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Ca2+ binding to F-ATP synthase β subunit triggers the mitochondrial permeability transition

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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 26 September 2016

Thank you for the submission of your manuscript to EMBO reports. I have now read and discussed your work with my colleagues here and also consulted an external advisor about your manuscript, and I regret to say that we all agree that it is not well suited for our journal.

We appreciate that your study reports that calcium binding to the catalytic site of the F1 ATP synthase is required for PTP opening. You find that a T163S mutation in the beta subunit decreases Ca2+-ATP hydrolysis and calcium sensitivity of the PTP in HeLa cells. Molecular dynamics simulations indicate that the mutation prevents calcium-induced conformational changes and affects OSCP motility. Moreover, your data indicate that the T163S mutation decreases apoptosis in developing zebrafish embryos and protects from PTP-induced cell death in cell culture.

We acknowledge that your findings indicate that calcium binding to the F-ATPase catalytic domain induces PTP opening. However, we also note that the evidence is based on a single mutation and MD simulations and no further evidence for the functional significance of the proposed conformational changes is provided. The expert advisor consulted shares our concerns. While we certainly appreciate the interest that this study will have for those working closer to the field, we overall feel that the advance and molecular insights provided are not sufficient for consideration for

publication in EMBO reports. We have therefore decided not to proceed with in depth peer review.

Please note that we can only publish a very small fraction of the many manuscripts that are submitted to our journal and that we therefore have to make a rather stringent selection on which ones to send out for peer review. I am sorry to have to disappoint you on this occasion, and hope that this will not prevent you from considering EMBO reports for publication of your work in the future.

Author appeal letter 28 September 2016

Thank you for your reply. We are a little surprised about your comment that "the evidence is based on a single mutation." Based on the literature in prokaryotes the T>S replacement at the catalytic site is the ONLY option because it allows normal function of F-ATP synthase with MgATP but not CaATP. All the other tested replacements prevent MgATPase activity, which means lethality. Mutagenesis at the catalytic site has not even been attempted before, and to the best of our knowledge this is the first example that this is possible with an eukaryote. The functional implications (decreased PTP-dependent cell death) are documented in the manuscript, so we feel that also the statement "no further evidence for the functional significance of the proposed conformational changes is provided" is not fully justified.

We would be grateful if you could bring our point to the attention of your external advisor and the board, because we are convinced that this issue substantially contributed to your negative decision. We should have been more explicit, which will be easy to do if we will be allowed to resubmit. Thank you in advance for your attention.

2nd Editorial Decision 09 November 2016

Thank you for the submission of your research manuscript to EMBO reports. It has been sent to three referees, and so far we have received reports from two of them, which I copy below. As both referees feel that the manuscript is interesting and provide constructive suggestions on how to improve the study, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this.

In the current reports, referee 1 asks to address the effect of the T>S mutation on ATP hydrolytic activities and to confirm the expression levels of the shRNA-resistant ATP5B constructs. This referee also suggests to test the effect of exogenous calcium addition on WT and T>S cells to confirm that the effects of Ca2+ are mediated through the beta subunit of the ATPase. Moreover, this referee indicates that the observed effect on apoptosis in zebrafish is inconsistent with previous findings. Referee 2 also recommends to reassess ATPase activity and to measure if there are differences in calcium uptake between the wt and the T>S protein. Moreover, the referees suggest to tone down some of the statements and to carefully review the introduction.

Overall, given the present referee comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

1) Introduction, pge 3: the authors state that "Concerning the molecular nature, each of the proposed PTP constituents (adenine nucleotide translocase, voltage-dependent anion channel, Pi carrier and peripheral benzodiazepine receptor) was ruled out by targeted gene deletion [7-11] and the only

established protein involved in the PT is cyclophilin D (CyPD), a modulator rather than a structural component as revealed by analysis of CyPD-null mitochondria [12-15]." This is incorrect: the adenine nucleotide translocator, specifically isoform 1, has been recently shown to mediate voltage sensing of the permeability transition (PMID: 27221760), thus being a modulator of the pore.

2) Introduction, page 3: the authors report that "The channel-forming properties of F-ATP synthases, and their match to the corresponding PTPs, have been also established in yeast and drosophila [19,20] and are supported by independent studies by other laboratories [21,22]." However, the authors have been claiming for a PTP formed in-between two adjacent F-ATP synthases (PMID: 23530243, PMID: 23675351), while the independent studies by other laboratories support the idea that the pore is formed within the c rings structure of one F-ATP synthase. I recommend to the authors to elaborate on these concepts more clearly, especially in view of the fact that they reference this work of theirs (reference 17), while the dimeric requirement of their earlier proposed model of the PTP is not even mentioned in the present manuscript.

3) Introduction, page 3: the authors mention that "Matrix Ca2+ is a unique "permissive" factor as the PTP cannot form in its absence". This is incorrect: cationic uncouplers (such as the organic divalent cationic cyanine dye tri-S-C4(5) and the sulfhydryl cross-linker Cu(OP)2) are also inducers of mitochondrial permeability transition, in a manner unrelated to Ca2+ (PMID: 9645482). Mastoparan is also inducing permeability transition in the absence of calcium (PMID: 25039402).

4) Introduction, page 4: the authors cite the work of Papageorgiou et al (reference 38), stating that "Binding of Ca2+ has major functional consequences, as Ca2+-ATP hydrolysis does occur yet there is no buildup of the H+ gradient [38]", and in the next sentence conclude that this means that "Ca2+ causes a conformational change in the enzyme complex that impairs energy conservation and that could be accounted for by PTP opening". I fail to see the logical connection between the statement regarding reference 38, and the evocation of Ca2+-dependent conformational change in the complex; since Ca2+ ATP hydrolysis is not associated with a buildup of proton gradient, why can't it be that Ca2+ leads to changes in pH or exerts on other bioenergetic parameters (such matrix ADP/ATP levels, membrane potential) which are cited and characterized in hundreds of publications, that could confer alterations in the threshold for permeability transition opening?

5) In Results and Discussion, page 4, I could not locate figure S1, but I think the data they describe are in figure EV1, panels A and B.

6) In figure EV1, please include positions from a molecular weight marker, for results shown in panel C.

7) Please show in a figure or mention in the text the ATP hydrolytic activities (WT vs T>S for both Ca2+ and Mg2+) in the same scale. From figure panels 1A and 1B one cannot deduce what is the effect of T>S in case of Ca2+ ATP hydrolytic activity, compared to the WT Mg2+ ATP hydrolytic activity (as they are on different y-axes), which is the native situation. Also, in reference 39, mutation of T159 in Rhodospirillum led to the complete loss of Ca2+ ATP hydrolytic activity, while the authors show an ~50% loss of Ca2+ ATP hydrolytic activity in HeLa cells from the T163S, which makes me wonder how relevant this model is to test the hypothesis of a Ca2+ site on the beta subunit being critical for PTP induction. Perhaps the Ca2+ ATP hydrolytic activity in HeLa cells is much smaller than the Mg2+ ATP hydrolytic activity, which is also why I suggest to the authors to show data on ATP hydrolytic activities (WT vs T>S for both Ca2+ and Mg2+) in the same scale. Since the methodology following NADH oxidation requires Mg2+, I suggest to the authors to use the molybdate assay, but with a lot less cells, so that they will not reach saturation of the assay.

