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The role of deimination in ATP5b mRNA transport in a transgenic mouse model of multiple sclerosis

Di Ding, Mabel Enriquez-Algeciras, Kunjan R. Dave, Miguel Perez-Pinzon and Sanjoy K. Bhattacharya

Corresponding author: Sanjoy K. Bhattacharya, University of Miami

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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 15 September 2011

Thank you for the submission of your research manuscript to our journal. We have now received the full set of three referee reports that is copied below.

As you will see, all three referees consider that your work is interesting and might be published in EMBO reports. However, all of them raise a number of technical concerns that should be addressed, particularly regarding the experiments assessing mitochondrial transport of ATP5b by REF. According to the referees, this is the central part of your manuscript.

Given that, according to the referees, the current data do not seem to sufficiently support the main conclusions of the manuscript, we believe that, as it stands, publication of the study in our journal cannot be considered at this stage. However, as the referees are positive and agree in the potential interest of your findings, I would like to give you the opportunity to address the reviewers' concerns and I would be willing to consider a revised 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.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

In this manuscript Ding and colleagues explore the role of reduced deimination of the RNA binding export factor (REF) in a mouse model of multiple sclerosis (ND4). The authors focused on the ATP5b mRNA which has been shown previously to localize onto the mitochondrial surface and detected an impaired ATP5b transport in ND4 mice. The authors provide solid evidence supporting a connection between decreased deimination of REF and reduced ATP5b mRNA transport to the mitochondrial surface. Furthnermore, authors suggest that reduced ATP5b mRNA transport to mitochondrial surface and may impair mitochondrial biogenesis and function and thus contribute to the pathogenesis of multiple sclerosis.

The study comprises several solid elements (microarray analysis, localization of REF on mitochondrial surface, mass spectrometry). However, despite the authors' assertion, it falls short of providing a real mechanistic insight as to how REF deimination affects mitochondrial mRNA transport and thus mitochondrial health. The authors propose a hypothetical mechanism, but this is not directly supported by data. Addressing the comments bellow would strengthen their conclusions.

Comments
The stronger association of components of translational machinery with deiminated REF does not guarantee increased translation levels. Monitoring of ATP5b protein synthesis in association with deiminated REF vs. non-deiminated REF will lead to a better supported model.

ATP5b and ATM1 are detected in mitochondria isolated from both WT and ND4 mice by RT-PCR (Fig 1B-a'). Double immunofluorescent staining for porin and ATP5b followed by confocal microscopy could provide a more direct evidence for the ATP5b presence at mitochondrial surface.

Fig. 1B-b' shows the relative abundance of ATP5b and ATM1 in cytosolic and mitochondrial fractions in normal mouse cDNA derived from three IP products. A Figure with ND4 mouse is needed for comparison.

In addition to the biochemical assays, the involvement of REF selectively in ATP5b mRNA transport, could also be tested by determining the effects of REF-directed siRNAi on ATB5b and ATP1 expression in mitochondrial and cytosolic fractions.

The lower levels of ATP5b in mitochondria of ND4 mice compared to WT controls (Fig. 2C) may be due to mutations affecting the sequences required to guide perimitochondrial localization of ATP5b mRNA (Garcia et al., 2010) in transgenic ND4 mice and not to reduced deimination of REF, which may occur independently.

In addition, only the state 3 mitochondrial respiratory rates differ significantly between CD1(normal) and ND4 synaptic mitochondria as shown in Fig. 3A-a' (considering the error bars). Overall, the mitochondrial defects of ND4 mice are not well documented (see Fig. 3A, FigS3, Table 1).

Finally, it would be interesting to know if the transportation efficiency of other mRNAs associated with REF is also reduced.

Referee #2:

In this study, Ding et al investigate the role of deimination (conversion of protein bound arginine to citrulline) of the RNA binding export factor (REF) in the ND4 mouse model (transgenic overexpressing DM20) of multiple sclerosis. They suggest that impaired deimination in neurons in these mice reduces binding of ATP5b mRNA at the mitochondrial surface, thus decreasing the transport of this mRNA. Mitochondria from these ND4 mice show respiratory defects as compared to mitochondria from control animals, an effect more prominent in synaptic mitochondria. By way of additional support, the authors show that inhibiting deimination in PC12 cells inhibits ATPase synthase activity.

