EMBO reports

Mfn2 localization in the ER is necessary for its bioenergetic function and neuritic development

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1st Editorial Decision 15th Dec 2020

Dear Dr. Soriano,

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

I apologize for this unusual delay in getting back to you. It took longer than anticipated to receive the referee reports.

We concur with the referees that the proposed role of ER localized Mfn2 in bioenergetic function in principle very interesting. However, referees also raise significant concerns that need to be addressed to consider publication here. In particular,

- The methods used for calcium measurement and release from the ER are not appropriate (referee #1 points 1 and 2, referee #2 2nd major point).
- The method used to measure ER-mitochondria proximity is not appropriate (referee #1 point 5, referee #2 1st major point).
- Cell specificity of the findings needs to be ruled out (referee #3 paragraph 2)
- In general, causality and the order of the events are not sufficiently established. Provided controls are insufficient (all referees).

Should you be able to address all referee concerns, we would like to invite you to submit a revised manuscript. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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

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

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

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

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

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

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

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

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

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The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

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

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

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

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

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

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The manuscript by Casellas-Díaz et al. studies the impact of Mfn2 on mitochondrial bioenergetics. They observe that Mfn2 KO MEFs suffer from grave bioenergetics defects, which is not the case for Mfn1 KO MEFs. These defects are not correlated to mitochondrial shape, Instead, the authors propose a model whereby Mfn2 stabilise ER-mitochondria contact sites, increasing calcium transfer from the ER to the mitochondria and thus promoting respiration. Indeed, the bioenergetics defects of MFN2 KO MEFs can be rescued by expressing an artificial ER-mitochondria tether, or a version of Mfn2, which is specifically targeted to the ER. Strikingly, this later rescue only works when there is a mitochondrial binding partner for ER-Mfn2 on mitochondria (either a mitochondrial version of Mfn2 or Mfn1). Finally, this rescue only works if Calcium release from the ER is unhinhibited. The work is interesting and would likely be an important contribution to the field. There are, however, currently shortcomings in the establishment of causal relationships. For instance, is an impediment in Calcium uptake by mitochondria causing a defect in bioenergetics, or a defect in bioenergetics impeding mitochondrial calcium uptake? These shortcomings and ways to address them are listed below. These are all very important points in the light of the fact that while Mfn2 KO, which moderately affects mitochondrial calcium, leads to mouse embryonic lethality and a strong bioenergetic defect, MCO KO, which abrogate mitochondrial calcium entry, is viable and leads to a mild bioenergetics defect at most. This is hard to reconcile with the proposed model.
1. Mitochondrial Calcium is measured using Rhod-2, a probe that depends on the membrane potential across the inner mitochondrial membrane. Therefore, any change in fluorescence may relate to changes in potential, rather than changes in Calcium. Indeed, Figure 2D shows that CCCP treatment, which collapses the membrane potential, leads to a decrease in rhod2 fluorescence. This is interpreted as a release of resting mitochondrial calcium, but this doesn't make sense since the

Kd of Rhod2 is higher than the resting mitochondrial calcium concentratio. It is expected that in resting conditions, most Rhod2 would be calcium-free already. It is instead more likely that Rhod2 itself is released from mitochondria. If Mfn2 KO cells indeed have a lower potential, they may accumulate less Rhod2, explaining why the decrease in fluorescence upon CCCP treatment is less pronounced in these cells.
2. Another potential issue is with the use of caffeine to stimulate calcium release from the ER. The molecular target of caffeine is the ryanodine receptor, which is not supposed to be expressed in cell lines such as mouse embryonic fibroblasts. There is no reference for the mode of action of caffeine in MEFs. The signals observed herein are long-lived, unlike the normal calcium transients observed upon receptor stimulation, and look more like what would be observed upon SERCA inhibition. How does caffeine work here? The authors should confirm that caffeine works as it is supposed to, and if not, use a more appropriate pharmacological intervention (e.g. ATP). Of note, the original report on Mfn2 as an ER-mitochondria tether (de Brito and Scorrano 2008) went into the problem that Calcium concentrations in the ER of Mfn2 KO MEFs was much higher than in control MEFs. They had to resort to using different concentrations of agonist to obtain similar calcium releases across genetic background. Is the same observed here? How does that impinge on the results? And if it is not the same as in the de Brito paper, how is this explained?
3. It is never tested if the calcium that accumulates in mitochondria upon caffeine treatment originates from the ER, and whether the defects observed in mitochondrial calcium import are specific for calcium that originates from the ER. Mitochondrial calcium import is electrophoretic, and thus relies on the membrane potential. If Mfn2 KO cells indeed have a lower mitochondrial membrane potential, this might be the cause (and not the consequence) of reduced calcium uptake.
4. One supremely important aspect is to tease apart the order of events and the causes and consequences. To support their claim that membrane potential defects in the Mfn2 knockout cells are caused by perturbed calcium transfer from the ER to the mitochondria (and not, for instance, the other way around), the authors pharmacologically block IP3R or knockdown MCU, and show that an artificial ER-mitochondria tether is ineffective at restoring normal bioenergetics in these conditions. These data are crucial to their current model and yet are currently buried in the supplementary (Supp. Fig. 3B-C & H-I). Furthermore, the normalisation for these data is confusing. It is important to show non-normalized raw data for each conditions. Moreover, since the author's model is that calcium import problems cause the bioenergetics defects in MFN2-KO cells, knockdown of MCU alone in wild-type cells should phenocopy MFN2-KO in terms of bioenergetics. Is this the case? If yes, isn't it at odd with the fact that MCU KO yields viable mice?
5. ER-mitochondria proximity is assessed using the Mander's coefficient. Yet this measure is likely very sensitive to the shape of the organelles. Bulky round mitochondria might push ER away much more than fine tubular ones, creating an impression of non-overlap at the resolution of light microscopy. Therefore, it is unclear whether Mander's coefficients herein report on organelle contacts or mitochondrial shapes. There are two quick fixes to that. 1) measure the Mander's coefficient in Mfn1 KO cells (globular mitochondria but intact ER-mitochondria contacts and organelle bioenergetics), and in Mfn2-KO expressing Mfn1 or a DN-Drp1 (tubular mitochondria but defective ER-mitochondria contacts and organelle bioenergetics).

Other important considerations:
1. A rescue of Mfn2 knockout cells with wild-type Mfn2 is never performed (e.g. Fig. 1C and Fig. 5F).
2. An important control for the luciferase assay would be to show no change in luciferase activity when both parts of the split probe are on the same organelle, to account for any other confounding effects of the Mfn2 knockout (e.g. defective import of coelenterazine or reduced expression of one of the rLuc hemiprotein. Moreover, the assembly of these split proteins is said to be reversible. There is no reference for this claim.

3. "Through this period of neurite outgrowth, neurons increase ER-mitochondria contacts (Fig. 6A), which correlates with increased Mfn2 expression (Fig. 6B)." This sentence is problematic since 1) the increase in mitochondria contact is claimed based on Mander's coefficient only (see above) and 2) it appears that all mitochondrial proteins (Hsp60, VDAC1) increase to the same extent during this period, indicating rather a general increase in mitochondrial mass.
4. Intracellular ATP levels are measured after a 2DG challenge. It is not shown, however, whether ATP levels are identical before the challenge. If ATP levels were different when cells can use glycolysis, it would point to a non-mitochondrial cause for the phenomena herein.

Minor points

1. The authors should cite Baughman ... Mootha Nature. 2011 Jun 19;476(7360):341-5 in addition to de Stefani et al.
2. The ChiMERA construct from Kornmann et al. (2009) expresses GFP yet the authors are using ER-GFP their colocalization experiments. Therefore, how do the authors calculate the Mander's coefficient of the ER and mitochondria when expressing ChiMERA? It would also be informative to see representative images for the ChiMERA experiments.
3. Line 187: 'in mitochondria' should read 'on mitochondria'.

