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LRPPRC is necessary for polyadenylation and coordination of translation of mitochondrial mRNAs

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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 05 September 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. As discussed, it has been evaluated by three knowledgeable referees whose comments are shown below.
I am sorry for the slow decision making process while we were consulting further with the referees and editorial colleagues.

The three referees generally agree that the data presented is of high quality and state of the art, although they raise a number of relatively minor issues that should be addressed experimentally. As also discussed in the referee reports, it will be essential to work on the text, to clearly introduce the current state of knowledge (largely based on cultured cell work), to discuss the contradictions with the previous literature openly and as impartially as possible, and to highlight the new insights provided in the discussion. It is clear that even the expert referees find the current manuscript somewhat complex and confusing. We would also recommend a description of the questions/hypotheses that this dataset throws open that need to be addressed in subsequent papers. Evidently some of the additional complexities in the data, such as effects on complexes II (ref 2) and V (ref 1) should also be discussed more prominently.
Please note that referee 3 recommends a more elaborate description of the methods. Please also accurately define the statistical parameters (such as what n represents) in the appropriate panels. Please describe/add scale bars (e.g. fig 1c, 2a).

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I will not list the textural issues raised by all the referees in detail, but we would expect them to be addressed comprehensively.

I would therefore like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html
Please note that we have just started to encourage the publication of 'source data' to the key experimental data in the paper - that is uncropped and unedited images of the gels, blots and micrographs underlying key data panels, including molecular weight markers. These will be published as supporting source data.
In particular this would likely address referee 3's points regarding figures 2 and 3.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Thank you for sending me this manuscript concerning the role of LRPPRC in mitochondrial gene expression. The authors report a substantial number of experiments on the analysis of ts KO LRPPRC mice and on cultured cell lines. The data is compelling and confusing. Once again we find that mechanisms of mitochondrial gene expression appear to be far from predictable. The authors show that depletion of LRPPRC leads to a profound loss of complex IV, as has been reported previously, with a partial loss of complex V and sparing complexes I and III. As has also been previously described, LRPPRC forms a complex with SLIRP that is essential for the maintenance of mitochondrial mRNA. A third observation, also previously reported is that LRPPRC depletion leads to the loss of steady state levels of all mitochondrial mRNAs but this paper reports the novel sparing of the L-strand encoded ND6. The authors have extended this work by showing clearly that mRNA remaining in the LRPPRC KO cells are not polyadenylated, consistent with the stability of ND6, the only nonpolyadenylated transcript. Perhaps the most interesting and surprising aspect of this work is the variability of transcript translation. This is particularly odd as it disagrees with the work of Sasarmann on the LRPPRC patient cell lines, where mRNA is depleted but translation is down across the board in fibroblasts. Why is this so different in the tissue from the KO mice ? Could this somehow be an artefact caused in part by isolation of mitos prior to 35S labelling ? Overall, this is an intriguing paper and should be published. The Group has an excellent international reputation and although several pieces of data are surprising and differ from other reports, I think the manuscript adds substantially to the debate about mitochondrial gene expression. I have one or two other points that the authors might wish to address and perhaps alter the manuscript accordingly:

P8.the authors state that a previous report by Sasarmann et al of knockdown of LRPPRC in cell lines causes a generalised resp chain deficiency, unlike their observation of a specific and profound complex IV loss (and partial V), suggesting this discrepancy may due to continuous proliferation of tc cells. However, the Sasarmann paper also shows that primary fibroblasts from patients demonstrate a very similar complex IV-specific defect. This is complicated, suggesting that partial

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depletion causes complex IV loss in man, whilst further depletion leads to global OXPHOS defects. This does not appear to be the case in mice.

I'm slightly confused about the LRPPRC/SLIRP complex, which is around 250 kDa. The authors report evidence that mRNA is found in the complex, as has been reported previously. However, if we assume that the average Mr of a murine mitochondrial transcript is approx. 250kDa one wonders where the apparent RNA is hiding ? I assume this means that most of the bound RNA is degraded mRNA ?

P12, Fig 6A, contrary to what is stated that complex V subunits are unaffected, there definitely looks to be a diminution of ATP8 levels in the KO. Further, as NDUFA9 is present at reasonable steady state levels, even in rho0 cells, the absence of any effect on this protein does not necessarily tell us much about fully functional complex I in the KO.

With the 35S labelling in isolated mitos, it is of course difficult to be sure of the identity of some translation products as some products could possibly be stalled intermediates. For example, the very intensely labelled products running just below ND2 in the controls. What are they ? If they are truly ND1 and ND2, why is there so much translation of these mRNAs ? This doesn't appear to be random translation as the authors suggest in the discussion. In fact, the steady-state levels of ND1 in the KO cells are not particularly high, yet if this is really one of the translation products that is selectively increased it would be interesting to know why the authors think this is the case. Why is this data so different to the paper of Sasarmann, where fibroblasts from patients with LRPPRC depletion show a generalised defect in mitochondrial protein synthesis.