The key finding of this study is that REF is present at the mitochondrial surface, where it binds ATP5b in a deimination-dependent manner, a regulatory mechanism impaired in a mouse model of multiple sclerosis. Several points in this study are clearly of interest, but I have several substantial concerns regarding the strength of evidence supporting a number of them:

1. In Figure 1A, a control IgG IP should be shown to confirm the specificity of the data shown. The REF signal in the IP is very weak, making proper IP controls essential.

2. My understanding of the fractionation using the Dunkley et al protocol is that it emphasizes the isolation of synaptosomes. Do the authors perform a lysis step on the synaptosomes, with subsequent fractionation to isolate the synaptosomal mitochondria from other synaptosomal components?

3. Regarding Figure 1C and 1D (and Supplemental Figure S1), I am not convinced there is any colocalization of REF with mitochondrial markers. In Figure 1C there is only one faint yellow spot (representing colocalization) in the neuronal processes. In Supplemental Figure S1 the REF staining in panels B and B' looks completely different -- what is the reason for this? Finally, in the EM image in Figure 1D, there is one isolated gold particle abutting a mitochondrion, but there are no controls shown -- this could be background.

4. As discussed above, the mechanistic points here rely heavily on the quality of the fractionations described. In Figure 2A, it would be helpful to show the nuclear fraction, and the exclusion of nuclear markers from the mitochondrial and cytoplasmic fractions.

5. As a follow up point to demonstrate that the REF is on the mitochondrial surface, the authors should perform protease protection experiments (e.g., proteinase K), as are frequently done in mitochondrial protein import studies. In this case, one would expect the REF to be protease-sensitive (but IMS or matrix proteins to be resistant).

6. The authors have not shown that REF protein is required for ATP5b transport or ATP synthase activity. This seems fundamental to their arguments, and could be done using siRNA.

7. I like the idea of the experiments in Figure 3, panels C and D. Could the authors show a panel demonstrating equal REF levels as well as a citrulline immunoblot confirming the in vitro deimination?

8. The PAD2 siRNA in Figure 3E appears to result in only about a 50% knock down based on protein levels. Thus, though I understand what the authors are trying to show, one expects a better level of knockdown to be convinced.

9. Overall, I found some of the points quite interesting, though the data need to be much more compelling to support them. I would emphasize more rigorously demonstrating REF on the mitochondrial surface and solidifying the in vitro data in Figure 3. I think Supplemental Figure S4 could be removed to focus the study a bit more.

Referee #3:

Revision Comments:

This manuscript investigates the ND4 mouse model of multiple sclerosis for a potential mechanism that leads to mitochondrial dysfunction. The authors propose that underlying cause is that of decreased ATP synthase activity that is caused a defect in trafficking of the ATP5b RNA to the mitochondrial outer membrane. Specifically, they show that the ATP5b RNA binds to the the mRNA carrier, RNA binding export factor (REF), and that deimination (conversion of arginine residues to citrulline residues) of REF is required ATP5b RNA binding. Overall, the manuscript is very interesting, but the model is quite extensive, from REF deimination to a decrease in ATPase activity, and the manuscript does not have adequate data to support all aspects of the model. Particular points to address are how does REF specifically select RNA cargo that is mitochondrial and how does REF direct these RNAs to the mitochondria, especially as multiple trafficking mechanisms (a different one for ATM1) are proposed? In addition, do changes in RNA targeting of ATP5b correlate into a decrease in the protein in the ATPase?

The following points would improve their study.

1. The authors propose that ATP5b RNA binding to the mitochondrial outer membrane results in a decrease in ATP synthase activity. However, other mechanisms may decrease OXPHOS. The authors should investigate the steady state levels of the ATP5b protein in mitochondria and confirm that it is decreased. In addition, blue native gels should be included to determine if the respiratory complexes are decreased in abundance or fail to assemble. If the F1 head is not assembly correctly, it may be dissociated from the F0 head, which can be detected on blue-native gels. Also, the authors propose that targeting of ribosomal RNAs may be affected, so a general decrease in mitochondrial translation could contribute to a defect in OXPHOS.

2. How does deiminated REF select specific cargo RNAs when it seems to be a general RNA binding factor?

3. Published studies by the Jacq laboratory show that the 3'UTR is used to localize RNAs to the mitochondrial outer membrane. If the authors delete the 3'UTR ofATP5b RNA, does it correlate with a decreased association with mitochondria?

