In vivo inhibition of the mitochondrial H\(^+\)-ATP synthase in neurons promotes metabolic preconditioning

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Transaction Report:

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

1st Editorial Decision 06 September 2013

Thank you for submitting your manuscript on the effects of neuron-specific conditional IF1 overexpression for consideration by The EMBO Journal, and please excuse the delay in its evaluation during the summer vacation period. We have now received the reports of three expert referees, which are copied below for your information. I am afraid that none of these referees offers strong support for publication in our journal. Although they acknowledge the potential interest of your conclusions, none of them appears convinced that your present set of experiments and data provides sufficiently definitive and compelling evidence to justify these conclusions. I will not repeat all the individual points of criticism in detail here, but you will see that key concerns pertain both to the experimental strategy (overexpression of a mutant IF1) and to the observed alterations being possibly due to IF1 effects directly on the respiratory chain and not linked to H\(^+\)-ATPase inhibition. In light of these critical opinions, we unfortunately had to conclude that we will not be able to offer publication of your study in our journal. As you hopefully understand, we can in light of the high number of submissions we receive really only proceed further with those few that are met with elevated enthusiasm from at least a majority of referees already upon initial review, which is unfortunately not the case here. I am sorry we cannot be more positive on this occasion, but hope that you will in any case find our referees' detailed comments helpful.

REFEREE REPORTS:

Referee #1:
The study provides convincing evidence that expression of the H49K mutant of human F-ATP synthase inhibitor protein IF1 (which is less prone to be released from the catalytic core) induces a glycolytic shift in vivo, and causes protection from the damaging effects of quinolinic acid injection into the striatal region of 1 year old mice. The Authors have also analyzed a series of pro-survival and preconditioning effects in neurons and brain (increase of ROS, activation of Akt/p70S6K and of PARP, increased Bcl-xL expression). The manuscript is well written and organized, but several points need to be addressed.

1. A general question that needs to be clearly discussed is whether it is permissible to draw general conclusions about endogenous IF1 based on the experiments with the hIF1 mutant. Whether endogenous IF1 binds the F-ATP synthase under phosphorylating conditions is still debated, as IF1 is reported to be released from F1 at high protonmotive force created by respiration. The contribution remains potentially important as a proof of principle, but a word of caution is essential.

2. There is no experiment to show that the overexpressed protein localizes (exclusively?) to mitochondria. A protease digestion/protection experiment in permeabilized cells is needed.

3. In Fig. 1A the Authors show the expression levels of hIF1 in H+, T+ and H+/T+ transgenic mice. They should check the level of endogenous IF1 in the same western blot, in order to follow changes in the total amount of IF1 (human + mouse) bound to ATP synthase.

4. In Fig. 1C I see increased levels of NDH9 and Core II and decreased levels of Cox IV, at variance from the statement at page 5 "The expression of hIF1 did not affect the expression of other mitochondrial proteins (Figure 1C)". It seems to me that in H+/T+ mice a rearrangement in OXPHOS complexes occurs (see also native PAGE in Fig. 2F), and I think that activity of respiratory chain complexes should be addressed in the Authors' own interest (see next point).

5. There is a major problem with Figure 2. In the traces (2A, left panel) I see virtually no stimulation of respiration by ADP in H+/T+ mitochondria (upper trace), yet in the bar graph (2A, middle panel) the average stimulation is twofold, so the experiment in the left panel is not representative. FCCP-stimulated respiration is also decreased substantially, and there is no easy way to explain why maximal respiration should be decreased by hIF1 expression (see also Fig. 3E) unless something also happens to respiratory chain complexes. This needs to be addressed also because from the bar graph (2A, middle panel) the ratios of state III/state IV and FCCP/state 4 appear to be the same for the 2 genotypes, which could mean that the purity of the 2 preparations is not the same. How was the amount of mitochondrial protein normalized?

6. Page 5 and 6 "The lower oxygen consumption elicited by OL over the maximal respiratory rate observed in mitochondria of H+/T+ mice (Figure 2A, histogram to the left) is consistent with the inhibition of the activity of the ATP synthase by the expression of hIF1." I don't understand the sentence.

7. Fig. 3H, the intensity of TMRM fluorescence is not adequate to evaluate differences in membrane potential between different cell cultures. More instructive would be the response to oligomycin, rotenone and FCCP.

8. Page 15, in Giorgio et al. (2013) I could not find evidence for an interaction of the ATP synthase with complex I, which would conflict with the distribution of complex V and complex I as defined by cryoEM.

Additional points:

1. In Fig 1B legend "granular cytoplasmic staining" is unclear and should be better explained.

2. Page 6, there is no comment to Fig. 2C.

3. In Fig. 4C (histogram) the value "0.06" in the graph is not clear and should be explained in the figure legend.
Referee #2:

In this work, the authors have generated a transgenic mouse model expressing a mutant of human ATPase Inhibitory Factor (hIF1) in neurons with the assumption that this mutant (H49K) has a higher binding affinity for the ATP synthase. They report that overexpression of mutated hIF1 affects the OXPHOS, induces a mild oxidative stress, and protects neurons from apoptosis. They conclude that ATP synthase is a target to prevent neuronal cell death.

Major points

Considering that IF1 is largely expressed in mouse neurons (Figure 7), the authors' conclusion requires an indisputable demonstration that H49K hIF1 does exclusively inhibit the ATP synthase. Unfortunately, if H49K hIF1 overexpression affects OXPHOS, little evidence indicates that it directly affects the ATP synthase, whereas it clearly affects Complex IV (Figure 2 G, 2F), which can easily explain why uncoupled respiration is decreased (Figure 2 A, 3E) while ATP concentration decreases (Figure 1D, 3A).

The observation that ADP addition leads to a smaller mitochondrial depolarization (Figure 2B) is the only result supporting the hypothesis that H49K hIF1 inhibits ATP synthase. A direct measurement of ATP-ase activity using isolated mitochondria would dramatically reinforce this conclusion. The observation of a mitochondrial depolarization after the addition of a respiratory chain inhibitor in intact cells (a particular situation where electrical membrane potential is sustained by ATP hydrolysis) would also confirm that H49K hIF1 inhibits ATP synthase. These experiments are easy to perform and are of particular importance considering that many results can also be the consequence of respiratory chain inhibition.