8) Page 5, regarding the results of qPCR analysis of mRNA for the shRNA -resistant beta sequences, the authors report values as +/- S.E. Is that of the mean?

9) One of the most important part of the manuscript is the suppression of the endogenous beta subunit transcript, while expressing shRNA-resistant WT or mutant ATP5B cDNA in Hela cells. Yet, I don't see any figure showing the results of qPCR comparing the endogenous ATP5B levels, with the reexpressed shRNA-resistant WT or mutant cDNA for the same gene. Also, for results shown in panels 1A and 1B the ATPase activity of the WT is the one in which endogenous ATP5B has been suppressed and shRNA reexpressed with the WT cDNA of ATP5B, or is it the native WT?

It is not clear from the text nor the legend.

10) In figure panel 1E, the PTP inhibitors ADP, CsA and compound 60 abolished the difference between WT vs T>S (although for the case of CsA, this is not so obvious from the corresponding bars). How do the authors explain that? Would this mean that the T163 is unrelated to the actions of ADP, CsA and compound 60?

11) In figure 3, please include positions from a molecular weight marker, for results shown in panel B. Same panel, please replace the arrow with the caption "caspase 3, cleaved form".

12) Page 6, regarding the experiments addressing T163S mutation and apoptosis in developing zebrafish embryos, how do the authors reconcile their reasoning and findings (figure 4, panels E and F) with the publications showing that the calcium-induced, cyclophilin D-dependent permeability transition is not associated with apoptotic, but necrotic death (PMID: 15800627, PMID: 15800626, PMID: 16103352)?

13) In figure 4, please include positions from a molecular weight marker, for results shown in panel D.

14) Regarding the effect of T163S diminishing apoptosis resulting in curly tails and decreased skin pigmentation, I suggest to the authors to check the effect of known apoptotic inhibitors, and test if they also reproduce the phenotypes of curly tails and decreased skin pigmentation.

15) Finally, I suggest to the authors to test the effect of exogenous calcium addition on WT and T163S HeLa cells on a host of bioenergetic parameters, such as mitochondrial membrane potential, respiration (also comparing the effects of NAD+-linked vs FADH2-linked substrates), DeltapH, ATP output, overall mitochondrial morphology. That would indicate if the effects of Ca2+ are at least partially mediated through an effect on the beta subunit of the ATPase.

Referee #2:

The authors describe analysis of a point mutant in a subunit of the mammalian beta subunit of the mitochondrial F1-ATP synthase (T163S). This site had been previously described as involved in the divalent cation sensitivity of the ATPase activity of this important protein complex at the heart of oxidative phosphorylation. They include biochemical measurements of ATPase, oxygen consumption and Ca uptake capacity prior to mPTP opening. They show molecular dynamic simulations of beta subunit structure, as well as cell death/apoptosis results in cells and zebrafish. There is much nice data presented, and they make a good case for this amino acid in F1-ATP synthase being important in mPTP. However, there are some aspects that limit the present version of the manuscript.

1. The results here are consistent with the authors contention that the F1/F0 ATP synthase is the mPTP channel, but it certainly does not prove it or demonstrate the molecular mechanism. While this is a nice set of observations, it may only be a step in this important mechanistic question. The authors should be a bit more conservative about how these results are consistent with a mechanistic role for this mutated site in mPTP control, but that the directness of this link in the real molecular mechanism of mPTP activation is not yet clear.

2. The ATPase activity should be specified as oligomycin-sensitive (assuming that was the case). It is not clear from the description, but it would be important that this is really mitochondrial F1/F0 ATPase (and not other ATPase activity that includes e.g. Na/K-ATPase or SERCA).

3. In Figure 1D, presumably the data is vertically offset (and baseline [Ca] was the same for both WT and T>S). This should be indicated somehow. It would also be worthwhile to know whether there was any difference in the apparent Ca uptake rate during [Ca]i decline of the first couple of pulses. At this point there should be no mPTP opening and the data might be a useful test for unaltered Ca uptake in the T>S vs. WT beta protein.

4. Related to the above, it would seem like mPTP inhibition by CsA and compound 60 should

virtually abolish the difference in Ca uptake between WT and T>S. The fact that it doesn't raises the possibility that uptake also differs in the T>S mitochondria.

5. Fig 2C does not communicate very well the points about movement and structure raised in the Results. This should be improved either by wording of text, improved graphics (or both). Fig EV3 seems to imply that the protein is 'stiffened" by Ca in WT, but that the opposite is true for the T>S mutant. If this is this right, the consequence should be discussed.

6. The study falls short of demonstrating how the structural effects on the beta subunit could propagate to the IMM region and where the actual mPTP pore structure is. Acknowledging this limitation is important.

7. The cell death data in Figures 3 and 4 is useful data to support the potential functional effects of the T>S mutation, but the mechanistic link with mPTP circumstantial rather than direct.

2nd Editorial Decision 15 November 2016

Editorial decision part two:

We have meanwhile received the third referee report on your manuscript that I herewith forward to you (see below). Also this referee thinks that the findings are potentially interesting but is also concerned that the data are somewhat preliminary. He/she suggests specific experiments to strengthen the conclusions and I think that all of them should be addressed. Given this overall positive evaluation I maintain my preliminary decision and invite you to revise your manuscript with the understanding that also the concerns of referee 3 must be fully addressed in your revision and in the point-by-point response. I wish you success with the work ahead and look forward to seeing a revised version of your manuscript when it is ready.

REFEREE REPORTS

Referee #3

This is a very interesting hypothesis being tested here on the role of Ca in generating a PTP type channel in the F-ATPase synthtase. Experiments are conducted on multiple levels from isolated mitochondria, cells and whole animals. Molecular dynamic simulations are provided that are outside of my expertise and I will not comment on them. In general, these studies seem a bit preliminary. Mutation introductions are being used with no quantitative information on PROTEIN expression in the cells or tissues. Secondly, testable hypothesis of the Ca level in the cells prior to death in the WT and mutant was stated but not tested. While measuring intracellular Ca is now very straightforward in HELA cells. Finally, though not a necessary addition to the manuscript, a more general proteome study should be conducted to assure that other protein changes, in addition to the introduced mutations, are not occurring over the rather long time course of these studies.

My specific comments follow in order of presentation in the manuscript and not of importance:

Page 4: "These results perfectly match those obtained in Rhodospirillum rubrum [39,40] and are of particular relevance because they demonstrate that the subunit bearing the T163S mutation had been incorporated in the F-ATP synthase complex."

I would suggest that these data "match" but nothing is perfect. Secondly these data are consistent and do NOT demonstrate that the T163S mutation has been incorporated. I would suggest these conclusions be modified.

