

Manuscript EMBO-2016-43602

Mitochondrial permeability transition involves dissociation of F1FO ATP synthase dimers and C-ring conformation

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Editor: Esther Schnapp

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

19 December 2016

Thank you for the submission of your manuscript to our journal. I am sorry for the unusual delay in the decision process of your manuscript; it was sent to three referees, but we have only received the reports from two of them, as referee 1 got sick and did not send her/his report. I am therefore making a decision based on the two reports we have.

As you will see, both referees acknowledge that the findings are potentially interesting and novel. However, both of them also raise important concerns that would need to be addressed for publication of the study by EMBO reports. The referees point out that it needs to be validated that the PLA assay can indeed be used to report changes in ATP synthase dimerization. Referee 2 further remarks that the correlations between ATP synthase dimer dissociation and MPT opening are not convincing, and raises a few other points. Both referees also note several mislabelings of the figures.

Given these constructive comments, we would like to invite you to significantly revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of 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.

REFeree REPORTS

Referee #2:

The authors report on the role of ATP synthase for the formation of the MPT pore and thus address a timely and in part controversially discussed topic. They propose that MPT opening involves dissociation of ATP synthase dimers (in contrast to previous models) while the c-ring is involved in pore formation. Experiments aiming to stabilize or destabilize ATP synthase dimers and mutational analysis of c-ring subunits are described to support of this model.

Although the proposed model is intriguing, the experimental support provided is not in all parts convincing. Most importantly, the experiments describing a correlation between ATP synthase dimerization and MPT opening are not convincing. The authors introduce PLA to monitor ATP synthase dimerization but the validation of this assay with established procedures such as BN-PAGE is poor, which raises doubts about the validity of experiments assessing ATP synthase dimerization.

Specific points:

1. The advantage of PLA over BN-PAGE is not obvious to me. However, the authors need at least to validate this assay using well-established BN-PAGE analysis. In Fig. 1C,D, the correlation between ATP synthase dimers and MPT sensitivity is not apparent. The quantification shown in D is difficult to reconcile with the gel analysis shown in C. For instance, HEK293T and A549 show the same ratio between dimers and monomers, but completely different calcein quenching rates (1A), questioning the correlation between the amount of ATP synthase dimers and PTP opening. Similarly, only monomers are present in A549 cells, but cells are protected from MPT induction. Finally in Fig. 1G, there is strong increase of dimer formation after Ca²⁺ and CsA treatment, which it is not paralleled by an increase in PLA intensity in Fig. 1K. Together, this raises significant doubts about the proposed correlation between the dissociation of ATP synthase dimers and MPT opening.

2. Despite the fact that a stabilizing effect of ATPIF5 on ATP synthase dimers has already been reported, it was already discussed in the original manuscript by Bason et al. that ATPIF5 might interact with other partners. The authors should therefore provide evidence that the major part of overexpressed ATPIF1 is binding to F1FO dimers and that the protective effect of over-expressed ATPIF1 is independent of secondary changes in matrix pH that can affect the ability to trigger MPT independent of dimer formation.

3. Does ATP5GI overexpression affect ATP synthase dimer formation? The authors should perform BN gels of ATP5GI overexpressing cells to examine whether overexpression of this protein increases the amount of the c ring without affecting the ratio between dimers and monomers. It has already been reported that excess the c subunits forms aggregates in the IMM that do not increase the membrane permeability but the authors need to show if this is also the case in their conditions. Moreover, the author should also comment on why overexpression of ATP5IG26L partially protects against H₂O₂ treatment (3I-J).

4. The authors should carefully assess the labeling of the figures as they appear to be a number of mislabelings in various figures.

Referee #3:

Bonora et al have thoroughly studied the link between dimerization of F1FO ATP synthase and mitochondrial permeability transition. The authors build quite a compelling case that formation of the MPTP requires the dissociation of ATP synthase dimers and involves remodeling of the c ring. The paper is very well written and the data are properly presented including statistical information. There are however several points that need to be addressed:

Title: the two ATPG1 mutations studied have opposite effects on MPTP opening (Fig. 4F). Therefore, implying in the title that MPT involves "correct C-ring conformation" is misleading. This can be fixed by simply removing the word "correct" from the title.