4. Are the total levels of ATP5b mRNA affected in the mouse model compared to a wild-type mouse?

5. The introduction should include the studies by the Jacq laboratory showing 3' UTR targeting to mitochondria (Sylvestre, J., et al., The role of the 3' untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells. Mol Biol Cell, 2003. 14(9): p. 3848-56 and Marc, P., et al., Genome-wide analysis of mRNAs targeted to yeast mitochondria. EMBO Rep, 2002. 3(2): p. 159-64.)

6. The THO/TREX complex should be explained in the introduction

7. Binding of REF was found for both ATM1 and ATP5b RNA, but ATM1 RNA targeting was not affected. What makes REF bind to specific RNAs or does it bind to all RNAs and what is the difference in affinity of deiminated vs iminated? Is a 2-fold increase in an RNA bound to deiminated REF going to translate to a difference in protein levels in the mitochondria for the mitochondrial targets? How do the authors propose that REF guides ATP5b RNA to the mitochondrial outer membrane?

8. The co-localization of REF with mitochondria by immunofluorescence and electron microscopy is not very conclusive. Also, additional RNAs other than ATP5b should be tested in Fig. 2C.

9. In Fig. 3E, the imination status of REF should be tested when PAD2 and PAD4 are down-

regulated by siRNA. Also is it possible that other mitochondrial proteins are deiminated and this contributes to the decrease in respiration? What other proteins are deiminated in the cell?

1st Revision - authors' response 03 November 2011

Response to reviewer's comments

Reviewer 1

We thank the reviewer for his/her comments. These reviews have immensely helped us improving our manuscript and we thank the reviewer for the same.

"In this manuscript Ding and colleagues explore the role of reduced deimination of the RNA binding export factor (REF) in a mouse model of multiple sclerosis (ND4). The authors focused on the ATP5b mRNA which has been shown previously to localize onto the mitochondrial surface and detected an impaired ATP5b transport in ND4 mice. The authors provide solid evidence supporting a connection between decreased deimination of REF and reduced ATP5b mRNA transport to the mitochondrial surface. Furthermore, authors suggest that reduced ATP5b mRNA transport to mitochondrial surface and may impair mitochondrial biogenesis and function and thus contribute to the pathogenesis of multiple sclerosis.

The study comprises several solid elements (microarray analysis, localization of REF on mitochondrial surface, mass spectrometry). However, despite the authors' assertion, it falls short of providing a real mechanistic insight as to how REF deimination affects mitochondrial mRNA transport and thus mitochondrial health. The authors propose a hypothetical mechanism, but this is not directly supported by data. Addressing the comments bellow would strengthen their conclusions."

We thank the reviewer for his/her comments. Additional new experimental data has been incorporated in light of the reviewer's comments.

"The stronger association of components of translational machinery with deiminated REF does not guarantee increased translation levels. Monitoring of ATP5b protein synthesis in association with deiminated REF vs. non-deiminated REF will lead to a better supported model."

Reviewer's point is well taken. In light of his/her comments in the revised manuscript we have incorporated new data (Supplemental Figure S5C) supporting effect of deiminated REF on ATP5b synthesis compared to control non-deiminated recombinant REF.

"ATP5b and ATM1 are detected in mitochondria isolated from both WT and ND4 mice by RT-PCR (Fig 1B-a'). Double immunofluorescent staining for porin and ATP5b followed by confocal microscopy could provide a more direct evidence for the ATP5b presence at mitochondrial surface."

As per the reviewer's suggestion, we have performed double immunoflorescent labeling with anti-Tomm20/anti-REF, anti-ATP5b/anti-Tomm20 and have included a confocal microscopy panel for each double labeling (Supplemental Figure S1D, E) in the revised manuscript. We would like to emphasize that these are representative figures.

"Fig. 1B-b' shows the relative abundance of ATP5b and ATM1 in cytosolic and mitochondrial fractions in normal mouse cDNA derived from three IP products. A Figure with ND4 mouse is needed for comparison."

In the revised manuscript, we have included a new figure for ND4 mouse (Supplemental Figure S1C) as suggested by the reviewer.