Page 4: " By qPCR analysis, we found that mRNA levels of the rescued shRNA-resistant sequences were approximately one-third of the total. Assuming that the mutant subunits are distributed randomly, these findings suggest that one mutant copy per assembled complex is sufficient to affect F-ATP synthase catalysis."(special characters removed)

This is very speculative. The relationship between mRNA levels and mitochondria protein is very tenuous as can be seen from the work of Peipei Ping and others. Since only a 50% reduction in Ca-ATPase activity was detected, it could be that only 50% of the protein had the mutation. What the mutation did to protein trafficking and complex assembly makes the extrapolation of mRNA to protein level in a given matrix complex tenuous. Direct determination is necessary.

Page 4: The observation that the mutation of only 1/3 of the peptides in the F-ATP synthase changes the Ca sensitivity to PTP by a factor of 2 in isolated mitochondria is the most interesting and direct finding in the manuscript. Hopefully, no other protein expression levels or PMT's were affected by the introduction of the mutation.

Molecular Dynamic Simulations: I am not an expert in molecular simulations thus I will not comment on this aspect of the study.

Page 6: The effect of the mutation on the intact cell viability was modest, this could be due to the lack of penetration of the mutation as discussed above or other changes in the cells associated with the mutation. The authors, in addition to outlining the non-specificity of the pharmacological challenges, state "Second, mitochondria expressing the T163S mutant displayed an increased CRC (i.e. increased resistancev to Ca2+) yet they still eventually opened the PTP at higher Ca2+ loads (Fig 1D,E)." This should be validated by monitoring the cytosolic calcium in these cells to show they can tolerate higher calcium levels as extrapolated from the isolated mitochondria studies. This is rather an easy experiment to conduct and could even be done in conjunction with the cell sorting or the cells prior to sorting.

Page 6: It is important in the Zebra fish studies to understand the extent of protein expression in the human WT and mutated forms. The authors state: "Whole-mount immunofluorescence and Western blotting (Fig 4C,D)indicate that the expected replacement of the endogenous subunits took place to a similar extent for the wild-type and T163S mutant species." However figure 4D is of the alpha subunit and TOM 20 which were not affected (data is consistent with that conclusion) but no information on the beta subunit. Figure 4C is immunohistology of the beta subunit and TOM. It is unclear looking at the images or the merged data whether they can state what the "replacement" was. Please provide quantitative data on the relative expression levels of the WT and mutation T163S in these studies.

Page 16: "Data information: In panels A-E one representative experiment of xx is shown." Please fill in "XX".

2nd Revision - authors' response 08 February 2017

We are submitting a revised version of our manuscript EMBOR-2016-43354V2-Q “Ca2+ binding to F-ATP synthase subunit triggers the mitochondrial permeability transition.”

First of all, we would like to express our gratitude to the 3 Reviewers for their fair and useful critiques. We have addressed all their comments, and we are much happier with the revision, in particular with the new experiments on ATPase activity, in situ Ca2+-dependent mitochondrial depolarization and in vivo effects of z-VAD-fmk.

Our detailed comments are reported below. We hope that they will be found adequate and we look forward to hearing from you about your decision.

Referee #1:

1) Introduction, pge 3: the authors state that "Concerning the molecular nature, each of the proposed PTP constituents (adenine nucleotide translocase, voltage-dependent anion channel, Pi carrier and peripheral benzodiazepine receptor) was ruled out by targeted gene deletion [7-11] and the only established protein involved in the PT is cyclophilin D (CyPD), a modulator rather than a structural component as revealed by analysis of CyPD-null mitochondria [12-15]." This is incorrect: the adenine nucleotide translocator, specifically isoform 1, has been recently shown to mediate voltage sensing of the permeability transition (PMID: 27221760), thus being a modulator of the pore.

Answer: We have modified the sentence as follows: "The PT is affected by the adenine nucleotide translocase isoform 1 [12] through an effect on voltage sensing [13] and by the outer membrane through an undefined mechanism [14]. The best characterized protein involved in the PT, however, is cyclophilin D (CyPD), a modulator rather than a structural component as revealed by analysis of CyPD-null mitochondria [15-18]."

2) Introduction, page 3: the authors report that "The channel-forming properties of F-ATP synthases, and their match to the corresponding PTPs, have been also established in yeast and drosophila [19,20] and are supported by independent studies by other laboratories [21,22]." However, the authors have been claiming for a PTP formed in-between two adjacent F-ATP synthases (PMID: 23530243, PMID: 23675351), while the independent studies by other laboratories support the idea that the pore is formed within the c rings structure of one F-ATP synthase. I recommend to the authors to elaborate on these concepts more clearly, especially in view of the fact that they reference this work of theirs (reference 17), while the dimeric requirement of their earlier proposed model of the PTP is not even mentioned in the present manuscript.

Answer. We have now mentioned dimers in the Introduction (page 3) and acknowledged the existence of different opinions by adding the sentence that "....there is no agreement on the mechanism of pore formation (see ref [3] for discussion)" at the end of the paragraph mentioned by the Reviewer. The present manuscript does not directly bear on the issue, but we have now added a brief (prudent) comment about the dimer hypothesis in the conclusions to read "Although they cannot predict whether F-ATP synthase dimers are required for PTP formation [20], our MD simulations provide a potential mechanism through which the Ca^{2+}-dependent effect on the catalytic sites is transmitted via OSCP to the peripheral stalk and thence to the inner membrane, a testable hypothesis for future studies".

3) Introduction, page 3: the authors mention that "Matrix Ca2+ is a unique "permissive" factor as the PTP cannot form in its absence". This is incorrect: cationic uncouplers (such as the organic divalent cationic cyanine dye tri-S-C4(5) and the sulfhydryl cross-linker Cu(OP)2) are also inducers of mitochondrial permeability transition, in a manner unrelated to Ca2+ (PMID: 9645482). Mastoparan is also inducing permeability transition in the absence of calcium (PMID: 25039402).

Answer: We are familiar with this problem, as we have shown that phenylarsine oxide induces the PTP in the absence of ***added*** Ca^{2+} as early as 1991 (Lenartowicz et al 1991, J. Bioenerg. Biomembr. 23, 679). Although we never published this, it turns out that the effect may depend on endogenous matrix Ca^{2+} (swelling could be significantly inhibited by incubation with EGTA). This may well be the case for cationic uncouplers as EGTA was not tested. Mastoparan is a special case (swelling is completely insensitive to CsA and appears to occur via perturbation of the lipid phase). Given that all common pore inducers strictly depend on Ca^{2+} (as does opening of the megachannel at the patch clamp and of the gel-purified F-ATP synthase in monolayers) we think that Ca^{2+} is essential, yet we have toned down the sentence to "Matrix Ca^{2+} is a key factor for PTP opening".

