EMBO reports

Chemical targeting of NEET proteins reveals their function in mitochondrial morphodynamics

Diana Molino, Irene Pila-Castellanos, Henri-Baptiste Marjault, Nivea Dias Amoedo, Katja Kopp, Leila Rochin, Ola Karmi, Yang-Sung Sohn, Laetitia Lines, Ahmed Hamaï, Stephane Joly, Pauline Radreau, Jacky Vonderscher, Patrice Codogno, Francesca Giordano, Peter Machin, Rodrigue Rossignol, Eric Meldrum, Damien Arnoult, Alessia Ruggieri, Rachel Nechushtai, Benoit De Chassey and Etienne Morel
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Corresponding author(s): Etienne Morel (etienne.morel@inserm.fr) , Benoit De Chassey (bdc@enyopharma.com)

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1st Editorial Decision 19th Sep 2019

Dear Dr. Morel,

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 the delay in getting back to you, it took longer than anticipated to receive the full set of referee reports due to this busy time of the year.

As you can see, the referees express interest in the proposed role of Mito-C in regulation of mitochondrial morphology and Dengue virus replication. However, they also raise a number of concerns that need to be addressed to consider publication here.

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

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

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

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

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

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

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

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

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

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

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

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

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

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

9) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

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

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

Data availability

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

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

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

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

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

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 Molino and colleagues reports the role of a new heterocyclic compound, MITO-

C, in mitochondrial dynamics regulation. They proposed that MITO-C induces a rapid and reversible fragmentation of the mitochondrial network without affecting mitochondrial respiration, membrane potential and cell death. Using a photo-affinity labelling based method, the authors report that MITO-C can interact with the NEET proteins and impact their capacity to regulate mitochondrial iron-sulfur transport. Using immunofluorescence analysis, they propose that MITO-C follows the same subcellular localization of NAF1 and that the fragmented network is directly due to the MITO-C -NAF1 interaction and an increase of Drp1 recruitment to the mitochondrial membrane. Finally, they show that MITO-C decreases mitochondrial hyperfusion induced by Dengue virus and also decreases its viral replication.

The main novelty of this study is the synthesis and characterization of the compound MITO-C leading to a rapid mitochondrial fragmentation without impacting other mitochondrial functions. However, the description of MITO-C synthesis, its characterization and the identification of potential partners are lacking in the paper. In addition, the subcellular localization of MITO-C and its effect on mitochondrial dynamics specifically via NAF-1 interaction are not fully convincing. Finally, even if the role of NAF1 (or MitoNEET) in mitochondrial morphology and the role of mitochondrial morphology in virus infection are interesting, they have already been reported (PMID: 28335035; PMID: 28716905; PMID: 23703906; PMID: 27545046, PMID: 27816895). There are other critical points to address in order for the work to be considered in EMBO reports as quality of the images, conclusions should be dampened...

Major points
1. The authors should show in figures and in the results section the different steps of production and purification of both compounds, MITO-C and MITO-N. A better characterization of both compounds should be done on mitochondrial morphology with different time and dose points. Authors should show that MITO-N does not induce mitochondrial fragmentation. Also, why? Does MITO-N interact with the NEET proteins? Does MITO-N induce mitochondrial fragmentation at higher concentration or time point? Does MITO-N localized to mito, ER or both? If MITO-N interacts with NAF1 and modulates its function on iron-sulfur transport, why It has no effect on mitochondrial morphology? How the authors explain the difference of functions between MITO-C and MITO-N?

2. The authors used a new parameter to analyse mitochondrial morphology, "skewness" based on symmetry. Appropriate controls should be performed to validate this new method of analysis using for example siOPA1 and siDrp1. In any case, through the manuscript the authors should use other parameters to analyse mitochondria morphology as percentage of cells with phenotype and also calculate mitochondrial length and area, as it is usually performed in the field. In addition, the authors should perform z-stack 3D reconstitution of the mitochondrial network before quantification. Single plan is not relevant to analyse mitochondrial morphology by confocal microscopy.

3. Results of the protein binding assay should be shown in the results section for both compounds (MITO-C and MITO-N). This is a critical point as the following of the study is based on this result. What are the other hits? Do the compounds interact also with NAF1 and mitoNEET, or only MiNT? What are the experimental conditions? Controls? For example, the authors should perform knockdown experiments of other top hits and analyse mitochondrial morphology to further reinforce the specific link between MITO-C and NAF1 in mitochondrial dynamics.

4. The localization experiments of NAF1 and MITO-C by microscopy are not convincing. The quality of the images should be improved and z-stack images should be acquired. In addition, triple labelling of mitochondria, ER and NAF-1 (or MITO-C) should be performed in the same time (Fig S4, Fig S5).

PTPIP51 is a mitochondrial protein, which can be localized at the mito-ER interface, but it should not be used as a mito-ER contact marker. Finally, MITO-C localization is unclear. In the text, the authors say that MITO-C is localized at both mitochondria and ER (confirmed by Fig S5). However, they also said that NAF-1 and MITO-C colocalize and share the same compartment (perfect colocalization in Fig 1E). However, NAF-1 is clearly an ER protein, which only colocalize at the mito at the mito ER contact sites. How the authors explain the 100% colocalization?

5. A better characterization of how MITO-C and NAF-1 induce mitochondrial fragmentation should be performed. For example, does MITO-C (and MITO-N) treatment (1) modulate protein levels of the NEET family? (2) impact mitochondria/ER or MAM localization of NAF1 (by microscopy or biochemical MAM fractionation)? (3) increase fragmentation level induced by loss of NAF1? (4) Induce fragmentation in cells silenced for CISD1 or CISD3? Does NAF-1 interacts with Drp1? And does this interaction is lost upon MITO-C treatment (and not MITO-N)?

6. To reinforce that NAF-1 and MITO-C are in the same pathway, other functions associated to NAF1 and already described related to mitochondrial morphology regulation should be analysed (mitochondria-ER contacts, autophagy, ER stress). Indeed, loss of NAF1 has been associated to mitochondria-ER contacts (PMID: 28335035), which are involved in mitochondrial calcium and mitochondrial morphology regulation.

7. The authors should also analyse Drp1-P-637, which has been shown to regulate mitochondrial morphology (PMID: 18838687) to potentially explained their mechanism of fragmentation. Indeed, dephosphorylation of Drp1-P-637 by calcineurin increases its mitochondrial recruitment and mitochondrial fragmentation. NAF1 regulates intracellular calcium homeostasis (PMID: 24833725; PMID: 28335035) and its loss leads to increased cytosolic calcium and calcineurin activity (PMID: 24833725).

8. The authors observed a cleavage of OPA1 at 60/120 mns after addition of the compound, but observed mitochondrial fragmentation at 15 mns. As already mentioned, does the fragmentation is increased at 2 hours? What the conclusion of the authors? In addition, OPA1 has been involved in cristae organization stabilizing complexes of the respiratory chain. Does treatment of MITO-C and silencing of NAF1 lead to cristae disorganization by electron microscopy analysis.

9. As explained by the authors, the role of SAMM50 on mitochondrial morphology and Drp1 have already been shown. I don't see the relevance of this figure in the current version of the paper.

10. Mitochondrial morphology should be quantified in Figure 4. What is the effect of MITO-N, and silencing of NAF1 and MitoNEET on mitochondrial morphology after Dengue infection and on its viral replication?

Minor points
1. Production and purification of recombinant proteins used in figure 1D should be shown.

2. A more detailed mitochondrial bioenergetics analysis should be performed. In addition to basal respiration, ATP-linked respiration, maximal respiration, spare respiratory capacity and proton leak should also be measured using Olygomycin, FCCP, Antimycin A and rotenone treatments. What is the effect of NAF1 loss on mitochondrial bioenergetics? While Mitotracker can be used to estimate mitochondrial mass, mitochondrial membrane potential analysis should be performed using JC-1 and/or TMRM probes.

3. In figure 2, the authors should analyse the effect of each siRNA on the protein levels of the 2 other members of the family.

4. In figure 2, silencing of MitoNEET does not lead to mitochondrial morphology defects. However, it has been recently shown that MitoNEET-KO induces mitochondrial fragmentation (PMID: 28716905). Can the authors propose an explanation for this discrepancy?

5. Figure legend of Fig S6 should be modified according to panels.

6. In figure 3A, the authors should use both an ER and mitochondrial marker to demonstrate that NAF-1 and Drp1 colocalize together at these sites. Otherwise, the authors can use fluorescent mito-ER contact probe reporter (PMID: 29229997).

7. Figure 3D. Total Drp1 (input) should also be shown. Cytosolic loading control is also required. Does NAF-1 loss lead to increase Drp1 at mitochondria by IF and fractionation?

8. In figure 3F, it is difficult to observe effect of DN-Drp1 expression on mitochondria morphology. The authors should fuse directly the fluorophore to it and modify the panel.

9. References to other studies on NEET proteins and mitochondrial dynamics should be cited through all the manuscript and discussed.

10. In the abstract, the authors say "NAF-1... facilitating recruitment of Drp1...". Based on their data, NAF1 decreases Drp1 recruitment to mitochondria.

Referee #2:

In this manuscript, Molino et al, established the role of Mito-C in mitochondrial dynamics, and explored the involvement of NEET proteins in this process. The study is interesting, well-conducted and of interest as it directly 1) identifies a small molecule inhibitor (chemical) that can affect mitochondrial dynamics and 2) provides evidence that the mode of action is possibly mediated through NEET proteins.
In general, this study is well organized and well presented. However, there are few palces where the provided data either do not reconcile well with the proposed model or are in slight disagreement with the already established consensus in the field. Inclusion of few additional controls and explanation will definitely improve the impact of this article. Regarding viral assays- 2uM Mito-C in 15 min seems sufficient to cause fragmentation and for viral assays, the same concentration was kept for 72H. The concern is that- the dose/ duration parameters are not uniform. In other words, the authors need to find out minimum effective concentration that can cause fragmentation after 48H (it would possibly be much less than 2uM as 2uM conc. caused fragmentation within just 15 min) and use that conc. for viral assays. In viral assays, if the effect of Mito-C is indeed mediated through mitochondrial dynamics then that minimum conc. should affect flavivirus replication. And if not, then the authors need to convince the readers why it can not be a pleotropic effect.
My specific comments are given below-
1. The claim based on Fig S2a needs to be reconsidered. Even if 20uM compound is not cytotoxic, it could be cytostatic. For such experiments, Annexin V should be presented along with the cell proliferation data. Any simple experiment will serve this purpose, such as a flow cytometry with nuclear stain to see the proportion of cells in G1/G2, S or M phase. The cytostatic effect has to be