"In addition to the biochemical assays, the involvement of REF selectively in ATP5b mRNA transport, could also be tested by determining the effects of REF-directed siRNAi on ATB5b and ATP1 expression in mitochondrial and cytosolic fractions."

We have used the siRNA for REF and found a reduction on ATP5b on mitochondrial surface. A quantitative estimate has been provided in Supplemental Figure 5A.

"The lower levels of ATP5b in mitochondria of ND4 mice compared to WT controls (Fig. 2C) may be due to mutations affecting the sequences required to guide perimitochondrial localization of ATP5b mRNA (Garcia et al., 2010) in transgenic ND4 mice and not to reduced deimination of REF, which may occur independently."

We (and also independently Dr. Fabrizio Mastronardi; personal communications) have found no evidence of any mutation in ATP5b in ND4 mice. The sequence of ATP5b in ND4 mice exactly matches with control CD1 (either with restriction fragment length polymorphism or with sequencing) thus while this is definitely a possibility but in light of sequencing efforts from our two laboratories it appears unlikely. However, we have discussed this possibility in the revised discussion and cited Garcia et al. 2010 paper.

"In addition, only the state 3 mitochondrial respiratory rates differ significantly between CD1(normal) and ND4 synaptic mitochondria as shown in Fig. 3A-a' (considering the error bars). Overall, the mitochondrial defects of ND4 mice are not well documented (see Fig. 3A, FigS3, Table 1)."

We agree with the reviewer that we observed marginal decrease in state 3 respiration rates in most of the experimental conditions. State 3 respiration rates, when measured in presence of pyruvate + malate gives an idea about efficacy of mitochondrial respiratory chain complex I, III and IV. Our results thus demonstrate marginal decrease in efficacy of these three complexes together. These results also suggest that ATP5b (a part of complex V) deficiency is not affecting efficacy of complex I, III and IV. We confirmed these observations by measuring individual complex activity (see Supplemental Figure S3). This point has been included in the revised discussion section of the manuscript.

"Finally, it would be interesting to know if the transportation efficiency of other mRNAs associated with REF is also reduced."

In the accompanying manuscript we have reported reduced SNAP25 mRNA transportation efficiency in neuronal dendrites due to loss of REF deimination. This occurs along with functional consequences for vision, as shown by pattern electroretinogram. Although we have not investigated another mitochondrial surface transported mRNA but SNAP25 mRNA is an example of reduced transportation efficiency due to loss of REF deimination (see accompanying manuscript).

Reviewer 2

We appreciate all the constructive suggestions and thank the reviewer. Responses to the comments and criticisms are highlighted in blue below each individual comment.

"In this study, Ding et al investigate the role of deimination (conversion of protein bound arginine to citrulline) of the RNA binding export factor (REF) in the ND4 mouse model (transgenic overexpressing DM20) of multiple sclerosis. They suggest that impaired deimination in neurons in these mice reduces binding of ATP5b mRNA at the mitochondrial surface, thus decreasing the transport of this mRNA. Mitochondria from these ND4 mice show respiratory defects as compared to mitochondria from control animals, an effect more prominent in synaptic mitochondria. By way of additional support, the authors show that inhibiting deimination in PC12 cells inhibits ATPase synthase activity.

The key finding of this study is that REF is present at the mitochondrial surface, where it binds ATP5b in a deimination-dependent manner, a regulatory mechanism impaired in a mouse model of multiple sclerosis. Several points in this study are clearly of interest, but I have several substantial concerns regarding the strength of evidence supporting a number of them:"

We thank the reviewer for his supportive comments. The specific comments are addressed below.

"In Figure 1A, a control IgG IP should be shown to confirm the specificity of the data shown. The REF signal in the IP is very weak, making proper IP controls essential."

In Supplemental Figure S1B, we have presented a control IgG IP (anti-Aldehyde dehydrogenase; ALDH1 antibody). This control IP shows lack of REF further demonstrating specificity of the IP data presented in Figure 1A.

"My understanding of the fractionation using the Dunkley et al protocol is that it emphasizes the isolation of synaptosomes. Do the authors perform a lysis step on the synaptosomes, with subsequent fractionation to isolate the synaptosomal mitochondria from other synaptosomal components"

We apologize for not clearly stating the experimental conditions. All mitochondrial respiration experiments were performed in synaptosomes permeabilized with 0.007% digitonin (Dave et al. 2008). This description has been incorporated in the revised text as well as in the Supplemental Information.

