24 hours of Tg treatment.

Response: Studies at different times ranging from 0 to 24 h are shown for all markers. This is now shown as Figure 4A.

12. Fig 5C: authors should change loading control or exposure time: tubulin immunodetection is saturated.

Response: Done. This is now shown as Figure 5D.

13. Plot of figure 5B and E lack measurement of DEVDase activity in wt cells. In figure 2 wt cells have a 20 fold increase of DEVDase activity. The level of the DEVDase activity in Mfn2-/- treated with tg and Xbp1 siRNA reaches about 3.5, which is still far from the level of wild type cells. Authors should add Mfn2 KO + Mfn2 as a control in this experiment. In addition authors should also add immunodetection of caspase3 cleavage for both knockdown experiments performed;

Response: Data on wild type cells are now included in Figure 5B and 5E. In addition, we also include data on the effects of reconstitution with Mfn2 in Mfn2 KO MEF cells on apoptosis (Suppl. Figure 3B).

14. Figure 5C and F lack immunodetection of LC3b for wt cells or Mfn2 -/- + Mfn2;

Response: Data on wild type cells are now included in Figure 5D and 5G. In addition, we also include data on the effects of reconstitution with Mfn2 in MEF cells (Suppl. Figure 5A).

15. Figure 6A lacks the immunodetection of not-phosphorylated PERK

Response: Done

16. Figure 6B: same of figure 5C: authors should change loading control or exposure time: tubulin WB is saturated.

Response: Done

17. Figure 7D and H: OCR for wt is about 550pmol/min, while for Mfn2 KO is about 200 in fig7D; in 7H scr Mfn2 KO cells show an increase of two times of OCR (from 200 to 400 pmol/min); authors should add Mfn2 KO + Mfn2 as a control in this experiment. The same has to be performed for figure 7G. In addition, authors should add oligomycin as a control of coupling of mitochondrial electron chain and cyanide to completely block respiration.

Response: The reviewer is right regarding the variability of the oxygen consumption data that was due to the fact that the expression was different in the different figures (in some studies total respiration was shown and in some other panels only mitochondrial respiration was graphed). We have modified the figures so only mitochondrial respiration has been represented, i.e., after substraction of oxygen consumption present after addition of the inhibitors rotenone and antimycin A. In some of the series, we now show routine respiration, oligomycin-resistant respiration, and total uncoupled respiration (Figure 8B, 8C, 8D). In addition, studies on the effect of Mfn2 reconstitution in Mfn2 KO cells are now shown (Figure 7F and 7G).

Referee #2.

This manuscript describes a role for MFN2 in modulation of the ER stress response in a cell. The manuscript is presented in a fragmented manner, jumping from a description of vacuolization and defective apoptosis and autophagy, transitioning to changes in UPR signaling and ending with analysis of mitochondrial function. Within this presentation, data are not internally consistent. In addition, data often directly contradict a recent study reporting that MFN2 is upregulated in response to ER stress and that loss of MFN2 leads to an amplified ER stress and an increase in ER-stress induced apoptosis. The authors do not offer an explanation for these potential discrepancies. The most novel and interesting component of the story is the direct interaction of PERK with MFN2 and the changes in mitochondrial function in Mfn2-/- cells with PERK silencing. however, these observations are underdeveloped.

Response: In the revised version of the manuscript we have resolved inconsistencies present in the first version. We decided to maintain the order in the presentation of the data as it was in which we carefully analyze the impact of ER stress in Mfn2 deficient cells on: a) ER morphology and documentation of ER expansion, b) classical biological processes induced by ER stress: apoptosis and autophagy, c) the UPR response, and d) impact of manipulation of UPR branches on the prior process and on mitochondrial morphology and function. In the revised version we also provide

evidence that the effects detected in mfn2 KO cells are rescued by Mfn2 re-expression. We explain the potential discrepancies between our data and prior data in the Discussion section, and we have developed further the interaction between Mfn2 and PERK.

Comments:

1. The arrows indicating autophagosomes in the thin section, negative stain EM images in Figure 1A do not show characteristics typical of autophagosomes. In addition, the fluorescent images of ER morphology in MEFs are not consistent with previously published data including deBrito 2008 and Ngoh 2012 - there should be some ER tubules visible at the edges of the cell. This could be a problem with the resolution of the images in the file.

Response: We agree with the reviewer and we have decided to concentrate into ER morphology. We now include better images of ER tubules in Figure 1A and 1B.
As to the ER morphology, our studies show a fragmentation of ER in Mfn2 KO cells when a luminal ER marker (ER-RFP) was used (Supplementary Figure 1A), which is in agreement with prior reports (de Brito & Scorrano, 2008). We have also used Sec61b as a marker of ER membrane, which does not permit to detect the same pattern of changes in response to Mfn2 deficiency, and indeed, Sec61b shows the existence of ER tubules in Mfn2 KO cells (Figure 1C). These data are consistent with the findings by Friedman et al., (2011) in Mfn2-depleted COS-7 cells. These data are better explained in the first section of Results.