ruled out as the authors are finally validating this finding in viral model which is highly sensitive to cell proliferation.
2. The authors need to categorically mention/ or provide evidence pertaining to the effects of various modifications they incorporated in Mito-C. The specific concerns here are- have the authors evaluated the effects of modified Mito-C? In other words, if a photo-reactive group (please provide specific group) or nitrobenzofurazan group is added, these modified Mito-Cs still exert similar effects as Mito-C. This question becomes pertinent in the light of Fig S5 where the extent of fragmentation does not appear to be as significant as I see in Fig 1B. Please also provide details of Mito-N.
3. For Fig S6, addition of FCCP or CCCP as positive control would have been better.
4. Fig S7 appears to be a major deviation from the proposed model here and the data here do not reconcile well with the data given elsewhere in this article. 2uM Mito-C for 15 minutes causes mitochondrial fragmentation (Fig 1b). In contrast, Drp-1-S616 phosphorylation is not affected at this time points (in fact any time point). Do the authors have any explanation to support this claim where merely upregulation of Drp1 (without S616 phosphorylation) can cause fragmentation?
5. In addition, the authors also included Opa-1 expression, which also remains unchanged (long or short) till 30 min of exposure with no change in p-DRP1 level. How the mitochondrial fragmentation is occurring at 15 min of Mito-C exposure without phosphorylation of DRP1 (Fig S7a); no change in OPA1 (Fig S7d) and also no change in mitochondrial membrane potential (Fig S6b, c)? This needs to be clarified. Do the authors propose some other hypothesis for Mito fragmentation without all these?
6. Please examine the Drp-1-S616 phosphorylation levels in the context of Fig 2a, b, c and Fig S8a. This will make sure that the events reported here, such as involvement of NEET, SAMM50 are indeed comparable to Mito-C and should also not affect S616 phosphorylation.
7. Any specific reason to opt for 10uM Mito-C while 2uM appeared to be sufficient? Although, 2uM Mito-C caused enough mitochondrial fragmentation, the virus titer for all three viruses went down only with 10uM Mito-C (five times more concentrated). How to explain this? Also, at which point Mito-C was added in these expts. (how long was the Mito-C treatment done?).
8. Fig. 4a, please make it clear what time point the samples were analyzed. As per the legend (72h post infection) while the methods say 48 h.
9. Fig 3d- Could the authors check level of p-DRP1 level in the cytosolic and Mito fractions?
10. Fig 1b- a better representative blot for NAF-1 knockdown would be more appropriate.
11. Figure legends for S3 and S4 and S5 are switched.
12. In the methods section- "Flavivirus virus" change it to Flavivirus infection

Referee #3:

This is an exciting, well written study.In this study the authors present a novel benzothiophene compound, Mito-C, which interferes with cellular iron metabolism mediated by a member of the family of NEET proteins, NAF1. The study is well-conducted and the research is overall conclusive. The reduction of viral replication (Dengue, West Nile, Zika) by Mito-C is especially intriguing giving rise to the possibility of novel approaches to combat neglected tropical diseases. There are some remaining points of criticism.
Fig 1c. The choice of skewness as a measure of mitochondrial fragmentation is rather unorthodox. It is unclear how the skewness values correlate with more intuitive morphology measurements such as circularity and aspect ratio. A direct comparison with these measurements would be helpful to establish the validity of the skewness as a read-out of mitochondrial fragmentation.
The effect of Mito-C treatment of cellular NAF1 protein levels has not been established making it

impossible to determine whether a reduction in NAF1 levels or iron-sulfur cluster transfer is responsible for Mito-C mechanism of action.
Authors mention the use of mass spectrometry to identify binding-partners of Mito-C, but do not present supporting data. The binding is inferred through a functional assay of NEET protein-iron binding. It would be helpful if the authors could include the relevant mass spectrometry traces.
It is unclear whether the real effect on mitochondrial fragmentation is based on DRP1 activity given the lack of increase in active DRP1-S616 under Mito-C treatment or changes in OPA1 isoform homeostasis. In order to conclusively prove the dependence of NAF1 driven mitochondrial fragmentation on DRP1 the authors should conduct a knock-down of NAF1 under DRP1-DN overexpression similar to the experiment with Mito-C in Fig 3.f. In addition, levels of Mfn2 should be monitored during Mito-C treatment.
Testing MitoC in models such as NAF1 Knockdown or over expression can further strengthen the claims
Testing MitoC in OMA1-KD could potentially be used to establish the role of OPA1 processing in the observed result.
The authors claim that the compound is non-toxic; however, a reduction in long-form Opa1 levels as seen in Fig.S7d etc. has been linked to mitochondrial respiratory super-complex disassembly and reduction in complex I driven respiration and several mitochondrial diseases. HeLa cells can completely rely on glycolysis or switching to complex II driven respiration to avoid mitochondrial toxicity. The authors should present ECAR data for the runs conducted for Fig. S2A. The ratio between OCR and ECAR can serve as an indicator for how much these cells are indeed relying on OXPHOS. Additionally, if the compound is indeed not toxic to mitochondria, the cells should be able to grow in Galactose media under compound treatment, if there is a mitochondrial toxicity growth in galactose would unmask it based on the absolute reliance of the cells on mitochondrial respiration for ATP generation. Similarly, it would be valuable to show that there is no increase in mitochondrial ROS production.
Fig.S8 The relevance of SAMM50 in the context of the present study is unclear. Unless SAMM50 levels or functionality can be influenced by Mito-C, I do not see the reason for these data to be included in the present study.

Referee #1:

The manuscript by Molino and colleagues reports the role of a new heterocyclic compound, MITO-C, in mitochondrial dynamics regulation. They proposed that MITO-C induces a rapid and reversible fragmentation of the mitochondrial network without affecting mitochondrial respiration, membrane potential and cell death. Using a photo-affinity labelling based method, the authors report that MITO-C can interact with the NEET proteins and impact their capacity to regulate mitochondrial iron-sulfur transport. Using immunofluorescence analysis, they propose that MITO-C follows the same subcellular localization of NAF1 and that the fragmented network is directly due to the MITO-C -NAF1 interaction and an increase of Drp1 recruitment to the mitochondrial membrane. Finally, they show that MITO-C decreases mitochondrial hyperfusion induced by Dengue virus and also decreases its viral replication.

The main novelty of this study is the synthesis and characterization of the compound MITO-C leading to a rapid mitochondrial fragmentation without impacting other mitochondrial functions. However, the description of MITO-C synthesis, its characterization and the identification of potential partners are lacking in the paper. In addition, the subcellular localization of MITO-C and its effect on mitochondrial dynamics specifically via NAF-1 interaction are not fully convincing. Finally, even if the role of NAF1 (or MitoNEET) in mitochondrial morphology and the role of mitochondrial morphology in virus infection are interesting, they have already been reported (PMID: 28335035; PMID: 28716905; PMID: 23703906; PMID: 27545046, PMID: 27816895). There are other critical points to address in order for the work to be considered in EMBO reports as quality of the images, conclusions should be dampened...

Thanks to the helpful comments and detailed suggestions of this reviewer, we believe that our revised manuscript now fulfills the required quality levels of experimental data, controls and mechanisms' discussion needed for the readers of Embo reports, to highlight the interest of using a new compound such as MITO-C to investigate molecular events that participate in mitochondrial morphodynamics regulation. More particularly, as described in the point-by-point answer to reviewer's comments (below), we are now showing information about the MITO-C synthesis, the detailed procedure about identification of MITO-C cellular targets and a better characterization of subcellular localization of MITO-C and relationship with NAF-1/NEET protein(s). Importantly, our new data about bioenergetics characterization (respiration, toxicity, ATP, etc.) of cells treated with MITO-C also provide evidence that the mitochondrial fragmentation induced by our compound is not a consequence of mitochondrial respiration impairment. Finally, we characterized the impact of MITO-C on mitochondria membranes by data unveiling the complex interplay of MITO-C and NAF1 with ER-mitochondria contact sites dynamics.

Major points:

1. The authors should show in figures and in the results section the different steps of production and purification of both compounds, MITO-C and MITO-N. A better characterization of both compounds should be done on mitochondrial morphology with different time and dose points.

Authors should show that MITO-N does not induce mitochondrial fragmentation. Also, why? Does MITO-N interact with the NEET proteins? Does MITO-N induce mitochondrial fragmentation at higher concentration or time point? Does MITO-N localized to mito, ER or both? If MITO-N interacts with NAF1 and modulates its function on iron-sulfur transport, why It has no effect on mitochondrial morphology? How the authors explain the difference of functions between MITO-C and MITO-N?

1. In agreement with this reviewer we now fully describe and show the different steps of MITO-C chemical production (see Fig1a). We agree with this reviewer that the mode of action of MITO-N is very interesting but not on the focus of this paper. Considering this and the fact that MITO-N related experiments were not essential for the readouts we used with MITO-C, we removed all MITO-N related experiments. Finally, we show time point treatments and increasing doses of MITO-C in new version of FigS1.

2. The authors used a new parameter to analyse mitochondrial morphology, "skewness" based on symmetry. Appropriate controls should be performed to validate this new method of analysis using for example siOPA1 and siDrp1. In any case, through the manuscript the authors should use other parameters to analyse mitochondria morphology as percentage of cells with phenotype and also calculate mitochondrial length and area, as it is usually performed in the field. In addition, the authors should perform z-stack 3D reconstitution of the mitochondrial network before quantification. Single plan is not relevant to analyse mitochondrial morphology by confocal microscopy.

2. We now removed all analyses made by the measurement of skewness parameter. Every experiment related to mitochondrial morphology characterization was thus addressed via classical quantification of fragmented vs non-fragmented (globular vs elongated) mitochondria in different conditions. (See Fig1c, Fig2e, Fig3e, Fig5c, FigS1c and FigS2e).

We understand that the remark of the reviewer about systematic Z-stack and 3D reconstruction was justified by the (automated) skewness initially used in the previous version of our manuscript. Now that skewness measurements have been removed and replaced by “manual” morphology counting, we respectfully disagree with this reviewer concerning the obligated requirement of 3D acquisitions to study the mitochondrial morphology by light fluorescence. Quantified data that we obtained concerning mitochondrial fragmentation (based on TOM20 staining) were highly reproducible, from confocal single plans or from apotome/wide-field acquisitions. In agreement, several recent key-papers dealing with mitochondria morphology analyzes were done without a systematic 3D approach, as notably underlined in the following examples: Zhang et al (EMBO reports 2014, 24719224), Pyakurel et al (Mol Cell 2015, 25801171), Ban et al (Nat Cell Biol 2017, 28628083), Naon et al (PNAS 2016, 27647893), Sautel et al (J Cell Science 2001, 11181170), Gomez-Suaga et al (Curr Biol 2017, 28132811), Loubiere et al (Scientific reports 2017, 28698627), Kauerkar et al (Nat Comm 2018, 30531964), Song et al (Mol Biol Cell 2009, 19477917).

Finally the mitochondrial morphology changes which we observed were confirmed by electron microscopy (Fig2f and Fig3g).

3. Results of the protein binding assay should be shown in the results section for both compounds (MITO-C and MITO-N). This is a critical point as the following of the study is based on this result. What are the other hits? Do the compounds interact also with NAF1 and mitoNEET, or only MiNT? What are the experimental conditions? Controls? For example, the authors should perform knockdown experiments of other top hits and analyse mitochondrial morphology to further reinforce the specific link between MITO-C and NAF1 in mitochondrial dynamics.