4) Introduction, page 4: the authors cite the work of Papageorgiou et al (reference 38), stating that "Binding of Ca2+ has major functional consequences, as Ca2+-ATP hydrolysis does occur yet there is no buildup of the H+ gradient [38]", and in the next sentence conclude that this means that "Ca2+ causes a conformational change in the enzyme complex that impairs energy conservation and that could be accounted for by PTP opening". I fail to see the logical connection between the statement regarding reference 38, and the evocation of Ca2+-dependent conformational change in the complex; since Ca2+ ATP hydrolysis is not associated with a buildup of proton gradient, why can't it be that Ca2+ leads to changes in pH or exerts on other bioenergetic parameters (such matrix ADP/ATP levels, membrane potential) which are cited and characterized in hundreds of publications, that could confer alterations in the threshold for permeability transition opening?

Answer: We agree that there are alternative explanations and we have therefore deleted the second part of the sentence, which mentioned the PTP only. The sentence now reads, "Thus, Ca^{2+} causes a conformational change in the enzyme complex that impairs energy conservation".

5) In Results and Discussion, page 4, I could not locate Figure S1, but I think the data they describe are in Figure EV1, panels A and B.

Answer: Sorry for the mislabeling, this was indeed meant as EV1, thanks.

6) In Figure EV1, please include positions from a molecular weight marker, for results shown in panel C.

Answer: done, thank you.

7) Please show in a Figure or mention in the text the ATP hydrolytic activities (WT vs T>S for both Ca2+ and Mg2+) in the same scale. From Figure panels 1A and 1B one cannot deduce what is the effect of T>S in case of Ca2+ ATP hydrolytic activity, compared to the WT Mg2+ ATP hydrolytic activity (as they are on different y-axes), which is the native situation. Also, in reference 39, mutation of T159 in Rhodospirillum led to the complete loss of Ca2+ ATP hydrolytic activity, while the authors show an ~50% loss of Ca2+ ATP hydrolytic activity in HeLa cells from the T163S, which makes me wonder how relevant this model is to test the hypothesis of a Ca2+ site on the beta subunit being critical for PTP induction. Perhaps the Ca2+ ATP hydrolytic activity in HeLa cells is much smaller than the Mg2+ ATP hydrolytic activity, which is also why I suggest to the authors to show data on ATP hydrolytic activities (WT vs T>S for both Ca2+ and Mg2+) in the same scale. Since the methodology following NADH oxidation requires Mg2+, I suggest to the authors to use the molybdate assay, but with a lot less cells, so that they will not reach saturation of the assay.

Answer: We are very grateful for this extremely important comment. As suggested, we have used the molybdate method also for Mg^{2+}-ATP hydrolysis and, prompted by Reviewer 2, also included ouabain in the assays. In order to reliably monitor the kinetics of both Mg^{2+}-ATPase (which is very fast) and Ca^{2+}-ATPase in the same plate we lowered the temperature to 25°C and this turned out to be a key point, because it minimized the contribution of non mitochondrial ATPases, as clear from new Figure 1A. The results indeed show that the mutation has a nearly complete inhibitory effect on Ca^{2+}-ATPase activity. The latter enzymatic activity is insensitive to oligomycin (the statement at the end of the original legend only referred to Mg^{2+}-ATPase activity) but it is 50% inhibited by resveratrol, which is in line with the literature [Gladhill et al (2007) PNAS 104 13632-7; Sassi et al. (2014) Biochim Biophys Acta 1837, 1781-9; Madreiter-Sokolowski et al. Cell Physiol Biochem (2016) 39, 1404-20].

8) Page 5, regarding the results of qPCR analysis of mRNA for the shRNA -resistant beta sequences, the authors report values as ± S.E. Is that of the mean?

Answer. SE is the standard error of the mean (s.e.m.) as now indicated.

9) One of the most important part of the manuscript is the suppression of the endogenous beta subunit transcript, while expressing shRNA-resistant WT or mutant ATP5B cDNA in Hela cells. Yet, I don't see any Figure showing the results of qPCR comparing the endogenous ATP5B levels, with the reexpressed shRNA-resistant WT or mutant cDNA for the same gene.

Answer. The results are specified in the text at page 4 “By qPCR analysis, we found that mRNA levels of the rescued shRNA-resistant β sequences were approximately one-third of the total (WT: 26.6 ± 5.1%, n=24; mutant: 36.2 ± 4.8%, n=20, ± s.e.m.)”. We felt that this is clear enough and did not add a Figure with the results.

(continued) Also, for results shown in panels 1A and 1B the ATPase activity of the WT is the one in which endogenous ATP5B has been suppressed and shRNA reexpressed with the WT cDNA of ATP5B, or is it the native WT? It is not clear from the text nor the legend.

Answer. Yes, this is the reexpressed wild-type sequence from the shRNA-resistant construct. We have stated this in the text where the experiments are presented “.... compared with cells reexpressing the wild-type sequence”.

10) In Figure panel 1E, the PTP inhibitors ADP, CsA and compound 60 abolished the difference between WT vs T>S (although for the case of CsA, this is not so obvious from the corresponding bars). How do the authors explain that? Would this mean that the T163 is unrelated to the actions of ADP, CsA and compound 60?

Answer. In our hands there is always a maximum CRC in any type of mitochondria and permeabilized cells, independent of the inhibitor(s) used. The CRC assay isn't perfect, and the limit may also reflect matrix Ca^{2+} toxicity, so we prefer not to speculate on this point.

11) In Figure 3, please include positions from a molecular weight marker, for results shown in panel B. Same panel, please replace the arrow with the caption "caspase 3, cleaved form".

Answer. The requested changes have been made, thank you, but we kept the arrow as well.

12) Page 6, regarding the experiments addressing T163S mutation and apoptosis in developing zebrafish embryos, how do the authors reconcile their reasoning and findings (Figure 4, panels E and F) with the publications showing that the calcium-induced, cyclophilin D-dependent permeability transition is not associated with apoptotic, but necrotic death (PMID: 15800627, PMID: 15800626, PMID: 16103352)?

Answer. While this may be true in the cited models of ischemia-reperfusion injury (which specifically relied on cyclophilin D ablation), previous work indicated that depending on the intensity and duration of the stimulus, and on the levels of ATP, pore opening can induce apoptosis, necrosis or both (PMID:11134038, PMID:11134037, PMID:15070903). Of particular relevance is our *in vivo* study with CsA demonstrating that in fulminant hepatitis both necrosis and apoptosis were prevented when the pore was inhibited, in spite of cleavage of BID upstream of mitochondria (PMID:15201276).

13) In Figure 4, please include positions from a molecular weight marker, for results shown in panel D.

Answer. Done, thank you.

14) Regarding the effect of T163S diminishing apoptosis resulting in curly tails and decreased skin pigmentation, I suggest to the authors to check the effect of known apoptotic inhibitors, and test if they also reproduce the phenotypes of curly tails and decreased skin pigmentation.

Answer. Excellent suggestion, thank you. z-VAD-fmk induced a curly tail phenotype like the T163S mutation, as shown in revised Figure 4B and mentioned in the text which now reads "At 48 hpf the expression of T163S subunit caused a curly tail phenotype (which was also observed by treatment of the wild-type with the pancaspase inhibitor z-VAD-fmk, Fig 4B)". This makes us feel more confident in our conclusions.