2. In contrast to previously published observations (Ngoh 2012), this manuscript describes a decrease in caspase activation and a decrease in cell death, as measured by PI staining following induction of ER stress. The western blot analysis should be quantified and normalized to loading controls to strengthen the argument. Authors must comment on possible explanations for the direct contradiction to published data

Response: We have quantified all studies shown in Figure 2 and Suppl. Figure 3. We have also performed studies in which we have analyzed apoptosis by staining cells with Annexin V and Propidium Iodide and performing flux cytometric assays, and we have studied apoptosis in Mfn2 deficient C2C12 and 3T3-L1. Indeed, our data indicate that apoptosis is reduced in Mfn2 KO cells upon ER stress. Our data are at odds with data from Ngoh et al. (Ngoh et al, 2012) indicating that ER stress exacerbated ER stress-induced apoptosis in Mfn2 deficient MEF cells.
As to the study by Ngoh et al. (2012), they used concentrations of thapsigargin 10-fold lower than in our study, and as a result they only found a 2-fold activation of caspase (in the range of 10-fold in our study). It is likely that the conditions used by Ngoh et al. did not completely engage apoptosis in wild type cells as the UPR response was sufficient to deal with and resolve a mild ER stress. However, when a more severe ER stress is induced, cells are induced to undergo a full apoptotic response. We provide some explanations in the Discussion section regarding why our data are different from those of other authors (page 17, second paragraph).

3. Autophagy is examined by western blot analysis of LCB3 cleavage and confocal imaging of GFP-mCherry-LC3. The gel in 3A is overloaded and all western analysis must be quantified.

Response: We now provide better western blots and we provide quantification of all studies in Figure 3 and in Suppl. Figure 5.

Authors conclude from these data that both autophagosomal and autophagolysosomal formation are impaired in MFN2 knock out cells. However, from the thin section negative stain EM data, authors conclude that autophagosomes accumulate in MFN2 knock out cells. If formation is impaired, why would they accumulate?

Response: We agree with the reviewer that our interpretation was not right. In fact, we can not say, based on EM observations, that autophagosomes accumulate in Mfn2 KO cells in response to ER stress. Additionally, our data indicate that the abundance of lysosomes is reduced, and that formation of autophagosomes and of autophagolysosomes is impaired (Figure 3). Overall, autophagy is markedly impaired in Mfn2-deficient cells.

4. Consistent with published data, authors report a sustained activation of PERK, which

phosphorylates eIF2, leading to lower levels of cellular translation. In contrast to published data, this report shows increased expression of the eIF2 dephosphorylase, GADD34, and a subsequent decrease in eIF2 phosphorylation. However, they also observe an increased expression of the eIF2 target, CHOP. If eIF2 is dephosphorylated by GADD34, how is CHOP activated? If CHOP is activated, why isn't there increased caspase activation? If ATF6 is promoting cell survival, this must be tested.

Response: We document that in Mfn2 deficient cells or tissues, a chronic ER stress develops which leads to a sustained activation of PERK and enhanced phosphorylation of eIF2alpha (Figure 6). A recent report has demonstrated that chronic phosphorylation of eIF2alpha promotes resistance to ROS-mediated cell stress (Zeng et al. 2011), which may be useful as an ER stress preconditioning mechanism through the selective expression of survival genes. Under acute ER stress, Mfn2 KO cells undergo less autophagy and a paraptosis-like cell death is promoted.
Mfn2 deficient cells undergo a reduction in the phosphorylation of eIF2alpha (compared to wild type cells) in response to acute ER stress due to an enhanced induction of CHOP, and GADD34b, which may turn into an enhanced protein synthesis, and this may be relevant in the ER expansion (Figure 1). In addition, this enhanced protein synthesis may be key in the activation of paraptosis, which is inhibited by cycloheximide (Figure 2).
The explanation of why CHOP is induced in response to ER stress in Mfn2 KO cells under conditions in which GADD34 is over-induced and eIF2alpha is inactivated remains to be demonstrated. However, it has been reported that other UPR branches (ATF6 and XBP-1) also induce CHOP during ER stress (reviewed in Oyadomari and Mori, 2004). Based on that, we propose that CHOP is induced during ER stress by Mfn2 KO cells through another UPR branch.
CHOP has been classically associated to enhanced apoptosis, an effect mediated by Bim (Tabas and Ron, 2011). Nevertheless, CHOP and ATF4 have also been associated to cell death protection in cells (Halterman et al., 2010; Rouschop et al., 2010; Sun et al., 2013). Our results show an enhanced expression of CHOP under conditions in which apoptosis is reduced (Figures 2 and 4). Based on this, we propose that Mfn2, through its activity in the mitochondrial-ER contact sites, is relevant for the coordination of the processes between ER and mitochondria that lead to apoptosis during ER stress. In this regard, it has been reported that the loss of proteins involved in the formation of the mitochondrial-ER contact sites causes a reduced apoptotic response upon ER stress (Chami et al, 2008; Giorgi et al, 2010; Simmen et al, 2005).
Our data suggest that the enhanced activation of ATF6 in Mfn2 deficient cells that occurs during ER stress is oriented to promote cell survival. Thus, Mfn2 KO cells silenced for ATF6 show a drastic increase in PI permeability under ER stress (Suppl. Figure 6D).

Also in contrast to previous reports (Ngoh 2012), Figure 4 suggests that all branches of the ER stress pathways are induced. Please address the discrepancy.