Referee #2:

The study by Casellas-Diaz and colleagues describes the specific role for the OMM protein Mfn2 in mitochondrial bioenergetics by its capacity to control mitochondria-ER contacts, independently of its function in outer mitochondrial membrane fusion. Mfn2 is a complex protein not only involved in OMM fusion, but also in the regulation of mitochondria-ER contacts. Loss of Mfn2 or Mfn2 mutations lead to numerous alterations of cellular functions and have been widely associated to human diseases including Charcot Marie tooth disease. However, if cellular defects are all associated to mitochondrial morphology deregulation or to mitochondria-ER contacts defects, is not fully understood.
In this study Casellas-Diaz and colleagues proposed that mitochondrial bioenergetics defects observed in Mfn2-KO MEFs are associated to its mitochondria-ER contact regulation function and thus calcium signalling. The authors corroborate their conclusions in more specialized cell lines, and show the contribution of mitochondria-ER contacts Mfn2-dependent to neurite development.

While this study could be interesting, the methodology used by the authors is not always optimal and further experiments/controls have to be performed to support authors conclusions.

Major points

- The overexpression system used by the authors to target Mfn2 to mitochonria or ER raises concerns about the physiological relevance. Indeed, as stated by the authors in introduction, only a small fraction of Mfn2 can be found at the ER. However, in the present study the authors used transient overexpression to depict the complex mechanism of how Mfn2 regulate mitochondria-ER contacts. Based on their methods (48h of overexpression), the authors need to compare the endogenous mito or ER Mfn2 levels with their overexpression system by fractionation and IB analysis. The authors should use a better system to finely target their mutants to different organelles. While CRISPR could be a solution, the authors could also establish stable cell lines expressing their different mutants by specifically selecting cells with low levels of expression, similar

to endogenous level.

- The analysis of the mitochondria-ER contacts is weak. Due to the discrepancy observed for the role of Mfn2 in the regulation of the contacts, colocalization analysis by manders coefficient should not be used. In addition, the images presented in the paper are really low quality raising the significance of the manders coefficient analysis calculated. Transmission electron microscopy analysis needs to be performed to analyse how the different conditions impact the distance, number and mitochondria-perimeter involved in these contacts. The authors propose a new method to analyse these contacts based on a split retina luciferase system, however this method has not been validated. There is no information about the correct localization of the 2 different plasmids, if they really detect mitochondria-ER contacts, if overexpression of the system forces the contacts... This system also needs to be tested on other cells silenced for others identified tethering prot/complex known to regulate mitochondria-ER contact (e.g: PTPIP51/VAPB, PDZD8....).

- Calcium signalling analysis needs to be strongly reinforced. The authors need to stimulate ER-induced Ca++ release via IP3R (e.g. ATP, histamine) which is localised at the mito-ER contacts interface to analyse ER to mitochondria calcium fluxes. However, in this study the authors have used caffeine. Caffeine is widely associated to RYR stimulation (PMID: 18518861) and has been proposed to inhibit IP3R-induced calcium release (PMID: 1312323; PMID: 18518861; PMID: 20103623). The authors need also to analyse in the same time cytosolic calcium.

- The authors used in their study an artificial mito-ER tether characterized in the yeast model. Does this tether have been validated in mammal cells? In figure 3, how the authors explain that the tether does not increase mito-ER contacts when overexpressed in WT cells? Same question for mitochondrial calcium? Recently, different groups have used and validated artificial tether in mammal cells (e.g PMID: 29097544), why the authors decide to use the one characterized in yeast?

- In Fig 5 and S5, while I appreciate the rescue expts performed with ER-Mfn2 and mito-Mfn2 in Mfn1/2-DKO cells, the authors also need to perform these expts with WT-Mfn2.

- The authors need to confirm their bioenergetics results using other methods that the Agilent kit e.g Seahorse analysis

- In Sup Figure 1, the authors used the overexpression of DN-Drp1 or Mfn1 to rescue mitochondrial morphology and conclude that the effect of the loss of Mfn2 on bioenergetics, mb potential, is morphology independent. The authors need to perform rescue experiments using the mito-targeted Mfn2 to really confirm their statement.

- It has been proposed that the effects of Mfn2KO on calcium signaling was due to decreased level of the mitochondrial calcium uniporter, which is also associated to cell confluency (PMID: 27647893; PMID: 25870285). The authors need to check levels for the mitochondrial calcium uniporter complex machinery in their conditions of experiments, e.g cell confluency.

- In all the manuscript, it is difficult to confirm authors results based on the low quality of the images. In addition, representative images need to be shown in all the experiments based on microscopy analysis. The authors also need to show single channel as well as the overlay.

- For mitochondrial morphology, the authors need to calculate different mitochondria parameters as length, number and area from their confocal images.

- For membrane potential analysis using TMRM, the authors need to use a second mitochondrial fluorescent marker as control and perform a ratio TMRM/mito marker to exclude any potential impact of mitochondrial mass variation.

Minor points
- S1F: Mfn2 blot needs to be done. In all the manuscript, there is no IB to confirm the cell lines used by the authors (MFN2KO, MFN1Mfn2-DKO and overexpression).

- In S3, the authors also need to analyse calcium fluxes in their different conditions of XeC, 2APB and MCU silencing.

- In Figure 6, the authors need to show the representative confocal images they used to analyse the different parameters.

Referee #3:

This is an interesting manuscript presenting an intriguing set of results to support ER targeted Mfn2 role in mitochondrial function. The manuscript is clearly written. The main shortcoming is by the lack of mechanistic information.

The finding that Mfn2 Ko cells have impaired maximal bioenergetics capacity has been demonstrated in MEF cells. However in other cell types the results were mixed. On the other hand various studies show that Mfn2 deletion is increasing proton leak. Therefore, authors should look carefully at the possibility that this is a cell specific phenomenon.

It is important to present the maximal respiratory capacity values in the main figures so that one can conclude if there is a respiratory dysfunction.

The respirometry results cannot be interpreted without an assessment of mitochondrial mass

In figure 1 the authors show reduction in TMRM fluorescence in Mfn2 KO in panel C, but the image shows reduction in Mfn1 KO cells. The experiment in panel C of this figure is lacking a positive and negative controls using Oligomycin and CCCP. Authors should also verify that TMRM in cell did not reach the level that induces quenching.

The restoration of Mfn2 using ER -targeted Mfn2 is an interesting approach. However, one has to demonstrate that indeed the protein reached the ER and not the mitochondria.
Can the authors isolate ER and mitochondria and demonstrate that the transfected Mfn2 was in the ER and not in the mitochondria?

As with figure 1, it is essential in figure 4 to show the values of basal, maximal and leak respiration.

The manuscript does not provide any insight into the mechanism by which these constructs restore bioenergetics functions.

Referee #1:

The manuscript by Casellas-Díaz et al. studies the impact of Mfn2 on mitochondrial bioenergetics. They observe that Mfn2 KO MEFs suffer from grave bioenergetics defects, which is not the case for Mfn1 KO MEFs. These defects are not correlated to mitochondrial shape, Instead, the authors propose a model whereby Mfn2 stabilise ER-mitochondria contact sites, increasing calcium transfer from the ER to the mitochondria and thus promoting respiration. Indeed, the bioenergetics defects of MFN2 KO MEFs can be rescued by expressing an artificial ER-mitochondria tether, or a version of Mfn2, which is specifically targeted to the ER. Strikingly, this later rescue only works when there is a mitochondrial binding partner for ER-Mfn2 on mitochondria (either a mitochondrial version of Mfn2 or Mfn1). Finally, this rescue only works if Calcium release from the ER is unhinhibited. The work is interesting and would likely be an important contribution to the field. There are, however, currently shortcomings in the establishment of causal relationships. For instance, is an impediment in Calcium uptake by mitochondria causing a defect in bioenergetics, or a defect in bioenergetics impeding mitochondrial calcium uptake? These shortcomings and ways to address them are listed below. These are all very important points in the light of the fact that while Mfn2 KO, which moderately affects mitochondrial calcium, leads to mouse embryonic lethality and a strong bioenergetic defect, MCO KO, which abrogate mitochondrial calcium entry, is viable and leads to a mild bioenergetics defect at most. This is hard to reconcile with the proposed model.