15) Finally, I suggest to the authors to test the effect of exogenous calcium addition on WT and T163S HeLa cells on a host of bioenergetic parameters, such as mitochondrial membrane potential, respiration (also comparing the effects of NAD+-linked vs FADH2-linked substrates), DeltapH, ATP output, overall mitochondrial morphology. That would indicate if the effects of Ca2+ are at least partially mediated through an effect on the beta subunit of the ATPase.

Answer. As suggested, we tested the effect of exogenous Ca^{2+} by increasing it to 10 mM and measuring basal, oligomycin-sensitive and maximal respiration. High Ca^{2+} decreased both basal and maximal respiration but this was true in both WT and T>S HeLa cells (see attached Figure 1 for Reviewers). In order to address the Reviewer's point we have therefore measured the response of cytosolic [Ca^{2+}] (with Fluo-4FF) and of the mitochondrial membrane potential (with TMRM) to ionomycin in parallel measurements. The results are presented in new panel A of Figure 3 (we have replaced the less informative original panel) and show that the T>S mutants are resistant to

depolarization following the cytosolic Ca^{2+} rise, which is instead identical in WT and T>S cells. The mitochondrial network/morphology was not altered as now shown in the same revised Figure. The title and beginning of the paragraph now read:

"**β subunit T163S mutation protects from Ca^{2+}-dependent mitochondrial depolarization and from PTP-dependent cell death in HeLa cells**

We next tested the prediction that the T163S β mutation should have a protective effect against Ca^{2+} dependent depolarization and cell death in established Ca^{2+}- and PTP-dependent paradigms [49,50]. Cells were incubated in Hank's balanced salt solution (HBSS) supplemented with 1 mM Ca^{2+}, and intracellular Ca^{2+} and mitochondrial membrane potential monitored with Fluo-4FF-AM and tetramethylrhodamine methyl ester (TMRM), respectively. Staining with TMRM did not reveal major differences in mitochondrial morphology and extension of the mitochondrial network (Fig 3A, upper panels). Upon addition of 2 μM ionomycin intracellular [Ca^{2+}] increased rapidly in both wild-type and T>S mutant cells, while after a lag phase mitochondria depolarized in wild-type cell only (Fig 3A, lower panels). The PTP-inducing effects of Ca^{2+} ionophores are extremely well documented [49,50], and these results are thus consistent with resistance to PTP opening of the mutant cells".

Respiration was reported in original Figure 1 and it is identical in the reexpressed wild-type sequence and T>S mutant, as is the oligomycin-sensitive fraction (which means identical ATP requirements and production). All in all, we hope that we have addressed all the key concerns raised by the Reviewer.

Referee #2:

The authors describe analysis of a point mutant in a subunit of the mammalian beta subunit of the mitochondrial F1-ATP synthase (T163S). This site had been previously described as involved in the divalent cation sensitivity of the ATPase activity of this important protein complex at the heart of oxidative phosphorylation. They include biochemical measurements of ATPase, oxygen consumption and Ca uptake capacity prior to mPTP opening. They show molecular dynamic simulations of beta subunit structure, as well as cell death/apoptosis results in cells and zebrafish. There is much nice data presented, and they make a good case for this amino acid in F1-ATP synthase being important in mPTP. However, there are some aspects that limit the present version of the manuscript.

Answer: We appreciate the overall positive comments.

1. The results here are consistent with the authors contention that the F1/F0 ATP synthase is the mPTP channel, but it certainly does not prove it or demonstrate the molecular mechanism. While this is a nice set of observations, it may only be a step in this important mechanistic question. The authors should be a bit more conservative about how these results are consistent with a mechanistic role for this mutated site in mPTP control, but that the directness of this link in the real molecular mechanism of mPTP activation is not yet clear.

Answer. We have toned down the last sentence of the Introduction by removing "mechanistic" and changing "can" to "could". At the end of the manuscript we have removed "key insights into" and "that holds great promise". The final sentence now reads "Our MD simulations provide a potential mechanism through which the Ca^{2+}-dependent effect on the catalytic sites is transmitted via OSCP to the peripheral stalk and thence to the inner membrane, a testable hypothesis for future studies."

2. The ATPase activity should be specified as oligomycin-sensitive (assuming that was the case). It is not clear from the description, but it would be important that this is really mitochondrial F1/F0 ATPase (and not other ATPase activity that includes e.g. Na/K-ATPase or SERCA).

Answer. As suggested by Reviewer 1 we have now used the molybdate method for both Mg^{2+}-ATP and Ca^{2+}-ATP hydrolysis. Prompted by your comment, we also included ouabain in the assays. In order to reliably monitor the kinetics of both Mg^{2+}-ATPase (which is very fast) and Ca^{2+}-ATPase in the same plate we lowered the temperature to 25°C and this turned out to be a key point, because it minimized the contribution of nonmitochondrial ATPases, as clear from new Figure 1A. The results

indeed show that the mutation has a major inhibitory effect on Ca^{2+}-ATPase activity. The latter enzymatic activity is insensitive to oligomycin (the statement at the end of the original legend only referred to Mg^{2+}-ATPase activity) but it is 50% inhibited by resveratrol, which is in line with the literature [Gladhill et al (2007) PNAS 104 13632-7; Sassi et al. (2014) Biochim Biophys Acta 1837, 1781-9; Madreiter-Sokolowski et al. Cell Physiol Biochem (2016) 39, 1404-20].

3. In Figure 1D, presumably the data is vertically offset (and baseline [Ca] was the same for both WT and T>S). This should be indicated somehow. It would also be worthwhile to know whether there was any difference in the apparent Ca uptake rate during [Ca]i decline of the first couple of pulses. At this point there should be no mPTP opening and the data might be a useful test for unaltered Ca uptake in the T>S vs. WT beta protein.

Answer. Yes, the two traces were offset and baseline [Ca^{2+}] was the same as now specified in the Figure legend. The rate of Ca^{2+} uptake before onset of the PT was unaffected as now specified in the text.

4. Related to the above, it would seem like mPTP inhibition by CsA and compound 60 should virtually abolish the difference in Ca uptake between WT and T>S. The fact that it doesn't raises the possibility that uptake also differs in the T>S mitochondria.

Answer. In our hands there is always a maximum CRC in any type of mitochondria and permeabilized cells, independent of the inhibitor(s) used. The CRC assay isn't perfect, and the limit may also reflect matrix Ca^{2+} toxicity, so we'd rather not speculate on this point. As mentioned above the rate of Ca^{2+} uptake before onset of the PT was unaffected.

5. Fig 2C does not communicate very well the points about movement and structure raised in the Results. This should be improved either by wording of text, improved graphics (or both). Fig EV3 seems to imply that the protein is 'stiffened" by Ca in WT, but that the opposite is true for the T>S mutant. If this is this right, the consequence should be discussed.