The observation that H49K hIF1 overexpression affects the respiratory chain (Figure 2A, 2F, 2G, 3E, 6C, and may be core II, figure 1A) is an interesting but embarrassing observation. Whether these effects are secondary to ATP-synthase inhibition or direct binding of H49K hIF1 on the respiratory chain is not clarified in this manuscript. The resolution of this issue is beyond the scope of this manuscript, but this should be carefully considered since the respiratory chain has also been involved in the control of apoptosis.

Other concerns

The material and method section should be carefully revised and sometime explained with more details.

Page 16: If mitochondria were permeabilized with digitonin, they were supposed to be uncoupled.

Page 17: Propidium iodide and calcein are mentioned, but I did not find any result with this products.

Page 17: The technique used to quantify TMRM and Mitosox must be detailed. In particular, the fluorescence dramatically depends on the number of cells observed. How this point was considered?

Page 18: "spectrophotometric determination of complex I and IV activities" is vague. Many pitfalls exist with these techniques and the precise method used must be detailed.

Referee #3:

Formentini et al. describe a mouse model for studying the function of IF1 (ATPase inhibitory factor 1), which is reported to associate with the F1Fo ATP synthase and to inhibit its ATPase activity. A transgenic mouse was generated that overexpresses a mutant of human IF1 (H49K with enhanced affinity for the F1 beta subunit) in neurons under the control of a doxycycline-repressible promoter. Using this mouse model, the role of the H+--ATP synthase on neuronal metabolism and cell death in vivo and in vitro was investigated. The authors found that hIF1 induces a partial inhibition of oxidative phosphorylation in brain mitochondria and an increase in the rate of aerobic glycolysis. In addition to this metabolic reprogramming, hIF1 expression also increased production of ROS. This mild oxidative stress was suggested to induce a state of preconditioning in the brain, which in turn activates several protective signaling pathways. Consistent with this hypothesis, hIF1 expression protected against quinolinic acid-induced excitotoxic damage. This protection was associated with activation of several pro-survival (Akt/p70S6K), repair (PARP), and anti-apoptotic (Bcl-xL) proteins. Bcl-xL is reported to be essential for hIF1-mediated neuronal survival as its silencing reduced hIF1-induced protection against glutamate and oxygen deprivation-induced death in vitro.

Overall, the study supports the concept of targeting H+--ATP synthase to prevent neuronal death. These authors have several reports of similar effects on metabolism and ROS of overexpressed and
knockdown of IF1 using cell culture models and human tumors.

Although this study provides interesting evidence supporting the central hypothesis, there are complications with interpreting the findings. One difficulty is the overall strategy itself, IF1 overexpression, and the seemingly opposite to expected effects of overexpressed IF1 on ATP levels (IF1 overexpression dramatically decreased rather than increased ATP, the latter being expected by preventing ATP consumption by the ATPase). Thus, there is concern for overexpression artifacts. The Discussion explains that the evidence indicates that IF1 also inhibits ATP synthase activity of F1Fo (not just reversal of the synthase/ATPase activity), but it is not clear if this is due to direct inhibition of H⁺-ATP synthase by IF1. The relevant blots are not probed for the presence of IF1. Nevertheless, an interesting finding of this study is the apparent metabolic reprogramming of neurons in vivo by hIF1 overexpression, with a relative increase of glycolysis, although it is unclear if this specific change has any beneficial impact following stress. Although the abstract indicates that the neurons shift to an enhanced aerobic glycolysis, it is not clear if changes induced by hIF1 could occur at other mitochondrial locations, e.g. the observed effects on complex IV activity. This issue may be important as the authors suggest that neuroprotection afforded by increased expression of hIF1 in the brain is specifically mediated through inhibition of H⁺-ATP synthase. Otherwise any agent that would nonspecifically boost ROS production could be expected to precondition and to be neuroprotective.

Additional comments
1. Depleting Bcl-xL in stressed cells is well known to cause cell death. Therefore, if Bcl-xL was depleted only to the level of WT (not below WT), only then could one assess whether Bcl-xL was an effector of IF1.
2. The text could better relate these findings to other studies, for example regarding examples of impaired ATP synthase activity, and how to reconcile with findings that IF1 increases ATP by decreasing ATP consumption.
3. The claim is that cytoplasmic but not mitochondrial factors are altered in IF1-expressing brain: "expression of hIF1 did not affect the expression of other mitochondrial proteins". However, the Core II protein (and possibly also NADH9) appears to be substantially increased, while COXIV and F1-beta may be slightly decreased in the H+/T+ samples. These results should be quantified as in Fig. 1E.
4. How was the "basal ATP synthase activity" calculated in Fig. 2A? The y-axis label implies a rate, but sufficient information was not provided. In Fig. 2D, the text indicates that a difference exists, but there is only a trend (not a difference) without statistical significance.
5. Figure abbreviations should be explained in all of the legends.
6. The time points after the injury at which the indicated measurements were performed should be indicated in the figure legend Fig. 4B-E and Fig. 5A-E.
7. Bcl-xL levels in the H+/T+ samples are similar (not increased) relative to wild type samples in Fig. 6D, contrary to the central conclusion and in Fig. 5D where Bcl-xL appears to be increased in the H+/T+ samples compared to wt.

Appeal 16 September 2013

Thank you for your kind Editor's Decision letter on our EMBO Journal submission (EMBOJ-2013-86392) and the appreciation and interest in our work.

After reading the comments of the reviewers, who also appreciate the novelty and interest of our data, I have realized that they are requesting clarifications and minor experiments that have already been done or could be easily addressed in a short-time period.

Regarding the two major points that you have pinpointed, you will convene with me that if the cell type you want to study is flooded with endogenous IF1 (which is the case of neurons, see Fig. 7) and you want to inhibit the ATP synthase you have to express a protein whose binding to the ATP synthase is insensitive to the regulation exerted by the proton motive force: that protein is the H49K mutant of IF1 (hIF1) as it was demonstrated years ago (Cabezon et al., JBC 2000). We agree with reviewers 2 and 3 that we have to provide additional data supporting that hIF1 is indeed inhibiting
the ATPase in our system. The results of these experiments (that were not included in the original submission) confirm that that is the case.

Regarding the concerns about possible direct effects of IF1 on the respiratory chain we should mention that there is no record in the literature suggesting such fact, what most likely indicates that hIF1 is indirectly affecting the assembly and activity of complex IV. This finding is really of upmost importance in cell physiology because it underlines the feedback regulation that the ATPase exerts on the respiratory chain under a stressful situation. However, and as stated by reviewer 2, this issue is beyond the scope of the manuscript but provides the first in vivo experimental system to analyze the underlying molecular mechanism.