"Regarding Figure 1C and 1D (and Supplemental Figure S1), I am not convinced there is any colocalization of REF with mitochondrial markers. In Figure 1C there is only one faint yellow spot (representing colocalization) in the neuronal processes. In Supplemental Figure S1 the REF staining in panels B and B' looks completely different -- what is the reason for this? Finally, in the EM image in Figure 1D, there is one isolated gold particle abutting a mitochondrion, but there are no controls shown -- this could be background"

In light of reviewer's comments we have presented two new and representative confocal images in Supplemental Figure S1D and E. We would like to emphasize that these are representative figures. We have replaced the old EM figure with new figures to compare wild type (CD1) and ND4 mouse optic nerve (revised Figure 1D upper and lower panels). The negative control EM figures have been presented in Supplemental Figure S1F, G clearly showing lack of gold particles with no primary antibody controls.

We attribute the difference in REF staining between Figure 1C and previous Supplemental Fig. S1 to following reasons: (1) the Figure 1C and previous Supplemental Fig. S1 are probably from two different layers, Fig. 1C is a single layer from a confocal image where as

Fig. S1 was a z- stack image (2) we also have used two different REF antibodies obtained from two different vendors. The differences in signal intensities could also be due to minor factors such as slight difference in concentration. We have performed several repeats to confirm the specificity of the signal that has been presented in the manuscript.

"As discussed above, the mechanistic points here rely heavily on the quality of the fractionations described. In Figure 2A, it would be helpful to show the nuclear fraction, and the exclusion of nuclear markers from the mitochondrial and cytoplasmic fractions."

In light of reviewer's comment, different fractions with markers have been presented in the revised Fig. 2A.

"As a follow up point to demonstrate that the REF is on the mitochondrial surface, the authors should perform protease protection experiments (e.g., proteinase K), as are frequently done in mitochondrial protein import studies. In this case, one would expect the REF to be protease-sensitive (but IMS or matrix proteins to be resistant)."

We thank the reviewer's suggestion, we have presented a figure with partial proteinase K digestion using IR-700 and IR-800 coupled antibodies for ATP5b and REF presented in Fig. 1G. Whereas REF is completely digested, ATP5b is largely left intact.

"The authors have not shown that REF protein is required for ATP5b transport or ATP synthase activity. This seems fundamental to their arguments, and could be done using siRNA"

The reviewer's point is well taken. We have subjected REF siRNA treatment and presented the new data in Supplemental Figure S5A and B.

"I like the idea of the experiments in Figure 3, panels C and D. Could the authors show a panel demonstrating equal REF levels as well as a citrulline immunoblot confirming the in vitro deimination?"

Using IR-coupled antibodies we show equal level and deimination of in vitro recombinant REF from an identical blot that was used for gel mobility shift assay (Fig. 3D, lower panel).

"The PAD2 siRNA in Figure 3E appears to result in only about a 50% knock down based on protein levels. Thus, though I understand what the authors are trying to show, one expects a better level of knockdown to be convinced."

We have repeated transfection experiments and have achieved up to ~80% reduction. We have replaced the Figure 3E with a new figure and now provided the new quantitative average data from a larger data set (Supplemental Figure S4D).

9. Overall, I found some of the points quite interesting, though the data need to be much more compelling to support them. I would emphasize more rigorously demonstrating REF on the mitochondrial surface and solidifying the in vitro data in Figure 3. I think Supplemental Figure S4 could be removed to focus the study a bit more.

We thank the reviewer for his/her suggestions. We have presented improved EM figures (Fig. 1D upper and lower panels). We have provided control EM picture that shows lack of gold particles (Supplemental Fig. S1F, G). We have provided improved confocal pictures for co-localization (Supplemental Fig. S1D, E). If the reviewer strongly feels that we should remove Fig. S4, then during final submission, we will omit it.

Reviewer 3

Our sincere thanks are to this reviewer for his/her comments, which have helped us improving our manuscript. Responses to the comments and criticisms are highlighted in blue below each individual comment.