Answer. Thank you for your very useful comment. We have modified Figures 2C and EV2B by reducing the threshold to 5.5Å both for (red) coloring and for backbone thickness. This should better highlight the flexibility of the β subunit in the presence of Mg^{2+}, minimizing outlier values generated by the broken OSCP chain. Furthermore, we added new supplementary Figure EV4 containing the plots obtained from the RMSF values for both OSCP (panel A) and β subunits region 9-163 (panel C). We believe that this supplementary Figure now clearly shows the higher flexibility of the Mg^{2+} binding complexes as well as the partial rescue of the Ca^{2+} stiffness (in particular for OSCP) in the presence of the T>S mutation.

6. The study falls short of demonstrating how the structural effects on the beta subunit could propagate to the IMM region and where the actual mPTP pore structure is. Acknowledging this limitation is important.

Answer. We have toned down the conclusions to read "Although they cannot predict whether F-ATP synthase dimers are required for PTP formation [20], our MD simulations provide key insights into a potential mechanism through which the Ca^{2+}-dependent effect on the catalytic sites is transmitted via OSCP to the peripheral stalk and thence to the inner membrane, . This is a testable hypothesis that holds great promise for future studies."

7. The cell death data in Figures 3 and 4 is useful data to support the potential functional effects of the T>S mutation, but the mechanistic link with mPTP circumstantial rather than direct.

Answer. We think that the resistance to Ca^{2+}-dependent depolarization of the T>S mutants (which is now presented in new panel A of Figure 3) is a good step in this direction. The data also fits previous published work both by ourselves and others demonstrating a mechanistic link between treatement with Ca^{2+} ionophore, arachidonic acid, PTP opening and cell death.

Referee #3

This is a very interesting hypothesis being tested here on the role of Ca in generating a PTP type channel in the F-ATPase synthtase. Experiments are conducted on multiple levels from isolated mitochondria, cells and whole animals. Molecular dynamic simulations are provided that are outside of my expertise and I will not comment on them. In general, these studies seem a bit preliminary. Mutation introductions are being used with no quantitative information on PROTEIN expression in the cells or tissues.

Answer. Unfortunately it is not possible to distinguish the relative protein levels of reexpressed wild-type and mutant, yet the total protein is the same (Fig EV1) and we have quantitated the difference by qPCR as reported in the first paragraph of the Results and Discussion Section. To address this problem, however, we have also assessed the expression levels of a flagged version and found no difference between wild-type and mutant protein level (see attached Figure 2 for Referees at the end of the document).

Secondly, testable hypothesis of the Ca level in the cells prior to death in the WT and mutant was stated but not tested. While measuring intracellular Ca is now very straightforward in HELA cells. Finally, though not a necessary addition to the manuscript, a more general proteome study should be conducted to assure that other protein changes, in addition to the introduced mutations, are not occurring over the rather long time course of these studies.

Answer. In order to address this point we have measured the changes of cytosolic [Ca^{2+}] with Fluo-4FF and of the mitochondrial membrane potential with TMRM after addition of ionomycin. The results are presented in new panel A of Figure 3 (we have replaced the less informative original panel) and show that the T>S mutants are more resistant to depolarization following the cytosolic Ca^{2+} rise. Thank you for not demanding that we carry out the proteomic study at this stage! a good suggestion for the future.

My specific comments follow in order of presentation in the manuscript and not of importance:

Page 4: "These results perfectly match those obtained in Rhodospirillum rubrum [39,40] and are of particular relevance because they demonstrate that the subunit bearing the T163S mutation had been incorporated in the F-ATP synthase complex." I would suggest that these data "match" but nothing is perfect. Secondly these data are consistent and do NOT demonstrate that the T163S mutation has been incorporated. I would suggest these conclusions be modified.

Answer. We have removed “perfectly” and changed as suggested to read “These results match those obtained in *Rhodospirillum rubrum* [42,43] and suggest that the β subunit bearing the T163S mutation had been incorporated in the F-ATP synthase complex”.

Page 4: " By qPCR analysis, we found that mRNA levels of the rescued shRNA-resistant sequences were approximately one-third of the total. Assuming that the mutant subunits are distributed randomly, these findings suggest that one mutant copy per assembled complex is sufficient to affect F-ATP synthase catalysis."(special characters removed) This is very speculative. The relationship between mRNA levels and mitochondria protein is very tenuous as can be seen from the work of Peipei Ping and others. Since only a 50% reduction in Ca-ATPase activity was detected, it could be that only 50% of the protein had the mutation. What the mutation did to protein trafficking and complex assembly makes the extrapolation of mRNA to protein level in a given matrix complex tenuous. Direct determination is necessary.

Answer. When measured with Pi release as suggested by Reviewer 1, the reduction of Ca^{2+}-ATP ase activity was almost complete in the T>S mutant (new Figure 1A), a finding that should address the Reviewer’s concern (please see answer to question 7 of Reviewer 1 for further details). We feel confident that the F-ATP synthase is correctly assembled because (i) the amount of subunits alpha, beta, OSCP, c and IF1 is identical (Fig EV1); and (ii) oligomycin-sensitive respiration is identical, indicating the the complex is correctly assembled and functional. As also mentioned above, direct determination of wild-type and mutant protein levels is not possible even in 2D electophoresis

because T and S have the same isoelectric point, and antibodies recognize both wild-type and T>S proteins. In order to address the question we have attached a C-terminal flag to the WT and T>S beta subunits. We could detect the flagged species both in cells and in zebrafish irrespective of whether the mutation was present (see attached Figure 2 for Reviewers; apologies for the apparent low quality, the anti flag antibody is not very good).

Page 4: The observation that the mutation of only 1/3 of the peptides in the F-ATP synthase changes the Ca sensitivity to PTP by a factor of 2 in isolated mitochondria is the most interesting and direct finding in the manuscript. Hopefully, no other protein expression levels or PMT's were affected by the introduction of the mutation.

Answer. We appreciate the comment. The second point would of course require a full proteomic analysis and we understand that this is more of a recommendation for future studies than a request for the current manuscript.

Molecular Dynamic Simulations: I am not an expert in molecular simulations thus I will not comment on this aspect of the study.

Page 6: The effect of the mutation on the intact cell viability was modest, this could be due to the lack of penetration of the mutation as discussed above or other changes in the cells associated with the mutation. The authors, in addition to outlining the non-specificity of the pharmacological challenges, state "Second, mitochondria expressing the T163S β mutant displayed an increased CRC (i.e. increased resistancev to Ca2+) yet they still eventually opened the PTP at higher Ca2+ loads (Fig 1D,E)." This should be validated by monitoring the cytosolic calcium in these cells to show they can tolerate higher calcium levels as extrapolated from the isolated mitochondria studies. This is rather an easy experiment to conduct and could even be done in conjunction with the cell sorting or the cells prior to sorting.

Answer. This is an excellent point raised also by the other Reviewers. We have measured the changes of cytosolic [Ca^{2+}] with Fluo-4FF and of the mitochondrial membrane potential with TMRM after addition of ionomycin. The results are presented in new panel A of Figure 3 (we have replaced the less informative original panel) and show that the T>S mutants are more resistant to depolarization following the cytosolic Ca^{2+} rise. Please see answer to question #15 of Reviewer 1 for the new text.

