

APOPT1/COA8 assists cox assembly and is oppositely regulated by UPS and ROS

Alba Signes, Raffaele Cerutti, Anna S. Dickson, Cristiane Benincá, Elizabeth C. Hinchy, Daniele Ghezzi, Rosalba Carrozzo, Enrico Bertini, Michael P. Murphy, James A. Nathan, Carlo Viscomi, Erika Fernandez-Vizarra and Massimo Zeviani

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

1st Editorial Decision

17 September 2018

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

As you will see from the referees' reports pasted below, the study should in principle be published. However, both referees after cross-commenting feel that the mechanistic side of the data should be developed and ref. 2's suggestions should be followed. We also would like to encourage you to discuss the disease relevance and impact that these data have for patients to better fit with our scope.

Therefore, we would welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

I did not mark "High" on novelty because the group published recently in AJHG that this gene has a role in COX assembly. However, this submission is much more mechanistic and I think it advances our knowledge in this relatively new protein (APOPT1/COA8). They used both patient cells and a KO mouse.

Referee #1 (Remarks for Author):

The manuscript by Signes and colleagues describe the characterization of APOPT1, which was previously shown by the same group to cause cavitating leukoencephalopathy associated with mitochondrial cytochrome c oxidase (COX) deficiency. In this study they generated an Apopt1 knockout mouse model, which showed decreased motor coordination, and endurance associated with reduced COX levels in several tissues. Expression of wild-type APOPT1 in control and patient derived cultured cells showed that this protein does not have a direct role in apoptosis and that this protein is necessary for proper COX assembly.

Overall the study is well performed and it adds to our knowledge of COX assembly. It shows clearly that COA8 has a role in COX assembly in vivo. I have relatively minor comments.

1. If they concluded that M14 is the endogenous initiation methionine, why use the M1 in the constructs?
2. In figure 4. Why is the stoichiometry between precursor/mature is so different when using HA or APOPT1.
3. I am not sure what is the physiological relevance of observing an over-expressed HA-tagged protein accumulating ubiquitinated forms when the proteasome is inhibited (Figure 6B). May be I am missing the point.
4. The following statement: "however, when the patient-derived fibroblasts were oxidatively challenged they showed a significant increase in ROS production compared to the controls (Melchionda et al., 2014), which argues in favor of a 'pro-oxidant state' in the absence of APOPT1." I am not sure I agree with the conclusion. The patient derived fibroblasts may have less ROS because of less COX. However, that led to an adaptation where less ROS scavenging mechanisms are required. These cells, when challenged, may be more sensitive to the oxidative stress.

Referee #2 (Remarks for Author):

In 2014, this group reported patient mutations in APOPT1 causing a COX deficiency. This study revealed a marked COX deficiency in muscle biopsy samples and in patient fibroblasts. The APOPT1-GFP fusion was reported to be mitochondria and suggested to be matrix localized based on the MTS cleavage. One highlight of the characterization of APOPT1 was that the protein was unstable in cells unless proteasomal degradation was blocked or the cells were treated with hydrogen peroxide. They concluded this study with the suggestion that APOPT1 exerts a protective role on COX during oxidative stress.

The present study builds on these 2014 observations and provides greater details in the various observations. They now generate an APOPT1 knockout mouse model, which shows the same COX deficiency associated with neurologic deficits. Thus, the APOPT1 KO mouse is an excellent model for neurologic testing studies. They demonstrate that APOPT1 depleted mouse tissues and human patient fibroblasts exhibit a diminution in COX subunits and MT-CO1 assembly intermediates. These studies are very well executed and the effects are marked. However, no major new insights are provided on the function of APOPT1. The investigators report again the oxidative accumulation of APOPT1 and show that APOPT1-deficient cells are oxidatively challenged with enhanced turnover of COX subunits. The significance of this present study is the more thorough

characterization of APOPT1-deficient cells, but many observations are reflected in their 2014 publication, which diminishes the novelty of the report.