Abstract, last sentence: The current study does not provide direct evidence showing that the C ring is actually part of the pore. Since polymerization of ATP synthase dimers is crucially important for cristae morphology, their stability is expected to have major impact on the mitochondrial remodeling that goes along with MPT. It still cannot be excluded that this may indirectly affect other constituents of the inner mitochondrial membrane which then actually form the "pore". Based on the data presented, it can only be concluded that the C-ring is somehow involved in MPT, but not necessarily that it is part of the pore itself. Therefore, the last sentence of the Abstract should be toned down.

Page 7: the validity of the proximity ligation assay is critical to judge on the reliability of a significant portion of the results presented, especially since it is used for the first time to monitor ATP synthase dimerization in cells. Considering the common problems that may occur with siRNA experiments, it would be important to provide another control to show that the PLA signal indeed faithfully reports changes in ATP synthase dimerization. Eg. it should be shown that the signal is essentially absent in NIH3T3 or rho0 cells, which contain no or hardly any dimer. Furthermore, it would be helpful for readers not familiar with the PLA approach to move the explanatory sentence "This system allows...." further up to the second line of the previous paragraph.

Fig 1 and 5: in the legend for panels K, p-values are given for two and three asterisks. However, in the respective panel only on asterisk is shown. Similarly, in Fig. 5B and D, four asterisks are shown, but no corresponding p-value is found in the legend. This needs to be checked carefully and corrected.

1st Revision - authors' response

18 March 2017

Thank you again for your comments regarding MS EMBOR-2016-43602V1. We are extremely pleased that you are in principle interested in publishing an appropriately revised version of the manuscript. Please find enclosed here a revised version of the paper, which incorporates the answers to the referees' queries, your editorial changes and requests of clarification, and is in accord with the editorial checklist.

POINT BY POINT RESPONSE

Thanks for allowing us to submit a revised version of the manuscript "Mitochondrial permeability transition involves dissociation of F₁F₀ ATP synthase dimers and C-ring conformation" by Bonora et al. (# EMBOR-2016-43602V1) for publication in *EMBO Reports*. In this version of the paper, we have addressed most, if not all, the issues raised by reviewers, as detailed here below. Please note that our revision includes source data for figure 1B-C, 2A and 3A due to the particular nature of these results. If the provision of additional source data is required, we will be happy to accommodate.

Referee #2 (*Italicized*)

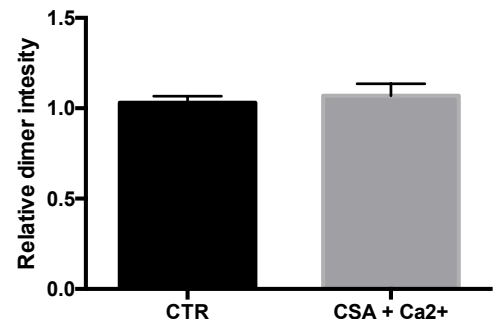
1. The advantage of PLA over BN-PAGE is not obvious to me. However, the authors need at least to validate this assay using well-established BN-PAGE analysis. In Fig. 1C,D, the correlation between ATP synthase dimers and MPT sensitivity is not apparent. The quantification shown in D is difficult to reconcile with the gel analysis shown in C. For instance, HEK293T and A549 show the same ratio between dimers and monomers, but completely different calcein quenching rates (1A), questioning the correlation between the amount of ATP synthase dimers and PTP opening. Similarly, only monomers are present in A549 cells, but cells are protected from MPT induction. Finally in Fig. 1G, there is strong increase of dimer formation after Ca²⁺ and CsA treatment, which it is not paralleled by an increase in PLA intensity in Fig. 1K. Together, this raises significant doubts about the proposed correlation between the dissociation of ATP synthase dimers and MPT opening.