3. The photo-affinity labeling assay revealed the interaction of the capture compound with five proteins. The five captured proteins identified in area of significance are listed in the table (see FigS3a). LFQ (label free quantification) intensities are used to calculate fold change (FC) and p-values for comparison between assay (A) and competition (C) and no capture compound controls (B). Specific criteria for consideration as specific binders are FC>2 and $p<0.05$. As MiNT (CISD3) is the only interacting candidate that is localized at the mitochondria, we prioritized our study on this protein, as the preferential target. When the photo-affinity labeling assay was performed with an excess of “free” compound MiNT capture was dramatically inhibited showing the high specificity of the interaction between the compound and MiNT (see FigS3b). These data have been obtained according to experimental conditions described in Köster et al. (Assays Drug Dev Tech, 2007, PMID: 17638538).

MiNT protein belongs to the NEET protein family and MiNT, MitoNEET and NAF1 are structurally very similar. Since MitoNEET and NAF1 were not identified using the photo-affinity labeling approach we validated the interaction between MITO-C and these proteins with the Fe-S cluster transfer assay (Fig1d and FigS4). In addition, the distribution of the signal of the fluorescently-tagged version of Mito-C largely overlaps with the subcellular localization of NAF1 in living cells (Fig1e). Given that all the other interacting candidates are localized in compartments such as extracellular space and plasma membrane (as indicated in FigS3), different location from where MITO-C is identified, and their localization and function are difficult to conciliate with observed mitochondrial phenotype, we thus excluded these interacting candidates from further analysis.

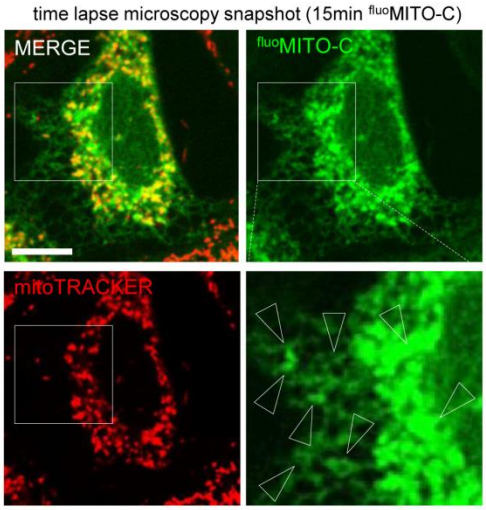
4. The localization experiments of NAF1 and MITO-C by microscopy are not convincing. The quality of the images should be improved and z-stack images should be acquired. In addition, triple labelling of mitochondria, ER and NAF-1 (or MITO-C) should be performed in the same time (FigS4, FigS5). PTPIP51 is a mitochondrial protein, which can be localized at the mito-ER interface, but it should not be used as a mito-ER contact marker. Finally, MITO-C localization is unclear. In the text, the authors say that MITO-C is localized at both mitochondria and ER (confirmed by FigS5). However, they also said that NAF-1 and MITO-C colocalize and share the same compartment (perfect colocalization in Fig1E). However, NAF-1 is clearly an ER protein, which only colocalize at the mito at the mito ER contact sites. How the authors explain the 100% colocalization?

4. We understand that the set-up of this series of experiments was misleading, especially concerning the fact that the data which this reviewer refers to were obtained by live-cell microscopy using a fluorescent version of MITO-C (see below).

First of all, we apologize for having stated that PTPIP51 is a protein of ER-mitochondria contact sites. In fact, as clearly stated by a recent review (Scorrano et al, Nat Comm 2019 (PMID 30894536)), identification and classification of contact sites, and proteins associated with contact sites, is still an evolving research field and “markers” of contact sites (and possibly different kind of contact sites at the same organelle-to-organelle interface) are not yet positively identified. Rather, we used PTPIP51 as a mitochondrial protein which sub-localized at ER-mitochondria interface, to help to discriminate tethering events involving the two different compartments which are, as this reviewer knows, particularly intertwined one with each other.

The fluorescent version of MITO-C, shown used in live-cell imaging experiment described in Fig1e, is not fixable and highly sensitive to photo-bleaching, which makes high resolution live microscopy (and 3D / live acquisitions) with multiple labeling rather impossible. When observed in live microscopy short time points (from 0 to 5min of treatment) the Fluo-MITO-C signal appears like a reticular pattern (similar to ER), as partially illustrated by FigS6b (with Sec61β ER marker) and Fig1e (with NAF1). Overall this suggests that the immediate and primary location of Fluo-MITO-C (and thus presumably MITO-C itself) is not the existing mitochondrial compartment itself, but rather ER associated membranes, arguing for the presence of MITO-C target NAF1 at ER-mitochondria interface, as suggest by data showing a partial co-distribution of NAF1 with PTPIP51 and DRP1 (FigS9a). These observations make sense with our new electron microscopy data showing, as detailed further in our rebuttal letter (point #5), that MITO-C treatment increases ER-mitochondria tethering events (Fig3g and 3h), which we know correlate or cause mitochondria to become fragmented.

However, later on time, starting from 5 minutes of treatment and obviously after 15 minutes, the Fluo-MITO-C pattern appears much more globular and overlaps with mitochondria (themselves being fragmented by Fluo-MITO-C), as showed by its codistribution with Red-OX mitotracker at 15 minutes of treatment (see rebuttal figure 1, below), a situation in which mitochondria are already fragmented.



Rebuttal figure 1: fluo-MITO-C and mitochondria morphology

time lapse video-microscopy on HeLa cells treated with 2µM of fluo-MITO-C (green channel) 15min and labeled with mitoTRACKER (red channel). Arrowheads indicate Fluo-Mito-C positive reticular membranes negative for mitotracker. Scale bar = 10µm.

Importantly, some of the (green) Fluo-MITO-C signal still appears as a reticular pattern (see arrowheads in cropped picture), probably reflecting presence of Fluo-MITO-C at ER and/or ER/mitochondria interface.

NAF1 being itself a protein present at ER and ER/mitochondria contact sites, the data showed in Fig1e illustrates the NAF1 binding by Fluo-MITO-C at ER/mitochondria interface subcellular locations in living cells.

5. A better characterization of how MITO-C and NAF-1 induce mitochondrial fragmentation should be performed. For example, does MITO-C (and MITO-N) treatment (1) modulate protein levels of the NEET family? (2) impact mitochondria/ER or MAM localization of NAF1 (by microscopy or biochemical MAM fractionation)? (3) increase fragmentation level induced by loss of NAF1? (4) Induce fragmentation in cells silenced for CISD1 or CISD3? Does NAF-1 interacts with Drp1? And does this interaction is lost upon MITO-C treatment (and not MITO-N)?

5. We thank the reviewer for the detailed propositions concerning the re-enforcement of our readouts and analyzes concerning the complex relationship existing between NEET proteins, mitochondrial dynamics and our MITO-C compound.

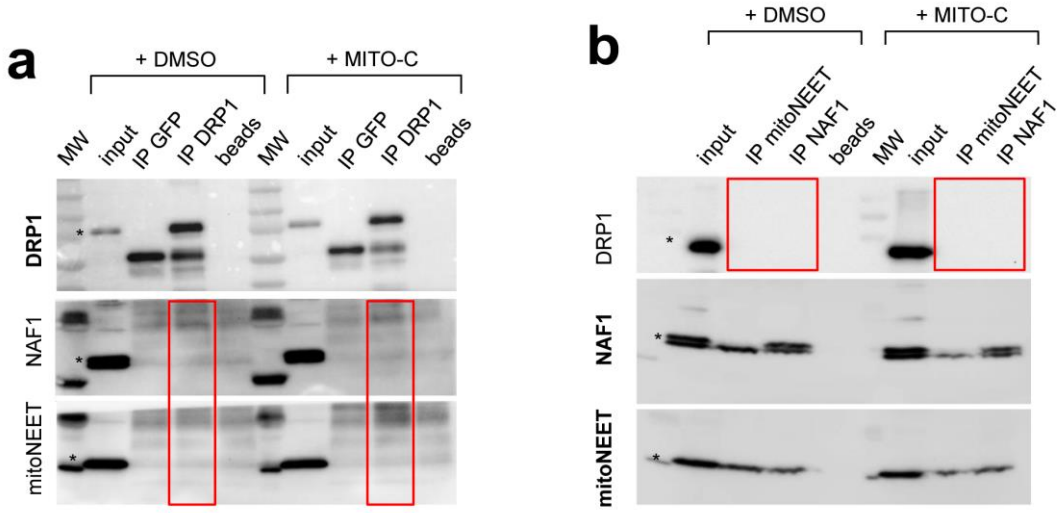
Data from Figure 3 (3a, 3b, 3c and 3d) show that MITO-C treatment induces a massive and fast (within minutes) recruitment of DRP1 at mitochondria. Moreover, based on this reviewer suggested experiments, we now report the following data:

- While MITO-C does not alter NAF1 protein cellular amount (See FigS9b), the subcellular localization of NAF1 was severely modified by MITO-C, as shown by the quantified increase of NAF1 presence at immediate vicinity of mitochondria (See S9c and S9d).

- Importantly, and based on the reviewer suggestion, we now show by electron microscopy that MITO-C stabilizes (or increases) ER-mitochondria membrane tethering events (Fig3g and 3h), arguing for the implication of MITO-C target(s) (NEET proteins, including NAF1) in the regulation of ER-mitochondria contact sites in relationship with mitochondrial fission machinery. Interestingly, similar results were previously reported for NAF1 mutation associated with Wolfram syndrome human disease: in their paper, Rouzier C et al (Hum Mol Gen 2017, PMID 28335035) demonstrated that NAF1 functional alteration increases contact sites between ER and mitochondria, a situation close to the one we are now reporting with the use of MITO-C.

- Concerning the putative adding effect of MITO-C on NEET proteins knockdown, it is pretty much unlikely to see one since the (acute, strong and reversible) effect we observed is reached within minutes, and is not comparable with genetic-based biological function alteration as obtained with siRNA transfection.

- Following the important suggestion by this reviewer, we performed immunoprecipitation experiments to address the putative DRP1-NAF1 (or mitoNEET) interaction. As this reviewer can see (see rebuttal figure 2) while we confirm already reported interaction between NAF1 and its partner mitoNEET, we were not able to detect any interaction between NAF1 and DRP1, either by using anti-DRP1 nor by using anti-NAF1 (or mitoNEET) antibodies. This result suggests that, at least in our hands, NAF1 and DRP1 are not part of the same stable complex and that the interplay existing in between NEET protein(s) and DRP1 fast recruitment and mobilization at the mitochondria membrane requires specific studies to be unraveled in details.