In the following, I briefly summarize our answers to reviewers’ comments and the state of the requested experiments in order to appeal for the possibility of a further consideration of a revised version of our manuscript in EMBO Journal accommodating all the experiments and suggestions requested by the reviewers if you consider it appropriate.

Reviewer 1:
We thank the reviewer for the positive comments towards our work.

1.- It is not our intention to draw general conclusions about the endogenous IF1. Our aim is to inhibit the ATPase by the most efficient way especially in neurons which is a cellular type that shows a very high expression of the endogenous IF1. As far as it has been published the most active inhibitory protein of the ATPase is the H49K mutant of IF1. In any case, we will make this point clearer in the revised version.

2.- We have already published the exclusive localization of IF1 in mitochondria as reveal by the co-localization of the protein with ?-F1-ATPase (JBC 2010). Moreover, the over-expression of IF1 in HeLa cells and of the H49K mutant in mouse brain confirms that the protein is exclusively localized in mitochondria as revealed by the co-localization with ?-F1-ATPase in immunofluorescence microscopy assays. These results are already available and will be incorporated in the revised version as a supplementary Figure.

3.- The western blot requested will be carried out and incorporated in the revised version.

4.- Quantification of the bands from different experiments reveal no significant differences in the content of proteins from respiratory complexes as a result of the expression of hIF1. In the revised version we will incorporate these data and provide new blots more clearly supporting this point. In addition, we will determine the activity of complex II + III of the respiratory chain which are the missing ones from our paper.

5.- We thank the reviewer because she/he has done an excellent job with Fig. 2A. We didn't realize that in the upper trace we have marked the addition of ADP incorrectly. As it can be observed, the addition of ADP in the upper trace should be labeled at the point where the slope starts to increase, that is 1 cm to the left with respect to the addition of ADP in the lower trace. We apologize for this error. The revised version of figure 2 will incorporate this correction. As pointed out in the manuscript the expression of hIF1 does affect the activity and assembly of complex IV of the respiratory chain explaining the reduction of the maximum respiratory rates. However, as suggested by R#2, these studies are out of the scope of the present work and will be addressed in the future. We should mention that brain mitochondrial preparations from wild type and H+/T+ mice have essentially the same degree of contamination as reveal by western blots against different mitochondrial and cytosolic proteins. A sentence and a blot illustrating this point will be included in the revised version of the manuscript.

6.- The sentence will be rewritten to indicate that oligomycin has less inhibitory effect on respiration in isolated mitochondria of H+/T+ mice than in wild type mitochondria. The results are expressed as % of maximal respiration. The additional data requested by R#2 (see point 1) emphasize the inhibition of the ATPase by hIF1.

7.- In agreement with the reviewer's suggestion the revised version will accommodate the response of TMRM loaded neurons to oligomycin, rotenone and FCCP.
8.- Evidence for the interaction of complex I and complex V is provided in Giorgio et al. PNAS 2013 under Fig. 4C in the western blot of the upper panel. In that blot it is shown that on SDS-PAGE the dimers of the F0F1-ATPase contain a subunit of complex I, although the identity of this subunit is not specified. However, following the comment of the reviewer we have decided to incorporate the argument that such interaction is not observed in CryoEM studies of the macromolecular organization of complex I and the ATP synthase as it has been documented in Davies et al., PNAS 2011.

Additional points.
1.- As indicated under point 2, hIF1 is exclusively localized in mitochondria of neurons. In the revised version we will mention in the legend "mitochondrial staining" 
2.- The revised version will amend this typographical error. 
3.- The revised version of Fig. 4C will incorporate p=0.06.

Reviewer 2 
We thank the reviewer for the positive comments and the suggested experiments.

Major points.
1.- We agree with the comment of the reviewer. It was very unfortunate on our side not to have included the two experiments requested. We thought that since this point has been demonstrated in the literature in different experimental settings, we didn't have to show the same results in our model. In the revised version of the manuscript we will incorporate the results that illustrate that hIF1 (i) inhibits the ATPase activity in isolated mitochondria and (ii) induces a higher mitochondrial depolarization after the addition of antimycin A.

2.- We agree with the reviewer on the importance of our finding that inhibition of the ATPase by hIF1 has a clear effect on the activity of the respiratory chain. Since no interaction of IF1 with components of the respiratory chain has been described this finding pinpoints a feedback regulation of the activity of complex IV by the activity of the synthase. In other words, we do not think it is an embarrassing observation but attractive future aim of investigation beyond the scope of this manuscript as indicated by the reviewer. However, following the reviewer's suggestion in the revised version of the manuscript the Discussion section will also contemplate the possible involvement of the respiratory chain in the control of apoptosis. Other concerns. The new version of the manuscript will be carefully revised in the material and methods section specifically addressing the permeabilization of synaptosomes (not mitochondria) with digitonin (p16); cell death detection with PI and calcein (p17 and Fig. 6D); membrane potential and ROS production protocols (p17) and the spectrometric determination of enzyme activities (p18).

Reviewer 3 
We thank the reviewer for the positive comments towards our work. However, we should mention that our model is not to study the function of IF1 on the ATPase but to inhibit as much as possible its activity in vivo in order to verify the relevance of the engine of oxidative phosphorylation in cell death. As far as we know, hIF1 is the only feasible alternative to promote a protein-mediated inhibition of the neuronal ATP synthase in vivo. As we have demonstrated in different cancer cell lines the over-expression of IF1 and of the H49K mutant of IF1 also inhibits the ATP synthetic activity of the enzyme. Hence, the decrease of ATP concentrations is not an artifact but the expected result by the expression of hIF1 in neurons. The inclusion of the experiments requested by R#2 (point 1) will further emphasize that IF1 directly inhibits the ATP synthase. The blots of IF1 requested (Fig. 1E/ Fig. 2D/ Fig. 3G and Fig. 5D) will be incorporated in the revised version. No interaction of IF1 with components of the respiratory chain has ever been described. However, IF1 and especially the H49K mutant definitively interact and inhibit the ATP synthase (previous data by us and others and the new experiments requested). Hence, our findings suggest that metabolic rewiring of neurons to an enhanced aerobic glycolysis is due to a limited cellular ATP availability by direct inhibition of the ATP synthase. The reduced activity of complex IV could further contribute to the reduction of ATP by a negative feedback regulation exerted by the ATP synthase on the respiratory chain (see point 2 of R#2). It is well established that ROS act as a double-edged sword and that cellular responses depend on the site and intensity in which ROS are being produced.
In the specific case of the pro-survival IF1-mediated signal of mitochondrial ROS we have demonstrated in different cell lines (Mol. Cell 2012; Oncogenesis 2013) that they are produced by blocking the activity of the ATP synthase.