We thank Reviewer #2 for the constructive critique and we apologize for not addressing the purpose of PLAs clearly in the previous version of the manuscript. The advantage of PLAs is to allow for the visualization of ATP synthase dimers in the context of small or non-homogeneous mitochondrial contents. Moreover, the very nature of this imaging technique (which can be applied to single cells)

allows for the determination dimeric ATP synthase in concomitance with the overexpression of specific cDNAs. Indeed, the overexpression of specific proteins – which has incomplete penetrance over a cell population – is not always compatible with the collection of mitochondrial samples that are sufficiently homogeneous to perform BN electrophoresis. Thus, we considered mandatory to develop an additional technique and PLA appeared to be the best candidate. We now included an improved explanation of purpose of PLAs in the result section. Additionally, with the specific purpose of further validating the sensitivity of PLA, we tested the technique in cells depleted of mitochondrial DNA (Rho 0), which have an impaired F_1F_0 ATP synthase dimeric status (as per the suggestions of Reviewer 3). As expected, Rho 0 cells displayed markedly reduced PLA signal compared to their WT counterpart (New Figure S1H).

Reviewer #2 points out that the increase in dimer content observed by BN electrophoresis is not paralleled by an increase in the PLA signal. Nonetheless, the increase in dimeric signal could not be considered as a transition between the two states as the monomeric form is increasing as well and the ratio (dimer/monomer) is substantially the same. To avoid data misinterpretation, we quantified the dimers in all replicates, but no significant variation was observed (see Appended Figure 1). The variation observed in former Figure 1 is most likely due to a variation in loaded protein amount. A new representative BN electrophoresis is now provided in Figure 1. Finally Reviewer #2 objects the correlation between dimer/monomer ratio and MPT measured in multiple cell lines. We understand that this correlation is not perfectly linear and not statistically significant. Indeed, our assay does not account for many variables as intracellular P_i , ADP, ATP and Mg^{2+} content as well as for the expression levels of members and regulators of PTPC. Therefore, to avoid confusion, we decided to exclude this experiment from the manuscript.

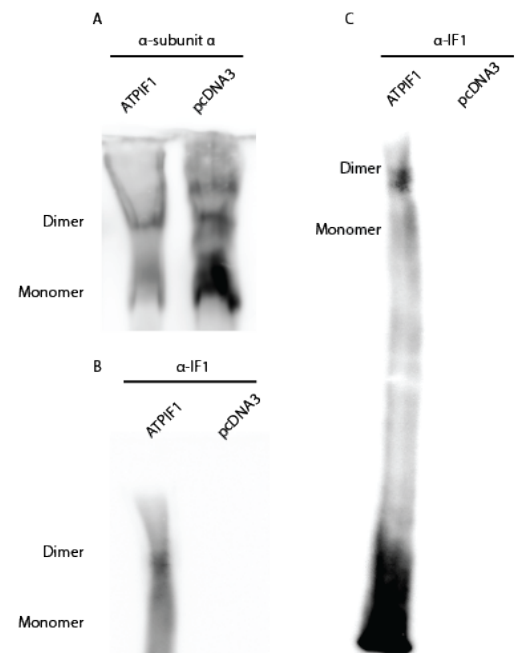
Appended figure 1: Dimer band quantitation from BN electrophoresis in control mitochondria (CTR) or mitochondria pretreated with CsA and exposed to Ca^{2+} (CsA + Ca^{2+}).



2. Despite the fact that a stabilizing effect of ATPIF5 on ATP synthase dimers has already been reported, it was already discussed in the original manuscript by Bason et al. that ATPIF5 might interact with other partners. The authors should therefore provide evidence that the major part of overexpressed ATPIF1 is binding to F_1F_0 dimers and that the protective effect of over-expressed ATPIF1 is independent of secondary changes in matrix pH that can affect the ability to trigger MPT independent of dimer formation.