The Cas9-mediated deletion of mouse APOPT1 is very well executed and discussed. Although they were unable to validate the KO by immunoblotting due to no protein detection, it is clear that a deletion was attained. The KO mice had reduced motor skills based on a myriad of solid studies. The COX biochemical studies on the KO tissues are well done, but it is surprising that no oxygen consumption measurements were done to address the consequences of reduced COX activity. They demonstrated that APOPT1-HA and GFP fusions expressed in HEK293 and 143B cells showed mitochondrial localization and loose association with the inner membrane. Using graded digitonin extractions followed by trypsin digestion, they conclude that the protein is IMS localized, but the data are not convincing and are not validated with other studies. The MTS of APOPT1 is cleaved in cells, so if APOPT1 is indeed IMS, it is one of the unusual IMS proteins with a cleaved MTS. Since vector borne-APOPT1 can rescue S2 or S6 skin fibroblast mutant cells, they could attempt to validate the IMS localization by testing whether replacing the APOPT1 MTS with a classical matrix MTS would give complementation. Is APOPT1 released by hypotonic solutions? It is important to validate the mitochondrial localization to better anticipate what functional role it may play in COX maturation under oxidative conditions. They postulate that COA8 exhibits a role in stabilizing the MT-CO2 module or the insertion of this module to the MT-CO1 module. A second way to validate an IMS localization is to assess interaction partners with affinity purified APOPT1. Does COA8 bind COX subunits or assembly factors such as SCO1, COA6, etc that affect the maturation of the MT-CO2 module? COA8 runs on a DDM BN gel with an apparent mass of ~40 kDa. Based on this apparent mass, they suggest that COA8 may interact with a protein of ~20 kDa. One cannot quantify the mass at the low end of BN gels, so this suggestion is not well based. However, a proteomic study of affinity purified COA8 might be very insightful and add impact to the current study. The BN study in Fig 4D revealed that under BN-PAGE conditions, COA8 does not associate with COX. However, BN conditions often dissociate weak interactions, so coIP studies are needed. The enhanced turnover of COX in APOPT1-deficient cells may arise from an oxidant-induced mechanism. One wonders whether OMA1 is activated in the deficient cells and contributes to the COX subunit turnover.

In summary, the description of the mouse model is excellent and worthy of publication. The apparent role of APOPT1 in stabilizing COX in oxidative conditions is striking and elucidating this mechanism would be a major contribution to the field. The impact of these studies would be markedly strengthened if some of the mentioned concerns were addressed.

A series of other concerns should be addressed.

1. The mature form of COA8 was stabilized by treatment with hydrogen peroxide. They conclude that this form must be mitochondrial, but many IMS proteins (e.g. COX19) are not stably localized in the IMS under stress conditions. They should validate that COA8 remains in the IMS under oxidant treatment. Under normal culture conditions, COA8 levels are labile suggesting proteolytic degradation. If COA8 is IMS localized, perhaps iAAA is responsible for the degradation or if matrix perhaps the LON protease mediates its diminution. Such studies could help validate the mitochondrial localization of COA8.
2. The APOPT1-deficient cells exhibit a down-regulation of COX subunits and subcomplexes. If COA8 is indeed IMS-localized, it could affect COX maturation through the association of COXVIb, which is oxidatively folded in the IMS. Is COXVIb association with COX impaired in mutant cells?
3. The mature human COA8 has four Cys residues, but it doesn't appear that any of them are totally conserved. They should comment on this. Are these Cys residues important for the candidate IMS localization of COA8?
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Overall the study is well performed and it adds to our knowledge of COX assembly. It shows clearly that COA8 has a role in COX assembly in vivo. I have relatively minor comments.

We thank the Reviewer for the positive view about our work.