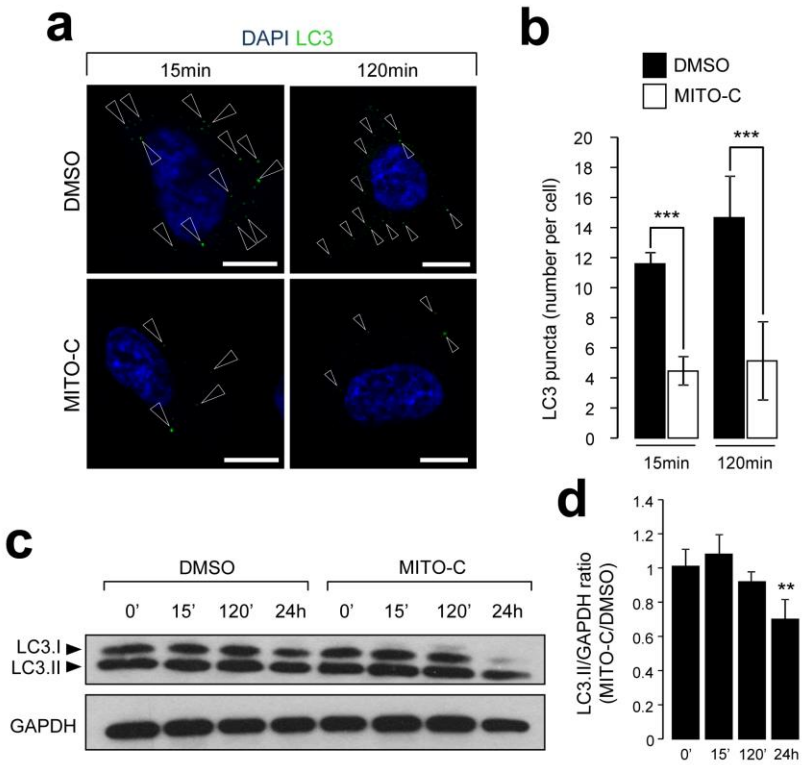


Rebuttal figure 2: NAF1 and DRP1 immunoprecipitation assay in MITO-C treated cells
HeLa cells were treated with 2µM of MITO-C or DMSO for 24h. Cells were lysed and prepared for immunoprecipitation using anti DRP1 antibody (a) or anti-NAF1 and anti-mitoNEET antibodies (b). Red rectangles indicate immunoprecipitates in a and b.

6. To reinforce that NAF-1 and MITO-C are in the same pathway, other functions associated to NAF1 and already described related to mitochondrial morphology regulation should be analysed (mitochondria-ER contacts, autophagy, ER stress). Indeed, loss of NAF1 has been associated to mitochondria-ER contacts (PMID: 28335035), which are involved in mitochondrial calcium and mitochondrial morphology regulation.

6. We thank the reviewer for this series of useful suggestions.

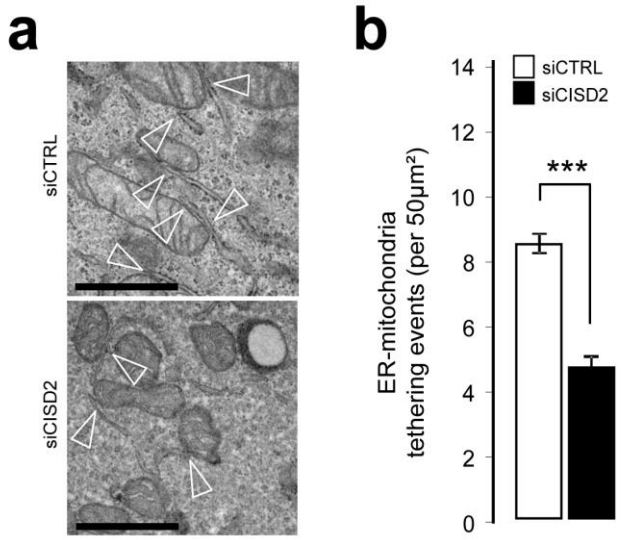
First, we now show that MITO-C treatment negatively regulates autophagy (see rebuttal figure 3, below) as assessed by LC3 analyses by western blot and immunofluorescence. This suggests that MITO-C not only perturbs direct mitochondrial related membrane events (such as fusion/fission) but also indirect mitochondrial membrane associated functions, such as autophagosome biogenesis. The latter being know to occurs, even partially, at ER/mitochondria interface (Molino et al. Comm Int Biol 2017, PMID: 29259731, and Hamasaki et al, Nature 2013, PMID: 23455425), our data about MITO-C associated autophagy downregulation are in favor of a general modification of ER-mitochondria contact sites, as we now show directly (Fig3g and 3h).



Rebuttal figure 3: MITO-C and autophagy

HeLa cells were treated with 2µM of MITO-C at indicated times. Cells were fixed and prepared for immunofluorescence analyse using anti-LC3 antibody and DAPI (a, b) or lysed to analyze the protein content by western blot, using anti-LC3 and anti-GAPDH antibodies (c, quantified in d). Scale bar = 10µm.

Moreover and importantly, as suggested by the reviewer and as stated above, we now analyzed the effect of MITO-C treatment on ER-mitochondria contact sites. Interestingly, while siRNA-mediated depletion of NAF1 reduces these contact sites (see rebuttal figure 4, below), MITO-C treatment stabilizes these events, as assessed by electron microscopy (Fig3g and 3h). Interestingly, similar results were previously reported for NAF1 mutation associated with Wolfram syndrome human disease: in their paper, Rouzier C et al (Hum Mol Gen 2017, PMID 28335035) indeed reported that NAF1 functional alteration (but not its depletion) increases contact sites between ER and mitochondria, in association with Ca^{2+} homeostasis modification. While we obtained similar results on contact sites with MITO-C treatment (which does not affect NAF1 expression levels (FigS9b)), the genetic knockdown of NAF1 reduces these contact sites, showing that the acute and reversible effects observed with MITO-C are more subtle than the one obtained via genetic mediated complete depletion of the protein.



Rebuttal figure 4: NAF1 knock-down and ER-mitochondria contact sites

HeLa cells were transfected with siRNA targeting CISD2 transcript (coding for NAF1 protein) or control siRNA (siCTRL). Cells were fixed and prepared for electron microscopy analyses (a). ER-mitochondria membrane tethering events quantified (b). Arrowheads in a indicated ER-mitochondria tethering events. Scale bar 200 nm.

These results however re-enforced the fact that MITO-C alters the function(s) of NAF1 (and putatively other NEET proteins) similarly to what was observed in mutated NAF1 patients suffering from Wolfram syndrome, thus arguing for a stabilization of ER-mitochondria contact sites in cells treated with MITO-C. Such a phenomenon could explain, at least partially, the increased recruitment of DRP1 that we report to be essential in MITO-C induced mitochondrial

fragmentation (Fig3). We believe that these data are important for a better understanding of our study and it is now discussed in the dedicated section of the revised manuscript.

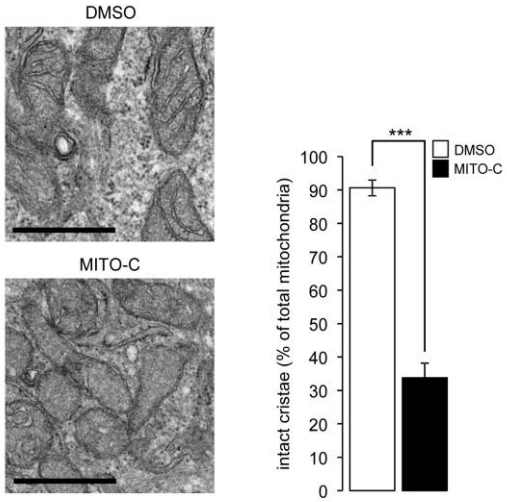
7. The authors should also analyse Drp1-P-637, which has been shown to regulate mitochondrial morphology (PMID: 18838687) to potentially explained their mechanism of fragmentation. Indeed, dephosphorylation of Drp1-P-637 by calcineurin increases its mitochondrial recruitment and mitochondrial fragmentation. NAF1 regulates intracellular calcium homeostasis (PMID: 24833725; PMID: 28335035) and its loss leads to increased cytosolic calcium and calcineurin activity (PMID: 24833725).

7. We agree with the referee that DRP1 phosphorylation on S637 has been demonstrated by Luca Scorrano's group to be involved in the regulation of mitochondrial morphology in the context of mitochondrial depolarization (Cereghetti et al, PNAS 2008, PMID: 18838687). In our hands, treatment with MITO-C very rapidly induces a fragmentation of the mitochondrial network (FigS1 and Fig1) without affecting mitochondrial transmembrane potential (FigS7). Moreover, the involvement of DRP1 phosphorylation on serine 637 during regulating mitochondrial fission has been recently challenged by Monica Nister's laboratory (Yu et al, J Biol Chem, PMID: 31533986). Indeed, this study reported that the status of DRP1 phosphorylation on S637 does not determine DRP1 recruitment to mitochondria to exert its pro-fission activity.

Importantly, in agreement with the fact that MITO-C promotes mitochondrial network fragmentation within few minutes, DRP1 recruitment is also very rapid (Fig3), does not require phosphorylation on S616 (FigS8) and the migration mobility/size of this GTPase does not seem to be modified (FigS8) by MITO-C treatment, suggesting that its targeting to mitochondria could be phosphorylation-independent. In addition to phosphorylation, the fission activity of Drp1 has been described to be modulated by other post-translational modifications such as ubiquitylation, SUMOylation and S-nitrosylation (Chang et al, Ann. NY Acad. Sci., PMID: 20649536) so whether MITO-C affects the post-translational state of DRP1 will be of high interest in future studies.

8. The authors observed a cleavage of OPA1 at 60/120 mns after addition of the compound, but observed mitochondrial fragmentation at 15 mns. As already mentioned, does the fragmentation is increased at 2 hours? What the conclusion of the authors? In addition, OPA1 has been involved in cristae organization stabilizing complexes of the respiratory chain. Does treatment of MITO-C and silencing of NAF1 lead to cristae disorganization by electron microscopy analysis.

8. We now show that mitochondrial fragmentation induced by MITO-C is detectable as soon as 5min of MITO-C treatment and is maintained at 120min of treatment (FigS1a). As suggested by the reviewer, we analyzed the cristae organization of fragmented mitochondria observed in MITO-C treated cells. Interestingly, we observed that the number of intact cristae in fragmented mitochondria is significantly reduced in MITO-C treated cells (Rebuttal figure 5).