Reviewer #2 suggested investigating in deeper detail the overexpression of ATPIF1 to exclude the possibility that it could alter PTPC activity in an ATP synthase-independent fashion. First, to confirm the relationship between ATPIF1 overexpression and PTPC opening, we investigated MPT by the calcein/ Co^{2+} assay upon ATPIF1 silencing with specific siRNAs. These data are provided in Figure 2, and confirm the requirement for ATPIF1 for normal PTPC opening.

Appended Figure 2: Immunoblot for subunit C (A) and IF1 (B) on BN electrophoresis. IF1 antibody was incubated after subunit C antibody stripping. Whole lane from BN electrophoresis (same samples as A) incubated with IF1 antibody



Moving forward, the most straightforward method to identify the specific binding of ATPIF1 to dimeric F_1F_0 ATP synthase was immunoblotting for IF1 upon transferring proteins from BN electrophoresis on a dedicated membrane. In this setting, we confirmed PLA data (see Figure 2E-F). Indeed, the overexpression of ATPIF1 led to a consistent increase in dimer/monomer ratio. These data is provided in new Figure EV2L-M. Additionally IF1 immunoreactivity on BN products

display a clear band in the proximity of the dimeric complex, while the extent in proximity of ATP synthase monomers was significantly smaller (See Appended Figure 2A-B). This suggests that - in our experimental conditions - ATPIF1 overexpression promotes its association with F_1F_0 ATP synthase, which in turns stimulates dimerization. Quite expectedly, the largest amount of ATPIF1 was detected at the electrophoresis front, and a minor amount of bands at intermediate height were detected (See Appended Figure 1C). This does not allow us to distinguish between free ATPIF1 and ATPIF1 bound to other partners.

However, AFTPIF1 overexpression is able to completely overcome the MPT-promoting effects of ATP5G1^{4GL} mutant (see Figure 5A-B). Mutations in the glycine-rich domain of the c subunit have previously been shown to change the properties of MPT-like currents in reconstituted proteoliposomes (Alavian et al., 2014), where no additional binding partners are expected, strongly suggesting that (in our experimental conditions) the observed effect of ATPIF1 are not mediated by additional partners of the PTPC.

Reviewer #2 also proposed to verify that ATPIF1 overexpression does not indirectly affect PTPC by altering mitochondrial matrix pH. We thank Reviewer #2 for highlighting this important point. Indeed, ATPIF1 overexpression was previously demonstrated to increase mitochondrial membrane potential in resting conditions (Sánchez-Cenizo et al 2010), an effect that was confirmed in our model (Figure EV2E-F). Such an effect on membrane potential would imply an increase in mitochondrial matrix pH. We investigated this assumption with a GFP-based pH sensor developed in Tullio Pozzan's lab, mtAlphi (Abad et al, 2004). We observed a non- significant increase in matrix pH (Figure EV2 I-J). The lack in statistical significance is likely due to the extent of the alteration. This could be due either to a reduced sensitivity of mtAlphi in detecting pH variation as compared to the sensitivity of TMRM in detecting transmembrane potential, or to the fact that the relationship between mitochondrial matrix pH and transmembrane potential also depends on other ions (specially K^+ and Ca^{2+}). Reviewer 2 will agree with us that investigating in detail such equilibrium lays outside the purpose of this study.

The relationship between mitochondrial pH and MPT is not perfectly linear. Indeed, an acidic pH was reported to inhibit PTPC opening (e.g. see Bernardi, 1991; Petronili et al., 1992). This effect is expected up to pH 7.4, a point at which basification should also reduce the PTPC opening probability (Basso et al., 2005). Nonetheless, inhibition of MPT at matrix pH above 7.4 appears lower than the inhibition at acidic pHs, and this relationship at pH above 8 (our experimental conditions) has never been tested (Matsuyama et al., 2000). These considerations, together with the minimal variation in mitochondrial pH observed upon ATPIF1 overexpression, led us to conclude that ATPIF1 inhibited PTPC opening via a mechanism that does not depend on alterations in mitochondrial pH. Overall, the new data suggest that ATPIF1 overexpression is unlikely to alter PTPC opening probability by interacting with unidentified partners or by altering mitochondrial pH. Conversely, we observed a consistent and reproducible increase in dimer/monomer ratio. We are positive that Reviewer #2 will agree with our conclusions.