1. If they concluded that M14 is the endogenous initiation methionine, why use the M1 in the constructs?

We did not see any changes in expression levels or protein size using the constructs starting from 'M1' or 'M14'. However, we reasoned that the sequence in between those two positions is the APOPT1 mRNA 5'-UTR so that by keeping it we would maintain a more physiological structure and possibly expression of the protein.

2. In figure 4. Why is the stoichiometry between precursor/mature is so different when using HA or APOPT1.

The samples ran in lanes 2 and 3 (labelled as 'S6' and 'S2') in the Western blot image shown in figure 4A are from the patients described in Melchionda et al. 2014. The mutations predict truncated APOPT1 protein products, and, therefore the absence of the full-length precursor protein. However, the anti-APOPT1 antibody recognizes in the patient samples a protein band of the same size as the APOPT1 precursor. Obviously, the signal detected in the patients' samples cannot correspond to the APOPT1 precursor but to another protein. The band in the wild type may well correspond to both the Apopt1 precursor and to the unknown protein shared with the patients samples. Conversely, the anti-HA antibody detects only the tagged precursor and mature species with no "noise" derived from cross-reacting contaminants.

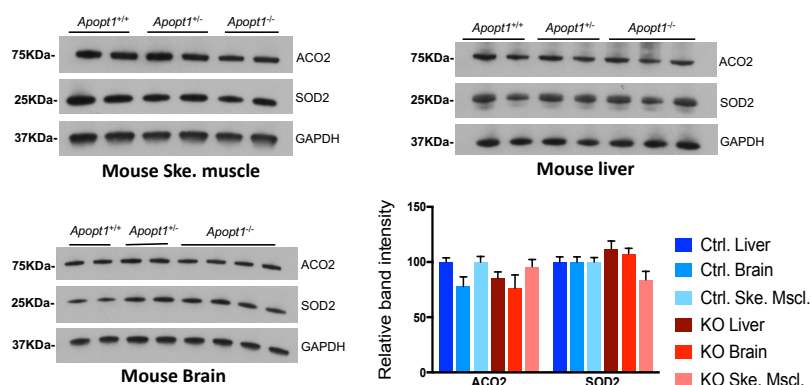
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The experiment demonstrates that HA tagged APOPT1 is functional and the accumulation and ubiquitination of the APOPT1-HA precursor following proteasome inhibition shows that this protein is very tightly regulated within the cytosol, which is in contrast to the effect of H2O2 where the cleaved mitochondrial form accumulates. These experiments also show that APOPT1 stabilisation following proteasome inhibition is a direct effect on the protein (as it is ubiquitinated), rather than an indirect effect of pharmacological treatment.

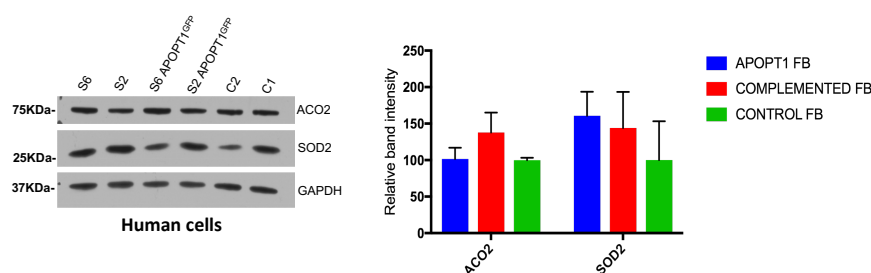
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because of less COX. However, that led to an adaptation where less ROS scavenging mechanisms are required. These cells, when challenged, may be more sensitive to the oxidative stress.

This could indeed be a valid hypothesis. However, the basal H₂O₂ production in patient-derived fibroblasts was not lower than in control fibroblasts (Melchionda et al., 2014). Also, we could not detect differences in basal ROS production in isolated mitochondria from heart and brain of KO and WT mice. In addition, we tested the steady-state levels of SOD2 as well as those of ACO2 (mitochondrial aconitase, which serves as a marker of oxidative stress) by Western blot in mouse liver, brain and skeletal muscle and we did not find differences between Apopt1^{wt} (wt = +/+ and +/-) and Apopt1^{-/-} (see image below showing representative blots obtained with female mice samples and the result of the densitometric quantification summarized in the graph).