We are pleased the referee considers our work as interesting and that would likely be an important contribution to the field. We thank the referee for the feedback that has contributed improve our study.

1. Mitochondrial Calcium is measured using Rhod-2, a probe that depends on the membrane potential across the inner mitochondrial membrane. Therefore, any change in fluorescence may relate to changes in potential, rather than changes in Calcium. Indeed, Figure 2D shows that CCCP treatment, which collapses the membrane potential, leads to a decrease in rhod2 fluorescence. This is interpreted as a release of resting mitochondrial calcium, but this doesn't make sense since the Kd of Rhod2 is higher than the resting mitochondrial calcium concentratio. It is expected that in resting conditions, most Rhod2 would be calcium-free already. It is instead more likely that Rhod2 itself is released from mitochondria. If Mfn2 KO cells indeed have a lower potential, they may accumulate less Rhod2, explaining why the decrease in fluorescence upon CCCP treatment is less pronounced in these cells.

We have now used two new additional approaches to show that Mfn2 KO cells have lower mitochondrial Ca^{2+} levels:

1.- We have uncoupled mitochondria with CCCP to induce mitochondrial Ca^{2+} release and observed lower cytoplasmic Ca^{2+} rise in Mfn2 KO cells (new Fig. EV2G).

2.- We have used the genetic Ca^{2+} sensor targeting mitochondria mt-Cepia, also showing reduced Ca^{2+} levels in mitochondria (new Fig. EV2H).

2. Another potential issue is with the use of caffeine to stimulate calcium release from the ER. The molecular target of caffeine is the ryanodine receptor, which is not supposed to be expressed in cell lines such as mouse embryonic fibroblasts. There is no reference for the mode of action of caffeine in MEFs. The signals observed herein are long-lived, unlike the normal calcium transients observed upon receptor stimulation, and look more like what would be observed upon SERCA inhibition. How does caffeine work here? The authors should confirm that caffeine works as it is supposed to, and if not, use a more appropriate pharmacological intervention (e.g. ATP). Of note, the original report on Mfn2 as an ER-mitochondria tether (de Brito and Scorrano 2008) went into the problem that Calcium concentrations in the ER of Mfn2 KO MEFs was much higher than in control MEFs. They had to resort to using different concentrations of agonist to obtain similar calcium releases across genetic background. Is the same observed here? How does that impinge on the results? And if it is not the same as in the de Brito paper, how is this explained?

RyR are abundantly expressed in excitatory cells, however expression in of RyR has also been detected at low levels in other types of cells, including fibroblasts and MEFs (Augusto *et al*, 2020; Giannini *et al*, 1995; Huang *et al*, 1998; Mukherjee *et al*, 2012; Ruas *et al*, 2015).

Mitochondrial Ca^{2+} uptake after caffeine stimulation is unsurprising since rat brain proteome has identified RyR2 as a component of the MAM (Poston *et al*, 2013), in fact, it has been previously reported Ca^{2+} transfer from the ER to mitochondria via the RyR (Guidarelli *et al*, 2019; Santulli *et al*, 2015; Seidlmayer *et al*, 2016; Szalai *et al*, 2000). In our experiments, as expected, caffeine works through the RyR, since treatment with the RyR inhibitor dantrolene blocks mitochondrial Ca^{2+} rise after caffeine treatment (new Fig. EV2K).

Following the referee's suggestion, we also show that ATP and histamine treatment produced higher mitochondrial Ca^{2+} transfer from the ER to the mitochondria in WT than in Mfn2 KO cells, which could be rescued by ChiMERA (new Fig. EV2L, EV2M and EV3C).

As in De Brito and Scorrano's paper, we also observe increased ER Ca^{2+} levels in Mfn2 KO cells, using the Ca^{2+} sensor targeting the ER ER-LAR-GECO1, and in the revised version also measuring cytoplasmic Ca^{2+} after emptying the ER with thapsigargin treatment (new Fig. EV2I). Moreover, we also show that caffeine treatment produces higher Ca^{2+} levels in the cytoplasm of Mfn2 KO cells (new Fig. EV2J). However, despite releasing more Ca^{2+} from the ER, mitochondrial Ca^{2+} uptake is lower in Mfn2 KO MEFs, supporting the notion that ER and mitochondria are more separated and as a result of Ca^{2+} diffusion in the cytoplasm, the concentration in the proximity of the MCU is too low for Ca^{2+} uptake.

3. It is never tested if the calcium that accumulates in mitochondria upon caffeine treatment originates from the ER, and whether the defects observed in mitochondrial calcium import are specific for calcium that originates from the ER. Mitochondrial calcium import is electrophoretic, and thus relies on the membrane potential. If Mfn2 KO cells indeed have a lower mitochondrial membrane potential, this might be the cause (and not the consequence) of reduced calcium uptake.

The possibility that reduced mitochondrial Ca^{2+} uptake is diminished in Mfn2 KO after ER Ca^{2+} release may be due to reduced mitochondrial membrane potential is a very interesting point. To determine whether reduced Ca^{2+} uptake is the cause or consequence of lower mitochondrial Ca^{2+}, we treated Mfn2 MEFs with methyl-pyruvate, which freely permeates the OMM and IMM,

to increase MMP to the level of WT MEFs. An increase in MMP produced increased mitochondrial Ca^{2+} uptake after caffeine treatment in both, WT and Mfn2 KO; however, although MMP in Mfn2 KO cells treated with methyl-pyruvate was the same as in WT (untreated), the mitochondrial Ca^{2+} uptake in treated Mfn2 KO cells remained lower than in WT cells. These results indicate that reduced mitochondrial Ca^{2+} uptake in Mfn2 KO cells is not primarily due to reduced MMP. These results are shown in new Fig. 2J and 2K.

4. One supremely important aspect is to tease apart the order of events and the causes and consequences. To support their claim that membrane potential defects in the Mfn2 knockout cells are caused by perturbed calcium transfer from the ER to the mitochondria (and not, for instance, the other way around), the authors pharmacologically block IP3R or knockdown MCU, and show that an artificial ER-mitochondria tether is ineffective at restoring normal bioenergetics in these conditions. These data are crucial to their current model and yet are currently buried in the supplementary (Supp. Fig. 3B-C & H-I). Furthermore, the normalisation for these data is confusing. It is important to show non-normalized raw data for each conditions. Moreover, since the author's model is that calcium import problems cause the bioenergetics defects in MFN2-KO cells, knockdown of MCU alone in wild-type cells should phenocopy MFN2-KO in terms of bioenergetics. Is this the case? If yes, isn't it at odd with the fact that MCU KO yields viable mice?

We agree with the reviewer that these are important data and we have moved the MCU results to the main figures (Fig. 3Q-U). We have also included data showing that knock down of MCU is enough to impair bioenergetics parameters in WT MEFs.

These results are in contrast with the MCU KO mice being viable and with no remarkable bioenergetics deficit. These results of the MCU KO mice generated by Finkel's lab aroused considerable surprise among researchers in the field and raised questions that remain unanswered. MCU KO mice grown in a mixed DC1 background were viable (although born in non-Mendelian ratio), however they could not produce viable inbreed mice within a C57BL/6 background, which died at E101.5-13.5. It is unknown why MCU KO mice are viable in the outbread CD1 background but not in the inbreed C57BL/6 background. The different background could explain the metabolic phenotype in Mfn2 KO MEFs, which come from a C57BL/6 background. In fact, liver specific MCU KO in the C57BL/6 background showed reduced respiratory capacity (Tomar *et al*, 2019). The fact that MCU KO mice from a DC1 background is not born at Mendelian rate also suggests a significant amount of embryonic lethality and adaptations in the surviving embryos during development. Indeed, extramitochondrial adaptations, including transcriptional reprogramming, has been described in transgenic mice expressing dominant negative-MCU in myocardium from a CD1 background (Rasmussen *et al*, 2015).