Response: There are two relevant studies regarding the issue mentioned by the reviewer: Ngoh et al (2012) and Zhao et al. (2012).
Ngoh et al. (2012) analyzed the impact of ER stress on the PERK pathway in MEF cells transduced with adenoviruses 24 or 48 h before exposure to ER stress. In this study, the authors did not analyzed the ATF6 or the IRE-1 branches but the PERK pathway (phosphorylation of eIF2alpha, and the expression of GADD34 and p58IPK). They found that at short times (0.5 h) upon ER stress the phosphorylation of eIF2alpha or the expression of GADD34 or p58IPK were similarly induced in control and Mfn2 deficient cells. However, at longer times (18 h) of ER stress, the induction was reduced in Mfn2 deficient cells (Ngoh et al., 2012. No intermediate times were studied.
Zhao et al. (2012) studied ER stress signals in Mfn2 KO mouse cardiac cells. They found an increased phosphorylation of eIF2alpha and increased expression of UPR target genes in Mfn2 KO cells. Our data are coherent with data from Zhao et al. (2012) and not from Ngoh et al. (2012). At this point, it is difficult to understand the reasons that explain the discrepancies between Ngoh et al. and Zhao or our own data. However, a potential interference of inflammatory factors secondary to the adenoviral transduction may not be discarded. Explanation of these results is now included in the Discussion section (page 15 , line 14).

5. Authors reduce PERK expression in MFN2 null cells and examine mitochondrial activity and report that cells lacking both PERK and MFN2 have less severe defects in ROS production, respiration and calcium overload. Is this due to a relief in translational repression? Does silencing of PERK alone alter mitochondrial function?

Response: We have performed studies in which we repressed PERK in wild type cells and in Mfn1 KO cells and we have monitored mitochondrial morphology and function. Our data indicate that PERK silencing in wild type cells or in Mfn1 KO cells does not modify mitochondrial morphology (Figure 7C, and D) and it does not reduce ROS levels (Figure 7F, and G). However, we now document that PERK silencing generates a general stimulatory effect on mitochondrial respiration (wild type, Mfn1 or Mfn2 KO cells). Similarly, overexpression of Mfn2 reduces respiration and causes mitochondrial fragmentation (Figure 8). Thus, PERK has a key impact on mitochondrial function and on mitochondrial morphology.

Minor concerns:
1. There are no sizes indicated for any scale bars. The fluorescent images in Figure 1C would benefit from being displayed in greyscale to improve contrast. Also, the insets should be more clearly separated from the original image and should contain scale bars. Finally, the original images could be slightly larger so that the cell fills the panel.

Response: Figure 1 has been extensively modified. Size of scale bars are included. Figure 1C has been changed and cells are larger.

2. Quantification/normalization of western analysis would greatly strengthen the conclusions.

Response: Western blot analyses have been quantified throughout the different figures of the manuscript.

3. For the PERK silencing experiments, wild type should be included in parallel with scramble RNA controls.

Response: In the revised version of this manuscript we include PERK silencing studies performed in wild type cells. This is now shown in Figures 5, 7, and 8.

Referee #3.

The manuscript by Juan Pablo Muñoz et al entitled "Mfn2 operates upstream of PERK to modulate the UPR and mitochondrial function" described novel findings indicating that Mfn2-deficient cells experiment an impaired response to drugs that induces ER stress. Due to the known role of Mfn2 in the interaction between ER and mitochondrial membrane, this study may have important implications. However, in some experiments the author miss important controls. Data should be confirmed by reconstituting in Mf2 KO cells and by using RNAi. Some Western blot fail to show the phenotype that the authors describe in the text. Due to these problems, quantifications is necessary to strength the conclusions of the study. There are not functional experiments in vivo or in a physiologic condition of ER stress to assess the importance of Mfn2 over the PERK pathway. There is only one inmunoprecipitation assay using a PERK-myc ectopic expression but endogenous immunoprecipitation are absent. This is also
a correlative study, no mutagenesis is provided to conclude causality. In summary, I believe the story has great potential but has to be improved.

Response: In the revised version we have performed the changes suggested by the reviewer: a) quantification of the data, b) addition of relevant controls (reconstitution with Mfn2), c) coimmunoprecipitation of endogenous PERK and Mfn2, and d) we have added extensive additional evidence on the implications of PERK on mitochondrial morphology and function.

Major points:

Figure 1:
Is necessary to compare WT and KO mfn2 cells. The images show difference in the distribution of ER-RFP, but not an expansion of the ER. This is a problem of the interpretation. To show the effects of Tg, Tm or BrefA in ER expansion, could be made with Brefeldine A_bodipy and FACS analysis as previously reported. The author should consider to do similar experiments with a mfn2

knockdown strategy in WT cells and also reconstitution of mfn2 in KO mfn2 cells. This is very important to discard putative clonal effect between both WT and KO mfn2 cells.

Response: We have performed studies using Brefeldin-bodipy FACS analysis in cells upon ER stress induced by thapsigargin. Secretory pathway expansion was quantified by fluorescence-activated cell sorting (FACS) in living cells stained with a red fluorescent version of brefeldin A as reported (Hetz et al, 2006). ER-Golgi content was similar in WT and Mfn2 KO cells under unstressed conditions. However, increased brefeldin A–BODIPY (boron dipyrromethene difluoride) staining was observed in Mfn2 KO cells undergoing ER stress induced by thapsigargin (Figure 1D). These data together with observations of ER morphology in cells expressing SEC61beta-GFP or ER-RFP strongly support the view that Mfn2 ablation induces ER expansion .
In addition, we have generated cells that are deficient in Mfn2 or Mfn1 by lentiviral-mediated expression of shRNAs, and we have analyzed ER vacuolization upon induction of ER stress with thapsigargin. Data indicate that Mfn2 deficiency causes ER expansion upon ER stress as detected by Sec61b which is not seen in Mfn1 deficient cells (Suppl. Figure 2). In consequence, we conclude that Mfn2 depletion causes ER expansion in response to ER stress and that this is not a clonal effect of Mfn2 KO MEF cells.
We have also performed reconstitution assays by re-expressing Mfn2 in Mfn2 deficient cells. Data are now shown as Supplementary Figure 1B-D.