Why are there two peaks of mRNA that are not in the monosome (Fig 8)? This suggests that the free mRNA can associate both with the large and small subunit alone. What does this mean ? One assumes that there must be a well defined mechanism for generating a pre-initiation complex. In most systems, this normally includes the small ribosomal subunit and Professor Spremulli has published that at least in vitro mitochondrial mRNA preferentially binds to the small subunit. Does this mean that the monosome dissociates in some way on isolation ?

Referee #2

The manuscript by Ruzzenente and colleagues reports new studies on the role of LRPPRC in the stabilization of mitochondrial mtRNAs and their translational regulation. The results presented here on LRPPRC constitutive and conditional KO in muscle contain many novel and unexpected elements. The loss of LRPPRC not only leads to decreased steady levels of several mt-mRNAs but this appears to occur in a mRNA specific manner with sparing of the single L-strand transcript ND6 and the two rRNAs. Most intriguingly, the translational dysregulation of mitochondrial peptides appears unrelated to the respective mRNA levels and involves extreme decrease of some transcripts (COX genes and complex V genes) and up regulation of others. Complex IV deficiency is predominant possibly explaining why patients with LRPPRC mutations present with severe complex IV defects in liver and brain (LSFC). The extensive biochemical studies in this work confirm that LRPPRC forms a complex with SLIRP, which requires mRNA to form. In the absence of mRNA and SLIRP binding, LRPPRC forms high molecular weight structures of unclear nature, possibly homo or hetero oligomers. LRPPRC absence decreases mRNA polyadenylation, which may partly be responsible for the decrease stability of some species of message. However, this may not be a main cause of defective translation, since this is unaffected for some mRNAs, which are not polyadenylated.
The work is very well conducted, looking at LRPPRC function from different angles and many different types of approaches. The results are convincing and shed new light on the function of LRPPRC in mammalian mitochondria. Some important questions, beyond the scope of the present work, remain to be answered. For example, why the turnover of the COX mRNAs is so different (faster) than other genes? How does this correlate with the turnover of the assembled proteins in comparison with complex I and III?
I have a few points, mostly for discussion.
1) The authors review the genes involved in mtDNA transcription in the introduction and discussion, but the role of LRPPRC appears more related to mRNA maintenance and translation. On the contrary they do not introduce the role of SLIRP, which is a binding partner of LRPPRC. The

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introduction and discussion can focus more of the protein complex at hand.
2) A decline of complex II function in the LRPPRC KO is shown in figure 2 but not described or discussed. Complex II is not mtDNA encoded; what could be reason of this finding.
3) What could be the reason of the different interpretation of the role of mRNA in allowing the binding of LRPPRC and SLIRP between this study and the previous one by the Shoubridge group?
4) What could be the nature of the high molecular weight complex containing LRPPRC in the absence of mRNA binding?

Referee #3

Mutations in LRPPRC, a mitochondrial pentatricopeptide repeat protein, cause a recessive mitochondrial respiratory chain disorder characterised by deficiency of cytochrome c oxidase (COX), but the exact function of LRPPRC that is perturbed in these patients is unclear. Using cellular systems, Shoubridge's group (Sasarman et al. 2010) recently showed that LRPPRC interacts with another mitochondrial protein, SLIRP, to regulate mitochondrial mRNA, but not tRNA or rRNA, levels; Avadhani's group (Sondheimer et al., 2010) made a similar observation. Notably, mammalian LRPPRC appears to behave somewhat differently than does Pet309, its counterpart in yeast mitochondria, which affects only (or mainly) the translation of Cox1 mRNAs.

In this manuscript, Ruzzenante et al. have not only expanded upon, but have made an important extension of the in vitro work, by analysing the consequences of the loss of LRPPRC in conditional-KO mice. They confirm much of what is already known, but also show that mt-mRNA polyadenylation is severely reduced, and that, for unknown reasons, transcript levels are fairly poor predictors of the corresponding polypeptide levels. I particularly liked the analysis of the relationship of LRPPRC and SLIRP to ribosome structure. The issue of what and how LRPPRC regulates steady-state mt-mRNA levels is still controversial (some of the data reported here disagrees with those of others), but my overall view is that the work, while covering much ground already reported in the literature, extends our understanding of this enigmatic protein in important ways. I note that LRPPRC has now also been implicated in apoptosis (Michaud et al, 2011) and autophagy (Xie et al., 2011). Do the authors' findings fit in or help explain these observations?

My main concerns are technical. I found the methods to be terribly skimpy. For example, there is no presentation of the BN-PAGE methods and probes, or of the in-organello transcription and translation protocols, the immunoprecipitation conditions, the sucrose gradients, the measurement of mito mass in EM's, the sources of the antibodies, and the like. Details of experiments may not be important to 95% of readers, but to the other 5% failure to provide critical experimental details is a real disservice.

Fig. 1: In the maps Fig. 1A, please show predicted band sizes for the Southerns. In the Western in Fig. 1D, the asterisk denotes a non-specific band. How was this determined? By size only? By competition (i.e. did added LRPPRC protein block the real band but not this band)?