5. ER-mitochondria proximity is assessed using the Mander's coefficient. Yet this measure is likely very sensitive to the shape of the organelles. Bulky round mitochondria might push ER away much more than fine tubular ones, creating an impression of non-overlap at the resolution of light microscopy. Therefore, it is unclear whether Mander's coefficients herein report on organelle contacts or mitochondrial shapes. There are two quick fixes to that. 1) measure the Mander's coefficient in Mfn1 KO cells (globular mitochondria but intact ER-mitochondria contacts and organelle bioenergetics), and in Mfn2-KO expressing Mfn1 or a DN-

Drp1 (tubular mitochondria but defective ER-mitochondria contacts and organelle bioenergetics).

In the first version of the manuscript, former Fig. S2J (new Fig. EV2A) showed that there were not differences in Mander's coefficient beteween WT and Mfn1 KO cells. Moreover, we also showed that expression of ChIMERA or ER-targeted Mfn2 increased Mander's coefficient values in Mfn2 KO cells without modifying mitochondrial morphology (old Fig. 3A and J and Fig. 4A and C). These results argue against the possibility that the differences in Mander's coefficient between WT and Mfn2 KO are due to the difference in the shape of the organelles. Now, following the referee's suggestions, we show that restoration of mitochondrial morphology in Mfn2 KO cells with the expression of DN-Drp1 did not correct the differences in Mander's coefficient between WT and Mfn2 KO cells (new Fig. EV2B).

Other important considerations:

1. A rescue of Mfn2 knockout cells with wild-type Mfn2 is never performed (e.g. Fig. 1C and Fig. 5F).

Now, Appendix Fig. S4 and S5 show the rescue of mitochondrial morphology, ER-mitochondria contacts (Mander's and RLuc reconstitution), OCR, ATP, MMP and Ca^{2+} fluxes in Mfn2 KO and DKO, respectively, by expression of full length Mfn2.

2. An important control for the luciferase assay would be to show no change in luciferase activity when both parts of the split probe are on the same organelle, to account for any other confounding effects of the Mfn2 knockout (e.g. defective import of coelenterazine or reduced expression of one of the rLuc hemiprotein. Moreover, the assembly of these split proteins is said to be reversible. There is no reference for this claim.

Now, we show no change in RLuc activity between WT and Mfn2 KO cells when these cells are transfected with full length RLuc driven by the CMV promoter, the same promoter driving the ERMITO-RLuc construct (new Fig. EV2E).

The reference for the claim that RLuc split proteins assembly is a reversible way is also cited (Stefan *et al*, 2007).

3. "Through this period of neurite outgrowth, neurons increase ER-mitochondria contacts (Fig. 6A), which correlates with increased Mfn2 expression (Fig. 6B)." This sentence is problematic since 1) the increase in mitochondria contact is claimed based on Mander's coefficient only (see above) and 2) it appears that all mitochondrial proteins (Hsp60, VDAC1) increase to the same extent during this period, indicating rather a general increase in mitochondrial mass.

As expected, there was an increase in mitochondrial mass during neuronal maturation. This may indicate that increased ER-mitochondria colocalization shown by Mander's coefficient could be the effect of increased mitochondrial mass. However, Fig 6C shows that deletion of Mfn2 at DIV7 did not affect the mitochondrial mass, but Mander's coeeficient was lower in Mfn2 KO neurons. The lower Mander's coefficient in Mfn2 KO neurons could be completely rescued by ChiMERA, the expression of ER-Mfn2 plus mt-Mfn2, partially by ER-Mfn2 alone and not at all by mt-Mfn2

(Fig. 6D), which is in agreement with the results obtained in MEFs. In conjunction with the results obtained with MEFs, these results suggest that Mfn2 plays an important role in stablishing ER-mitochondria contacts during neuronal maturation. Nonetheless, we agree with the referee that other approaches are required before we can confidently to state that there are more contacts during differentiation. Consequently, we have reworded the sentence as follows: "Throughout this period of neurite outgrowth, neurons increase ER-mitochondria colocalization".

4. Intracellular ATP levels are measured after a 2DG challenge. It is not shown, however, whether ATP levels are identical before the challenge. If ATP levels were different when cells can use glycolysis, it would point to a non-mitochondrial cause for the phenomena herein.

Cells adjust metabolic pathways to maintain relatively constant total ATP levels. There are not differences in total ATP levels between WT and Mfn2 KO cells when metabolic plasticity is allowed by not blocking any metabolic pathway (new Fig. EV1A). However, as we showed in the first version of the manuscript, when glycolysis is blocked to force ATP production by mitochondria Mfn2 KO cells show reduced levels of ATP.

Minor points

1. The authors should cite Baughman ... Mootha Nature. 2011 Jun 19;476(7360):341-5 in addition to de Stefani et al.

We thank the referee for pointing this unpardonable oversight.

2. The ChiMERA construct from Kornmann et al. (2009) expresses GFP yet the authors are using ER-GFP their colocalization experiments. Therefore, how do the authors calculate the Mander's coefficient of the ER and mitochondria when expressing ChiMERA? It would also be informative to see representative images for the ChiMERA experiments.

We choose this yeast expression vector because we wanted low expression to avoid cell death produced by excessive ER-mitochondria contact. New Fig. EV3A shows weak expression of ChiMERA compared to GFP driven by the CMV promoter. Consequently, GFP signal is not detectable using fluorescence microscopy although three steps immunofluorescence using anti-GFP antibody showed weak staining (new Fig. EV3B).

3. Line 187: 'in mitochondria' should read 'on mitochondria'.

We apology for this mistake.

Referee #2:

The study by Casellas-Diaz and colleagues describes the specific role for the OMM protein Mfn2 in mitochondrial bioenergetics by its capacity to control mitochondria-ER contacts, independently of its function in outer mitochondrial membrane fusion. Mfn2 is a complex

protein not only involved in OMM fusion, but also in the regulation of mitochondria-ER contacts. Loss of Mfn2 or Mfn2 mutations lead to numerous alterations of cellular functions and have been widely associated to human diseases including Charcot Marie tooth disease. However, if cellular defects are all associated to mitochondrial morphology deregulation or to mitochondria-ER contacts defects, is not fully understood.

In this study Casellas-Diaz and colleagues proposed that mitochondrial bioenergetics defects observed in Mfn2-KO MEFs are associated to its mitochondria-ER contact regulation function and thus calcium signalling. The authors corroborate their conclusions in more specialized cell lines, and show the contribution of mitochondria-ER contacts Mfn2-dependent to neurite development.

While this study could be interesting, the methodology used by the authors is not always optimal and further experiments/controls have to be performed to support authors conclusions.

We are pleased that the referee appreciates the potential of our study. We thank the referee for the helpful comments.

Major points

- The overexpression system used by the authors to target Mfn2 to mitochonria or ER raises concerns about the physiological relevance. Indeed, as stated by the authors in introduction, only a small fraction of Mfn2 can be found at the ER. However, in the present study the authors used transient overexpression to depict the complex mechanism of how Mfn2 regulate mitochondria-ER contacts. Based on their methods (48h of overexpression), the authors need to compare the endogenous mito or ER Mfn2 levels with their overexpression system by fractionation and IB analysis. The authors should use a better system to finely target their mutants to different organelles. While CRISPR could be a solution, the authors could also establish stable cell lines expressing their different mutants by specifically selecting cells with low levels of expression, similar to endogenous level.

Although deletion of a given gene does not usually occur in nature and rescue normally requires protein expression above the physiological levels, it is undeniable that the KO technology and rescue experiments have greatly contributed to the advancement of our understanding of biological processes. However, we agree that there will always be a concern about the physiological relevance when using these approaches. The experiments proposed by the reviewer are good alternative to design experiments as close as possible to a physiological situation. Unfortunately, CRISPR knock in experiments are more challenging than KO ones, and the cell lines used are immortalized ones expressing neomycin as our constructs. Consequently, performing these experiments would require much more time than that given for a revision.