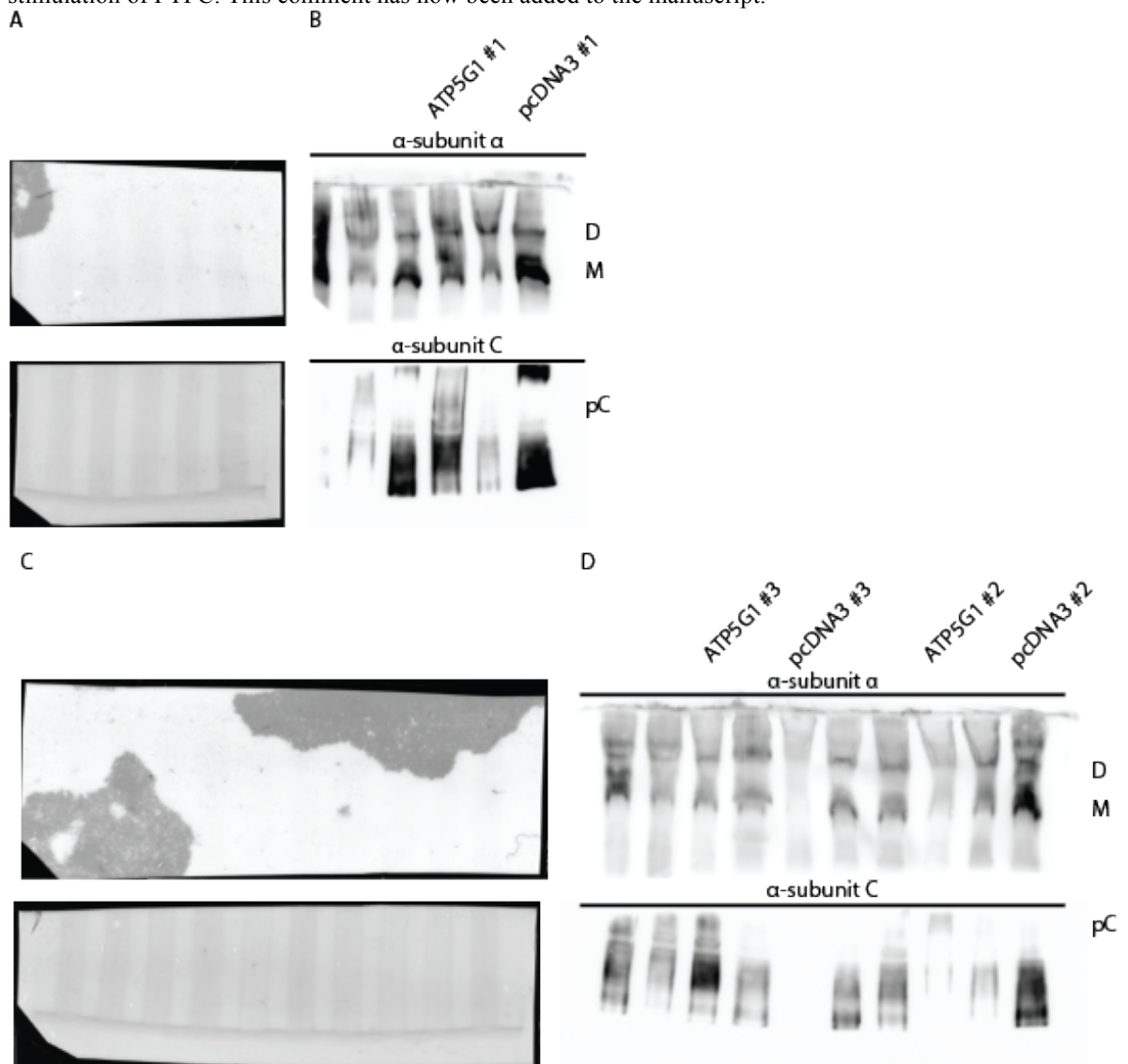
3. Does ATP5G1 overexpression affect ATP synthase dimer formation? The authors should perform BN gels of ATP5G1 overexpressing cells to examine whether overexpression of this protein increases the amount of the c ring without affecting the ratio between dimers and monomers. It has already been reported that excess the c subunits forms aggregates in the IMM that do not increase the membrane permeability but the authors need to show if this is also the case in their conditions. Moreover, the author should also comment on why overexpression of ATP5IG26L partially protects against H2O2 treatment (3I-J).