"The authors propose that ATP5b RNA binding to the mitochondrial outer membrane results in a decrease in ATP synthase activity. However, other mechanisms may decrease OXPHOS. The authors should investigate the steady state levels of the ATP5b protein in mitochondria and confirm that it is decreased. In addition, blue native gels should be included to determine if the respiratory complexes are decreased in abundance or fail to assemble. If the F1 head is not assembly correctly, it may be dissociated from the F0 head, which can be detected on blue-native gels. Also, the authors propose that targeting of ribosomal RNAs may be affected, so a general decrease in mitochondrial translation could contribute to a defect in OXPHOS."

We really appreciate the reviewer's suggestion pertaining to the blue gel and 2D gel detection of ATP5b. We compared ATP5b components with NUDSF4 and found insignificant differences between ND4 and CD1 mice (Fig. 1E). Although level of expression of ATP5b showed some decrease in ND4 mice (Fig. 1F). We cannot completely rule out the impaired mitochondrial translation of several mRNAs and this point has been discussed in the revised text in light of reviewer's comment. Steady state level of ATP5b is decreased but not that of ATM1.

"How does deiminated REF select specific cargo RNAs when it seems to be a general RNA binding factor?"

The REF may have different specificity towards different mRNA based on their secondary structure and also contributed by other cofactors. While REF binds to ATP5b, the binding is lacking for ATM1. Consequently it appears that REF affects ATP5b transport on mitochondrial surface but the ATM1 transport is not affected(Fig. 1B). The binding specificity for mRNA cargos by REF may also be imparted partly by co-binding partners. This is a question that will necessitate separate investigation, which we plan to do subsequently. In light of reviewer's comments we have revised the discussion.

"Binding of REF was found for both ATM1 and ATP5b RNA, but ATM1 RNA targeting was not affected. What makes REF bind to specific RNAs or does it bind to all RNAs and what is the difference in affinity of deiminated vs iminated? Is a 2-fold increase in an RNA bound to deiminated REF going to translate to a difference in protein levels in the mitochondria for the mitochondrial targets? How do the authors propose that REF guides ATP5b RNA to the mitochondrial outer membrane?"

The biochemical studies (Fig. 3C and D) revealed a greater dissociation constant for ATP5b mRNA and non-deiminated REF compared with deiminated REF, suggesting stronger binding between ATP5b mRNA and the deiminated protein (0.28 versus 0.15 nM) under our in vitro test conditions. These results show that deimination alters the RNA binding properties of REF, strengthens ATP5b mRNA binding, and enhances protein translation of select mRNAs. The difference in mRNA binding between deiminated and non-deiminated protein is structural but in vivo other than mRNA and REF structure, the co-binding factors (such as translocases) also may confer part of the binding specificity. We think REF is part of a complex that helps contained handling of the mRNA species from

nucleus to mitochondrial surface where other co-binding proteins confers additional specificity and mobility. This report pertains to demonstration that deimination of REF is involved in the process.

"Published studies by the Jacq laboratory show that the 3'UTR is used to localize RNAs to the mitochondrial outer membrane. If the authors delete the 3'UTR of ATP5b RNA, does it correlate with a decreased association with mitochondria?"

We would respectfully like to submit that this is a different question than what our present report is centered around. However, this question has independently occurred to us as well largely due to published work from Jacq laboratory. At animal model level several levels of complexities will necessitate large number of different experiments to properly evaluate this question. With cell culture systems, our preliminary experiments, just as from Jacq laboratory, suggests 3'UTR is important and it deletion correlates with decreased association with mitochondria. Even at cell culture level our preliminary experiments suggest that a knock out of ATP5b followed by knock in of 3'UTR deletion is necessary to get a cleaner outcome. We would respectfully submit to the reviewer that the stipulation for the current manuscript/report as stated in the editorial comments is, "Please take into account that revised manuscripts should be submitted within three months; they will otherwise be treated as new submissions. Also, the length of the revised manuscript may not exceed 28,000 characters (including spaces) and maximally 5 figures may be presented in the main manuscript file", inclusion of UTR deletion experiments now under progress and when properly completed is likely to exceed if considered with current data and focus. We humbly submit that we think that a manuscript with UTR deletion experiments (currently under progress) will likely to be having a different focus than the work presented here.

"Are the total levels of ATP5b mRNA affected in the mouse model compared to a wild-type mouse?"

Fig. 1B and 2C are updated with our new result of comparison of total levels of ATP5b mRNA between ND4 and wild type mice. There is very slight difference at expression level normalized by ATM1, which is consistent with our observation that was presented in our previous version.