However, some data suggest that the effects observed are not merely an effect of overexpression:

1) The bioenergetics effect of ER-Mfn2 is lost when there is no mitochondrial Mfn, i.e. in Mfn1/2 DKO.

2) mt-Mfn2 overexpression does not have a bioenergetic effect on Mfn1/2 DKO cells, but its co-expression together with ER-Mfn2 restore the bioenergetics defect.

3) Mfn1 overexpression does not rescue bioenergetics defects in Mfn2 KO cells.

4) Restoration of ER-Mitochondria contacts in Mfn2 KO cells to physiological levels with ChiMERA also rescues bioenergetics defects.

- The analysis of the mitochondria-ER contacts is weak. Due to the discrepancy observed for the role of Mfn2 in the regulation of the contacts, colocalization analysis by manders coefficient should not be used. In addition, the images presented in the paper are really low quality raising the significance of the manders coefficient analysis calculated. Transmission electron microscopy analysis needs to be performed to analyse how the different conditions impact the distance, number and mitochondria-perimeter involved in these contacts. The authors propose a new method to analyse these contacts based on a split retina luciferase system, however this method has not been validated. There is no information about the correct localization of the 2 different plasmids, if they really detect mitochondria-ER contacts, if overexpression of the system forces the contacts... This system also needs to be tested on other cells silenced for others identified tethering prot/complex known to regulate mitochondria-ER contact (e.g: PTPIP51/VAPB, PDZD8....).

Now, we have analysed ER-mitochondria contacts by electron microscopy (EM) and found that, in agreement with Mander's colacalization and RLuc complementation assay, mitochondria of Mfn2 KO cells stablish less contacts with the ER that WT cells. Moreover, when there are contacts, these show more distance between the organelles in Mfn2 KO cells. Confirming that in our conditions Mfn2 is an ER-mitochondria tether.

mTORC2 promotes ER-mitochondria contacts (Betz *et al*, 2013), thus, we have manipulated mTOR pathway to prove that ERMITO-RLuc can sense changes in ER-mitochondria contacts. New Fig. EV2C and EV2D show that inhibition of mTOR diminished RLuc signal meanwhile activation of mTOR increases RLuc signal. The EM results also indicate that ERMITO-RLuc is a good reporter of ER-mitochondria contacts. Mfn2 KO cells have less contacts determined by EM and lower RLuc signal (Fig. 2) and expression of ChiMERA restores ER-mitochondria contacts and RLuc signal (Fig. 3).

- Calcium signalling analysis needs to be strongly reinforced. The authors need to stimulate ER-induced Ca++ release via IP3R (e.g. ATP, histamine) which is localised at the mito-ER contacts interface to analyse ER to mitochondria calcium fluxes. However, in this study the authors have used caffeine. Caffeine is widely associated to RYR stimulation (PMID: 18518861) and has been proposed to inhibit IP3R-induced calcium release (PMID: 1312323; PMID: 18518861; PMID: 20103623). The authors need also to analyse in the same time cytosolic calcium.

Now, we also show that IP3R stimulation also results in lower mitochondrial Ca^{2+} uptake in Mfn2 KO cells and its rescue by ChiMERA expression (Figs. EV2L, EV2M and EV3C).

In point 2, referee 1 has also raised some concern about the use of caffeine in our experiments. Please, read the response above for further clarification.

- The authors used in their study an artificial mito-ER tether characterized in the yeast model. Does this tether have been validated in mammal cells? In figure 3, how the authors explain that the tether does not increase mito-ER contacts when overexpressed in WT cells? Same question for mitochondrial calcium? Recently, different groups have used and validated artificial tether in mammal cells (e.g PMID: 29097544), why the authors decide to use the one characterized in yeast?

Excessive ER-mitochondria contact causes cell death. Thus, we reasoned that weak rather than strong expression of the tether might be more appropriate. Obviously, a yeast plasmid could produce very low expression. In fact, ChiMERA that consist in GFP flanked by ER and mitochondria location signals shows low expression (Figs. EV3A and EV3B). We tested it and found that, indeed, expression of ChiMERA restored ER-mitochondria contact both physically and functionally.

The simple answer to why ChiMERA does not increase mitochondrial Ca^{2+} in WT cells is that it does not increase ER-mitochondria contacts in WT cells. But why not? For now, we can only speculate as to the reason. As there are ER-mitochondria tethers, there are also spacers. Membrane proteins that bulge out from the ER or mitochondria outer membrane can limit distances between ER and mitochondria. In the case of Mfn2 KO cells, despite its low expression, ChiMERA can bring the ER and mitochondria closer together until it reaching a distance where it enters into competition with the spacers. In the case of WT, ChiMERA must compete from the outset with the spacers, and due to its low expression the balance is in favour of the spacers.

- In Fig 5 and S5, while I appreciate the rescue expts performed with ER-Mfn2 and mito-Mfn2 in Mfn1/2-DKO cells, the authors also need to perform these expts with WT-Mfn2.

Now, we have rescued the phenotype with full length Mfn2 in Mfn1/2 DKO and Mfn2 KO. These results are now shown in Appendix Figs. S4 and S5.

- The authors need to confirm their bioenergetics results using other methods that the Agilent kit e.g Seahorse analysis

We have analysed three bioenergetics parameters (OCR, MMP and ATP levels), so we assume the referee is referring to the OCR results. The MitoXpress oxygen-sensitive probe (Agilent kit) is a validated method to analyse oxygen consumption. It has been used by other researchers publishing in well-respected journals with rigorous peer-review procedures (Greene *et al*, 2012; Kalia *et al*, 2017; Lee *et al*, 2021; Namba, 2019; Will *et al*, 2006). It has also been accepted as a method to assess mitochondrial dysfunction in cellular models of neurodegenerative diseases in the guidelines adopted by prestigious researchers in the field (Connolly *et al*, 2018). Most importantly, in our experiments, it responded as expected to mitochondrial inhibitors and the results obtained are consistent, reproducible and similar to those obtained by other researchers studying the same cell model using the Seahorse (Hu *et al*, 2020). Having said that, to allay any concerns the referee may have about the Agilent kit, we ran Seahorse experiments on the four cell lines used in this study and observed similar results to these obtained using the Agilent kit (Appendix Fig. S1B).

- In Sup Figure 1, the authors used the overexpression of DN-Drp1 or Mfn1 to rescue mitochondrial morphology and conclude that the effect of the loss of Mfn2 on bioenergetics, mb potential, is morphology independent. The authors need to perform rescue experiments using the mito-targeted Mfn2 to really confirm their statement.

In the first version of the manuscript, we already showed that mt-Mfn2 restored mitochondrial morphology to the same extent as full length Mfn2 in Mfn1/2 DKO but had no bioenergetic effect.

- It has been proposed that the effects of Mfn2KO on calcium signaling was due to decreased level of the mitochondrial calcium uniporter, which is also associated to cell confluency (PMID: 27647893; PMID: 25870285). The authors need to check levels for the mitochondrial calcium uniporter complex machinery in their conditions of experiments, e.g cell confluency.

We have run protein extracts obtained from cultures in the same confluency used in our experiments and observed that Mfn2 KO cells express less MCU than WT cells. However, despite ChiMERA rescues mitochondrial Ca^{2+} uptake in Mfn2 KO cells, ChiMERA expression does not alter MCU levels (Fig. EV3N). These results indicate that, although reduced, MCU levels in Mfn2 KO cells are enough to uptake as much Ca^{2+} as in WT cells as far as the ER and mitochondria have the appropriate separation.

- In all the manuscript, it is difficult to confirm authors results based on the low quality of the images. In addition, representative images need to be shown in all the experiments based on microscopy analysis. The authors also need to show single channel as well as the overlay.

At the request of the referee, we have included new representative images. Because the figures are pretty overcrowded, these are mainly in EV or appendix suplementary figures. If the referee and editor consider that is necessary to move any figure to the main text we will be happy to do so.