To answer this intriguing question we performed BN electrophoresis of samples from HEK293 cells overexpressing ATP5G1 or an empty vector (pcDNA3). We observed that ATP5G1 overexpression does not affect the ATP synthase dimerization status, indicating that the effect of ATP5G1 overexpression on MPT is not due to a destabilizing effect on F_1F_0 ATP synthase super complexes. These data is presented in Figure EV3.

We used the same BN electrophoresis samples to verify if the C-ring (or at least c subunit aggregates) was detectable, upon immunoblotting with a specific antibody. We detected some band largely below the position of the dimer and monomer, only in samples overexpressing ATP5G1. This suggests that C-ring can exist outside of the complex in our experimental conditions (see

Appended Figure 3). We were unable to obtain an appropriate marker for detection of C-ring in BN gels, we thus incubated the upper part of the run with antibodies for ATP5A in order to detect F_1F_0 ATP synthase dimers and monomer. The lower part of the run was incubated with an antibody against the c subunit, where C-rings were expected. Unfortunately, our present study cannot generate a precise model by which the c subunit (especially the C-ring) can generate the current responsible for MPT. Therefore, we cannot establish if the c subunit detected outside of ATP synthase might participate in PTPC formation. Based on these considerations, we are happy to share the data with Reviewer #2 in this document, but we consider inappropriate to include them in the revised version of the manuscript.

We would also comment on the effect of ATP5I^{G26L} on H_2O_2 challenges. Reviewer #2 observed a partial protection as compared to cells transfected with an empty vector (pcDNA3). Unfortunately, the statistical analysis does not allow us to conclude on this comparison, as the ANOVA test provides non-significant results. Nonetheless, it should be noted that H_2O_2 challenges are milder MPT inducers as compared to ionomycin, which we used for calcein/ Co^{2+} assays. Thus, we could only speculate that the G26L substitution does not result in a total inhibition of ATP5I activity, contrary to what observed for the yeast orthologue (TIM11), and detectable only with mild stimulation of PTPC. This comment has now been added to the manuscript.



Appended Figure 3: Membranes (A, C) and immunoblot (B, D) from two different BN electrophoresis for a total of 3 sample replicates. F_1F_0 ATP synthase dimers (D) and monomers (M) were detected by anti ATP5A antibodies while a band corresponding to a putative C-ring (pC) was detected using anti ATP5G1 antibodies.

4. The authors should carefully assess the labeling of the figures as they appear to be a number of mislabelings in various figures.

We thank the reviewer for the suggestion. Extensive revision of the figures has been run and this issue has been corrected

Referee #3: (*Italicized*)

Bonora et al have thoroughly studied the link between dimerization of F1FO ATP synthase and mitochondrial permeability transition. The authors build quite a compelling case that formation of the PTPC requires the dissociation of ATP synthase dimers and involves remodeling of the c ring. The paper is very well written and the data are properly presented including statistical information.

We thank Reviewer #3 for the positive comment as well for the suggestions for increasing the quality of our manuscript. An extensive revision has been run according to these recommendations. In particular, the revised paper underlines that the current study does not identify the C-ring as the pore-forming unit of the PTPC, but rather suggests the dynamic nature of PTPC formation and the requirements (even indirect) for the C-ring for proper execution of the process.