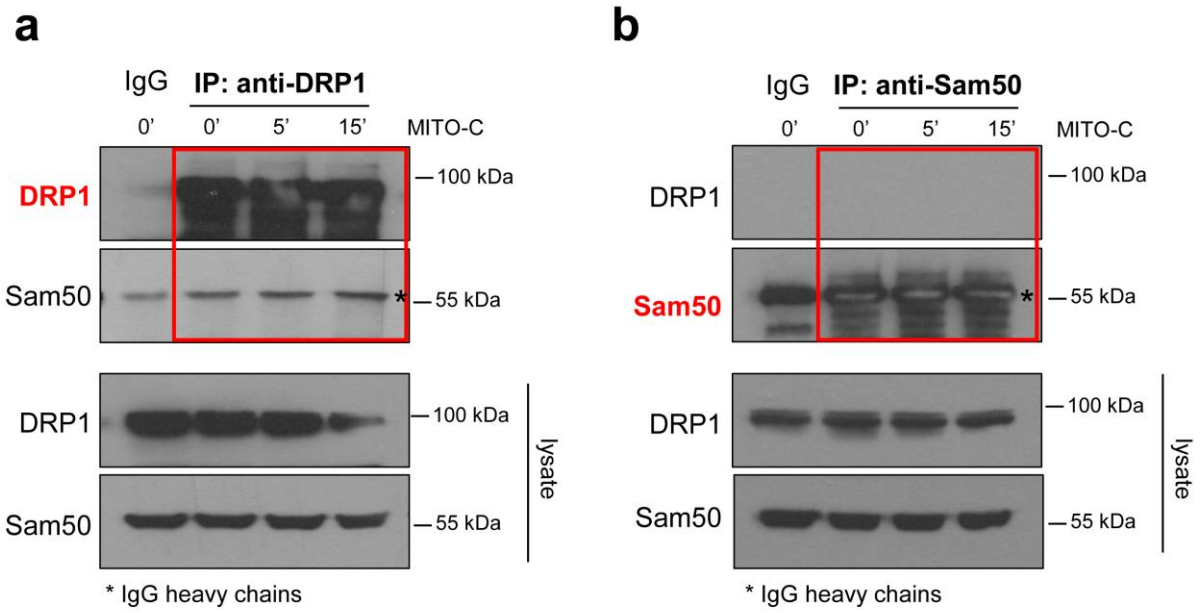


Rebuttal figure 5: MITO-C and mitochondria cristae

HeLa cells were treated with 2µM of MITO-C, or DMSO, for 24h. Cells were fixed and prepared for electron microscopy analyses (left panel). Intact mitochondrial cristae were quantified (right panel). Scale bar 200 nm.

9. As explained by the authors, the role of SAMM50 on mitochondrial morphology and Drp1 have already been shown. I don't see the relevance of this figure in the current version of the paper.

9. We perfectly understand the point of view of the reviewer. Our initial goal was to show that perturbation of ER-mitochondria contact sites (using Sam50 levels modulation) led to mitochondrial fragmentation, as we observed with our MITO-C compound. To go further, we wondered whether the previously reported interaction (Liu et al. FEBS Lett 2016 (PMID: 27059175) of Sam50 and DRP1 was modified by MITO-C treatment in our experimental conditions. However, as shown here (see rebuttal figure 6), we failed to detect any interaction by immunoprecipitation (using either anti-Sam50 or anti-DRP1 antibodies) between the two proteins.



Rebuttal figure 6: Sam50 and DRP1 immunoprecipitation assay in MITO-C treated cells

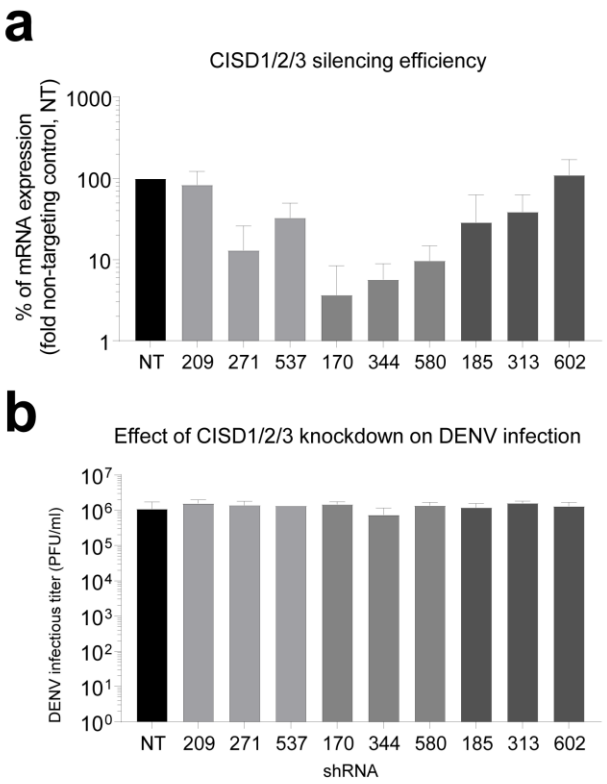
HeLa cells were treated with 2µM of MITO-C at indicated times. Cells were lysed and prepared for immunoprecipitation using anti DRP1 antibody (a) or anti-Sam50 antibody (b).
Red rectangles indicate immunoprecipitates in a and b.

Thus, in agreement with this reviewer's comment and with reviewer #3 suggestion, we removed all data related to Sam50 from the revised version of our paper.

10. Mitochondrial morphology should be quantified in Figure 4. What is the effect of MITO-N, and silencing of NAF1 and MitoNEET on mitochondrial morphology after Dengue infection and on its viral replication?

10. The mitochondrial morphology quantification is now shown in the Fig4c.

Concerning the effect of NAF1 and MitoNEET knockdown on dengue virus infection, and as proposed by this reviewer, all three members of the family were silenced with lentiviral shRNA vectors prior to dengue virus infection. Three different shRNAs were purchased for each gene and comparatively analyzed in three replicate experiments. As shown here (see rebuttal figure 7), although silencing efficiency was variable for the different shRNAs, depletion of NAF1 (CISD2), MitoNEET (CISD1) and MiNT (CISD3) did not significantly impact on dengue virus replication and virus production. This result is in line with the results obtained in response to comment #6 of this reviewer and supports the substantial difference between genetic depletion and chemical modification of a protein, which does not modify its expression level but may alter the network of its interactions and specific molecular features (such 2Fe-2S clusters in the case of NAF1 (Fig1d)).



Rebuttal figure 7: effect of NEEt proteins KD on dengue virus infection

a, Huh7 cells were infected with lentiviruses coding for indicated sRNA (209, 271 and 537 for CISD1, 170, 344 and 580 for CISD2 and 185, 313 and 602 for CISD3). Cells were then infected with dengue virus (DENV) 72h later. **b**, Infectious titers are presented as % of DMSO in Mito-C concentration of 2 and 10µM conditions, upon 24h of viral infection (n=3)

Furthermore, as shown in rebuttal figure 4, the genetic knockdown of NAF1 reduces ER-mitochondria contact sites. This has been reported to naturally occur upon dengue virus infection likely to counteract MAVS-dependent innate signaling and the activation of the interferon response, which takes place at ER–mitochondria interface (Chatel-Chaix et al., Cell Host & Microbes 2016, PMID: 27545046). This rather confirms the importance of ER-mitochondria contact sites in the antiviral response, as we now highlighted in the discussion section.

Minor points (reviewer #1)

m1. Production and purification of recombinant proteins used in figure 1D should be shown.

m1. Production and purification of NAF-1 (now detailed in Fig1a) was carried out according to the procedures detailed in “Crystal Structure of Miner1: The Redox-active 2Fe-2S Protein Causative in Wolfram Syndrome 2” by Conlan et al. (J. Mol. Biol. 2009) This reference is now quoted in the text.

m2. A more detailed mitochondrial bioenergetics analysis should be performed. In addition to basal respiration, ATP-linked respiration, maximal respiration, spare respiratory capacity and proton leak should also be measured using Olygomycin, FCCP, Antimycin A and rotenone treatments. What is the effect of NAF1 loss on mitochondrial bioenergetics? While Mitotracker can be used to estimate mitochondrial mass, mitochondrial membrane potential analysis should be performed using JC-1 and/or TMRM probes.

m2. We thank the reviewer for pointing out that detailed mitochondrial bioenergetics and respiration were indeed required in our manuscript. While we considered that studying the effect of NAF1 depletion on mitochondrial bioenergetics was beyond the scope of the present paper since similar studies have been reported already (Wiley et al, EMBO Mol Med 2013, PMID: 23703906), we now show complete analysis of mitochondrial bioenergetics function in cells treated with MITO-C using high-resolution respirometry dedicated experiments. Importantly, our results showed that MITO-C does not affect the different bioenergetics parameters assessed by high-resolution respirometry, which included routine respiration, ATP-linked respiration (oligomycin sensitive), uncoupled respiration (CCCP-induced) and spare respiratory capacity (FigS7f). Moreover, we confirmed that MITO-C compound does not impair MT respiration and thus ATP production by culture experiments on glucose-free/galactose medium (FigS7g), indicating a normal oxidative phosphorylation (OXPHOS) system. Altogether, these new analyses show that the mitochondrial fragmentation rapidly induced by MITO-C is not a consequence of mitochondrial respiration alteration.

m3. In figure 2, the authors should analyse the effect of each siRNA on the protein levels of the 2 other members of the family.

m3. We respectfully believe that such a minor-point related experiment is beyond the scope of the study.

m4. In figure 2, silencing of MitoNEET does not lead to mitochondrial morphology defects. However, it has been recently shown that MitoNEET-KO induces mitochondrial fragmentation (PMID: 28716905). Can the authors propose an explanation for this discrepancy?

m4. There is indeed an apparent discrepancy between our data and the results published by Pierre Cosson's lab. Experiments have been performed in two different species (mouse vs human) and in very different cells (embryonic fibroblast vs cervical cancer cell line) where MitoNEET impact on mitochondria morphodynamics could be different. In addition, the subcellular localization of NAF1 overlaps with the one of MitoNEET at the mitochondria. As both proteins are structurally similar, depending on its concentration at the mitochondria, NAF1 could be functionally redundant with MitoNEET in HeLa cells but not in MEFs.

The second important argument here is the technology used. While the genetic inactivation of MitoNEET led to a total/complete knock-out of its expression, this is not the case in our

situation, as often with the use of siRNA (Fig2a) shows residual MitoNEET expression. This expression could be sufficient to maintain normal mitochondria morphology.

m5. Figure legend of Fig S6 should be modified according to panels.

m5. Figure legend of New FigS7 (ex FigS6) has been corrected accordingly.

m6. In figure 3A, the authors should use both an ER and mitochondrial marker to demonstrate that NAF-1 and Drp1 colocalize together at these sites. Otherwise, the authors can use fluorescent mito-ER contact probe reporter (PMID: 29229997).

m6. As previously mentioned by this reviewer, PTPIP51 should not be considered itself as a direct "ER-mitochondria contact-sites marker" *per se*. However, in our hands and in recent literature, this protein was shown to be very useful to highlight the membrane interface between mitochondrial and ER compartment. DRP1 is mostly detectable by fluorescence when sequestered at mitochondrial membrane and can thus be used, with some restrictions, as a marker of mitochondrial membrane. FigS5 reports presence (at least partially) of NAF1 at the ER/mitochondria interface and the data presented in Fig3a is aimed at showing that some NAF1 protein directly colocalizes with DRP1 at specific zones of ER and mitochondria appositions, to illustrate the link between NEET proteins and mitochondria fission machinery.

m7. Figure 3D. Total Drp1 (input) should also be shown. Cytosolic loading control is also required. Does NAF-1 loss lead to increase Drp1 at mitochondria by IF and fractionation?