To further address the point raised by the Reviewer, we have analysed the steady-state levels of SOD2 and aconitase in the patient-derived immortalized fibroblasts, the same cell lines complemented with wild-type APOPT1, and in two different controls. As it can be seen below, the levels of SOD2 were not lower in the patient-derived fibroblasts and neither SOD2 nor ACO2 showed significant differences in abundance in the three types of analysed cell lines (APOPT1-null, COMPLEMENTED and CONTROL fibroblasts).



All these observations suggest that there is no decrease of ROS in basal states and no decrease in antioxidant stress defences in APOPT1-less cells and tissues.

Referee #2 (Remarks for Author):

In 2014, this group reported patient mutations in APOPT1 causing a COX deficiency. This study revealed a marked COX deficiency in muscle biopsy samples and in patient fibroblasts. The APOPT1-GFP fusion was reported to be mitochondria and suggested to be matrix localized based on the MTS cleavage. One highlight of the characterization of APOPT1 was that the protein was unstable in cells unless proteasomal degradation was blocked or the cells were treated with hydrogen peroxide. They concluded this study with the suggestion that APOPT1 exerts a protective role on COX during oxidative stress.

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observations. They now generate an APOPT1 knockout mouse model, which shows the same COX deficiency associated with neurologic deficits. Thus, the APOPT1 KO mouse is an excellent model for neurologic testing studies. They demonstrate that APOPT1 depleted mouse tissues and human patient fibroblasts exhibit a diminution in COX subunits and MT-CO1 assembly intermediates. These studies are very well executed and the effects are marked. However, no major new insights are provided on the function of APOPT1. The investigators report again the oxidative accumulation of APOPT1 and show that APOPT1-deficient cells are oxidatively challenged with enhanced turnover of COX subunits. The significance of this present study is the more thorough characterization of APOPT1-deficient cells, but many observations are reflected in their 2014 publication, which diminishes the novelty of the report.

The Cas9-mediated deletion of mouse APOPT1 is very well executed and discussed. Although they were unable to validate the KO by immunoblotting due to no protein detection, it is clear that a deletion was attained. The KO mice had reduced motor skills based on a myriad of solid studies. The COX biochemical studies on the KO tissues are well done, but it is surprising that no oxygen consumption measurements were done to address the consequences of reduced COX activity.

We did some measurements from knockout mouse heart and liver isolated mitochondria. We observed a reduction of 33% in oxygen consumption induced by succinate, compared with the wild type, which is consistent with the COX deficiency displayed by the mouse tissues. We did not perform a systematic set of measurements as we considered that the kinetic and histochemical analyses were sufficient to demonstrate the significant isolated COX deficiency associated with the absences of COA8.

They demonstrated that APOPT1-HA and GFP fusions expressed in HEK293 and 143B cells showed mitochondrial localization and loose association with the inner membrane. Using graded digitonin extractions followed by trypsin digestion, they conclude that the protein is IMS localized, but the data are not convincing and are not validated with other studies.

We think that there is some terminological confusion about the localization of APOPT1. In the experiments shown in figure 4 we demonstrate that APOPT1 is associated with the inner mitochondrial membrane, after cleavage of the MTS. This conclusion is provided by the evidence that the protein is present in the membrane fraction in basal conditions, and is partially extracted only by treatment with 0.1M Na₂CO₃, pH 10.5. In addition, the experiments based on the sensitivity of different proteins to trypsin proteolysis in the presence of increasing concentrations of the mild detergent digitonin, show that APOPT1 is partially sensitive to trypsin+digitonin, in a way very similar if not identical to AIF (apoptosis inducing factor), which is a protein bound to the inner membrane of mitochondria but protruding towards the intermembrane space. Other inner membrane-bound proteins, such as CO1, which is embedded in the membrane, or matrix proteins (Aco2) are instead completely protected by trypsin, unless the membranes are solubilized by Triton X100. This behaviour to trypsin digestion is indeed similar to a known IMS soluble protein (AK2) which however does not co-fraction with the membranes. Therefore, the combination of these two results, co-fraction with membranes and partial sensitivity to trypsin, indicates that, like AIF, APOPT1 is indeed an inner membrane protein facing the IMS. This concept has now been clearly explained in the manuscript.