Title: the two ATPG1 mutations studied have opposite effects on PTPC opening (Fig. 4F). Therefore, implying in the title that MPT involves "correct C-ring conformation" is misleading. This can be fixed by simply removing the word "correct" from the title.

The referee is right and the title has been corrected as suggested

Abstract, last sentence: The current study does not provide direct evidence showing that the C ring is actually part of the pore. Since polymerization of ATP synthase dimers is crucially important for cristae morphology, their stability is expected to have major impact on the mitochondrial remodeling that goes along with MPT. It still cannot be excluded that this may indirectly affect other constituents of the inner mitochondrial membrane, which then actually form the "pore". Based on the data presented, it can only be concluded that the C-ring is somehow involved in MPT, but not necessarily that it is part of the pore itself. Therefore, the last sentence of the Abstract should be toned down.

We thank Reviewer #3 for highlighting this point. The relationship between cristae remodeling and MPT is indeed quite controversial. Mitochondria isolated from Opa1-overexpressing mice (Opa1 is a major regulator of cristae remodeling) do not display significant differences in calcium retention capacity (Varanita et al., 2015). Haploinsufficient (Opa1^{+/−}) mice, however, display a dramatic loss in cristae morphology and a desensitization to PTPC opening, suggesting that loss of cristae integrity (that could be expected with overexpression of the c subunit) should be rather associated to MPT inhibition. We have corrected this concept according to the suggestion of Reviewer #3.

Page 7: the validity of the proximity ligation assay is critical to judge on the reliability of a significant portion of the results presented, especially since it is used for the first time to monitor ATP synthase dimerization in cells. Considering the common problems that may occur with siRNA experiments, it would be important to provide another control to show that the PLA signal indeed faithfully reports changes in ATP synthase dimerization. Eg. it should be shown that the signal is essentially absent in NIH3T3 or rho0 cells, which contain no or hardly any dimer. Furthermore, it would be helpful for readers not familiar with the PLA approach to move the explanatory sentence "This system allows...." further up to the second line of the previous paragraph.

We are indebted with Reviewer #3 for this excellent suggestion. A PLA assay has been performed in Rho 0 cells versus their WT counterparts (Fig. EV1 H-I). As expected, we observed a dramatic reduction of the PLA signal in Rho 0 cells, according with the reduced dimeric state of F1/FO ATP synthase.

Fig 1 and 5: in the legend for panels K, p-values are given for two and three asterisks. However, in the respective panel only one asterisk is shown. Similarly, in Fig. 5B and D, four asterisks are shown, but no corresponding p-value is found in the legend. This needs to be checked carefully and corrected.

The referee is right. We extensively reviewed all the statistical information. Also according to the author checklist, all p values have been inserted in figure legends where appropriate.

2nd Editorial Decision

11 April 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it and I am happy to tell you that both support the publication of your study now.

I only would like you to incorporate a few changes before we can proceed with the official acceptance of your manuscript.

The character count is a little too high for a scientific report, can you please try to shorten the text where possible? Please also change the text in the abstract to present tense (inhibit, sensitize, etc.).

Please note that all figures need to be in portrait format and need to fit on a single page, so some of the figures might need to be re-arranged.

All source data need labels of the gel bands and (hand-drawn) size markers if possible.

The EV figures need to be uploaded as individual files and the EV legends need to be added to the end of the main manuscript file.

EV4 legend does not list B or C, EV2 legend does not list C, EV4 legend and figure do not have K.

The other two corresponding authors, MW and LG need to link their ORCID IDs in their profile pages of our online manuscript submission system. We can unfortunately not do this for you.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

REFEREE REPORTS

Referee #2:

The Authors have made a significant effort to address all the concerns of the reviewers. They now describe additional control experiments in the revised version, which support the validity of the PLA assay that is used for monitoring ATP synthase dimerization in cells. Moreover, they have removed mainly correlative data from the manuscript, improved the presentation of the data and toned down their conclusions on the role of the c-ring during MPTP opening. The finding that mutations stabilizing ATP synthase dimerization can inhibit MPTP is intriguing and will be of broad interest, also considering recent findings of the Walker group (He et al., PNAS 2017) suggesting that the c-ring is dispensable for MPTP opening.