Fig. 2: In Fig. 2B, it is not clear how mitochondrial mass was determined from the EM data. There is nothing in the Methods to explain this. In Fig. 2D, please indicate in the legend or in the Methods what antibodies were used for the BN-PAGE westerns. It looks as if the filter for complexes III and V were probed twice (after stripping?). It might be easier for the reader if you showed the separate panels for each complex, as was done with the other complexes. F1 in this panel implies that the entire F1 subcomplex of ATPase was present (subunits alpha, beta, gamma, etc). Did you do experiments to verify this? In Fig. 2E, it is remarkable how clean the silver-stained 2-D gels are, given that there are more than 1500 proteins in mitochondria, and the legend states that total heart mitochondria, not isolated respiratory complexes, was the starting material. Please explain why the gels aren't more "messy". Better yet, please provide something in the Methods on exactly how this experiment was performed.

Fig. 3: The Northerns in Figs. 3B and 3D show each transcript in a "strip" on the figure, but was there any evidence of changes in unprocessed precursor transcripts in test vs control mice (e.g. the well-known RNA19 transcript containing 16S-tRNALeu(UUR)-ND1)? If not, LRPPRC presumably operates after, not before, maturation of the transcripts.

Fig. 5: In Fig. 5D, LRPPR should be LRPPRC.

1st Revision - authors' response 08 September 2011

We thank you for providing us with expert reviewers and we are grateful for the very positive comments from all of them:
" this is an intriguing paper and should be published. The Group has an excellent international reputation and although several pieces of data are surprising and differ from other reports, I think the manuscript adds substantially to the debate about mitochondrial gene expression." (Referee #1)
" The results presented here on LRPPRC constitutive and conditional KO in muscle contain many novel and unexpected elements."...." The work is very well conducted, looking at LRPPRC function from different angles and many different types of approaches. The results are convincing and shed new light on the function of LRPPRC in mammalian mitochondria." (Referee #2)
"In this manuscript, Ruzzenante et al. have not only expanded upon, but have made an important extension of the in vitro work, by analysing the consequences of the loss of LRPPRC in conditional-KO mice." (Referee #3)

Our comments are as follows:

Referee #1:

The authors have extended this work by showing clearly that mRNA remaining in the LRPPRC KO cells are not polyadenylated, consistent with the stability of ND6, the only nonpolyadenylated transcript. Perhaps the most interesting and surprising aspect of this work is the variability of transcript translation. This is particularly odd as it disagrees with the work of Sasarmann on the LRPPRC patient cell lines, where mRNA is depleted but translation is down across the board in fibroblasts. Why is this so different in the tissue from the KO mice ?
Response:
We use knockout mice as this experimental system has the advantage that it provides insights into the physiological action of the studied gene in a differentiated tissue. Continuously dividing transformed cell lines or primary culture cells have certainly provided insights into the role of different genes, but the physiological setting is very artificial. Tissue culture mammalian cells are mainly glycolytic and are usually grown at much higher oxygen tensions than those present in real tissues. The dependency of oxidative phosphorylation in tissue culture cells is not absolute as demonstrated by the fact that it is even possible to derive proliferating mammalian cells lacking mtDNA. In accordance with these statements, we have published two previous papers showing that the role of TFB1M and MTERF3, which are both key regulators of mtDNA expression, could only be clarified after tissue-specific disruption of the corresponding genes in the mouse (Park et al., Cell 2007:130:273-285 and Metodiev et al., Cell Metabolism 2009:9:386-397). The previous studies of the molecular roles of these two proteins in mammalian cell lines had given really confusing results and in both cases the mouse knockouts provided novel and unexpected insights, see detailed discussion in these two papers.
Concerning the studies of LRPPRC in cell lines we would like to point out that an important in vivo regulatory feature seems to be absent in the RNAi knockdown cells as these have no induction of mitochondrial biogenesis. In addition, Leigh syndrome French-Canadian variant (LSFC) patients have a point mutation in LRPPRC that has been reported to decrease the stability of the LRPPRC protein, however, this is unlikely to result in complete lack of the protein. In fact, the findings in our paper show that complete loss of LRPPRC causes embryonic lethality in mammals.

Could this somehow be an artefact caused in part by isolation of mitos prior to 35S labelling ?

Response:
We have extensively used this approach and never observed anything similar in isolated wildtype mitochondria or in other types of mutant mitochondria, see e.g. Fig. 6C of Metodiev et al., Cell Metabolism 2009:9:386-397 and Fig. 4C of Camara et al., Cell Metabolism 2011:13:527-539. We therefore feel confident that the observed pattern of aberrant translation in mitochondria lacking LRPPRC is not an artifact but a real phenomenon.