Additional comments 1 and 7.- From these comments of the reviewer it appears that there is some misunderstanding in the interpretation of Fig. 6D and Fig. 5D. The basal Bcl-xL levels in the non-stressed brain hemisphere of wild type and H+/T+ mice (R in Fig. 5D) are the same. Therefore, non-stressed primary cultures of neurons from wild type and H+/T+ mice reveal the same level of Bcl-xL (Fig. 6D). There is no contradiction with the central conclusion that Bcl-xL is increased in the stressed brain hemisphere of H+/T+ mice (L in Fig. 5D). The blot in Fig. 6D is just the western blot that illustrates that the silencing of Bcl-xL has worked.

2.- We really don't understand this point of the reviewer. In all the studies that we have done in cancer and stem cells or with animals in vivo we have never observed that IF1 expression increases ATP levels and that this could result by decreasing ATP consumption.

3.- As indicated under point 4 of R#1 the revised version will accommodate the quantification of the bands from different experiments that actually reveal no significant differences in the content of proteins from respiratory complexes as a result of the expression of hIF1.

4, 5 and 6.- The revised version of the manuscript will provide details for the determination of the basal ATPase activity, abbreviations in all the figure legends and time-points at which the determinations have been performed.

Thank you for your patience during our further expert consultations regarding the arguments in your appeal letter. With regard to our two main concerns, I realize that your arguments rationalizing the IF1 H49K overexpression approach are well taken, and I agree with your plans of further addressing this issue through inclusion of additional supporting data. With regard to the second major concern, the possibility of survival effects being related to direct IF1 effects on respiratory chain complexes, I discussed these criticisms as well as your response to them with an expert arbitrating advisor, whose relevant comments you will find copied below for your information. As you will see, the advisor for the most part agreed with your interpretation and too considers the raised alternative explanations unlikely in light of the current literature on the topic. Nevertheless, s/he offers some suggestions on how to further address the issue through additional experiments and/or discussions.

In this light, we shall be happy to consider a new version of the manuscript further for publication, following the revisions you proposed and taking into account also the additional comments of our expert advisor. Further guidelines for preparing your revision can be found at the end of this email. Should you have any further questions, please do not hesitate to contact us directly.

Arbitrating advisor's comments:

The issue of whether IF1 mutant used in this overexpressing mouse protects from cell death by a direct effect on the ATPase or by altering the respiratory chain is important. However, I am in favor of the interpretation of he authors for the following reason. The authors show that in the overexpressing mouse, less respiratory chain supercomplexes (RCS) are formed (especially when complex IV is probed, but this is likely an issue of antibody sensitivity) (Fig. 2). As recently published (Cogliati et al., Cell 2013), RCS disruption can be secondary to cristae shape changes (affected by IF1), and leads to increased, rather than decreased apoptosis (observed here in the IF1 mutant overexpressing mouse). I therefore believe that if anything, the effect of mutant IF1 on RCS would be detrimental for cell survival and that the protection reported here depends on some other effect (likely on the ATPase, as the authors state). I think that the confusion arises from the attempt of the authors to link mechanistically the observed increased ROS production (that ensues from the
respiratory chain and that is compatible with the issue in RCS reported here) to the protection from cell death (the "preconditioning" theory). I could not find a direct experiment where they tested if an antioxidant reverted the protection afforded by IF1 overexpression.

In conclusion: the suggestion by the reviewers that mutant IF1 would protect from apoptosis by affecting RCS is not supported by the published literature that conversely suggests increased apoptosis upon RCS impairment (see above). This solves the issue of whether the protection is caused by a direct ATPase effect (that shall be substantiated by the proposed experiments), but at the same time the authors shall provide a direct mechanistic link between tonic mild ROS production and protection from apoptosis, or drop this conclusion and stick to some alternative explanation (i.e. modulation of PTP that is composed by ATPase dimers, see Giorgio et al., PNAS 2013).

1st Revision - authors' response 05 December 2013

Reviewer 1:
We thank the reviewer for the positive comments towards our work.

1.- As indicated in our previous letter it is not our intention to draw general conclusions about the endogenous IF1. Our aim is to inhibit the ATPase by the most efficient way, especially in neurons, which is a cellular type that shows a very high expression of the endogenous IF1 (see Fig. 7 and S10). As far as it has been published the most active inhibitory protein of the ATPase is the H49K mutant of IF1. Following this comment of the reviewer, we have stressed this point in the revised version of the paper (see page-4, line-10).

2.- We have already published the exclusive localization of IF1 in mitochondria as reveal by the co-localization of the protein with β-F1-ATPase (JBC 2010). Moreover, the over-expression of hIF1 in HeLa cells and of the H49K mutant in mouse brain cortex confirms that the protein is exclusively localized in mitochondria as revealed by its co-localization with β-F1-ATPase in immunofluorescence microscopy assays. Per reviewer’s suggestion, these results have been incorporated in the revised version as a supplementary Figure S2.

3.- As suggested by the reviewer the western blot requested has been incorporated in the revised version of Fig. 1A and commented in the text (p-5, l-10). The commercial mAb recognizes both the mouse and human IF1 and hence the absolute content of IF1 in brain of double transgenic mice is roughly 3-fold higher than the levels found in controls.

4.- To address this point we have quantified the band intensity from different experiments of the alluded blots. The results reveal no significant differences in the content of the proteins from respiratory complexes as a result of the expression of hIF1. In the revised version we have incorporated these data (see new Supplementary Figure S3) and provided new blots (Fig. 1C) more clearly supporting this point.

In addition, we have completed the determination of the activities of the respiratory chain by assaying complex II + III activity in isolated mitochondria. The results (see new Fig. 2G) show that the only activity affected by expression of hIF1 is that of Complex IV of the respiratory chain as we initially reported.