"The introduction should include the studies by the Jacq laboratory showing 3' UTR targeting to mitochondria (Sylvestre, J., et al., The role of the 3' untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells. Mol Biol Cell, 2003. 14(9): p. 3848-56 and Marc, P., et al., Genome-wide analysis of mRNAs targeted to yeast mitochondria. EMBO Rep, 2002. 3(2): p. 159-64.)"

In light of reviewer's comments, we have added this in the introduction section in the revised text.

"The THO/TREX complex should be explained in the introduction"

In light of reviewer's comments we have incorporated this in the introduction section in the revised version.

"The co-localization of REF with mitochondria by immunofluorescence and electron microscopy is not very conclusive. Also, additional RNAs other than ATP5b should be tested in Fig. 2C."

In light of reviewer's comments, we have provided improved figures (Fig. 1A and revised Supplementary Figure S1) in the revised version. We have incorporated ARP mRNA results in Fig. 2C which does not show much difference at mitochondrial surface between CD1 and ND4 mice.

"In Fig. 3E, the imination status of REF should be tested when PAD2 and PAD4 are down-regulated by siRNA. Also is it possible that other mitochondrial proteins are deiminated and this contributes to the decrease in respiration? What other proteins are deiminated in the cell?"

In light of reviewer's comments, deimination level of REF has been determined in PAD-siRNA treated cells (revised Figure 3E, bottom panel). We appreciate reviewer's point. We have known existence of about 45 proteins during previous work from our and other laboratories [reviewed in Bhattacharya SK (2009) Retinal deimination in aging and disease IUBMB Life 61(5):504-9] that undergo deimination. However, this list is increasing. A small fraction of Porin 1 is found deiminated but heterogeneously. Available data for Porin 1 is consistent with deimination in astroglial cells not neurons. We aim to perform in near future a comprehensive mass spectrometry of the isolated purified cytosolic, nuclear and mitochondrial fraction using specific methods to detect this modification.

2nd Editorial Decision 01 December 2011

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

Referee #2 suggests that certain panels in figure 3 and figure S1 should be improved for better appreciation of the information presented. Also, I have noticed that the length of your manuscript is significantly higher than our 28000 characters limit. I understand the difficulties of reducing the main text, and I can exceptionally extend the limit to 30000 characters, but the manuscript would still need substantial shortening from the current ~35400 characters. I would recommend combining the "Results" and "Discussion" sections, as keeping them separate leads to redundancies that unnecessarily increase the character count.

Thank you for your contribution to EMBO reports.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors have adequately responded to my comments and the manuscript is now significantly improved. I believe that it is now suitable for publication in EMBO Reports.

Referee #2:

In this revised manuscript, the authors have performed a number of new experiments to address my concerns. Overall, they have done a creditable job of strengthening their claims, and the main points

are quite interesting and important. For a number of the immunoblots, such as the lower two panels of Figure 3E, longer exposures may be necessary to improve visibility. Also, in the confocal images in Supplementary Figure S1, panels D and E, it is hard for me to appreciate the co-localizations because some signals appear quite faint, and thus it will be important for the authors to ensure that the brightness/contrast, etc. of these images are optimized.

2nd Revision - authors' response 20 December 2011

Response to reviewers' comments

Referee #1:

The authors have adequately responded to my comments and the manuscript is now significantly improved. I believe that it is now suitable for publication in EMBO Reports.

We thank the reviewer for his/her encouraging comments.

Referee #2:

In this revised manuscript, the authors have performed a number of new experiments to address my concerns. Overall, they have done a creditable job of strengthening their claims, and the main points are quite interesting and important. For a number of the immunoblots, such as the lower two panels of Figure 3E, longer exposures may be necessary to improve visibility. Also, in the confocal images in Supplementary Figure S1, panels D and E, it is hard for me to appreciate the co-localizations because some signals appear quite faint, and thus it will be important for the authors to ensure that the brightness/contrast, etc. of these images are optimized.

We thank the reviewer for his/her encouraging comments. We have replaced the lower two panels of Figure 3E with a blot subjected to longer exposure. We have optimized the Panels D and E of Supplementary Figure S1 as recommended by the reviewer.

3rd Editorial Decision 21 December 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