The MTS of APOPT1 is cleaved in cells, so if APOPT1 is indeed IMS, it is one of the unusual IMS proteins with a cleaved MTS. Since vector borne-APOPT1 can rescue S2 or S6 skin fibroblast mutant cells, they could attempt to validate the IMS localization by testing whether replacing the APOPT1 MTS with a classical matrix MTS would give complementation.

We would like to draw the attention of Reviewer 2 to the fact that we never asserted that APOPT1 is a soluble IMS protein. Of course, we do not know whether the protein is completely translocated through the inner membrane, cleaved in the matrix and repositioned in the inner membrane, or simply inserted in the inner membrane where the N-terminal MTS is then cleaved off. Possibly, the latter is the most likely mechanism, known to act also for other proteins, for instance PINK1, MICU1 and possibly AIF itself.

Is APOPT1 released by hypotonic solutions? It is important to validate the mitochondrial localization to better anticipate what functional role it may play in COX maturation under oxidative conditions.

We would like to emphasize that we find APOPT1 only in the membrane fraction of isolated mitochondria even after the samples are vigorously sonicated, indicating a tight physical binding to the membrane that only strong alkalization and increase of the ionic strength by carbonate can partially disrupt.

They postulate that COA8 exhibits a role in stabilizing the MT-CO2 module or the insertion of this module to the MT-CO1 module. A second way to validate an IMS localization is to assess interaction partners with affinity purified APOPT1. Does COA8 bind COX subunits or assembly factors such as SCO1, COA6, etc that affect the maturation of the MT-CO2 module?

We indeed performed immunopurification experiments of APOPT1^{HA} and APOPT1^{GFP} and tested the purified fractions for the presence of COX structural subunits, but failed to detect any of them.

COA8 runs on a DDM BN gel with an apparent mass of ~40 kDa. Based on this apparent mass, they suggest that COA8 may interact with a protein of ~20 kDa. One cannot quantify the mass at the low end of BN gels, so this suggestion is not well based.

We thank the Reviewer for this remark and agree that this estimation of the molecular weight is not accurate enough. We have eliminated this statement from the text and substituted it with the following sentence in page 12: "This suggests that both versions of APOPT1 are not stably interacting in a high-molecular weight complex, including the COX assembly intermediates containing MT-CO1 or MT-CO2."

However, a proteomic study of affinity purified COA8 might be very insightful and add impact to the current study. The BN study in Fig 4D revealed that under BN-PAGE conditions, COA8 does not associate with COX. However, BN conditions often dissociate weak interactions, so coIP studies are needed.

In addition to the unlabelled co-IP experiments with the targeted detection of COX structural subunits, we also performed SILAC experiments combined with immunopurification of APOPT1^{GFP}. Again we could not find any significant interaction with mitochondrial proteins, suggesting that APOPT1 does not establish permanent or long-lasting stable interactions with partner proteins. This is also suggested by the fast migration of APOPT1 in BNGE with DDM and digitonin, which again indicates lack of stable interactions. It is possible that more subtle interactions can be detected by different techniques (e.g. cross-linking, bio-ID), which will require a dedicated optimization.

The enhanced turnover of COX in APOPT1-deficient cells may arise from an oxidant-induced mechanism. One wonders whether OMA1 is activated in the deficient cells and contributes to the COX subunit turnover.