5.- As indicated in our previous letter, we thank the reviewer because she/he has done an excellent job with Fig. 2A. We didn’t realize that in the upper trace we marked the addition of ADP incorrectly. The addition of ADP in the upper trace is now labeled at the point where the slope starts to increase, that is 1 cm to the left with respect to the addition of ADP in the lower trace. We apologize for this error that has been corrected in the revised version of Figure 2A. In addition, we have now incorporated in the Figure a mark for each addition.

As pointed out in the manuscript the expression of hIF1 does affect the activity and assembly of complex IV of the respiratory chain explaining the reduction of the maximum respiratory rates. However, as suggested by R#2, these studies are out of the scope of the present work and will be addressed in future studies.
We should mention that brain mitochondrial preparations from wild type and H+/T+ mice have essentially the same degree of contamination as reveal by western blots against different mitochondrial and cytosolic proteins. A sentence in this regard has been included in the revised version of the manuscript (p-18, l-20).

6. - The sentence has been rewritten. It now indicates that oligomycin sensitive respiration in isolated mitochondria from H+/T+ mice is lower than in wild type mitochondria, consistent with the inhibition of the ATP synthase by hIF1 (p-6, l-4). The additional data requested by R#2 (see point 1) emphasize the inhibition of the ATPase by hIF1.

7. - In agreement with the reviewer's suggestion the revised version of the manuscript now accommodates the response of TMRM loaded neurons to oligomycin, rotenone and FCCP (see new Figure S5). The results confirm our previous data (Fig. 3H) that hIF1 expression does not trigger a significant increase in basal ΔΨm. However, by the reviewer's suggestion we now document that the oligomycin induced changes in ΔΨm are more pronounced in neurons from control than in neurons of H+/T+ mice (see new Figure S5) supporting also the partial inhibition of the synthase activity of the ATP synthase by hIF1 (p-7, l-15). For additional experiments in this regard, see R#2 point 1.

8. - As indicated in our previous letter, evidence for the interaction of complex I and complex V is provided in Giorgio et al. PNAS 2013 under Fig. 4C in the western blot of the upper panel. In that blot it is shown that on SDS-PAGE the dimers of the F0F1-ATPase contain a subunit of complex I, although the identity of this subunit is not specified and it might be a contaminant. For this reason, and following the comment of the reviewer we have decided to delete this argument and to stress that such interaction is not observed in CryoEM studies of the macromolecular organization of complex I and the ATP synthase, as it has been elegantly documented in Davies et al., PNAS 2011 (p-16, l-15).

Additional points.
1. - As indicated under point 2, hIF1 is exclusively localized in mitochondria of neurons. In the revised version we now mention in the legend of Fig. 1B “mitochondrial staining”
2. - The revised version accommodates the correction of this typographical error.
3. - The revised version of Fig. 4C now states that the statistical difference is p=0.06

Reviewer 2

We thank the reviewer for the positive comments and the suggested experiments.

Major points.
1. - As indicated in our previous correspondence, we agree with the comment of the reviewer. It was very unfortunate on our side not to have included the two experiments requested. In the revised version of the manuscript we have incorporated these experiments and the results illustrate that hIF1: (i) inhibits the ATPase activity in isolated mitochondria (new Fig. 1F) and (ii) induces a higher mitochondrial depolarization after the addition of antimycin A in isolated mitochondria (new Fig. 1G) and in primary cultures of cortical neurons (Supplementary Fig. S6), thus reinforcing our argument that hIF1 is inhibiting the ATP synthase. In addition, the smaller ΔΨm changes observed in primary cultures of neurons after the addition of oligomycin (see new Supplementary Fig. S5) also support the hIF1-mediated inhibition of the ATP synthase reinforcing data on Fig. 2B. The results section has been modified accordingly (p-5, l-22) and (p-7, l-15)

2. - As indicated in our previous correspondence, we agree with the reviewer’s comment on the importance of the observation that inhibition of the ATPase by hIF1 has a clear effect on the activity of the respiratory chain. As she/he indicates the mechanistic insight into this observation is beyond the scope of the manuscript. As indicated by the arbitrating advisor the point here is whether hIF1 exerts neuroprotection by a direct effect on the ATP synthase or on the activity of the respiratory chain. Since no interaction of IF1 with components of the respiratory chain has been described we suggested that the effects of hIF1 on respiration results from a feedback regulation of Complex IV activity/assembly by the inhibition of the ATP synthase. As it has been stressed by the arbitrating advisor, most studies indicate that the impairment of the respiratory chain leads to an increase in apoptosis. Very recent findings further support this hypothesis (see Cogliati et al., Cell 155, 160-
In this paper Scorrano and colleagues have highlighted that genetic disruption of cristae shape morphology promotes the disassembly of respiratory chain supercomplexes leading to an increase and not to a decrease in apoptosis. In other words, our findings of an increased neuroprotection mediated by hIF1, despite the impairment of Complex IV activity and assembly, should be most likely mediated by an effect of hIF1 on the H+-ATP synthase. Hence, neuroprotection mediated by inhibition of the H+-ATP synthase could result from cristae structure stabilization and/or by a the induction of the pro-survival pathways as we initially hypothesized based on the activation of Akt/p70S6K, AMPK and NFκB and in previous findings in different cancer cells (Mol Cell 2012; Oncogenesis 2013). Following the reviewer’s suggestion in the revised version of the manuscript the Discussion section now contemplates both possibilities (see also new Fig. 7G and answers to Arbitrating Advisor).

Other concerns.
The new version of the manuscript has been carefully revised in the material and methods section. It now accommodates details of most of the methods employed that were initially omitted or presented as Supplementary Material.
Page 16: More specifically, we indicate that synaptosomes (not mitochondria) are permeabilized with digitonin (p-18, l-18).
Page 17: PI and calcein were used for the detection of cell death which is shown in Fig. 6C and 6E (p-20, l-8).
Page 17: To comply with the reviewer’s indication in the revised version of the paper we provide details on the TMRM and MitoSox determination of ΔΨm and ROS production, respectively.
Specifically, fluorescence intensity was always assessed in approximately the same number of cells per condition and at least in 15 different fields per condition tested using the “redistribution mode” as detailed in Duchen et al. 2003. This reference has also been included in the revised version of the paper (p-19, last paragraph).
Moreover, and as suggested by R#1 under point 7, we have also determined the sequential changes in ΔΨm in response to OL, rotenone and FCCP in the same cells that more appropriately document the changes in ΔΨm (see New Fig. S5).
Page 18: We now provide the reference of the detailed protocols for the spectrometric determination of the enzymatic activities of OXPHOS complexes (p-21, l-15).