P8.the authors state that a previous report by Sasarmann et al of knockdown of LRPPRC in cell lines causes a generalised resp chain deficiency, unlike their observation of a specific and profound complex IV loss (and partial V), suggesting this discrepancy may due to continuous proliferation of tc cells. However, the Sasarmann paper also shows that primary fibroblasts from patients demonstrate a very similar complex IV-specific defect. This is complicated, suggesting that partial depletion causes complex IV loss in man, whilst further depletion leads to global OXPHOS defects. This does not appear to be the case in mice.
Response:
As discussed above, LFSC patients likely have some remaining LRPPRC protein present. We feel our in vivo results showing a preferential defect of complex IV in knockout mice (Figure 2C) is actually reminiscent of the situation in LFSC patients, reported to have a complex IV deficiency. Our findings argue that the mouse model will be a much more useful future tool for dissection of disease pathophysiology than knockdown cell lines.

I'm slightly confused about the LRPPRC/SLIRP complex, which is around 250 kDa. The authors report evidence that mRNA is found in the complex, as has been reported previously. However, if we assume that the average Mr of a murine mitochondrial transcript is approx. 250kDa one wonders where the apparent RNA is hiding ? I assume this means that most of the bound RNA is degraded mRNA ?
Response:
We agree with the referee that the size exclusion chromatography procedure we use likely causes a partial degradation of mitochondrial RNA. It is not possible for us to work in an RNAse-free environment once the samples have been loaded onto the FPLC. We would like to point out three things. Firstly, our data from size exclusion chromatography provide strong evidence that the LRPPRC/SLIRP complex is RNA dependent as the complex is disrupted by procedures that degrade RNA or decreases RNA binding to protein (Fig. 5A). Secondly, size exclusion chromatography is a good method for establishing complex formation, but it is not a good method for determination of molecular weights as the migration of a protein is influenced by its shape (See e.g. Fig. 5 of Camara et al., Cell Metabolism 2011:13:527-539.) Thirdly, by using a completely independent approach (sucrose gradient sedimentation of ribosomes) we find that the LRPPRC/SLIRP complex comigrates with mRNA.

P12, Fig 6A, contrary to what is stated that complex V subunits are unaffected, there definitely looks to be a diminution of ATP8 levels in the KO. Further, as NDUFA9 is present at reasonable steady state levels, even in rho0 cells, the absence of any effect on this protein does not necessarily tell us much about fully functional complex I in the KO.
Response:
We agree with the referee that the levels of individual subunits do not necessarily tell us much about the functionality of the oxidative phosphorylation complexes. This is the reason why we have used a very stringent approach, including measurement of the activity of individual respiratory chain complexes (Fig. 2C), BN-PAGE electrophoresis of respiratory chain complexes (Figure 2D), two-dimensional gel electrophoresis of respiratory chain complexes (Fig. 2E), to access the function of the respiratory chain. All of these analyses show that complex IV is the most affected of the complexes.

With the 35S labelling in isolated mitos, it is of course difficult to be sure of the

identity of some translation products as some products could possibly be stalled intermediates. For example, the very intensely labelled products running just below ND2 in the controls. What are they ? If they are truly ND1 and ND2, why is there so much translation of these mRNAs ? This doesn't appear to be random translation as the authors suggest in the discussion. In fact, the steady-state levels of ND1 in the KO cells are not particularly high, yet if this is really one of the translation products that is selectively increased it would be interesting to know why the authors think this is the case. Why is this data so different to the paper of Sasarmann, where fibroblasts from patients with LRPPRC depletion show a generalised defect in mitochondrial protein synthesis.

Response:

As stated above, the difference from the Sasarman paper is likely due to the fact that we study the in vivo role of LRPPRC in a differentiated tissue. Just clarify, we do not argue that the translation is random, but rather that the normal coordination is lost. This means that some transcripts are translated more than others. The translation pattern in Figure 6 is clearly aberrant, supporting this conclusion. We admit that we cannot be sure of the identity of every individual band on this gel, but certainly the identity of the main bands we observe are non-controversial in the mitochondrial research community (Fernández-Silva et al., Methods Cell Biol. 2007:80:571-88). Another important finding is that we show that some of the newly translated products are unstable on a cold chase (Fig. S4A), further strengthening the conclusion that proper coordination of translation is lost in the absence of LRPPRC.

Why are there two peaks of mRNA that are not in the monosome (Fig 8)? This suggests that the free mRNA can associate both with the large and small subunit alone. What does this mean ? One assumes that there must be a well defined mechanism for generating a pre-initiation complex. In most systems, this normally includes the small ribosomal subunit and Professor Spremulli has published that at least in vitro mitochondrial mRNA preferentially binds to the small subunit. Does this mean that the monosome dissociates in some way on isolation ?

Response:

In the wild-type situation (Figure 8, L/L panel) we observe two main mRNA peaks: one bound to the LRPPRC/SLIRP complex and one (likely the translated portion) bound to the assembled ribosome. Our experiments were not designed to determine whether the mRNA bound to the assembled ribosome in wild-type mitochondria preferentially interacts with one of the two subunits, as reported by Spremulli. We like to clarify that the two mRNA peaks the reviewer discuss (i.e. association of mRNA with the free large and small subunit) are only present in the LRPPRC knockout mitochondria (Figure 8, L/L, cre panel). This finding further strengthens the conclusion that translation is uncoordinated in the absence of LRPPRC.