- For mitochondrial morphology, the authors need to calculate different mitochondria parameters as length, number and area from their confocal images.

Now, we also show mitochondrial aspect ratio and circularity.

- For membrane potential analysis using TMRM, the authors need to use a second mitochondrial fluorescent marker as control and perform a ratio TMRM/mito marker to exclude any potential impact of mitochondrial mass variation.

For the analysis of MMP, ROIs of the same surface were drawn in areas completely occupied by mitochondria; thereby the results obtained are independent of total mitochondrial mass. This is now indicated in material and methods section. In any case, in former Fig. S2I we showed that there is no change in the levels of two commonly used mitochondria markers (VDAC and HSP60) indicating the same mitochondrial mass.

Minor points

- S1F: Mfn2 blot needs to be done. In all the manuscript, there is no IB to confirm the cell lines used by the authors (MFN2KO, MFN1Mfn2-DKO and overexpression).

In former figure S2I we characterized WT and Mfn2 KO cells. Now, we also characterize Mfn1 KO and Mfn1/2 KO cells. These results are shown in appendix Fig. S1A.

- In S3, the authors also need to analyse calcium fluxes in their different conditions of XeC, 2APB and MCU silencing.

In new Figs. EV3F and EV3G we show that in WT and Mfn2 KO cells treated with XeC and 2ABP there is not Ca^{2+} transfer from the ER to mitochondria and in Fig. EV3E that MCU KD prevents mitochondrial Ca^{2+} uptake when cells are treated with ATP.

- In Figure 6, the authors need to show the representative confocal images they used to analyse the different parameters.

At the request of the referee, we have included representative images in Appendix Fig. S6.

Referee #3:

This is an interesting manuscript presenting an intriguing set of results to support ER targeted Mfn2 role in mitochondrial function. The manuscript is clearly written. The main shortcoming is by the lack of mechanistic information.

We appreciate the referee's positive feedback.

The finding that Mfn2 Ko cells have impaired maximal bioenergetics capacity has been demonstrated in MEF cells. However in other cell types the results were mixed. On the other hand various studies show that Mfn2 deletion is increasing proton leak. Therefore, authors should look carefully at the possibility that this is a cell specific phenomenon.

In general is well accepted that Mfn2 is a regulator of mitochondrial metabolism. Mfn2 expression is especially elevated in tissues with high energetic requirements (Bach *et al*, 2003; Eura *et al*, 2003), which suggests a bioenergetics role. Mfn2 KO or KD in different cell types has been shown to impair mitochondrial bioenergetics (Bach *et al.*, 2003; Boutant *et al*, 2017; Martorell-Riera *et al*, 2014; Schneeberger *et al*, 2013; Sebastián *et al*, 2012; Segales *et al*, 2013; Tur *et al*, 2020; Xu *et al*, 2020).

Although increased proton leak has been described in Mfn2 KD skeletal muscle cells (Segales *et al.*, 2013) or 10T1/2 fibroblast (Bach *et al.*, 2003) (which retain the potential for myogenic differentiation (Salvatori *et al*, 1995)), no increase in proton leak has been reported in neurons (Schneeberger *et al.*, 2013), brown adipocytes (Boutant *et al.*, 2017), chondrocytes (Xu *et al.*, 2020) or macrophages (Tur *et al.*, 2020). Indicating that increased proton leak in Mfn2 KO cells seem rather specific of the muscular linage.

To confirm that our findings are not limited to MEFs, we have made Mfn2 KO astrocytes and observed reduced MMP, ATP levels, basal and maximal oxygen consumption with no change in proton leak. As in MEFs, impaired bioenergetics was rescued by expressing ChiMERA (new Fig. EV3O-R).

It is important to present the maximal respiratory capacity values in the main figures so that one can conclude if there is a respiratory dysfunction.

Now, we have also included basal, maximal and proton leak respiration in the main figures.

The respirometry results cannot be interpreted without an assessment of mitochondrial mass

Respirometry has been normalized to the amount of protein. In Appendix Fig. S2H (former Fig. S2I) it is shown that there is no change in mitochondrial mass between WT and Mfn2 KO

In figure 1 the authors show reduction in TMRM fluorescence in Mfn2 KO in panel C, but the image shows reduction in Mfn1 KO cells. The experiment in panel C of this figure is lacking a positive and negative controls using Oligomycin and CCCP. Authors should also verify that TMRM in cell did not reach the level that induces quenching.

We fear that on this occasion the referee may not have properly understood the figure legend. Panel B show representative images of mitochondrial morphology, not MMP. We have now switched the order of the panels to avoid any further confusions.

Representative images of TMRM fluorescence in untreated and CCCP and oligomycin treated cells are shown in Fig. R1.

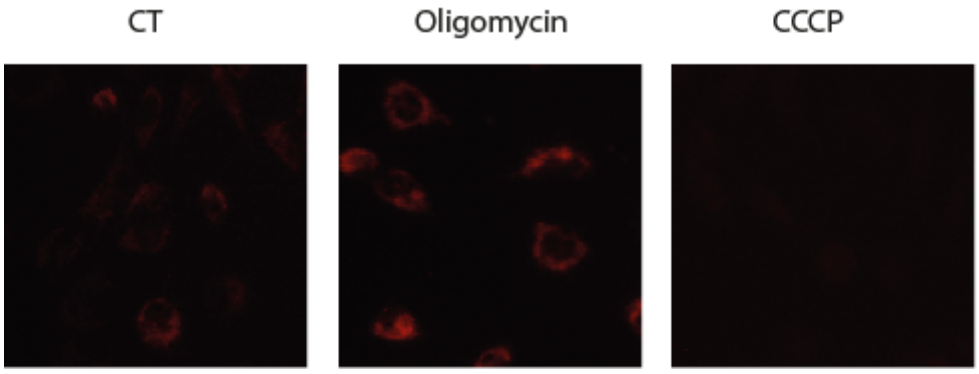

Fig. R1*. TMRM fluorescence after treatment with the indicated drugs.*

As stated in material and methods, the TMRM was used at a non-quenching concentration (6 nM). TMRM fluorescence absorbance and the effect of CCCP in the cell lines used in this study are shown in Fig. R2.

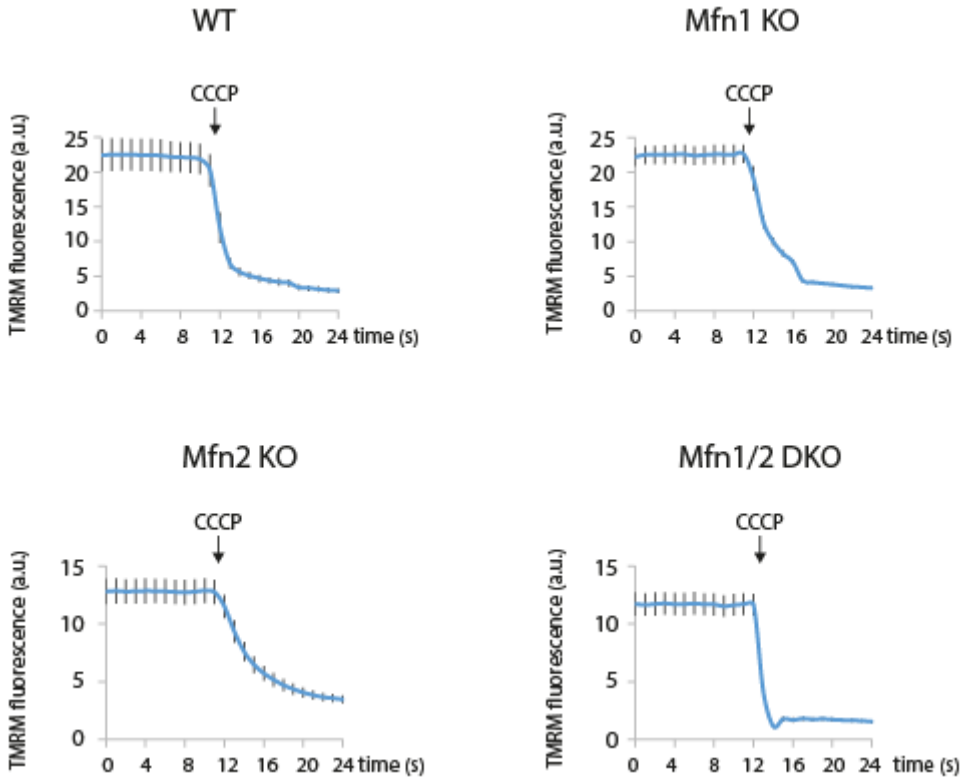

Fig. R2. *Mitochondrial depolarization causes loss of TMRM fluorescence, indicating non-quenching concentration.*

The restoration of Mfn2 using ER -targeted Mfn2 is an interesting approach. However, one has to demonstrate that indeed the protein reached the ER and not the mitochondria.