Reviewer 3
We thank the reviewer for the positive comments towards our work. However, as we indicated in our previous correspondence, we should mention that our model is not to study the function of IF1 on the ATPase but to inhibit as much as possible its activity in vivo in order to verify the relevance of the engine of oxidative phosphorylation in cell death. As far as we know, hIF1 is the only feasible alternative to promote a protein-mediated inhibition of the neuronal ATP synthase in vivo. As already mentioned, this point is now more clearly emphasized in the introduction of the paper (p-4, l-10).
As we have shown in different cancer cell lines the over-expression of IF1 and of the H49K mutant of IF1 also inhibits the ATP synthetic activity of the enzyme. As a result of the additional experiments requested by R#1 and R#2 we now more clearly document that hIF1 inhibits both the hydrolase (see new Fig. 1F and 1G and Supplementary Fig. S6) and synthase (Fig. 2A and 2B and Supplementary Fig. S5) activities of the H+-ATP synthase. Hence, the decrease of ATP concentrations is not an artifact but the expected result by the expression of hIF1 in neurons. The blots of IF1 requested (Fig. 1E/ Fig. 2D/ Fig. 3G and Fig. 5D) have been incorporated in the revised version of the new figures.
As already discussed under point 2 of R#2, our findings suggest that metabolic rewiring of neurons to an enhanced aerobic glycolysis is due to a limited cellular ATP availability by direct inhibition of the ATP synthase.
It is well established that ROS act as a double-edged sword and that cellular responses depend on the site and intensity in which ROS are being produced. In the specific case of the pro-survival IF1-mediated signal of mitochondrial ROS we have demonstrated in different cell lines (Mol. Cell 2012; Oncogenesis 2013) that they are produced by blocking the activity of the ATP synthase. In this regard, it might be possible that other mechanisms that could stimulate a mild ROS signal in mitochondria able to induce the pro-survival pathways that we document in this paper could promote preconditioning and neuroprotection.

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Additional comments

1 and 7.- As we indicated in our previous correspondence, from these comments of the reviewer it appears that there is some misunderstanding in the interpretation of previous Fig. 6D (now New Fig. 6E) and Fig. 5D. The basal Bcl-xL levels in the non-stressed brain hemisphere of wild type and H+/T+ mice (R in Fig. 5D) are the same. Therefore, non-stressed primary cultures of neurons from wild type and H+/T+ mice reveal the same level of Bcl-xL (New Fig. 6E). There is no contradiction with the central conclusion that Bcl-xL is increased in the stressed brain hemisphere of H+/T+ mice (L in Fig. 5D). The blot in New Fig. 6E is just the western blot that illustrates that the silencing of Bcl-xL has worked.

2.- As we have indicated in answer to the second comment of the reviewer, hIF1 is inhibiting both the synthase and hydrolase activity of the H⁺-ATP synthase, in agreement with previous studies done in cancer and in stem cells and with animals in vivo. We have never observed that IF1 expression increases ATP levels by decreasing ATP consumption.

3.- Following the reviewer’s indication and as indicated under point 4 of R#1 the revised version of the paper accommodates the quantification of the bands from different experiments (Supplementary new Fig. S3). The results obtained reveal no significant differences in the content of proteins from respiratory complexes as a result of the expression of hIF1. Moreover, the new Fig. 1C now contains a better selection of blots documenting this point.

4. - As indicated under “other concerns” of R#2, the revised version of the manuscript now accommodates details of most of the methods employed that were initially omitted or presented in Supplementary Material.

5.- Figure abbreviations are now explained in all the figure legends.

6.- Figure legends of Fig. 4 B-E and 5 A-E, now state that determinations have been performed at 21 days post-surgery.

ARBITRATING ADVISOR:

We thank the reviewer for his/her very positive comments towards our work. As clearly stressed by the reviewer the point is whether hIF1 exerts neuroprotection by a direct effect on the ATP synthase or on the activity of the respiratory chain. We agree with the interpretation offered by the reviewer in that the effects triggered by hIF1 expression result from the inhibition of the ATP synthase because we observed a reduction in cell death rather than an increase in apoptosis, which is the normal observation in case of impaired respiration as recently demonstrated by the group of Scorrano (see Cogliati et al., Cell 155, 160-171, 2013). In this paper these authors have highlighted that genetic disruption of cristae shape morphology promotes the disassembly of respiratory chain supercomplexes leading to an increase and not to a decrease in apoptosis.

It has been extensively documented that cristae shape depends on dimers of the ATP synthase which has been recently associated with the permeability transition pore (PTP). In this regard, it has been suggested that IF1 could mediate ATP synthase dimerization although this suggestion is still a matter of debate. Therefore, we agree with the reviewer in that hIF1 could mediate neuroprotection by affecting the structure and/or activity of the PTP. These arguments are now included in the revised version of the Discussion (see last paragraph on p-12 to p-13).

That ROS are involved in neuronal excitotoxicity is well established and, as we show in Fig. 5C, the brain of hIF1 expressing mice is more reduced than that of controls after induction of excitotoxicity. However, it has been documented that IF1 expression triggers a ROS signal in many different cell lines as a result of the partial arrest of the H⁺-ATP synthase, mimicking the effect of oligomycin. Moreover, we have shown that hIF1 expression in neurons induces the production of superoxide (Fig. 3I) leading to subtle but significant changes in the carbonylation of brain (Fig. 2C) and neuronal (Fig. 3J) proteins. ROS are well known activators of several of the pro-survival pathways that we have documented in this paper to be already activated in the unstressed brain of the hIF1 expressing mice (Fig. 1E, Fig. 5D and Fig. 6B), consistent with our previous observations that the IF1-mediated production of ROS signals to the nucleus programs aimed at the evasion from apoptosis. Therefore, we have to distinguish between ROS levels that induce cell-death or long-term effects of ROS that support cell survival by promoting changes in the cell phenotype. Hence, we cannot exclude this additional possibility in the interpretation of our findings that on the other hand, is not incompatible with the reviewer’s point of view.