Referee #3:

The authors have properly addressed my concerns.

2nd Revision - authors' response

22 April 2017

We are delighted of the positive evaluation of the reviewer of our manuscript (EMBOR-2016-43602V2) and glad that you are considering our manuscript for publication. Of course we understand that manuscript would require some additional editing and we would accommodate at our best. Will follow a point by point list reporting the editing conducted. Hopefully you will find the manuscript largely improved and suitable for publication. If additional editing would be required, we would be happy to provide better modifications.

The character count is a little too high for a scientific report, can you please try to shorten the text where possible? Please also change the text in the abstract to present tense (inhibit, sensitize, etc.).

As you will surely appreciate, we did our best to shorten the text down to the editorial limitations that apply to this type of article (25000 +/- 2000 characters). Now the main text of the paper (including Introduction, Results and Discussion and Figure Legends) count slightly more than 27,500 characters (including spaces). We hope that you will be indulgent on this minor excess.

Please note that all figures need to be in portrait format and need to fit on a single page, so some of the figures might need to be re-arranged.

Now all the figures are formatted in portrait format.

All source data need labels of the gel bands and (hand-drawn) size markers if possible.

Due to our digital system for immunoblot acquisition we do not allow hand made modification to the source data. Nonetheless, to accomplish at our best, we prepare a new figure for source data for figures 2A and 3A that include original picture of the membrane and digital annotation of both marker size and antibody hybridization. Due to the particular nature of blue native gels instead, there is no marker size available for figure 1A (indeed bands identification is well explained on the main text of our manuscript). Hopefully you will agree with this arrangement.

The EV figures need to be uploaded as individual files and the EV legends need to be added to the end of the main manuscript file.

EV4 legend does not list B or C, EV2 legend does not list C, EV4 legend and figure do not have K.

All the EV materials have been edited accordingly.

The other two corresponding authors, MW and LG need to link their ORCID IDs in their profile pages of our online manuscript submission system. We can unfortunately not do this for you.

The additional co-corresponding authors updated their ORCID information.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Summary, Highlights and Synopsis Image are now provided as two additional files attached with the revised manuscript.

3rd Editorial Decision

25 April 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Paolo Pinton
 Journal Submitted to: EMBO Reports
 Manuscript Number: EMBOR-2016-43602V1

Reporting Checklist for Life Sciences Articles (Rev. July 2015)

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 justified
- 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 χ^2 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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http://jill.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For ex-vivo ischemia/reperfusion injury experiment the number of animals was determined on the basis of the experimental conditions and on the statistical power that must have the final data. Those parameters were determined by using the GPower software (http://www.gpower.hhu.de/). We used a Multivariate analysis of variance (MANOVA) with "A priori" power analysis. We took into account an α err prob = 0.05; an effect size = 0.2 and a statistical power = 0.8. Page 18
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Following criteria were pre-established. 3 month old female C57 mice were used for mitochondria isolation liver. 6 weeks-old male Wistar rats were used for herth isolation. No other inclusion/exclusion criteria were used. Page 17 and 18
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.	NA (no treatment)
For animal studies, include a statement about randomization even if no randomization was used.	no randomization was used
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.	no blinding was adopted
4.b. For animal studies, include a statement about blinding even if no blinding was done	no blinding was adopted
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, page 19
Is there an estimate of variation within each group of data?	Yes, page 19
Is the variance similar between the groups that are being statistically compared?	Yes, page 19

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).	Yes, page 18
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were already available in the lab. Cell were routinely tested for micoplasma contamination. Page 14

* 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.	page 14
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 14
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(8), 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.	recommended guidelines has been consulted and, accordingly, relevant information about animal use has been reported.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA

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

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Proteins, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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).	NA
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).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: Primary Data Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 Referenced Data Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4Q26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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 Biomedelis (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.	NA

G- Dual use research of concern

23. 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.	Current study do NOT fall under dual use research restrictions
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