m7. Western blot analyses of total DPR1 levels are shown in FigS8a. Moreover, as suggested by this reviewer, we completed the mitochondrial fragmentation experiments by showing cytosolic marker (GAPDH) in the new version of Fig3c. We haven't checked the behavior of DRP1 by immunofluorescence and biochemistry after NAF1 knock-down in the time-course of the revision of the paper: beside being out of the scope of the present study, it is very important to stress out that the data we would like to report here indicate that the use of a chemical compound such as MITO-C induces mitochondrial fragmentation almost instantly - by targeting (some of) NEET proteins' local function, but not by inducing their degradation as show in the FigS9b with NAF1 - which is very different than what we could observe with a genetic knock down (with 24h, 48h or 72h siRNA transfection) or total knock-out of the suspected/involved protein(s), which could lead to compensations and cellular strategies to counteract the total (or partial) loss of a key protein. Our data point out that MITO-C allow a "fine tuning" of mitochondrial morphology by allowing a very fast recruitment of DRP1 at mitochondrial membrane, not by altering NAF1 stability or quantity, but presumably by altering a particular, and local, hallmark of NEET proteins, (possibly by modifying domain(s) interaction, specific folding, partners engagement, Fe-S clusters transfer, etc.). We believe that the first version of

our paper was not clear enough about this point and we now amended the whole revised manuscript to clarify the real focus of our study on the utility of chemical-mediated acute modification of mitochondrial morphodynamics, in comparison to classical up or down regulation of key proteins.

m8. In figure 3F, it is difficult to observe effect of DN-Drp1 expression on mitochondria morphology. The authors should fuse directly the fluorophore to it and modify the panel.

m8. The use of DRP1K38A was previously shown to work properly in conditions similar to the ones described in our previous paper (Zemirli N et al, FEBS J 2014, PMID: 24841215) and the presence of RFP is only used to detect transfected cells.

m9. References to other studies on NEET proteins and mitochondrial dynamics should be cited through all the manuscript and discussed.

m9. We thank the reviewer for this suggestion: we now carefully paid attention to cite – and discuss – papers involving NEET related processes about mitochondrial morphodynamics.

m10. In the abstract, the authors say "NAF-1... facilitating recruitment of Drp1...". Based on their data, NAF1 decreases Drp1 recruitment to mitochondria.

m10. We modified accordingly the abstract and the rest of the manuscript to clarify, as mentioned above, in the point m7.

Referee #2:

In this manuscript, Molino et al, established the role of Mito-C in mitochondrial dynamics, and explored the involvement of NEET proteins in this process. The study is interesting, well-conducted and of interest as it directly 1) identifies a small molecule inhibitor (chemical) that can affect mitochondrial dynamics and 2) provides evidence that the mode of action is possibly mediated through NEET proteins.

In general, this study is well organized and well presented. However, there are few places where the provided data either do not reconcile well with the proposed model or are in slight disagreement with the already established consensus in the field. Inclusion of few additional controls and explanation will definitely improve the impact of this article. Regarding viral assays- 2uM Mito-C in 15 min seems sufficient to cause fragmentation and for viral assays, the same concentration was kept for 72H. The concern is that- the dose/ duration parameters are not uniform. In other words, the authors need to find out minimum effective concentration that can cause fragmentation after 48H (it would possibly be much less than 2uM as 2uM conc. caused fragmentation within just 15 min) and use that conc. for viral assays. In viral assays, if the effect of Mito-C is indeed mediated through mitochondrial dynamics then that minimum conc. should affect flavivirus replication. And if not, then the authors need to convince the readers why it can not be a pleotropic effect.

We thank this reviewer for finding our study interesting and well presented. Thanks to the reviewer comments and suggestions, a better characterization of MITO-C effect on Dengue viral infection is now provided. In the revised version of our paper, we notably report that in our experimental conditions, MITO-C does not affect mitochondrial bioenergetics balance and is not cytostatic and that mitochondrial fragmentation, which participates directly in antiviral effect of MITO-C, is not a consequence of anti-proliferative effect. We hope that the additional controls and experiments suggested by this reviewer now improve both impact and clarity of our message.

My specific comments are given below:
1. The claim based on Fig S2a needs to be reconsidered. Even if 20uM compound is not cytotoxic, it could be cytostatic. For such experiments, Annexin V should be presented along with the cell proliferation data. Any simple experiment will serve this purpose, such as a flow cytometry with nuclear stain to see the proportion of cells in G1/G2, S or M phase. The cytostatic effect has to be ruled out as the authors are finally validating this finding in viral model which is highly sensitive to cell proliferation.

1. We thank the reviewer for this important suggestion. Interestingly, it was recently reported (Roth et al, mBIO 20107, PMID: 28074025.) that Dengue virus infection is itself responsible for altering cell proliferation by strongly repressing global host cell translation early post infection, and thereby precluding here any cytostatic effect of MITO-C in our experimental framework. Moreover, our own data suggest that a cytostatic/toxic effect of MITO-C is unlikely responsible

for the dengue virus infection reduction we observed, since MITO-C has no effect on HBV infection (FigS10).

However and importantly, as recommended by this reviewer, using BrdU staining experiments we now show that MITO-C compound treatment has no major cytostatic effect (FigS2c).

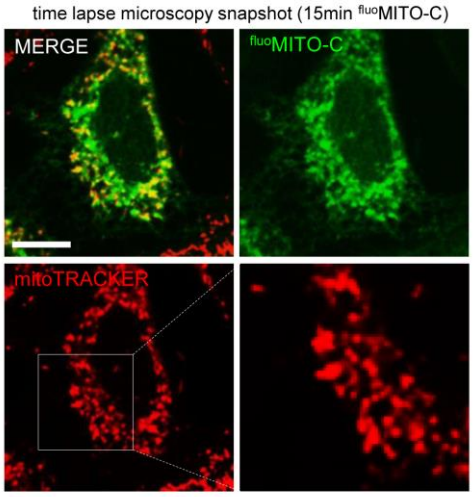
As reported in new Fig4a, we now show the antiviral effects of MITO-C on Dengue virus in a concentration range from 0.625 to 10 µM (at which point the effect of DENV infection titer was maximal).

We finally show that MITO-C is not affecting mitochondrial respiration and does not induce mitochondrial toxicity (FigS7f and S7g).

2. The authors need to categorically mention/ or provide evidence pertaining to the effects of various modifications they incorporated in Mito-C. The specific concerns here are- have the authors evaluated the effects of modified Mito-C? In other words, if a photo-reactive group (please provide specific group) or nitrobenzofurazan group is added, these modified Mito-Cs still exert similar effects as Mito-C. This question becomes pertinent in the light of Fig S5 where the extent of fragmentation does not appear to be as significant as I see in Fig 1B. Please also provide details of Mito-N.

2. We understand the concerns of this reviewer for a potential impact of the modified versions of MITO-C on the different phenotypes explored in this study. For the capture compound related experiment (FigS3), the specific compound was designed only for pull down experiment purpose. We now provide evidences that when the pull down is performed in the presence of an excess of free molecule, MiNT precipitation is prevented (FigS 3b). This demonstrates that the free molecule and the capture compound binding to MiNT are similar.

Concerning MITO-C and Fluo-MITO-C, we agree that the extent of mitochondria fragmentation is not similar for both molecules in Fig1. The colocalization between Fluo-MITO-C and NAF-1 is analyzed at 2min post-treatment (see Fig1e) while the fragmentation of mitochondria with MITO-C is shown at 15min (new Fig1b and rebuttal Fig8, below). In order to remove any concern, we present a rebuttal figure for this reviewer showing that at 15min post-treatment, mitochondria fragmentation induced by Fluo-MITO-C is similar to what is observed with MITO-C at this time point (see rebuttal figure 8, below). Conversely the new FigS1 shows that at 5min post-MITO-C treatment, the fragmentation of mitochondria is not yet visible. These data show that MITO-C and Fluo-MITO-C induce mitochondria fragmentation with similar kinetics. We finally removed all MITO-N related experiments, considering that it was not essential for the readouts we used with MITO-C.



Rebuttal figure 8: fluo-MITO-C and mitochondria morphology

time lapse video-microscopy snapshots from HeLa cells treated with 2µM of fluo-MITO-C (green channel) 15min and labeled with mitoTRACKER (red channel). Scale bar = 10µm.

3. For Fig S6, addition of FCCP or CCCP as positive control would have been better.

3. We thank the reviewer for this helpful suggestion. We now show complete analysis of mitochondrial bioenergetics function in cells treated with MITO-C using high-resolution respirometry (HRR) dedicated experiments. Importantly, our results showed that MITO-C does not affect the different bioenergetics parameters assessed by HRR, which included routine respiration, ATP-linked respiration (oligomycin sensitive), uncoupled respiration (CCCP-induced) and spare respiratory capacity (FigS7f). Moreover, we confirmed that MITO-C compound does not impair the oxidative phosphorylation system by culture experiments on glucose-free/galactose medium (FigS7g). Interestingly enough, altogether these new analyses show that the mitochondrial fragmentation rapidly induced by MITO-C is neither an immediate consequence nor an immediate cause of mitochondrial respiration changes.

4. Fig S7 appears to be a major deviation from the proposed model here and the data here do not reconcile well with the data given elsewhere in this article. 2uM Mito-C for 15 minutes causes mitochondrial fragmentation (Fig 1b). In contrast, Drp-1-S616 phosphorylation is not affected at this time points (in fact any time point). Do the authors have any explanation to support this claim where merely upregulation of Drp1 (without S616 phosphorylation) can cause fragmentation?

4. This is indeed an interesting observation: MITO-C triggers mitochondrial fission by local and very rapid DRP1 recruitment to mitochondrial membrane, and, as underlined by this reviewer, we did not detect any modifications in DRP1 phosphorylation status in these conditions. The

small increase observed in DRP1 levels after 30 min in the presence of MITO-C (FigS8) may be due to a reduction in its turnover once the GTPase is bound to its mitochondrial receptors Mff, Mid49 and Mid51 (PMID: 21149567, 18568013).

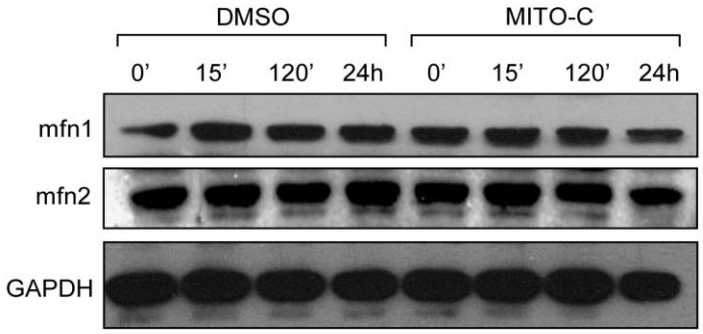
We now hypothesize that MITO-C induces stabilization in ER-mitochondria contact sites (now reported in Fig3g and 3h) leading to an “artificial” targeting/stabilization of DRP1 at mitochondrial membrane fission sites, which are directly associated with contact sites. Indeed, ER tubules wrapping around mitochondria have been reported to play an active role in defining the position of mitochondrial division sites (PMID: 21885730).

Importantly, in agreement with the fact that MITO-C promotes mitochondrial fragmentation within few minutes, DRP1 recruitment to mitochondrial membrane is also very rapid (Fig3), does not require phosphorylation on S616 (FigS8) and the migration mobility/size of this GTPase does not seem to be modified (FigS8) suggesting that DRP1 targeting to mitochondria could be phosphorylation-independent. In addition to phosphorylation, the fission activity of DRP1 has been described to be modulated by other post-translational modifications such as ubiquitylation, SUMOylation and S-nitrosylation (PMID: 20649536) so whether MITO-C affects the post-translational state of DRP1 will be of high interest in future studies.