To support our argument we have studied the effect of ROS scavenging on glutamate-induced cell death in primary neuronal cultures (see new Fig. 6C). The results obtained illustrate
Mito Q, a superoxide scavenger significantly diminished cell death in neurons from wild type mice indicating that ROS are indeed involved in this type of cell death. Consistent with our hypothesis of preconditioning the cell death response to glutamate in neurons from hIF1 expressing mice is much less than that observed in controls being undistinguishable from the levels of protection afforded by the antioxidant (Fig. 6C). These findings are interpreted on the grounds of the different basal phenotype of control and hIF1 expressing neurons. The activation of AMPK, Akt, p70s6K and PARP in hIF1 expressing neurons (Fig. 1E, Fig. 5D and Fig. 6B) presumably confers a more resistant phenotype (preconditioning) against ROS mediated cell death (new Fig. 6C and Fig. 6E). In fact, the silencing of Bcl-xL that is over-expressed as a result of the activation of pro-survival pathways during excitotoxicity reverted the protection afforded by IF1 overexpression.

Overall, and following the reviewer’s suggestion we have entirely revised the Discussion section accommodating both the structural and metabolic alternatives for neuroprotection afforded by the inhibition of the H^+-ATP synthase. The new Fig. 7G has been modified accordingly.

We thank the reviewer for her/his argument and advice that has improved the presentation of our contribution.

Thank you for submitting your revised manuscript on IF1 and neuronal survival signaling to our editorial office. It has now been assessed once more by the original referees 1 and 2, and I am pleased to inform you that they both consider the revised version significantly improved and now in principle suitable for publication in The EMBO Journal, pending addressing of a few remaining minor points. These points are listed below in their reports, but please note that referee 1 also sent an annotated PDF to indicate and explain necessary textual changes - please find this additional file attached to my decision letter for your attention.

In addition to these presentational changes, I would like ask you to provide (in your resubmission cover letter) 2-5 one-sentence 'bullet points' (complementary to the abstract) that summarize key aspects of the paper - they will accompany the online version of the article as part of a 'synopsis'. Please see the latest research articles on our renewed website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.

Finally, I notice that many of the gel/blot images in the figures suffer from low resolution, blurriness as well as contrast/brightness over-adjustments (see for example Fig 2F or 6D). I would therefore ask you to re-assemble these figure panels from the original data, making sure to increase the image quality and to keep digital adjustments to a minimum, so that the correlation of the final figures to the originals gels/blots can be directly assessed. In addition, please provide us with source image data for all gels, blots and autoradiographs. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files can be uploaded upon resubmission selecting "Figure Source Data" as object type, and they would be linked as such to the respective figures in the online publication of your article.

After having received these files and final revisions, we should hopefully be in a position to proceed further with acceptance and publication of your manuscript. Should you have any further questions in this regards, please do not hesitate contact me directly. I look forward to receiving your final version!

REFEREE REPORTS:

Referee #1:

I have made my suggested changes directly in the text below [notes are within square brackets].

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Please compare with the original text (I could not highlight the changes as formatting is lost by copying into the website form, but I am transmitting an edited version by email).

Page 3

... The point of no return in cell death is the permeabilization of the inner mitochondrial membrane to low molecular weight solutes through opening of the so-called permeability transition pore (PTP) (Di Lisa et al., ; Galluzzi et al., 2009).

Page 4

..... Studies in total brain, isolated mitochondria and primary neuronal cultures demonstrate that overexpression of hIF1 partially inhibits oxidative phosphorylation triggering an adaptive metabolic response that results in increased aerobic glycolysis. The IF1-mediated metabolic reprogramming results in brain preconditioning and involves a mild oxidative stress that protects neurons from excitotoxic damage, enhancing motor behavior.

Page 5

.... Western blots (Figure 1A) and immunohistochemistry of coronal brain slices (Figures 1B, S1 and S2) revealed that only the H+/T+ genotype expresses hIF1 in mitochondria of neurons, resulting in ~3-fold increase in the total cellular content of IF1 (Figure 1A). The expression of hIF1 did not affect the expression of other mitochondrial proteins (Figures 1C and S3). However, the concentration of ATP was significantly diminished in the brain of H+/T+ mice when compared to controls (Figure 1D). These changes were accompanied by an increased concentration of AMP (Figure 1D) and the concurrent phosphorylation of the metabolic sensor AMPK (Figure 1E) in the brain of H+/T+ mice. Interestingly, a significant increase in the expression of the glycolytic enzymes GAPDH and LDH-A (Figure 1E) was observed in brain extracts of H+/T+ mice. Despite these differences, H+/T+ mice were born in the expected Mendelian ratios and were normal in appearance, home-cage behavior, reproduction and longevity up to one year follow up.

Page 7

... Basal fluorescence did not show significant differences between H+/T+ and controls (Figures 3H, S5 and S6), suggesting that there is no major difference in m. Consistent with the inhibition of both the synthase and hydrolase activity of the H+-ATP synthase by hIF1, we observed that changes in response to OL were lower (Figure S5) whereas those to antimycin A were higher (Figure S6) in H+/T+ cultures than in control.

Pages 8-9

Neurological evaluation indicates a better performance in hIF1 mice. No differences in locomotor performance were observed between control and H+/T+ mice before quinolinic acid administration (Figure S9A-F). However, three locomotor tests showed that H+/T+ mice are partially protected from quinolinic acid-induced damage and maintained lower deviation from pre-surgical values than control animals (Figure S9 and Supplementary videos for control and hIF1 expressing mice). Paw usage contralateral to the brain lesion (right) was significantly diminished in control animals when compared to H+/T+ mice (Figure S9A). No differences were noted in the use of the ipsilateral forelimb (left) (Figure S9B). Gate analysis revealed that control mice showed an increased stride length of the contralateral forelimb (Figure S9C) and in the coefficient of variation for base width of the hind limbs (Figure S9D) when compared to H+/T+ mice in the "footprint" test (Carter et al., 1999). All animals showed an increase in the number of paw-slips in the grid test (Brooks & Dunnett, 2009) but as early as 4 days post-surgery H+/T+ mice were able to walk through the grid with a significant lower number of paw slips (Figure S9E). The motility index of H+/T+ 9 mice was unaltered by lesion (Figure S9F). However, control mice were less prone to explore than H+/T+ mice suggesting a severe motor impairment (Figure S9F) (Supplemental videos for control and hIF1 expressing mice).
hIF1 signals the activation of neuroprotection pathways. Phosphorylation of Akt is part of the survival signaling pathway induced in cells confronted with mitochondrial respiration defects and/or a mild oxidative stress (Leslie, 2006; Pelicano et al., 2006). Consistently, the phosphorylation of Akt was significantly augmented in both brain hemispheres of mice expressing hIF1 (Figure 5D). We also observed an enhanced phosphorylation of the pro-survival p70S6K in both brain hemispheres (Figure 5D), and enhanced expression of c-fos in the damaged area of H+/T+ mice (Figure 5E). In contrast, the expression of the antiapototic Bcl-2 was not affected by hIF1 expression or by quinolinic acid administration (Figure 5D). Remarkably, the ATP and ADP content in the damaged area of the left hemisphere was much lower in H+/T+ mice (Figure 6A). In contrast, the AMP content was significantly increased (Figure 6A). The larger drop in ATP content of H+/T+ mice can be explained by the enforced hIF1-mediated inhibition of the synthase activity of the H+-ATP synthase, but could also result from higher activation of ATP consuming reactions following activation of repair mechanisms. This is consistent with a higher basal PARP activity in the brain of H+/T+ mice (Figure 6B), a difference that was magnified after hemispheric damage (Figure 6B).