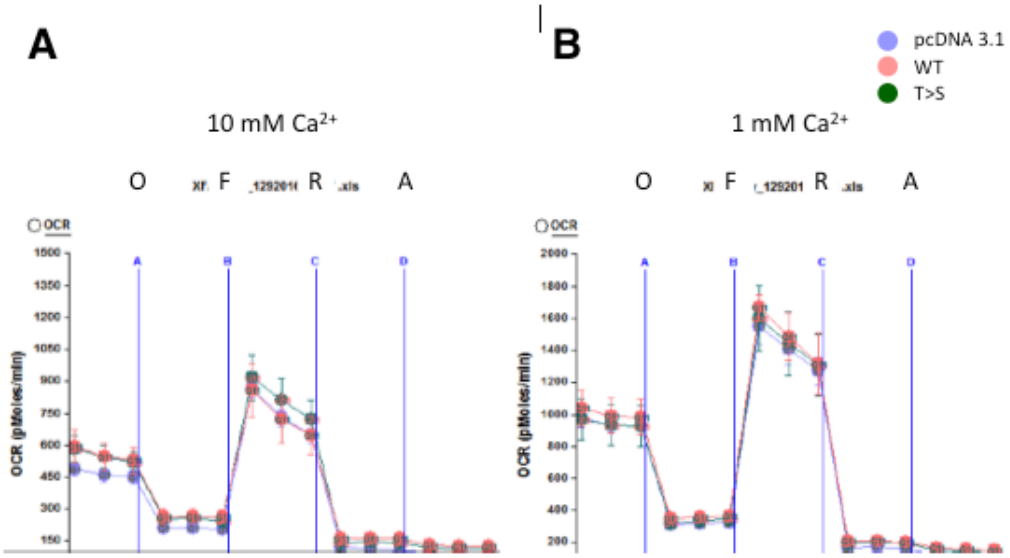
Page 6: It is important in the Zebra fish studies to understand the extent of protein expression in the human WT and mutated forms. The authors state: "Whole-mount immunofluorescence and Western blotting (Fig 4C,D) indicate that the expected replacement of the endogenous β subunits took place to a similar extent for the wild-type and T163S mutant species." However Figure 4D is of the alpha subunit and TOM 20 which were not affected (data is consistent with that conclusion) but no information on the beta subunit. Figure 4C is immunohistology of the beta subunit and TOM. It is unclear looking at the images or the merged data whether they can state what the "replacement" was. Please provide quantitative data on the relative expression levels of the WT and mutation T163S in these studies.

Answer. We had tried our beta antibody in zebrafish immunoblots but it does not react even at a titer of 1:50. In the sentence "Whole-mount immunofluorescence and Western blotting (Fig 4C,D) indicate that the expected replacement of the endogenous β subunits took place to a similar extent for the wild-type and T163S mutant species", mention to the Western blotting was a mistake and we have removed it, thank you for bringing this up. To address the last point we have attached a C-terminal flag to the WT and T>S beta subunits, and detected the flagged species in fish lysates irrespective of whether the mutation was present (see attached Figure 2 for Reviewers; apologies for the apparent low quality, the anti flag antibody is not very good).

Page 16: "Data information: In panels A-E one representative experiment of xx is shown." Please fill in "XX".

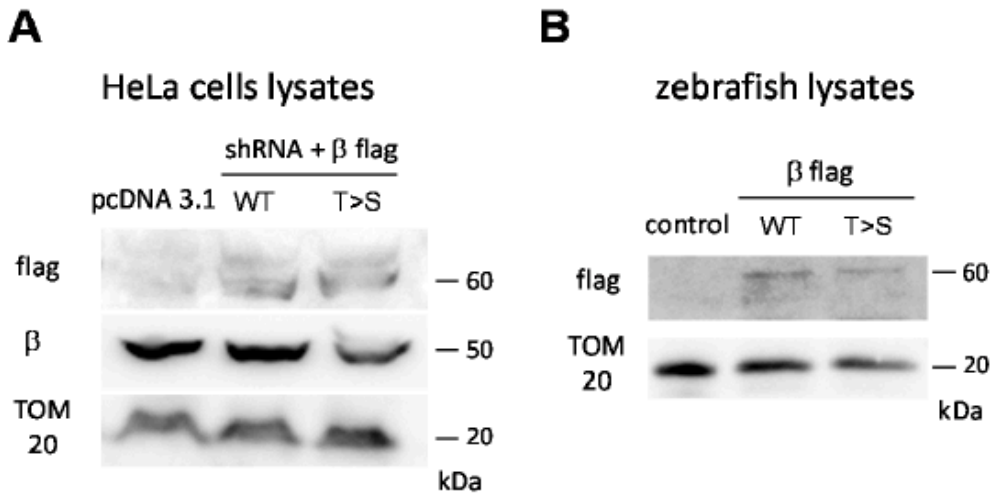
Answer. Done, thank you.

Figures for Reviewers



HeLa cells treated with shRNA against endogenous F-ATP synthase β subunit were transfected with vectors encoding sh-resistant wild-type β subunit (WT) or T163S β subunit (T>S). pcDNA 3.1 denotes cells treated with the empty vectors used for silencing and reexpression. Cells were kept for 72 h in normal growth medium, switched for to 2 h to DMEM containing 10 mM or 1 mM Ca^{2+} and oxygen consumption rate (OCR) measured.

Fig. 1R



(A) HeLa cells treated with shRNA against endogenous F-ATP synthase β subunit were transfected with vectors encoding sh-resistant wild-type β subunit (WT) or T163S β subunit (T>S) bearing a 3' sequence encoding the DYKDDDK flag. pcDNA 3.1 denotes cells treated with the empty vectors used for silencing and reexpression. A Triton X-100 extract of 10^6 cells was prepared, separated by SDS-PAGE, blotted onto nitrocellulose filters and probed with anti flag, β and TOM 20 antibodies. (B) Zebrafish fertilized eggs were injected with vectors encoding wild-type β subunit (WT) or T163S β subunit (T>S) bearing a 3' sequence encoding the DYKDDDK flag. Control denotes untreated zebrafish. Triton X-100 extracts from 30 embryos per condition were prepared, separated by SDS-PAGE, blotted onto nitrocellulose filters and probed with anti flag, β and TOM 20 antibodies.

Fig. 2R

3rd Editorial Decision 03 March 2017

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for my delayed response but we have only received the last referee report and all reports are copied below.

As you will see, all three referees are now very positive about the study. Referee 2 requests higher resolution images for Figure 4C to support the conclusion that the β subunit localizes to mitochondria. Referee 1 mentions a recent publication that should be cited and discussed.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please provide higher resolution versions for figures 3, 4 and EV1.
- Please reformat all figures in portrait orientation.
- Figure EV3 is never mentioned in the text. Please also change the callouts to the table from "supplemental table" to "table EV1".
- Data information in Fig. EV1C is missing.
- I suggest to move the data description currently listed at the end of each figure legend (n, SED, SD, p-value etc) to the respective description of the panel. It appears easier and faster to interpret the information contained in a graph, if the data information is listed next to the description of the results.
- Please edit the abstract so that present tense is used to conform to the style of EMBO reports.
- Remove the paragraph "Conclusions" and/or integrate it into the Discussion section.