In figure 1C, is necessary to improve the quality of the images to see the dramatic ER vacuolization in mfn2 KO cells, but not in Mfn1. Thus, would be important to see the effect of knocking down of mfn1.

Response: New images are now shown in Figure 1C to better visualize the differences between Mfn2 KO and Mfn1 KO cells. In addition, we have knocked down Mfn1 in cells and we have analyzed ER vacuolization upon ER stress (these data are shown as Supplementary Figure 2).

Figure 2:
Nogh G.A et al. (JBC 2012) show before that knock down of Mfn2 increases cell death in MEFs cells exposed to ER stress. However, the authors show that ER stress triggers less cell death in Mfn2 KO cells compared to WT cells. Experiment previously mentioned are then important. Other apoptosis assys should be performed (i.e. Caspase 3 stating).

Response: We have performed flow cytometry analysis of cells labeled with propidium iodide/annexin V. Data indicate that Mfn2 KO cells externalize phosphatidylserine in response to ER stress approximately the same percentage as wild type cells. The z-VAD inhibitor blocked PS externalization in wild type cells but not in Mfn2 KO cells. Furthermore, cycloheximide decreased propidium iodide uptake in Mfn2 KO cells but not in wild-type cells. In all, these data together with the lack of necrosis induction (Figure 2F) suggest the activation of paraptosis-like cell death in Mfn2-ablated cells after an ER insult. Data are shown in in Figure 2G and Supplementary Figure 3F.
In addition, we have performed studies in which we have triggered apoptosis with other agents such as TNF-alpha+cycloheximide or staurosporine. Data indicate that apoptosis triggered by those factors is smewaht higher in Mfn2 KO cells compared to wild type cells (Suppl. Figure 3).
We have also conducted studies in Mfn2-knockdown 3T3-L1 or C2C12 cells and data indicate a reduced ER stress-induced apoptosis in Mfn2 deficient cells (Figure 2 and Suppl. Figure 3). In all, our data indicate that ER stress-induced apoptosis (but not apoptosis in general) is reduced upon Mfn2 deficiency.
We should also mention that Mfn2 deficient cells show an enhanced rate of apoptosis under basal conditions (Supplementary Figure 3A), and that cell death upon ER stress is similar in wild-type or in Mfn2 ablated conditions, since Mfn2 KO cells undergo a paraptotic-like cell death.
In connection with data by Ngoh et al (2012), they used concentrations of thapsigargin which were 10-fold lower to the concentrations used in our study, and as a result they activated caspase activity 2-fold (in the range of 10-fold in our study). Our interpretation is that the conditions used by Ngoh et al. did not completely engage apoptosis in wild type cells as the UPR response is sufficient to deal with and resolve a mild ER stress. However, when a more severe ER stress is induced (our study), cells are induced to undergo a full apoptotic response. We think this may explain the discrepancy between our data and those published by Ngoh et al.

Figure 3:
A reduced induction of LC3-II in Mfn2 KO cells does not mean that the autophagy is deficient in these cells, because the autophagy flux could be higher than the controls. For this reason it is necessary to perform classical flux assays. The author did flux assay. However bafilomycin addition do not induce LC3-II accumulation. Evaluating if the absent of Mfn2 and not Mfn1 triggers impaired autophagy at basal levels or under ER stress condition could be shown with statistical analysis.

Response: We have now quantified the data of Figure 3 and Suppl. Figure 5 dealing on the impact of Mfn2 deficiency on ER stress-induced autophagy. Data indicate that Mfn2-deficient cells show a generalized reduction in autophagy upon ER stress which is characterized by reduced autophagosomal formation, lower abundance of acidic autophagolysosomal compartment, reduced lysosomal abundance and reduced expression of autophagy genes. In addition, we document that Mfn1 deficiency does not alter thapsigargin-induced autophagy, and enhances lysosomal abundance (Suppl. Figure 5A, 5B, and 5C).

Figure 4
To state that deficient of Mfn2 triggers an enhanced UPR, the best ways to study this is by analyzing basic signaling markers of the pathway: the ER stress sensor activity, the transcriptional factor regulated by this ER stress sensor and some key UPR gene target. From this perspective, the authors assayed PERK phosphorylation, ATF4 and CHOP induction. However, for IRE1-alpha signaling or XBP-1s induction, the analysis is poor and the XBP1 splicing assay is difficult to interpret. In figure 4D, the smaller band amplified in this assay do not correspond to xbp1 small transcript. This band is smaller because the absent of 26 nucleotides is translated into an smaller amplicon. So, is necessary that the author explain in a better way this assay to avoid misinterpretation. Quantifications of these analyses and the effect of the absent of Mfn2 in the expression of UPR target genes induced by ER stress is necessary to confirm that Mfn2 regulates PERK, IRE1alpha and ATF6 pathways.

Response: We now provide new data on the expression of the XBP-1s protein variant, which provides a more definitive evidence for an enhanced expression of the short form of XBP-1. These data are shown as Figure 4B, and 4C. Furthermore, we have quantified data on Figure 4, and we document the expression of UPR target genes. Data show an enhanced expression of UPR target genes in Mfn2-deficient cells (shown as Supplementary Table 1).

Figure 5:
The authors miss to knock down PERK, XBP-1 and ATF6 in WT cells. These controls are important. Another possibility is to evaluate the effect of Mfn1 in PERK, ATF4, IRE1, XBP1 or ATF6 KO cells.