Pages 10-11

Bcl-xL participates in hIF1-mediated protection of neuronal death. Blue-native gels confirmed the interaction of Bcl-xL with the H+-ATP synthase (Figure 6D) in agreement with recent findings (Alavian et al., 2011; Chen et al., 2011). However, and to our surprise, we found that Bcl-xL preferentially interacts with a native protein complex that migrates with Complex I of the respiratory chain (Figure 6D). Remarkably, silencing of Bcl-xL in neurons restored the glutamate or hypoxia-driven cell death in pre-conditioned neurons of H+/T+ mice (Figure 6E), further supporting the role of the anti-apoptotic Bcl-xL in neuroprotection and eventually its cross-talk with the activity of the H+-ATP synthase.

Page 12

........ These findings provide the first in vivo account highlighting the relevance of rewiring energy metabolism in brain preconditioning, and stress the potential value of the H+-ATP synthase as a target for therapeutic intervention [note that there was a logical jump as you didn't study stroke or other neurological diseases, hence the deletion]. Moreover, the transgenic H+ mouse developed offers a valuable tool to investigate the relevance of OXPHOS impairments in mammalian tissues in vivo. Until recently, IF1 was considered an inhibitor of the hydrolase activity of the ATP synthase that helps to preserve cellular ATP during hypoxia or ischemia (Faccenda et al., 2013; Gledhill et al., 2007). However, previous in vitro studies (Husain & Harris, 1983; Lippe et al., 1988) and more recent findings in vivo (Formentini et al., 2012; Sanchez-Arago et al., 2013b; Sanchez-Cenizo et al., 2010; Shen et al., 2009) have stressed the role of IF1 as an inhibitor of the synthase activity of the H+-ATP synthase. [I disagree with the deleted sentence because an oncogene must immortalize cells and promote tumorigenesis in vivo].

Page 14

...... (Zhao et al., 2011). Inhibition of the synthase activity of the H+-ATP synthase by IF1 is known to promote a mild ROS signal (Formentini et al., 2012; Sanchez-Arago et al., 2013b), which is consistent with the observed inhibition of the H+-ATP synthase ....

Pages 16-17

...... CryoEM studies of the macromolecular organization of the inner membrane reveal no interaction between Complex I and the H+-ATP synthase (Davies et al., 2011). However, cyclophilin D that favors PTP opening, binds to and regulates the activity of the H+-ATP synthase (Giorgio et al., 2009) and affects modulation of PTP by rotenone (Li et al., 2012). [As just mentioned by the Authors complex I does not "interact" with the ATP synthase, so the sentence should be removed; in the experiments of Giorgio the proteins migrate very close to one another in BN-PAGE, but this is not evidence of a direct interaction]. These findings suggest that the metabolic and/or the structural preconditioning mediated by hIF1 increases the threshold of PTP opening by preventing the channel formation capacity of the H+-ATP synthase (Giorgio et al., 2013) (Figure 7G). Overall, our data provide the first in vivo demonstration that the activity of the H+-ATP
synthase is necessary for the efficient execution of cell death. Hence, the H+-ATP synthase not only functions as the power plant of the cell but also as a pivotal regulator of cell death. [I have deleted the last sentence because, again, you can't extend your findings to other models].

Referee #2:

In this revised version, the authors have performed the requested experiments. H49K hIF1 overexpression inhibits ATP-ase activity (Figure 1F), while the addition of a respiratory chain inhibitor (Antimycin A) in intact cells induces a larger depolarization in neurons overexpressing H49K hIF1 (Figure S6). Figure S5 is also consistent with a partial inhibition of ATP-ase because oligomycin is less efficient at increasing mitochondrial membrane potential. On the other hand, the result in Figure 1G is unexpected, unless ATP was present in the medium, which is not specified in the legend or in material and methods. I suggest removing Figure 1G if ATP was not present in the medium.

2nd Revision - authors’ response

The revised manuscript incorporates all the few minor points stressed by the reviewers. Specifically, we have (i) incorporated all the text changes proposed on the annotated PDF (reviewer 1) and (ii) indicated under the legend to Figure 1G (reviewer 2) that the experiments are run in the presence of 10 mM ATP.

Following your indications, the sentences below provide relevant “bullet points” that summarize key aspects of the paper:


2. Inhibition of the H+-ATP synthase triggers metabolic reprogramming and ROS-mediated brain preconditioning that contribute to minimize neuronal cell death and preserve locomotor function of mice.

3. The H+-ATP synthase not only functions as the power plant of the cell but also as a pivotal regulator of cell death emphasizing its potential as a target for therapy.

4. The transgenic H49K mouse developed provides the founder of different models to inhibit oxidative phosphorylation in a tissue-specific and regulated manner to study pathologies involving the dysfunction of the bioenergetic activity of mitochondria.

In agreement with your observation, we have re-assembled the figure panels you suggested (Fig. 2F and 6D), as well as others that we considered could be improved (Fig. 1C and 1E), by increasing image qualities.

In addition, following your indications, the revised manuscript is now accompanied with an annotated source image data for all the blots presented organized in one PDF for each of the figures.

Thank you very much for your kind assistance.