We look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1: Good revision. In the follow up studies of the authors I suggest to them to address the findings of Zhou W et al, claiming that simulations indicate the c-subunit ring of the F1Fo ATP synthase is not the mitochondrial permeability transition pore, Elife. 2017 Feb 10;6. pii: e23781. doi: 10.7554/eLife.23781.

Referee #2: The authors have been responsive, and I am satisfied that this study is of great value to this field. Only on small correction is needed for me: On page 6 where it says that expressed beta "co-localized with mitochondrial TOM 20 (Fig 4C)." at this 100 um resolution one cannot say anything about it showing that the expressed beta is mitochondrial localized or really co-localized with anything at all (except that it seems to be in cells. I don't really doubt that it is there, but showing no expression of the exogenous beta in controls and also a magnification that allows resolution of TOM20 and beta being in what look like mito would be better. Please fix this.

Referee #3: The key issues for me was the Ca measures in the intact cells to support the conclusions be drawn. I am comfortable with the data being presented as well as the other corrections made throughout the manuscript. I agree with the points that this is a step not a proof of the mechanism under evaluation.

3rd Revision - authors' response 21 March 2017

Thank you again for your kind help. We have now addressed all the remaining issues (point-by-point response below). We would like to mention that we have also added the total number of zebrafish eggs used in the experiments of Figure 4 in the figure legend. Although the actual molecular dynamics simulation is of course not essential for the manuscript, we have a problem with its accessibility (item 22 of the author checklist) because the size is close to 1 TB, virtually impossible to download. We would be happy to make it available on our server as there is no way

that it can be hosted by public databases. Let us know if this point is essential and if you have any suggestions to solve the problem.

As you will see, all three referees are now very positive about the study. Referee 2 requests higher resolution images for Figure 4C to support the conclusion that the β subunit localizes to mitochondria. Referee 1 mentions a recent publication that should be cited and discussed.

We have provided high-resolution images and also carried out determinations of the Pearson correlation coefficient, which fully support our conclusions and are now reported in the text. As requested, the publication has been mentioned together with a key paper from the Walker group on the same issue that just appeared in PNAS.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.
- Please provide higher resolution versions for figures 3, 4 and EV1.
- Please reformat all figures in portrait orientation.

Done

- Figure EV3 is never mentioned in the text. Please also change the callouts to the table from supplemental table" to "table EV1."

Figure EV3 was mentioned at page 5; the requested change has been made.

- Data information in Fig. EV1C is missing.
- I suggest to move the data description currently listed at the end of each figure legend (n, SED, SD, p-value, etc.) to the respective description of the panel. It appears easier and faster to interpret the information contained in a graph, if the data information is listed next to the description of the results.
- Please edit the abstract so that present tense is used to conform to the style of EMBO reports.
- Remove the paragraph "Conclusions" and/or integrate it into the Discussion section.

All points have been addressed. I hope that everything is in good standing, and to get the final acceptance notice soon!

4th Editorial Decision 28 March 2017

Thank you for submitting your revised manuscript to EMBO reports. I have now looked at all the incorporated changes and the open issues.

Source data and molecular dynamics simulations: the submission to Zenodo is a good option. I have meanwhile received feedback from our production team. The DOI of your article is: 10.15252/embr.201643354. Could you please add a data availability section to your article that links to the data deposited on Zenodo? The final link to the data can then be added at proof stage to this data availability section.

Apart from that, could you please insert the legend for Table EV1 into the .doc or excel file and resubmit the table as .doc or .xls file?

I have also noticed that you have calculated the Pearson correlation coefficient for Fig 4C in response to the reviewer's request but have not provided higher resolution images of the co-localization of beta with TOM20. Could you please provide higher resolution images that make a potential mitochondrial localization visible?

4th Revision - authors' response 02 April 2017

Thank you for the DOI, I understand that we should add the availability section within the article at the end of the "Modeling" paragraph of "Materials and Methods" as follows: "The MD simulation data from this publication have been submitted to the zenodo database (https://zenodo.org/) with accession links 10.5281/zenodo.438997 (Magnesium, wild type); 10.5281/zenodo.438943 (Magnesium, T163S mutant); 10.5281/zenodo.438711 (Calcium, wild type); 0.5281/zenodo.438697 (Calcium, T163S mutant)." We obviously do not have the publication date yet, please provide it as soon as available. We will then complete the zenodo form and make the links available to the public.

About Fig. 4C, the image was taken with a 40x objective, and we do not have higher magnifications to show. The Pearson analysis was performed and reported in the text both for the wild type and the T>S mutant (there is no GFP in Fig. 4C, green is the antibeta and red the antiTOM20 antibody). We think that this representation of the findings is acceptable. Indeed, the occurrence and extent of colocalization does not rely on visual inspection but rather on image analysis at the confocal with dedicated software (and the visual impression of "merging" is consistent with the actual measurements).

We hope that this addresses all of your concerns. Thank you again for your kind help.

5th Editorial Decision 05 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.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

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Corresponding Author Name: Valentina Giorgio, Giovanna Lippe and Paolo Bernardi
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43354V3

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 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.

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://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?	A minimum of 3 replicates was initially performed to test whether a trend was present. Experiments were then repeated until an acceptable statistical significance (or lack thereof) was achieved.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	As a routine, 3 rounds of injection with at least 50 eggs per group were performed for each construct
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	no samples or animals were 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.	no randomization for measurements; for animal treatment see below
For animal studies, include a statement about randomization even if no randomization was used.	each batch of zebrafish eggs for injection is divided randomly in the subgroups for treatment
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 used
4.b. For animal studies, include a statement about blinding even if no blinding was done	blinding was not necessary as the differences due to expression of the various fluorescent constructs are impossible to miss, and it was essential to separate the various treatment groups for western blotting
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 used all appropriate corrections for the analyzed data sets/groups (Student t, One way Anova with Bonferroni correction when necessary, as specified in the legends)
Is there an estimate of variation within each group of data?	yes
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	all sources of antibodies are specified in the manuscript. Catalog numbers are: alpha subunit, ab-110273 Abcam; beta subunit ab-14730 Abcam; OSCP, ab-110276 Abcam; c subunit ab-181243 Abcam; IF1, ab-110277 Abcam; TOM20, sc-11415 Santa Cruz.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	ATCC, tested and free of 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.	Danio rerio AB strain from fertilized egg to 72 hours post fertilization. Adults for mating. Adult zebrafish were maintained in aerated, 28°C-conditioned saline water according to standard protocols. Fish were kept under a 14 hour light/10 hour dark cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All protocols and manipulations with zebrafish were approved by the Ethics Committee of the University of Padova and authorized by the Italian Ministry of Health
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.	Studies are adequately reported

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	
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.	
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	
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.	
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.	

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. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	
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).	
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).	
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 Shewanella oneidensis 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 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	
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 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.	

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.	