Response: We have performed studies in wild type cells upon silencing of PERK, XBP-1 and ATF6 which are now shown as Figures 5, 7, 8, Suppl. Figures 6, and 8. Data indicate that silencing of these proteins (PERK, XBP-1 or ATF6 did not alter the stimulatory effects of ER stress on autophagy or apoptosis in a wild-type cell context). However, PERK has a relevant influence on mitochondrial morphology and mitochondrial function in cells.

Figure 6: Mfn2 KO cells show and impaired eif2alpha phosphorylation. However, in samples of mice, western blot of eif2alpha phosphorylated show that this is increased. What is the possible explanation?

Response: Studies in Figure 4 were mainly oriented to analyze differences as a consequence of ER stress induced by thapsigargin. In those studies, it was difficult to obtain a precise estimation of basal values due to its very low level compared to maximal stimulation by thapsigargin so we mainly obtained information on the response to ER stress. In this connection, data indicate that PERK phosphorylation is enhanced and this leads to enhanced GADD34 induction, which causes dephosphorylation of eIF2alpha.
However, in figure 6 we focused our attention on the effect of Mfn2 deficiency under basal conditions in cells and in tissues from KO mice. Under those conditions, we clearly detected enhanced PERK phosphorylation and enhanced eIF2alpha phosphorylation in Mfn2-deficient cells

and in liver and muscle tissues upon Mfn2 ablation. This is now shown as Figures 6A-D. We have included additional explanation on this issue in the Discussion section (page 15, line 3).

In 6D, the authors show that ectopic PERK co-inmunoprecipitated with Mfn2. But the authors did not assay the possible interaction with IRE1alpha or ATF6. Endogenous inmunoprecipitation from cells or from tissues should be assayed.
Response: we now provide a more robust demonstration for the existence of physical interaction between Mfn2 and PERK in cells just expressing endogenous Mfn2 and PERK. These data are shown as Figure 6F. The methods of analysis of the interaction between Mfn2 and ATF6 or IRE1alpha has not been set up in the lab yet.

Figure 7: The effect of Knocking down of PERK in Mfn2 KO cells should be compared with WT cells. Surprisingly, PERK KD cells show reduced ROS levels. That is opposite to the data of several papers showing that the PERK-ATF4 branch has an antioxidant role. One possible explanation is that PERK KD was done only in Mfn2 KO cells. An important control here will PERK WT cells.

Response: We have analyzed the effect of PERK silencing on mitochondrial function of wild type and in Mfn1 KO cells. Data indicate that contrary to the data obtained in Mfn2 KO cell, silencing of PERK in wild type or in Mfn1 KO cells did not alter mitochondrial morphology or ROS production (shown as Figure 7C, 7D, 7F, and 7G). In addition, we have performed PERK overexpression assays in wild type cells. Data indicate mitochondrial fragmentation (Figure x).
Our data indicate the existence of a relevant function of PERK on mitochondrial biology. The potential antioxidant role of PERK has not been fully studied.

Minor points (but important):
Molecular weights of Western blots or PCRs are not shown in any figures.

Response: Done

The text is vague and many times "unspecific", especially when referring to mechanisms that have been proposed before. Is necessary to be more rigorous.

Response: We have revised the text and fixed this relevant criticism throughout the different sections of the manuscript.

Examples:
Line 16: "IRE1alpha activates the xbp-1 transcription factor", must say "IRE1alpha activates expression of xbp-1 transcription factor".

Response: Modified

Line 4: "three", must say "at least three"

Response: Done

Line 6: "UPR (unfolding protein response)", must say "UPR (Unfolded Protein Response)".

Response: Done

2nd Editorial Decision 15 March 2013

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees and their comments are provided below.

The reviewers find that most of their requests have been satisfied and are in principle supportive of publication in The EMBO Journal. However, they nevertheless feel that several important concerns - some of which will require additional experimentation - still have to be addressed. Given the overall

positive assessment by the referees, I would like to return your manuscript to you once more for the necessary amendments.

Please do not hesitate to contact me should any points require further clarification!

REFEREE COMMENTS

Referee #1

New data shown by authors satisfy most of our requests. However some minor points still shall be clarified:
Relative to response to major comment 7:
Figure 8E: From images showing mitochondrial morphology in WT cells overexpressing PERK it does not seem that organelles are fragmented (except the image on the right). Authors should change images and show quantification of mitochondrial morphology.

Relative to response to major comment 8:
Supplementary Figure 8B: For Mfn2KO cells, levels of OPA1 (both short and long) seem to increase in siPERK cells. Authors should provide another WB, to exclude this possibility.

Relative to response to minor comment 10:
Figure 4B: is the antibody against Xbp1 specific for active Xbp1s? If not authors should show also the band relative to inactive Xbp1u. In the same Figure, proteins at same molecular weight (elf2 α and ATF4, 42KDa) seem to run in a different way (elf2α line is like smiling, while ATF4 line is straight); authors should show the whole WB or show the tubulin marker for all the WBs they are reporting.

Relative to response to minor comment 17:
Figure 8C: WT MEFs do not seem to increase OCR in response to FCCP. It is not clear if this is due to plot scale or to experimental problem. Authors should clarify this point.

Referee #3

The authors did an effort to improve this work and answered many of our requests. However, in my opinion the data presented is still not sufficient to prove the initial hypothesis.

The manuscript is difficult to read and understand in terms of explanation of experiments. For example, it is not clear how e the LC3-II quantification was done or how the mCherry-LC3-II-GFP quantification was performed. The information provided in figures, legends and text, sometime does not fit. Therefore, interpretation of data and its analysis is very difficult.