Referee #2:

Some important questions, beyond the scope of the present work, remain to be answered. For example, why the turnover of the COX mRNAs is so different (faster) than other genes? How does this correlate with the turnover of the assembled proteins in comparison with complex I and III?

I have a few points, mostly for discussion.

1) The authors review the genes involved in mtDNA transcription in the introduction and discussion, but the role of LRPPRC appears more related to mRNA maintenance and translation. On the contrary they do not introduce the role of SLIRP, which is a binding partner of LRPPRC. The introduction and discussion can focus more of the protein complex at hand.

Response:

We agree with the reviewer that our results open up novel interesting avenues to research the interesting mechanism that seems to specifically regulate COX mRNA stability. We have carefully revised the introduction and followed the suggestion of the referee.

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2) A decline of complex II function in the LRPPRC KO is shown in figure 2 but not described or discussed. Complex II is not mtDNA encoded; what could be reason of this finding.
Response:
We have previously observed a similar reduction of complex II activity in mouse knockout models for other proteins involved in regulating mtDNA expression, see e.g. Fig. 2C in Park et al., Cell 2007:130:273-285 or Fig.2F Camara et al., Cell Metab. 2011:13:527-539. We do not know the reason for this reduction, but, as pointed out by the reviewer, it must be a secondary phenomenon. Speculative mechanisms include superoxide-induced damage of FeS clusters of complex II or impaired synthesis of FeS clusters due to the bioenergetic deficiency. We feel this finding is of peripheral interest for the current study, but could provide an interesting future project for anyone interested in the biogenesis and stability of complex II.

3) What could be the reason of the different interpretation of the role of mRNA in allowing the binding of LRPPRC and SLIRP between this study and the previous one by the Shoubridge group?
Response:
Please see response to first point raised by reviewer 1.

4) What could be the nature of the high molecular weight complex containing LRPPRC in the absence of mRNA binding?
Response:
The high molecular weight complex is only seen in size exclusion chromatography if the mRNA interaction is destroyed. It likely represents an aggregation form of LRPPRC due to instability of the LRPPRC/SLIRP complex in the absence of RNA.

Referee #3:

I note that LRPPRC has now also been implicated in apoptosis (Michaud et al, 2011) and autophagy (Xie et al., 2011). Do the authors' s findings fit in or help explain these observations?
Response:
There is convincing evidence that dysfunctional oxidative phosphorylation makes tissue-culture cells and real tissues more prone to apoptosis (Wang et al., PNAS 2001: 98:4038-43, Kujoth et al., Science: 2005 309:481-4). We therefore feel this is a secondary, downstream phenomenon. Autophagy can also be a secondary response as several papers by Youle and coworkers argue that dysfunctional mitochondria induce an autophagy response.

My main concerns are technical. I found the methods to be terribly skimpy. For example, there is no presentation of the BN-PAGE methods and probes, or of the inorganello transcription and translation protocols, the immunoprecipitation conditions, the sucrose gradients, the measurement of mito mass in EM's, the sources of the antibodies, and the like. Details of experiments may not be important to 95% of readers, but to the other 5% failure to provide critical experimental details is a real disservice.
Response:
For space limitation reasons we provided most of the Materials and Methods as supplementary Information. We have now carefully gone through this supplementary file and feel that the methods are described in sufficient detail to allow reproducing our experiments.

Fig. 1: In the maps Fig. 1A, please show predicted band sizes for the Southerns.
Response:
We have revised Fig. 1A so that the predicted sizes of DNA fragments now are indicated.

In the Western in Fig. 1D, the asterisk denotes a non-specific band. How was this

determined? By size only? By competition (i.e. did added LRPPRC protein block the real band but not this band)?
Response:
The identity of LRPPRC on western blots was confirmed by the mouse knockout. The additional neighboring band, which is clearly not LRPPRC, increases with time in LRPPRC heart knockout mitochondria. This cross-reacting band likely represents a mitochondrial protein that is induced as part of the strong mitochondrial biogenesis response in LRPPRC knockout mice.

Fig. 2: In Fig. 2B, it is not clear how mitochondrial mass was determined from the EM data. There is nothing in the Methods to explain this.
Response:
These methods are now included in the supplementary information.

In Fig. 2D, please indicate in the legend or in the Methods what antibodies were used for the BN-PAGE westerns.
Response:
We have revised the figure legend and named the antibodies we used to detect respiratory chain complexes.

It looks as if the filter for complexes III and V were probed twice (after stripping?). It might be easier for the reader if you showed the separate panels for each complex, as was done with the other complexes.
Response:
In the experiment shown in the manuscript we probed first with an antibody to detect complex III and thereafter, without stripping, we probed the same membrane with an antibody against the alpha-subunit of complex V. We have not included the first panel in the manuscript as it would consume space without providing any novel information.