5. In addition, the authors also included Opa-1 expression, which also remains unchanged (long or short) till 30 min of exposure with no change in p-DRP1 level. How the mitochondrial fragmentation is occurring at 15 min of Mito-C exposure without phosphorylation of DRP1 (Fig S7a); no change in OPA1 (Fig S7d) and also no change in mitochondrial membrane potential (Fig S6b, c)? This needs to be clarified. Do the authors propose some other hypothesis for Mito fragmentation without all these?

5. As shown in Fig1 and FigS1, the MITO-C compound triggers very rapid mitochondrial network fission without, indeed, any changes in OPA1 isoforms or modifications in DRP1 phosphorylation (FigS8). This is why, as discussed in the previous point of our rebuttal letter, we suggest that MITO-C induces stabilization/increase of ER-mitochondria contact sites, without ruling out the hypothesis that MITO-C may affect other post-translational modifications in DRP1 such as ubiquitylation, SUMOylation and S-nitrosylation. This hypothesis, based on the data obtained on MITO-C effects on ER-mitochondria contact sites (Fig3g and 3h), is now mentioned in the revised version of our manuscript.

We also analyzed the levels of Mfn1 and Mfn2 (see rebuttal figure 9, below) in the same experimental conditions and we report no obvious alterations of Mfns total levels in cells treated with Mito-C at early time points when mitochondrial network fragmentation is already observed. This suggests that MITO-C does not promote the fast fragmentation of the mitochondrial network by inducing the degradation of Mfns. However, 24hrs post MITO-C treatment, less Mfn1 and Mfn2 are observed, likely as a consequence of an “adaptive state” in response to mitochondrial fission.



Rebuttal figure 9: MITO-C and mitofusins

HeLa cells were treated with 2µM of MITO-C at indicated times. Cells were lysed to analyze the protein content by western blot, using anti-mitofusins (mfn1 and mfn2) and anti-GAPDH antibodies.

6. Please examine the Drp-1-S616 phosphorylation levels in the context of Fig 2a, b, c and Fig S8a. This will make sure that the events reported here, such as involvement of NEET, SAMM50 are indeed comparable to Mito-C and should also not affect S616 phosphorylation.

We understand the concern of this reviewer about challenging the mechanism of action of MITO-C. First, we would like to underline that NAF1, as a target of MITO-C, has been validated by different approaches in our manuscript, such as iron-sulfur cluster stabilization assay and live imaging sub-cellular based codistribution. As stated in the previous version of our paper, the mitochondria fragmentation is the central phenotype associated with both MITO-C and knock-down of essentially NAF1 and SAMM50 proteins. In both cases, at the molecular level, DRP1 is overexpressed. This increased amount/stability of ER/MT contact sites and the mitochondrial fast recruitment of DRP1 are the main drivers of fragmentation.

During the course of this reviewing we have demonstrated that NAF1 doesn't interact with DRP1, however DRP1 is expected to interact with SAMM50, as demonstrated in vitro and in vivo (Liu et al, FEBS Let. 2016, PMID: 27059175). At this we have no clue about a possible common mechanism implying both NAF1 and SAMM50. We agreed that our initial manuscript was not clear enough on that point, we can consider that a knock down is not a perfect surrogate of a treatment with a compound such as MITO-C, that induce acute and very rapid mitochondria morphology modification. Lowering the expression of NAF1 is not equivalent to inhibiting NAF1 interaction neither with a cellular protein/partner nor with the stabilization of its iron-sulfur clusters. Even if the essential phenotypes are similar (fragmentation of mitochondria and increased stability of DRP1) it can be expected that the fine-tuned mechanism may differ. We paid attention to clarify this in the revised version of our paper.

Finally, we point out the fact that in addition to the phosphorylation of DRP1 S616, several other DRP1 post-translational modifications, such as ubiquitylation, SUMOylation and S-nitrosylation (Chang et al, Ann. NY Acad. Sci., PMID: 20649536), have been shown to modulate its activity. A comprehensive investigation of the impact of NEET knock downs and MITO-C treatment on DRP1 post-translational modifications will thus be the topic of future investigations.

7. Any specific reason to opt for 10uM Mito-C while 2uM appeared to be sufficient? Although, 2uM Mito-C caused enough mitochondrial fragmentation, the virus titer for all three viruses went down only with 10uM Mito-C (five times more concentrated). How to explain this? Also, at which point Mito-C was added in these expts. (how long was the Mito-C treatment done?).

7. We are sorry if the details about our experimental framework were not clear in the previous version of the paper. MITO-C compound is indeed added upon cells at the very same time that the viral infection starts. The reason for keeping the compound and the infection for 48h relies on the experimental setup: at earlier time points in general viral titers are too low and too close from the input condition. Although closely related, dengue virus, Zika virus and West Nile virus show different replication kinetics, different host interactions and pathogenic effects. Therefore, previous results about Zika virus and West Nile virus were removed from the present version of the manuscript and we now add new data showing a dose-response effect of MITO-C on Dengue viral infection showing that MITO-C was already efficient at lower doses than 10µM (see Figure 4a).

8. Fig. 4a, please make it clear what time point the samples were analyzed. As per the legend (72h post infection) while the methods say 48 h.

8. We are sorry for this mistake. Importantly, we are now showing data with 48h post-infection (Fig4a). Corresponding legend has been modified accordingly.

9. Fig 3d- Could the authors check level of p-DRP1 level in the cytosolic and Mito fractions?

9. Classically, DRP1 phosphorylated form is considered to be associated with mitochondrial membrane compartment, while non-phosphorylated form of DRP1 should remain in the cytosol (PMID: 20649536). As indicated in the point 4 of our rebuttal letter to the referee, MITO-C does not seem to affect the phosphorylation state of DRP1 so that we respectfully consider that such an experiment is not required to improve the clarity of our results. Nevertheless while we hypothesize that MITO-C induces stabilization in ER-mitochondria contact sites leading to “artificial” targeting of DRP1 at mitochondrial membrane fission sites, we do not rule out the possibility that MITO-C alters ubiquitylation, SUMOylation or S-nitrosylation of DRP1 since these post-translational modifications have be shown to modulate DRP1 activity as well (PMID: 20649536). We therefore plan to investigate more precisely in futures studies the post-translational changes in DRP1 in response to MITO-C.

10. Fig 1b- a better representative blot for NAF-1 knockdown would be more appropriate.

10. We are sorry for the misunderstanding: the anti NAF1 antibody we are using (only a very small number of antibodies are available for NEET proteins so far) not only recognizes NAF1, but detects mitoNEET (CISD1) protein as well, as we illustrated on the Figure 2b (lower band is mitoNEET, not NAF1). The proper (and only) NAF1 band is indicated with an arrow.

11. Figure legends for S3 and S4 and S5 are switched.

11. We are sorry for this editing mistake. The revised version of our manuscript has been fully checked and updated.

12. In the methods section- "Flavivirus virus" change it to Flavivirus infection

12. We modified this section accordingly.

Referee #3:

This is an exciting, well written study. In this study the authors present a novel benzothiophene compound, Mito-C, which interferes with cellular iron metabolism mediated by a member of the family of NEET proteins, NAF1. The study is well-conducted and the research is overall conclusive. The reduction of viral replication (Dengue, West Nile, Zika) by Mito-C is especially intriguing giving rise to the possibility of novel approaches to combat neglected tropical diseases. There are some remaining points of criticism.

We thank this reviewer for finding our study exciting, well conducted and conclusive. Thanks to the reviewer suggestions, we now provide a robust characterization of MITO-C effect on Dengue viral infection. In the revised version of our paper, we show that the MITO-C compound does not affect mitochondrial bioenergetics balance and is not cytostatic. Importantly, this demonstrates that MITO-C induced mitochondrial fragmentation, which participates directly in antiviral effect of MITO-C, is not a consequence of cytostatic effect. We think that the additional experiments suggested by this reviewer now improve the clarity and the strength of our data.

1) Fig 1c. The choice of skewness as a measure of mitochondrial fragmentation is rather unorthodox. It is unclear how the skewness values correlate with more intuitive morphology measurements such as circularity and aspect ratio. A direct comparison with these measurements would be helpful to establish the validity of the skewness as a read-out of mitochondrial fragmentation.

1. We naively thought that an original and automatized method to classify a clear cut phenotype would have been a plus for the community. In accordance with this reviewer's comment and suggestion of reviewer #1, we now removed all analyzes made by the measurement of skewness parameter. Every experiment related to mitochondrial morphology characterization was thus addressed via quantification of fragmented versus non-fragmented mitochondria in different conditions (see Fig1c, Fig2e, Fig3g, Fig5c, FigS1c and FigS2e).

2) The effect of Mito-C treatment on cellular NAF1 protein levels has not been established making it impossible to determine whether a reduction in NAF1 levels or iron-sulfur cluster transfer is responsible for Mito-C mechanism of action.

2. This is indeed a central question in our study. We show that the MITO-C compound is able to alter almost instantly (see new data on FigS1a and Fig1c) the mitochondrial morphology by inducing a DRP1 dependent mitochondrial fission (Fig3e and 3f). We now show that MITO-C has no effect on the amount of NAF1 protein (see new FigS9b), but we noticed however that the presence and/or clusterization of NAF1 at mitochondria was increased in cells treated with MITO-C (new FigS9c and S9d).

Furthermore, we now report that MITO-C treatment rapidly increase ER-mitochondria tethering events as assessed by electron microscopy experiments (Fig3g and 3h) without affecting NAF1

protein stability (FigS9b). These data suggest that while targeting NEET proteins (including NAF1), as shown by Fe-S release ability (Fig1d and Fig S4), MITO-C is not altering the protein amount *per se*, but rather modifies one or several molecular features of the NEET proteins at the ER-mitochondria membrane interface, which also explains some of the differences that we could observe between NAF1 knock-down and MITO-C treated cells.

While further studies will be required for a deep understanding of MITO-C cellular mechanism of action, our new data highlight the importance of ER-mitochondria contact sites stabilization induced by MITO-C: it is thus reasonable to consider that an "artificial" stabilization of ER-mitochondria contact sites platforms directly participates in DRP1 massive recruitment to mitochondria fission sites. Importantly, our results shed light on a specific function of NAF1 (and/or other NEET proteins family members) in the dynamics of mitochondria associated contact sites and in their close interplay with mitochondria morphodynamics local regulation.

3) Authors mention the use of mass spectrometry to identify binding-partners of Mito-C, but do not present supporting data. The binding is inferred through a functional assay of NEET protein-iron binding. It would be helpful if the authors could include the relevant mass spectrometry traces.

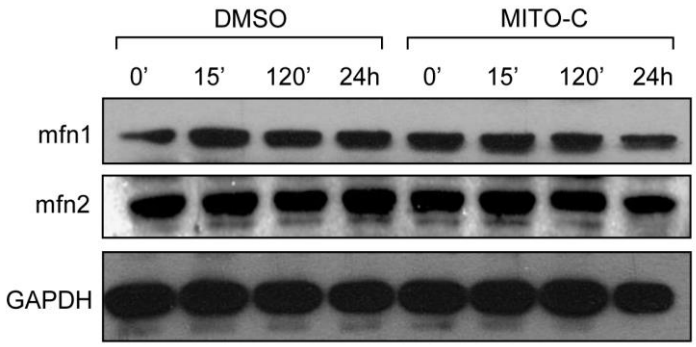
3. In order to avoid any concerns about the specificity of interaction between MiNT and the capture compound, we now show (FigS3b) the results of the photo-affinity labeling assay performed with an excess of "free" compound. MiNT capture was dramatically inhibited in these conditions showing the high specificity of interaction between the compound and MiNT.