In summary, the description of the mouse model is excellent and worthy of publication. The apparent role of APOPT1 in stabilizing COX in oxidative conditions is striking and elucidating this mechanism would be a major contribution to the field. The impact of these studies would be markedly strengthened if some of the mentioned concerns were addressed.

A series of other concerns should be addressed.

1. The mature form of COA8 was stabilized by treatment with hydrogen peroxide. They conclude that this form must be mitochondrial, but many IMS proteins (e.g. COX19) are not stably localized in the IMS under stress conditions. They should validate that COA8 remains in the IMS under oxidant treatment.

As we tried to explain above, we strongly believe that APOPT1 is bound to the inner membrane of mitochondria rather than free in the IMS. However, we performed an experiment based on immunofluorescence of APOPT1-GFP in cells under H2O2 treatment and did not observe any dispersion of the signal or change of the "mitochondrial pattern" of the APOPT1-specific IF.

Under normal culture conditions, COA8 levels are labile suggesting proteolytic degradation. If COA8 is IMS localized, perhaps iAAA is responsible for the degradation or if matrix perhaps the LON protease mediates its diminution. Such studies could help validate the mitochondrial localization of COA8.

This is clearly an interesting question but we think it deserves a dedicated project. AIF, for instance, is cleaved by calpain to produce the soluble form that eventually triggers apoptosis. We have clearly shown that APOPT1 is mainly degraded as a precursor in the cytosol. At this stage of the study, the further intramitochondrial degradation of the protein is clearly interesting but perhaps not priority.

2. The APOPT1-deficient cells exhibit a down-regulation of COX subunits and subcomplexes. If COA8 is indeed IMS-localized, it could affect COX maturation through the association of COXVIb, which is oxidatively folded in the IMS. Is COXVIb association with COX impaired in mutant cells?

As shown in Figure 3D, the steady-state levels of COX6B1 are very low in the knockout mouse liver and skeletal muscle. However, they are as reduced as other COX subunits that are assembled late (MT-CO3) or in the intermediate steps of COX assembly (MT-CO2 and COX5B). Therefore, the association of the subunit is impaired, but it does not seem to be a specific phenomenon for COX6B1.

3. The mature human COA8 has four Cys residues, but it doesn't appear that any of them are totally conserved. They should comment on this. Are these Cys residues important for the candidate IMS localization of COA8?

Thank you for this comment. The "oxidized cys" hypothesis has to be tested for instance by mutating the cys residues, again an experiment that is in the pipeline of future investigation on this intriguing protein. However, we notice that even in the human sequence the distribution of the cys residues does not follow the consensus of IMS proteins (Cx3C or Cx9C).

4. References need editing for consistency in journal abbreviations and word spacing.
OK, thank you.

2nd Editorial Decision

17 October 2018

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending minor editorial amendments and a response to referee 2. We would like to encourage you to add experimental evidence as suggested during the 1st review. Failing this, the conclusions have to be softened. Please make sure to discuss the referee comments in a point-by-point letter.

I look forward to reading a new revised version of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

It is an interesting and well-performed study of a disease-related cytochrome c oxidase assembly factor with mechanistic insights.

Referee #1 (Remarks for Author):

The authors have addressed my concerns

Referee #2 (Comments on Novelty/Model System for Author):

The impact is somewhat limited based on their previous report. The mouse model is excellent and it validated their earlier work. Beyond that, the impact of the work is only "medium"

Referee #2 (Remarks for Author):

This reviewer is disappointed that the investigators ignored all requests to substantiate their studies to gain rigor. Certain conclusions are based on weak data such as the protrusion of Coa8 into the IMS. In the revision, their statement "Taken together, these observations clearly indicate that APOPT1 is a protein tightly associated with the inner membrane, which is protruding into the IMS." is unacceptable without qualification since they did not validate the one observation. Without additional studies to substantiate observations, this revision is only partially above the bar for acceptance. They must soften their conclusion that Coa8 protrudes into the IMS. This is such an important point in considering how Coa8 may exert its stabilizing function.