F1 in this panel implies that the entire F1 subcomplex of ATPase was present (subunits alpha, beta, gamma, etc). Did you do experiments to verify this?
Response:
We detected complex V and the subcomplex by using an antibody against ATP5A1 (alpha-subunit) of the F1 portion of complex I. It is well established that ATP synthase can be almost fully assembled in the absence of mtDNA expression and it is even present in rho0 cells (Wittig et al, BBA 2010:1797:1004-11). The subcomplex we observe has the predicted size of the free F1 subunit and contains one of its main protein components. We therefore feel it is reasonable to argue that we indeed observe the F1 subcomplex on BN-PAGE analysis as depicted Fig. 2D. The exact protein composition of this observed F1 subcomplex is unknown, but this circumstance does not in any way affect the conclusions of the paper.

In Fig. 2E, it is remarkable how clean the silver-stained 2-D gels are, given that there are more than 1500 proteins in mitochondria, and the legend states that total heart mitochondria, not isolated respiratory complexes, was the starting material. Please explain why the gels aren't more "messy". Better yet, please provide something in the Methods on exactly how this experiment was performed.
Response:
The methods are presented in the supplementary information section. We would like to clarify that the first dimension of the depicted gel is a BN-PAGE electrophoresis. This initial separation is performed under non-denaturing conditions, which preserve the integrity of respiratory chain complexes. In mitochondria the respiratory chain complexes are abundant and furthermore constitutes the majority of the proteins retained on BN-PAGE gels (small and non-complexed proteins simply leave the gel). The BN-PAGE procedure thus leads to an enrichment of respiratory chain complexes and this explains why relative few other proteins are seen on the 2D gel.

Fig. 3: The Northerns in Figs. 3B and 3D show each transcript in a "strip" on the figure, but was there any evidence of changes in unprocessed precursor transcripts

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in test vs control mice (e.g. the well-known RNA19 transcript containing 16StRNALeu(UUR)-ND1)? If not, LRPPRC presumably operates after, not before, maturation of the transcripts.
Response:
We found no evidence of aberrant processing and we provide a depiction of a gel below to illustrate this fact. The loading is bit uneven, but it is quite clear that there is no evidence for any partly processed transcripts besides the RNA19. It is important to point out that although RNA19 levels are increased in LRPPRC knockout mitochondria the absolute levels of RNA19 are low in comparison with other transcripts. When exposing the blot longer the RNA19 is more clearly visible, but the ND1 is then too prominent. This explains why we chose to present RNA19 in a separate panel.

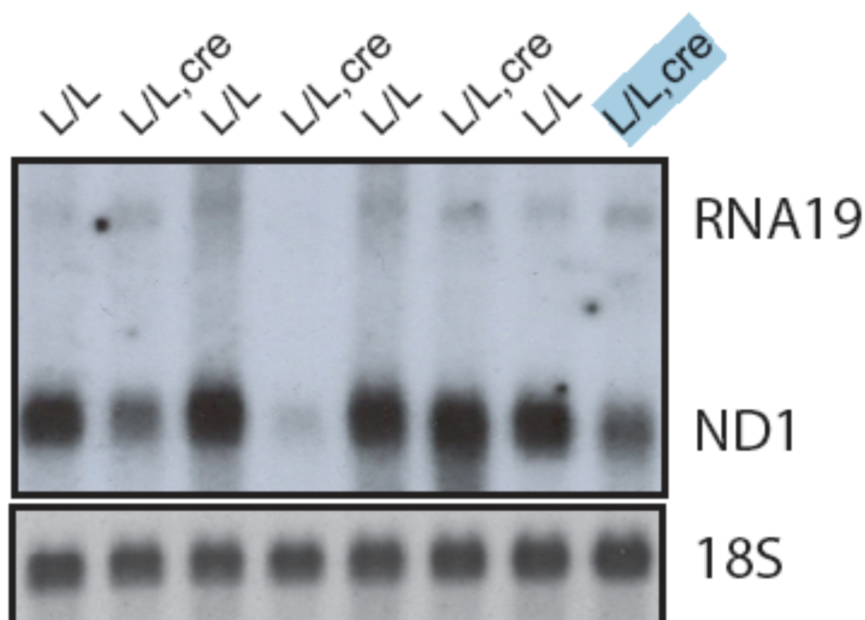

Fig. 5: In Fig. 5D, LRPPR should be LRPPRC.
Response:
We thank the referee for the careful reading of the pictures and we have corrected this mistake.

2nd Editorial Decision — 16 September 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have evaluated your response in detail and we will be happy to publish your manuscript pending some further textural revision, essentially to incorporate all substantive referee issues into the actual manuscript. These issues will also occur to many of our readers and - irrespective of the fact that these response will be posted as a review process file anyway - it is therefore valuable to discuss these matters in the paper.