4) It is unclear whether the real effect on mitochondrial fragmentation is based on DRP1 activity given the lack of increase in active DRP1-S616 under Mito-C treatment or changes in OPA1 isoform homeostasis. In order to conclusively prove the dependence of NAF1 driven mitochondrial fragmentation on DRP1 the authors should conduct a knock-down of NAF1 under DRP1-DN overexpression similar to the experiment with Mito-C in Fig 3.f. In addition, levels of Mfn2 should be monitored during Mito-C treatment.

4. MITO-C triggers mitochondrial fission by local and very rapid DRP1 recruitment to mitochondrial membrane, and, as underlined by the reviewer, we did not detect any changes in OPA1 isoform homeostasis or modifications in DRP1 phosphorylation status in these conditions. Nevertheless, based on the use of DRP1 dominant negative mutant, we confirmed that MITO-C-induced mitochondrial fragmentation is DRP1-dependent (Fig3e and 3f). We then suggest that MITO-C induces stabilization of ER-mitochondria contact sites (now reported in new Fig4g and 4h) leading to artificial targeting of DRP1 at mitochondrial membrane fission sites, which are directly associated with ER-mitochondria membrane interface. Indeed, ER tubules have been reported to play an active role in defining the position of mitochondrial division sites (PMID: 21885730). This hypothesis is now mentioned in the revised version of our manuscript.

In addition to phosphorylation, the fission activity of DRP1 has been described to be modulated as well by other post-translational modifications such as ubiquitylation, SUMOylation and S-nitrosylation (PMID: 20649536) so whether MITO-C affects the post-translational state of Drp1 will be of high interest in future studies.

As suggested by the reviewer, we analyzed the levels of Mfn1 and Mfn2 (see rebuttal figure 10, below) in the same experimental conditions and we report no obvious alterations of Mfns total amount in cells treated with MITO-C at early time points when mitochondrial network fragmentation is already observed. However, 24hrs post MITO-C treatment, less Mfn1 and Mfn2 are observed, likely a consequence of an “adaptive state” in response to mitochondrial fission.

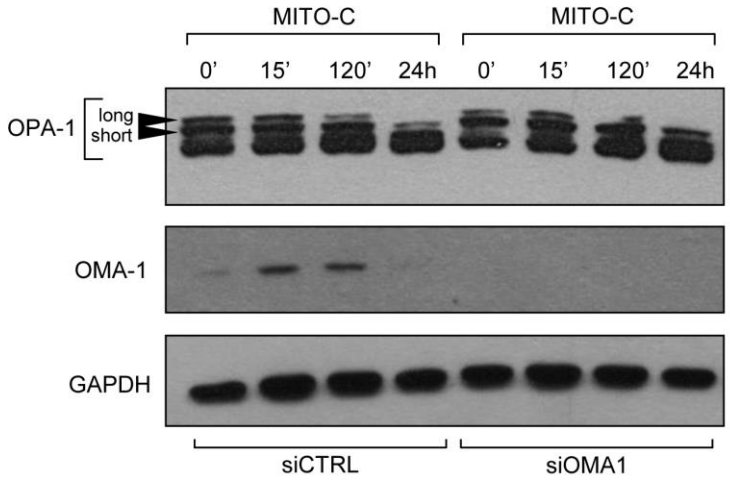


Rebuttal figure 10: MITO-C and mitofusins

HeLa cells were treated with 2µM of MITO-C at indicated times. Cells were lysed to analyze the protein content by western blot, using anti-mitofusins (mfn1 and mfn2) and anti-GAPDH antibodies.

5) Testing MitoC in models such as NAF1 Knockdown or over expression can further strengthen the claims. Testing MitoC in OMA1-KD could potentially be used to establish the role of OPA1 processing in the observed result.

5. We believe that the putative adding effect of MITO-C on NEET proteins knockdown is pretty much unlikely to happen, given the fact that the MITO-C treatment consequence on mitochondria is very strong and almost immediate. Thus, the MITO-C (acute, strong and reversible) effect we observed is not comparable (and thus probably not additional) with genetic-based biological function alteration as obtained with siRNA transfection. As suggested by this reviewer we perform the analyses of MITO-C effect in cells depleted for OMA-1. As shown in the figure below (rebuttal figure 11), we observe no differences in MITO-C associated OPA1 processing in cells transfected with siRNA targeting endogenous OMA1.



Rebuttal figure 11: MITO-C effect in OMA1 depleted cells

HeLa cells transfected with siRNA for OMA1 (siOMA1) or with siCTRL were treated with 2µM of MITO-C at indicated times. Cells were lysed to analyze the protein content by western blot, using anti-OPA-1, anti-OMA1 and anti-GAPDH antibodies.

6) The authors claim that the compound is non-toxic; however, a reduction in long-form Opa1 levels as seen in Fig.S7d etc. has been linked to mitochondrial respiratory super-complex disassembly and reduction in complex I driven respiration and several mitochondrial diseases. HeLa cells can completely rely on glycolysis or switching to complex II driven respiration to avoid mitochondrial toxicity. The authors should present ECAR data for the runs conducted for Fig. S2A. The ratio between OCR and ECAR can serve as an indicator for how much these cells are indeed relying on OXPHOS. Additionally, if the compound is indeed not toxic to mitochondria, the cells should be able to grow in Galactose media under compound treatment, if there is a mitochondrial toxicity growth in galactose would unmask it based on the absolute reliance of the cells on mitochondrial respiration for ATP generation. Similarly, it would be valuable to show that there is no increase in mitochondrial ROS production.

6. We thank the reviewer for this important point. We accordingly performed a news series of experiments about toxicity and putative cytostatic off target effect of MITO-C compound (FigS2c and S2b) and we analyzed as well the mitochondrial bio-energetic function using high-resolution respirometry (HRR, FigS7f). Our results showed no change in the different bioenergetics parameters assessed by HRR, which included routine respiration, ATP-linked respiration (oligomycin sensitive), uncoupled respiration (CCCP-induced) and spare respiratory capacity.

As suggested as well by this reviewer, we investigated the ability of HeLa cells treated with MITO-C to grow in glucose-free medium to assess the putative effect of MITO-C on mitochondrial ATP generation. Results shown in FigS7g demonstrate that no change in cell viability was observed in galactose (glucose-deprived) medium, indicating a valid oxidative

phosphorylation (OXPHOS) system. Previous studies demonstrated that OXPHOS is required for HeLa cells to survive in the galactose medium (Rossignol R. et al. 2004: PMID: 14871829). Based on reviewer suggestion, our new findings suggest that MITO-C modulates mitochondrial morphology without inducing direct mitochondrial toxicity and ROS generation.

Importantly, these new results show that the MITO-C induced mitochondrial fragmentation is not a consequence of mitochondria bioenergetics alteration.

7) Fig.S8 The relevance of SAMM50 in the context of the present study is unclear. Unless SAMM50 levels or functionality can be influenced by Mito-C, I do not see the reason for these data to be included in the present study.

7. In agreement with this reviewer and reviewer #1, we removed the data concerning SAMM50 from the revised version of our manuscript.

1st Revision - Editorial Decision 2nd Sep 2020

Dear Etienne,

Thank you for submitting the revised version of your manuscript. It has now been seen by two of the original referees. Referee #2 was not available for re-reviewing, therefore referee #3 also evaluated the response to the concerns of referee #2.

I apologize for this unusual 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. Before I can accept the manuscript, I need you to address some minor points below:

• Please address the remaining minor concerns of referee #1. Please let me know if you would like to discuss any of the points further.
• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.
• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.
• Please add a Conflict of Interests section.
• We notice that the Results and the Discussion sections are currently separate. Since your manuscript type is 'Scientific Report', the Results and the Discussion sections should be combined. Please see https://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide for more details.
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• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses 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 key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• 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.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

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:

The authors have performed extended revisions and have answered most of my concerns. I think this is a really interesting study and I m convinced by authors data showing that Mito-C induced mitochondrial fragmentation is Drp1-dependent. The effect of Mito-C on NAF-1 is also convincing. I also appreciate the data on viral replication and the different figures in their rebuttal letter.

However, few points need to be addressed before publication, which required only minor revisions.
1- The results presented in the rebuttal letter showing cristae analysis by TEM should be shown in the manuscript.
2- The authors need to dampened their conclusions. While it is clear that mitochondrial Drp1 is increased in mito-C treatment, the authors show no direct evidence that this increase occurs specifically at mito-ER contacts. A Drp1 labelling using both an ER and mitochondrial marker (as suggested in my next point 5) should answer this point.

Importantly, the authors should consider to analyse and present their data following the standard of the mitochondrial dynamics and contact sites fields before publication.
3- Mitochondrial morphology needs to be better quantified. While I appreciate that the authors do not present their new way to analyse mitochondrial morphology, which was missing controls, the authors need to present their results of morphology in the 3 main categories "Elongated",

"Fragmented" and "tubular". I don't know what is a "normal" mitochondrial morphology phenotype. In addition, as asked also by an other reviewer, they have to quantify the morphology by presenting different mitochondrial parameters as : "mitochondrial length" or mitochondrial area" and "mitochondrial number". These standard methods to describe mitochondrial morphology will really convinced the readers of the phenotype.
4- I appreciate that the authors have followed my recommendation and have analysed mitochondria-ER contacts. However, so far the results are weak (Fig 3g and h). Only showing the number of mito-ER contacts by 50 um2 of area is not enough, in particular when mitochondrial morphology is impacted by their treatment. Using their TEM images the authors have to analyse and quantify at least 3 other parameters: - the number of contacts / mitochondria, the percentage of the mitochondrial perimeter that the ER contacts represent, and the length of these contacts.
5- Finally, the fig S9 does not convincingly show that mito-ER contacts are increased by confocal analysis. While I acknowledge the increased colocalization of Naf-1 and TOM20, this data could be attributed by Naf1 mitochondrial localization increased upon treatment. The authors need to complement these data by showing an increase of mito-ER contacts by confocal using amother ER protein (or overexpressed probe as they used).

Referee #3:

The authors have addressed all of the comments including those of referee 2. I have only one minor issue that can be addressed in the discussion. It is surprising that such reduction in OPA1 and such impact on cristae structures is not affecting cellular viability.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 8th Oct 2020

Dear Etienne,

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 study!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports
--
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Corresponding Author Name: MOREL ETIENNE
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2019-49019V2

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 calculation was needed
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
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.	yes
Is there an estimate of variation within each group of data?	no

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	N/A

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).	all catalog number and information are in the text
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	sources have been identified

* for all hyperlinks, please see the table at the top right of the document

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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
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).	N/A
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.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	no