On the other hand, the presentations of the results could be improved. For example, knock down of perk was determined in two different western blots. Is the KD similarin both wt and ko mf2 cells? In the case of xbp-1 knock down, the expression of this protein is different in both mfn2 wt and mfn2 ko cells.

-The possible explanation of the effect of Mtf2 defficiency in Eif2alpha phosphorylation is not clear. Actually, it is difficult to interpret the UPR data if only Tg was used for these assays. This drug has complex effects on calcium signaling.

-If the PERK pathway mediates apoptosis in the system and the IRE1/XBP-1 pathway modulates autophagy, why both silencing do not rescue this phenotype?
Most of these experiments are not conclusive and the interpretation of the data is overstated.
-Basal levels of LC3-II and the basal flux of LC3-II is reduced in mf2 KO cells (F3A and F3B), but this phenotype is different in the control KD of PERK and XBP1 (Fig5D and F5H).

-In vivo characterization of p-eif2alpha and chop expression in liver and muscle looks great. The endogenous IP of PERK and Mfn2 is powerful. However, the authors did not evaluate XBP1 pathways as well as autophagy levels.

in summary, the paper could be improved.

2nd Revision - authors' response 12 June 2013

Referee #1:

New data shown by authors satisfy most of our requests. However some minor points still shall be clarified:
Relative to response to major comment 7:
Figure 8E: From images showing mitochondrial morphology in WT cells overexpressing PERK it does not seem that organelles are fragmented (except the image on the right). Authors should change images and show quantification of mitochondrial morphology.

Response: Images have been changed and quantification done (and shown as Figure 9E).

Relative to response to major comment 8:
Supplementary Figure 8B: For Mfn2KO cells, levels of OPA1 (both short and long) seem to increase in siPERK cells. Authors should provide another WB, to exclude this possibility.

Response: We now provide a clearer western blot that indicates that OPA1 expression is not altered in cells silenced for PERK (Supplementary Figure 8B).

Relative to response to minor comment 10:
Figure 4B: is the antibody against Xbp1 specific for active Xbp1s? If not authors should show also the band relative to inactive Xbp1u. In the same Figure, proteins at same molecular weight (elf2 α and ATF4, 42KDa) seem to run in a different way (elf2α line is like smiling, while ATF4 line is straight); authors should show the whole WB or show the tubulin marker for all the WBs they are reporting.

Response: The antibody used recognizes both forms of XBP1. We show a representative blot with both XBP1 bands in Figure 1 for Reviewer. The Xbp-1u form was expressed at low levels during the study. In addition, correction of ATF4 molecular weight and tubulin blots are now included in Figure 4B.

Relative to response to minor comment 17:
Figure 8C: WT MEFs do not seem to increase OCR in response to FCCP. It is not clear if this is due to plot scale or to experimental problem. Authors should clarify this point.

Response: MEF cells show a low mitochondrial respiration activity. In Figure 2 for the Reviewers, the mitochondrial respiration of MEF cells is compared to C2C12 myoblasts. It becomes clear that the routine oxygen consumption activity is lower in MEF cells compared to C2C12 myoblasts. In addition, FCCP causes a marked increase in mitochondrial respiration in C2C12 but not in MEF cells. The lack of response of MEF cells to FCCP is a characteristics of MEF cells in culture due to the low mitochondrial activity (Garcia-Cao et al., Cell 2012).
Referee #3:

The authors did an effort to improve this work and answered many of our requests. However, in my opinion the data presented is still not sufficient to prove the initial hypothesis.
The manuscript is difficult to read and understand in terms of explanation of experiments. For example, it is not clear how e the LC3-II quantification was done or how the mCherry-LC3-IIGFP quantification was performed. The information provided in figures, legends and text, sometime does not fit. Therefore, interpretation of data and its analysis is very difficult.

Response: We have rewritten the manuscript in an attempt to better explain the experiments performed, and major changes are in red color. We have also revised the

English style by an expert. In addition, we provide explanation in Methods and in Figure legends the quantification of GFP-mCherry-LC3 studies and the way to quantify LC3-II (please notice that we have displaced some of the methods to Supplementary Information). We have also performed a substantial number of studies to provide further proof to our hypothesis.

On the other hand, the presentations of the results could be improved. For example, knock down of perk was determined in two different western blots. Is the KD similar in both wt and ko mf2 cells? In the case of xbp-1 knock down, the expression of this protein is different in both mfn2 wt and mfn2 ko cells.

Response: We now provide a better presentation of the data concerning the knockdown of PERK and of XBP1 (now shown as Figures 5 and 6) in wild-type and in Mfn2 KO. Indeed, knockdown of PERK and of XBP1 was similar in wild-type and in Mfn2 KO cells (Figure 5A, and Figure 6A).

-The possible explanation of the effect of Mtf2 defficiency in Eif2alpha phosphorylation is not clear. Actually, it is difficult to interpret the UPR data if only Tg was used for these assays. This drug has complex effects on calcium signaling.

Response: We have performed new set of studies in which we have analyzed the response of wild-type and Mfn2 KO cells to tunycamicin. Under these ER stress conditions, Mfn2 KO cells showed a stronger activation of eIF2alpha and ATF4 (Figure 4D).
Furthermore, tunycamicin or brefeldin A-induced CHOP expression to a greater extent in Mfn2 KO cells compared to wt cells (Figure 3 for the Reviewer). This indicates that the PERK pathway was enhanced in Mfn2 KO cells independently of the ER stress stimulus. This is consistent with the global pattern of changes detected upon ER stress induced by thapsigargin.
In addition, our data suggest that the reduced activation of eIF2alpha in Mfn2 KO cells in response to thapsigargin may be explained by the complex effects that this drug has on calcium homeostasis (Inesi et al, 1998; Michelangeli & East, 2011). Further work will be required to clarify the mechanisms involved in the specific effects of thapsigargin in Mfn2 KO cells.