In particular,
1) referee, response 1 and referee 2: the discussion of the discrepancies with previous work (especially Sasarmann et al.) has to be substantially taken up in the manuscript (in slightly tighter form.
2) re2, response 2: add sentence on p14 of the manuscript stating this has not been observed in previous work.
3) ref 1 response 4: 250kD migration of LRPPRC/LIRP complex: this should be added in slightly abbreviated form on p.11 of the manuscript.
4) ref 1 response 5: again, the complexV response should be summarized in the main paper.
5) ref 1 response 6: a) 'random': you do use the word 'chaotic' prominently (for example, p 13, p20,

fig 6). In our view, this should be 'de-/mis-regulated' or 'uncoordinated'. b) the identity of translation product in the 35S-labelling experiment: the evidence for the identification of each labelled band needs to be added. Citation of a another paper is not sufficient (Fernandez-Silva eta al.).
6) ref 1 response 7:two monosome peaks: the penultimate sentence of this response should be in the manuscript.
ref 2, point2: complex II; the last sentence of the response ought to be be included.
ref 2, pt. 4: please mention hypothetical aggregation.
ref 3, response 1: please add a short statement about apoptosis and autophagy in condensed form.
ref 3, response 7: please include short note on reprobing.
ref 3, response 8, F1 complex: please add in condensed form.
ref 3, point 10: Please discuss this point. We strongly recommend to present all key data as unprocessed edited 'Source data' files within the paper (uncropped blots should include molecular weight markers and basic labelling to be able to match the blots with edited figure panels. Alternatively, this panel should be integrated into the supplementary information.

For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

As a matter of policy, competing manuscripts published during the revision period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

2nd Revision - authors' response 19 September 2011

Our comments are as follows:

In particular,
1) referee, response 1 and referee 2: the discussion of the discrepancies with previous work (especially Sasarmann et al.) has to be substantially taken up in the manuscript (in slightly tighter form.
Response:
We now include a discussion about the discrepancies from previous studies of cell lines, see Discussion p. 18.

2) re2, response 2: add sentence on p14 of the manuscript stating this has not been observed in previous work.
Response:
This has been added to the Results p.14

3) ref 1 response 4: 250kD migration of LRPPRC/LIRP complex: this should be added in slightly abbreviated form on p.11 of the manuscript.
Response:
We have followed your advice and added this point to the Results p. 12

4) ref 1 response 5: again, the complexV response should be summarized in the main paper.
Response:
We have added this to the Results p. 9.

5) ref 1 response 6: a) 'random': you do use the word 'chaotic' prominently (for example, p 13, p20, fig 6). In our view, this should be 'de-/mis-regulated' or 'uncoordinated'.
Response:
We have substituted the word chaotic with uncoordinated.

b) the identity of translation product in the 35S-labelling experiment: the evidence for the identification of each labelled band needs to be added. Citation of a another paper is not sufficient (Fernandez-Silva eta al.).
Response:
Mammalian mitochondrial translation products were first identified in the laboratory of the late Giuseppe Attardi at CalTech. Mammalian mitochondria only encode 13 proteins and the low complexity of the system allowed Attardi to identify the polypeptides by comparing the migration of radiolabelled translation products with western blots, see e.g. Matuno-Yagi et al., Nature 1985:314:592-597 and subsequent papers. The in organello translation system pioneered by Attardi is a widely used standard tool in mammalian mitochondrial genetics. It is well established that the in organello translation reaction monitors the synthesis of the 13 mtDNA-encoded proteins, whereas the synthesis of nucleus-encoded proteins are not detected, because all of the translation products disappear if mitochondrial translation is inhibited by drugs or genetic mutations (see e.g. Fig. 4C of Camara et al., Cell Metab. 2011). More than two decades of work has thus lead to the identification of the 13 different mitochondrial translation products, as summarized in the nice review by Fernandez-Silva et al. We have never in the past had any problems to publish in vitro translation gels based on this accepted interpretation of the migration of translation products, see e.g. Fig. 6C of Metodiev et al., Cell Metab. 2009 or Fig. 4C of Camara et al., Cell Metab. 2011. In essence, Sasarman et al and many other publications also use the same system. We have nevertheless added information to the Supplementary Material and Methods section clarifying that we reconfirmed the identities of three of the translation products with western blots (pag. 14). The other experiments in our paper are in fact based on wealth of established methods, molecular biology, bioenergetics, mouse knockout creation, etc., and we feel it would be an unreasonable burden to totally reconfirm the interpretation of in vitro translation patterns based on 25 years of experimentation. Additional experiments along these lines would surely not change the conclusions of our paper.