2nd Revision - authors' response

15 November 2018

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Our apologies for not addressing the question of the localization in a more rigorous way before. We thank Referee #2 for insisting in clarifying this point as it made us aware of the important functional implications related to APOPT1 topology within mitochondria. We have now re-addressed the question of APOPT1 sub-mitochondrial localization by performing an additional biochemical experiment of mitochondrial extraction, subfractionation by hypotonic shock and protease protection assays in APOPT1^{HA} cells. In addition, we have performed super-resolution fluorescence imaging, without using antibodies, in APOPT1^{GFP} cells (showed in the new Figure 4D). After treating the mitochondria with the hypotonic buffer, and replying to one of the Referee's original questions, we did not observe any release of APOPT1 to the supernatants while the soluble intermembrane space protein AK2 was clearly detectable (new Figure 4C). Also, a small peptide derived from SCO2 after trypsin digestion was released to the supernatant. The fluorescence-based imaging confirmed that APOPT1-GFP is contained within the matrix compartment and does not protrude beyond the contour of the inner membrane.

After evaluating these new data, we now conclude that the APOPT1 C-terminus is in the mitochondrial matrix. This part of the results has been re-written in pages 11 and 12 and the new results are shown in the new Figure 4.

Again, we thank Referee #2 for prompting us to obtain additional important information on this issue.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Massimo Zeviani and Erika Fernandez-Vizarra

Journal Submitted to: EMBO Molecular Medicine

Manuscript Number: EMM-2018-09582

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 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.

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?	We ran power analysis on the basis of preliminary data, assuming significance when $p < 0.05$ and power ≥ 0.8 .
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	In general, for each experiment we used ≥ 3 animals per genotype, in order to obtain statistically suitable values.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals were randomly allocated in the different groups based on the appropriate genotype. No animal or cell sample was excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes, the animals were randomly analysed in order to have similar numbers per genotype.
For animal studies, include a statement about randomization even if no randomization was used.	We used randomization as explained above.
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.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The animals and cell samples were randomized but the study was not based on blinding of the investigators.
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.	We assumed normal distribution. No particular method was used to determine whether the data met assumptions of the statistical approach.
Is there an estimate of variation within each group of data?	Yes data variation is expressed as Standard Error of the Mean (SEM)
Is the variance similar between the groups that are being statistically compared?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

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

<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-tun>

<http://datadryad.org>

<http://figshare.com>

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

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

<http://biomodels.net/>

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

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).	We added a suitable list in the main manuscript
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Patients fibroblasts have not been authenticated as they are derived from a certified Telethon Italy BioBank. HeLa and 143B cell have not been recently authenticated as the experiments were based on internal comparisons. Our cell lines are routinely tested for mycoplasma contamination.
* 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.	Mus musculus FVB/NJ, male and females, 3-12 months old, some animals were carrying one or both CRISPR/CAS9 modified APOPT1 alleles to obtain a constitutive knockout for this gene. The animals were maintained in a temperature- and humidity-controlled animal-care facility, with a 12 hr light/dark cycle and free access to water and food, and were sacrificed by cervical dislocation.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All procedures were conducted under the UK Animals (Scientific Procedures) Act, 1986, approved by Home Office license (PPL: 7538 and P6C97520A) and local ethical review. The mice were kept on a FVB/NJ background, and wild-type littermates were used as controls.
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.	We followed the ARRIVE guidelines for reporting animal experiments.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	We obtained cell lines from the cellular BioBank of Telethon Italy located at the Fondazione Istituto Neurologico Carlo Besta, Milan, Italy.
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.	The patients' cells and controls are the same as published in Melchionda et al. 2014 for which informed consent was obtained from the patients or parents as reported in the paper.
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.	No restrictions
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 Biocompare (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.	N/A
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