-If the PERK pathway mediates apoptosis in the system and the IRE1/XBP-1 pathway modulates autophagy, why both silencing do not rescue this phenotype? Most of these experiments are not conclusive and the interpretation of the data is overstated. Basal levels of LC3-II and the basal flux of LC3-II is reduced in mf2 KO cells (F3A and F3B), but this phenotype is different in the control KD of PERK and XBP1 (Fig5D and F5H).

Response: We have rewritten the studies in which wild type or Mfn2 KO cells were subjected to either PERK or XBP-1 silencing and caspase 3 processing, caspase activity or LC3b-II abundance were measured to avoid overinterpretation of the data. PERK silencing or XBP-1 silencing was similar in wild-type and in Mfn2 KO cells (Figure 5A and Figure 6A). In addition, we have performed additional studies oriented to further document the impact of PERK silencing (Figure 5E and F) or XBP-1 loss-of-function (Figure 6E, F, G, H) on autophagic activity.

-In vivo characterization of p-eif2alpha and chop expression in liver and muscle looks great. The endogenous IP of PERK and Mfn2 is powerful. However, the authors did not evaluate XBP1 pathways as well as autophagy levels.

Response: We have performed analysis of XBP-1s and measurement of autophagy in muscle and liver tissues from Mfn2 KO mice. Data are shown as Figure 7C, and indicate that XBP-1s protein expression is enhanced upon ablation of Mfn2 both in liver and in skeletal muscle. Autophagy seems to be inhibited both in muscle and in liver from KO mice as assessed by accumulation of LC3-II, and p62.

in summary, the paper could be improved.

Response: We do think that we have substantially improved the manuscript and we do thank the reviewer for the great help.

(Please see figures starting on next page)

Figure 1 for the Reviewers

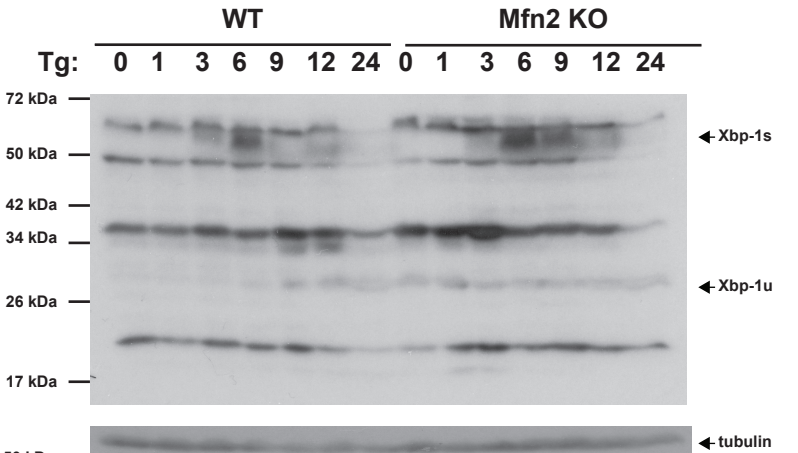
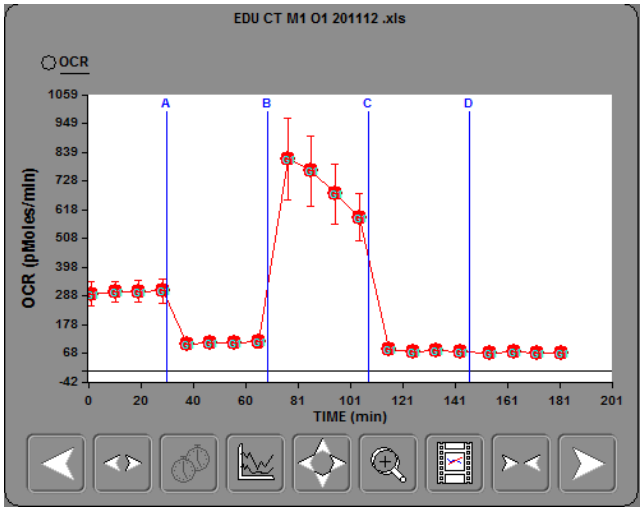
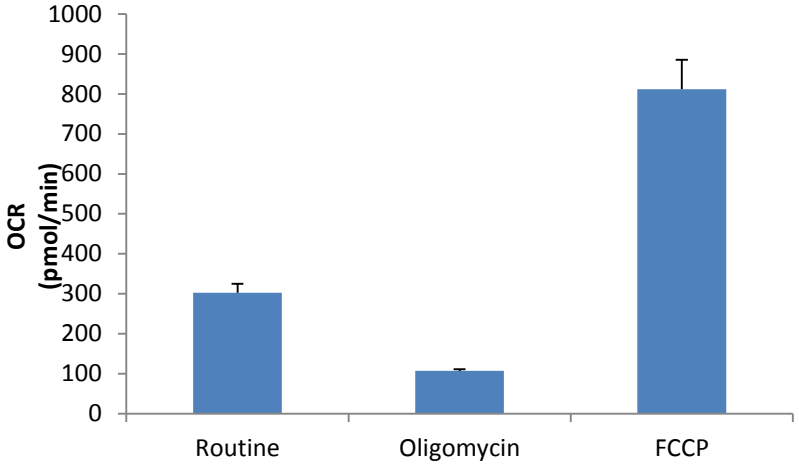