6) ref 1 response 7:two monosome peaks: the penultimate sentence of this response should be in the manuscript.
Response:
We have added this point to the Results p. 17

ref 2, point2: complex II; the last sentence of the response ought to be be included.
Response:
We have included this point in the Results p. 9.

ref 2, pt. 4: please mention hypothetical aggregation.
Response:
We added this to Results, p. 12.

ref 3, response 1: please add a short statement about apoptosis and autophagy in condensed form.
Response:
We revised the Introduction and we have added comment on autophagy and apoptosis on p. 6.

ref 3, response 7: please include short note on reprobing.
Response:
We clarified this point in the legend to Fig. 2

ref 3, response 8, F1 complex: please add in condensed form.
Response:
We mention this point in the Results section p. 9

ref 3, point 10: Please discuss this point. We strongly recommend to present all key data as unprocessed edited 'Source data' files within the paper (uncropped blots should include molecular weight markers and basic labelling to be able to match the blots with edited figure panels. Alternatively, this panel should be integrated into the supplementary information.

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Response:
We have added the RNA19 result as a supplementary figure (Figure S3, p. 5-6).

3rd Editorial Decision 23 September 2011

Thank you for resubmitting your manuscript. We are pleased to accept this manuscript for publication after a couple of minor editorial revisions:

1) please add references to your revised text on p. 18 and 14, addressing points 1 and 2 of the previous decision letter.
2) in your response to point 5 of the last decision, we would encourage you to show the Western identifying three translation products as an supplementary figure panel. Since SI is available these days, we discourage 'data not shown'. The legend to S 5 refers mistakenly to SI5D instead of B and there appears to be a discrepancy between the panel and the legend (MRPL13 vs. MRPL15). Finally, the blot and Coomassie gels in S 5A has been spliced between lanes 2 and 3: this has to be marked with a clear line and the legend has to state if this blot derived from a contiguous gel. Any other hypothetical splice sites in the paper have to be similarly marked.
3) The lanes of the Northern blots in figures 3B, 3D, 4A, S1D & S3 are not marked in sufficient detail to understand the nature of the repeated lane pairs. Please clarify in the panels or the respective figure legends.
4) please add a scale bar in 1C & 2A.
5) We would like to briefly reconsider the term 'uncoordinated' translation (replacing the previous 'chaotic'. The choice of words will be your, but please consider that there is a big difference between 'misregulated' and 'uncoordinated'. The former implies that certain specific translation products are induced and other are reduced - this seems to be the case. The term 'uncoordinated' implies there is no control i.e. translation products are induced or reduced in an uncoordinated, random manner.
6) To increase the accessability of the abstract, please add a very short description of LRPPRC's function.

Also, we will need a page charges form:
PAGE CHARGE AUTHORISATION AND OFFPRINT FORM
http://mts-emboj.nature.com/letters/page_charge_form.pdf

I look forward to to the final version of the manuscript, which will be forwarded immediately to our production department.

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<http://mts-emboj.nature.com/cgi-bin/main.plex?el=A5z5MHm1C3Wjx1I4A9McKNrPqcGVO4LvVv3OAZ>

best wishes,

Editor
The EMBO Journal

3rd Revision - authors' response 25 September 2011

Our comments are as follows:

In particular,
1) please add references to your revised text on p. 18 and 14, addressing points 1 and 2 of the previous decision letter.

Response:
The references of previous studies have been added on p. 14 and 18

2) in your response to point 5 of the last decision, we would encourage you to show the Western identifying three translation products as an supplementary figure panel.
Since SI is available these days, we discourage 'data not shown'.
Response:
We have added a supplementary figure depicting these westerns (Figure S6)

The legend to S 5 refers mistakenly to SI5D instead of B and there appears to be a discrepancy between the panel and the legend (MRPL13 vs. MRPL15).
Response:
We have now corrected these mistakes.

Finally, the blot and Coomassie gels in S 5A has been spliced between lanes 2 and 3: this has to be marked with a clear line and the legend has to state if this blot derived from a contiguous gel. Any other hypothetical splice sites in the paper have to be similarly marked.
Response:
All of the lanes of the autoradiograph and the Coomassie gel are derived from the same gel. We now clearly separate lane 1-2 and lane 3-4 in the Fig. S5A and state in the legend that all lanes are form a contiguous gel.

3) The lanes of the Northern blots in figures 3B, 3D, 4A, S1D & S3 are not marked in sufficient detail to understand the nature of the repeated lane pairs. Please clarify in the panels or the respective figure legends.
Response:
We have carefully checked all of the figure legends highlighted above and they all now explain that knockout mice are indicated with (L/L,cre) and controls with (L/L).

4) please add a scale bar in 1C & 2A.
Response:
The scale bars have been added to the figure panels and are explained in the figure legends of the indicated figures.

5) We would like to briefly reconsider the term 'uncoordinated' translation (replacing the previous 'chaotic'. The choice of words will be your, but please consider that there is a big difference between 'misregulated' and 'uncoordinated'. The former implies that certain specific translation products are induced and other are reduced - this seems to be the case. The term 'uncoordinated' implies there is no control i.e. translation products are induced or reduced in an uncoordinated, random manner.
Response:
We agree and now use “misregulated” throughout.

6) To increase the accessability of the abstract, please add a very short description of LRPPRC's function.
Response:
We have revised the abstract according to this suggestion.