Can the authors isolate ER and mitochondria and demonstrate that the transfected Mfn2 was in the ER and not in the mitochondria?

The location of the ER-Mfn2 Mfn2 has already been characterised by both imaging and cell fractioning (de Brito & Scorrano, 2008; Rojo *et al*, 2002). In the first version of the manuscript, we also confirmed the localization of ER-Mfn2 by immunofluorescence (former Fig. S4A, new Fig. EV4).

As with figure 1, it is essential in figure 4 to show the values of basal, maximal and leak respiration.

These results were presented in former Fig. S4A, now it is shown in the main Figure 4.

The manuscript does not provide any insight into the mechanism by which these constructs restore bioenergetics functions.

The proposed mechanism by which ER-located Mfn2 regulates mitochondrial bioenergetics is by stablishing contacts with mitochondria-located Mfns to allow Ca^{2+} transfer from the ER to mitochondria. Since the 1970's is known that Ca^{2+} regulate mitochondrial metabolism by

activating PDH via its dephosphorylation and activating dehydrogenases of the tricarboxylic acid cycle (TCA). Since then, many papers have shown Ca^{2+}-dependent increase in mitochondrial metabolism (reviewed in (Rossi *et al*, 2019)).

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1st Revision - Editorial Decision 4th Jun 2021

Dear Francesc,

Thank you for submitting your revised manuscript. It has now been seen by all of the original referees.

I apologize for the delay in getting back to you, it took longer than anticipated to receive the referee reports.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. However, referee #3 finds that currently, the data do not conclusively demonstrate the proposed mitochondrial calcium based mechanism explaining the reduced OCR of Mfn2 deficient cells and requests additional experimental support. I have discussed these concerns further with referee #1. We have decided that additional experimentation is not required, however, the text needs to be significantly revised to clarify that the bioenergetic defects associated with Mfn2 or mitochondrial calcium import are context-dependent and not as universal as the manuscript currently describes. For example, the data currently do not exclude that Mfn2 depletion might cause effects in the synthesis of ubiquinone as proposed by N Goran-Larsson's group.

Please address all of the remaining referee concerns and provide a point-by-point response. Please let me know if you would like to discuss any of the points further.

In addition, I need you to address the editorial points below:

• As per our guidelines, please add a 'Data Availability Section', where you give information about the primary datasets produced in this study that are deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>). If it is not applicable, make a statement that no data were deposited in a public database.
• Please complete the funding information into the manuscript submission system, too.
• We note the following regarding the figure callouts: panels of Fig EV4, Appendix Fig S4C, F, I and S5B-G,H,I are currently not called out in the text.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Casellas-Díaz et al. have made a substantial, and generally successful, attempt at addressing the previous concerns with the original manuscript. Of note, they have added supporting evidence to the causality of the bioenergetic defects in the Mfn2 knockout cells (showing for instance that, for bioenergetics, MCU knock down phenocopies Mfn2-KO, and that both perturbations are not additive) and strengthened their work on calcium dynamics. Before publication, a couple of text-based changes should be considered as they would further improve the understanding of some of the conflicting calcium data in the literature and this paper:

1. The conflicting data surrounding the viability of MCU knockout mice needs to be added to the discussion, especially if the authors are to link their work to human diseases (notably, CMT2A-associated mutations in Mfn2 being linked to bioenergetic defects). If the link between mitochondrial calcium uptake and bioenergetics is not reproducible in different mice genetic background, it makes extrapolation of this concept in humans much more tenuous than is currently discussed.
2. The authors add some key data regarding the difference in basal ER-calcium between wild-type and Mfn2 knockout cells, which is in agreement with de Brito & Scorrano (2008). The data are however very discrepant from those in de Brito & Scorrano (2008) when it comes to increased mitochondrial calcium uptake. As a reminder, something widely overlooked is that de Brito and Scorrano had to use 20-fold less ATP to stimulate Mfn2-KO cells. If they used the same amount, they actually observe an increase, in mitochondrial calcium uptake by Mfn2-KO cells. Here, with same ATP concentrations, a decrease is observed. While it is beyond the point of this manuscript to investigate this discrepancy, it should be thoroughly noted to allow readers to make their own idea of the reproducibility of such assays. This is very important given the argument surrounding the role of Mfn2 as ER-mitochondria tether. A lot of the disagreement in the field likely stems from this sort of non-reproducible assays. It is thus important to expose this clearly.
3. Line 175: "indicated that ER-mitochondria contact is lost in Mfn2 KO cells" would be more rigorous as "indicated that ER-mitochondria contact is reduced in Mfn2 KO cells".
4. Finally, the authors utilize caffeine to cause RyR-driven ER calcium release. They now have added controls that RyR is indeed responsible for the observed phenomenon, and they show that similar results can be obtained by stimulating IP3R instead. Nevertheless, It remains unclear why RyR stimulation was used in the first place since, the physiological source of calcium is in fact the IP3R (as shown by the XestC and 2ABP experiments). Therefore, the decreases mitochondrial calcium uptake upon IP3R (physiological) stimulation is more conceptually important than that observed upon RyR stimulation (the physiological relevance of which is not established). It is therefore odd that caffein experiments are in the main figures while ATP and Histamine experiments are in the supplement.

Referee #2:

Thanks to the authors who performed an extensive revision of their manuscript. They have addressed most of my concerns related to the function of ER-Mfn2 on different aspects of mitochondrial homeostasis, and from this aspect I support publication.

However, while I find the study interesting, I am still concerned, as already stated in my original report, about the quality of the images shown in the manuscript. The images are indeed very low quality and in some of them it is in fact really difficult to distinguish between signal and noise (in particular for the images shown in Appendix Sup Figures, S4A, S3B, S5A), raising the question of how the quantification has been performed. In addition, the authors should check that all the images in the same panel for each marker have the same contrast/luminosity settings, which is primordial for quantification, but also for the preparation of the figures.
Finally, an effort could be made in the presentation of these images, where we can only observe a very small area of the cell, which lead to difficulties to have a general idea of the phenotypes described by the authors. (Fig 1A, 3M, 4C, EV1C, G, EV5A, S3B, S4A, S5A).

Referee #3:

The authors only partially addressed referee#3.
The literature review provided by the authors is biased by the conclusion of the manuscript. There are a variety of studies that show no reduction in max-OCR in Mfn2-/-. This needs to be addressed in the text and in the conclusions.
The authors did not provide any insight into the mechanism of what the authors consider a well-established phenomenon (that Mfn2-/- has a bioenergetic defect and that the mechanism is the lack of Ca+2 accessibility to mitochondria, leading to mal-activation of the TCA cycle). Authors show no data to support the claim that Mfn2-/- bioenergeitc defect is due to reduced Ca activation of TCA cycle.
Previous studies reported that a bioenergetic defect in MEFs has nothing to do with Ca+2, but rather with synthesis of elements in the respiratory chain (Nils-Göran Larsson). As such, the assumption that the mechanism of impaired bioenergetics is Ca+2 transport is not backed up by previous publications and needs to be addressed experimentally.