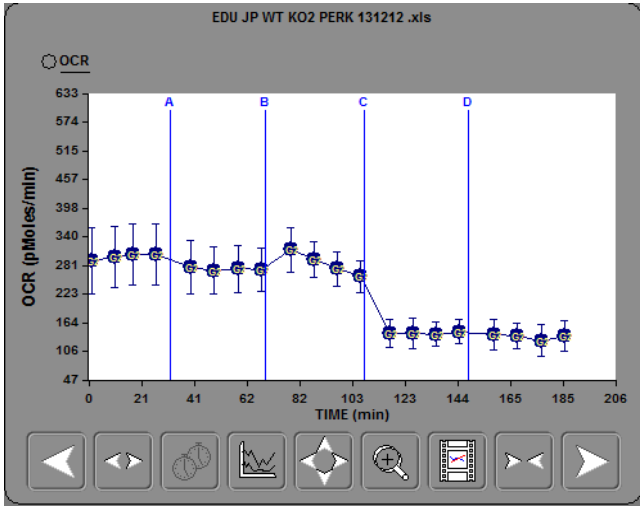
Figure 2 for the Reviewers

A) C2C12 myoblasts





B) MEF cells



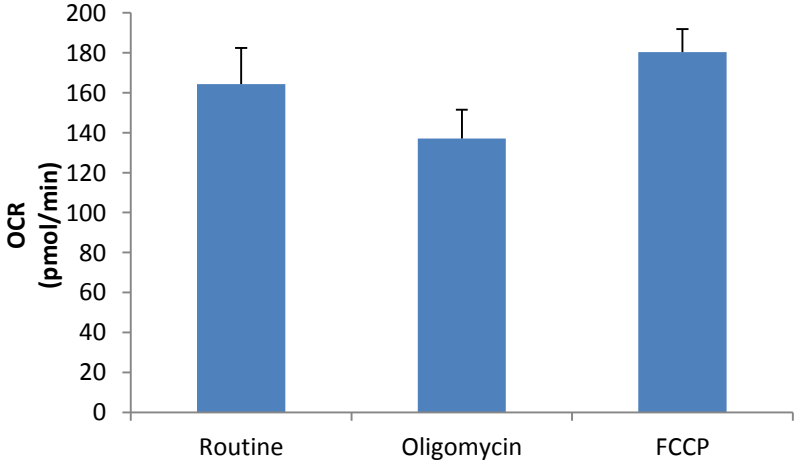
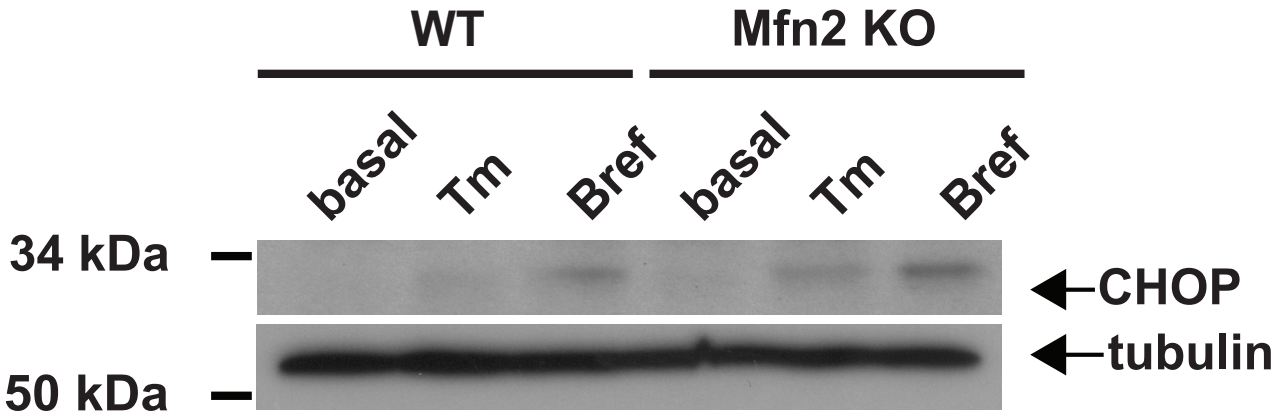


Figure 3 for the Reviewers



3rd Editorial Decision 18 June 2013

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the reviewers. I am happy to inform you that we are ready to proceed with acceptance of the paper, pending modification of a few minor points.

- Please add the number of biological replicates to all Figure legends.

- I would like to suggest changing the current title to "Mfn2 modulates the UPR and mitochondrial function via repression of PERK" in order to avoid the repeat of "response" and to add specificity.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I would therefore like to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be sent via e-mail and would be published online with the article as a supplementary "Source Data" file.

- Please let me know if you would prefer that the data provided for reviewers should be omitted from the Review Process File that we publish with each article.

Since you currently do not have access to your manuscript through our tracking system, please simply send the amended text file and the source data via email. We can then easily upload the information into our system. After that, we should be able to swiftly proceed with formal acceptance and production of the manuscript!

If you have any questions, please do not hesitate to contact me directly.

Additional correspondence (author) 02 July 2013

Enclosed you will find the revised versions of the text and supplementary data files containing information on the number of biological replicates and with the suggested new title. Enclosed you will find also a Source Data file of all gel/blots shown in the article.