Referee #1:

Casellas-Díaz et al. have made a substantial, and generally successful, attempt at addressing the previous concerns with the original manuscript. Of note, they have added supporting evidence to the causality of the bioenergetic defects in the Mfn2 knockout cells (showing for instance that, for bioenergetics, MCU knock down phenocopies Mfn2-KO, and that both perturbations are not additive) and strengthened their work on calcium dynamics. Before publication, a couple of text-based changes should be considered as they would further improve the understanding of some of the conflicting calcium data in the literature and this paper:

1. The conflicting data surrounding the viability of MCU knockout mice needs to be added to the discussion, especially if the authors are to link their work to human diseases (notably, CMT2A-associated mutations in Mfn2 being linked to bioenergetic defects). If the link between mitochondrial calcium uptake and bioenergetics is not reproducible in different mice genetic background, it makes extrapolation of this concept in humans much more tenuous than is currently discussed.

Now, we discuss the conflicting data surrounding the viability of MCU KO mice.

2. The authors add some key data regarding the difference in basal ER-calcium between wild-type and Mfn2 knockout cells, which is in agreement with de Brito & Scorrano (2008). The data are however very discrepant from those in de Brito & Scorrano (2008) when it comes to increased mitochondrial calcium uptake. As a reminder, something widely overlooked is that de Brito and Scorrano had to use 20-fold less ATP to stimulate Mfn2-KO cells. If they used the same amount, they actually observe an increase, in mitochondrial calcium uptake by Mfn2-KO cells. Here, with same ATP concentrations, a decrease is observed. While it is beyond the point of this manuscript to investigate this discrepancy, it should be thoroughly noted to allow readers to make their own idea of the reproducibility of such assays. This is very important given the argument surrounding the role of Mfn2 as ER-mitochondria tether. A lot of the disagreement in the field likely stems from this sort of non-reproducible assays. It is thus important to expose this clearly.

We now expose the discrepancies between ours and de Brito and Scorrano results.

3. Line 175: "indicated that ER-mitochondria contact is lost in Mfn2 KO cells" would be more rigorous as "indicated that ER-mitochondria contact is reduced in Mfn2 KO cells".

We thank the referee for pointing this mistake.

4. Finally, the authors utilize caffeine to cause RyR-driven ER calcium release. They now have added controls that RyR is indeed responsible for the observed phenomenon, and they show that similar results can be obtained by stimulating IP3R instead. Nevertheless, It remains unclear why RyR stimulation was used in the first place since, the physiological source of calcium is in fact the IP3R (as shown by the XestC and 2ABP experiments). Therefore, the decreases mitochondrial calcium uptake upon IP3R (physiological) stimulation is more conceptually important than that

observed upon RyR stimulation (the physiological relevance of which is not established). It is therefore odd that caffein experiments are in the main figures while ATP and Histamine experiments are in the supplement.

We have moved the ATP and histamine results to the main figures.

Referee #2:

Thanks to the authors who performed an extensive revision of their manuscript. They have addressed most of my concerns related to the function of ER-Mfn2 on different aspects of mitochondrial homeostasis, and from this aspect I support publication.

However, while I find the study interesting, I am still concerned, as already stated in my original report, about the quality of the images shown in the manuscript. The images are indeed very low quality and in some of them it is in fact really difficult to distinguish between signal and noise (in particular for the images shown in Appendix Sup Figures, S4A, S3B, S5A), raising the question of how the quantification has been performed. In addition, the authors should check that all the images in the same panel for each marker have the same contrast/luminosity settings, which is primordial for quantification, but also for the preparation of the figures.

Finally, an effort could be made in the presentation of these images, where we can only observe a very small area of the cell, which lead to difficulties to have a general idea of the phenotypes described by the authors. (Fig 1A, 3M, 4C, EV1C, G, EV5A, S3B, S4A, S5A).

Images within the same experiment were taken and analyzed with the same settings. Now, we specify it in Material and Methods section. Note that **t**he Mander's coefficient results correlate well with the split Rluc results; and, when analyzed, with EM results as wells.

To facilitate interpretation of the images, now we show entire cells.

Referee #3:

The authors only partially addressed referee#3.

The literature review provided by the authors is biased by the conclusion of the manuscript. There are a variety of studies that show no reduction in max-OCR in Mfn2-/-. This needs to be addressed in the text and in the conclusions.

I would have appreciate that the referee had cited some of the studies showing no reduction in max-OCR in Mfn2 KO cells to discuss them, since most of the results from different laboratories indicate that the absence of Mfn2 has a negative role on mitochondrial metabolism. This is logical, considering that Mfn2 show higher expression in tissues with higher energetic requirements [1, 2] or that it is induced in conditions where there is an increased energy demand [3, 4]. However, it is true that there are some conflicting reports such as Kaweleck et al [5] and Thaher et al [6] that we already discussed in the very first version of the manuscript. Now, we cite and discuss other articles.

The authors did not provide any insight into the mechanism of what the authors consider a well-established phenomenon (that Mfn2-/- has a bioenergetic defect and that the mechanism is the lack of Ca+2 accessibility to mitochondria, leading to mal-activation of the TCA cycle). Authors show no data to support the claim that Mfn2-/- bioenergeitc defect is due to reduced Ca activation of TCA cycle.

Previous studies reported that a bioenergetic defect in MEFs has nothing to do with Ca+2, but rather with synthesis of elements in the respiratory chain (Nils-Göran Larsson). As such, the assumption that the mechanism of impaired bioenergetics is Ca+2 transport is not backed up by previous publications and needs to be addressed experimentally.

The study by Nils-Göran Larsson's lab [7] is another example where it is observed impaired OCR in Mfn2 KO cells. In this article, the authors show reduction of mitochondrial coenzyme Q levels in Mfn2 cells with the consequent impairment in mitochondrial respiration.

In the first version of the manuscript we already discussed this article and stated that our results did not exclude the participation of Mfn2 in other mechanism described elsewhere and that they could be downstream effects of alterations in the ER-mitochondria contact. For instance, coenzyme Q biosynthetic proteins assemble into domains at ER-mitochondria contact [8]. What we propose is that the *primum movens* is the regulation of mitochondrial Ca2+ levels whose role in enhancing mitochondrial metabolism to feed the electron transport chain is well-documented in the literature. To reach this conclusion we are based in that the mitochondrial Ca2+ levels and bioenergetics defects in Mfn2 KO cells can be rescued by expressing ER-targeted Mfn2 and by artificially tethering ER and mitochondria, but blocking the Ca2+ transfer from the ER to the mitochondria (inhibiting IP3R or knocking down MCU) the rescue is lost.

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2nd Revision - Editorial Decision 23rd Jun 2021

Dear Francesc,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz Senyilmaz Tiebe
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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No sample size was chosen. If after 3-4 independent experiments there was a clear tendency to be significant but not reached yet because the inter-experimental variability another repeats were done
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Animals were only used to make primary cultures
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Neuronal morphometric and electron microscopy analyses were performed blindly
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Neuronal morphometric and electron microscopy analyses were performed blindly
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Kolmogorov-Smirnov Test was used to determine normality
Is there an estimate of variation within each group of data?	No

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Pregnant Sprague-Dawley rats were used to obtain neuronal primary cultures. C57BL/6J mice possessing loxP sites on either side of exon 6 of the Mfn2 gene expressing or not the CRE-ERT under the ubiquitin promoter. Embryos were used for primary cultures. Rats and mice were kept under specific pathogen-free conditions at the animal facility at the University of Barcelona
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All the experimental procedures were approved by the Research Ethics Committee at the University of Barcelona and they were carried out in accordance with Spanish and European guidelines (B.O.E, 18 March 1988, and 86/609/EEC European Council Directives).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	This study did not involve human research participants or samples.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	This study did not involve human research participants or samples.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	This study did not involve human research participants or samples.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	This study did not involve human research participants or samples.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	This study did not involve human research participants or samples.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	This study did not involve human research participants or samples.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	This study did not involve human research participants or samples.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No data was deposited in a public database
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	No data sets were generated
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	No computational models were used

G- Dual use research of concern

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