Nonsense mutations in the COX1 subunit impair the stability of respiratory chain complexes rather than their assembly

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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 30 September 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. After some delay due to the unusually high number of manuscripts we have received over the last few weeks, I have now had the opportunity to read it carefully and to discuss it within our editorial team. I am afraid that the outcome of these discussions is not a positive one.

We appreciate your careful and comprehensive analysis of the effect of a disease-relevant mutation within the mitochondrial COX1 gene, which results in a truncated COX1 protein, on respiratory chain complex and supercomplex assembly and integrity. You were able to provide evidence that this truncated version of COX1 does not lead to an assembly defect in complex IV of the respiratory chain, but rather to the formation of fully assembled, but intrinsically destabilised complex IV as well as destabilised respiratory chain supercomplexes that are degraded rapidly. However, in principle the general concept that the stability of the different respiratory chain (sub)complexes is interdependent and that mutations that affect the assembly/stability of one complex affects the stability of other complexes has been put forward before based on a number of different examples. We therefore think that the main novelty of the study is the clear discrimination between impaired complex assembly and the formation of unstable complexes as a result of the COI A7339G mutation. Clearly, we can see that this finding is certainly of interest and has disease-relevance. Still, this study is based on one specific mutation and we are concerned that the study remains at the level of an interesting phenomenon. Taking together all these considerations and if one looks at the study
from a broader and more general perspective we do not think that the extent of novel biological and mechanistic insight provided by this study reaches the level required for publication in The EMBO Journal. We have therefore decided not to send out the paper for in-depth peer review at this point.

Please note that we publish only a small percentage of the many manuscripts that we receive at The EMBO Journal, and that we can only subject those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. As in our carefully considered opinion, this is not the case for the present submission I am afraid to say that our conclusion regarding its publication here cannot be a positive one.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that we cannot be more positive on this occasion.

Yours sincerely,

Editor
The EMBO Journal

Rebuttal

04 October 2010

Thank you for considering our manuscript. However, for several reasons explained below we cannot share your conclusion that our manuscript does not provide novel and mechanistic insights and that it remains at the level of an interesting phenomenon. We therefore sincerely ask you to reconsider your decision and further consult expert opinions on this by sending out our manuscript for peer review.

The interdependency of respiratory chain complexes indeed has been described. The major reference for this phenomenon is Acin-Perez et al. (Mol. Cell 13: 805-815, 2004) who nicely showed that the absence of complex III leads to decrease of complex I. The generally accepted explanation for this phenomenon was and still is: "The natural form of complex I in the cell could be forming superstructures among complexes and if this is not achieved, because complex III is absent, complex I is directed to degradation" (Acin-Perez et al., 2004).

In our manuscript we show that in our cells this is NOT the case and that a different mechanism accounts for the interdependence of respiratory chain complexes. We demonstrate that the supercomplex assembly process is not affected at all, but instead, respiration deficient supercomplexes are degraded more rapidly and, interestingly, the first step in this degradation process is the disconnection of the electron influx module of complex I. Moreover, we show that decreased pool sizes of complex IV do not affect CI-CIII2-CIV supercomplex formation as long as heteroplasmy thresholds have not been surpassed, strongly suggesting that the cell uses the "reserve" pool of complex IV to maintain these large supercomplexes. These findings provide important novel mechanistic insights because they show that quality control does not occur at the level of individual subunits or complexes, but rather at the level of supercomplexes. The cell thus seems to be able to produce supercomplexes, but degrades them rapidly if it senses that their respiratory competence low. This also provides a much better explanation why complex IV, which is not interacting directly with complex I in the supercomplex, can lead to complex I deficiency (one would think that the association of complex III with complex I would be sufficient to stabilize this complex!).

Although we show this mechanism in two cell lines carrying two different mutations (not one as you claim, see Fig. 4 and Fig. S6, and more data would be available on the second cell line, if requested), this is a general mechanism, since similar observations, however lacking detailed mechanistic explanations are made in other systems (COX 10 k.o. mouse fibroblasts, mice and also patients with SCO2 mutations, but also our COX10 patient sample shown in Fig. S6).

We very much hope that we could convince you that our manuscript should be sent out for review and are happy to answer any further questions.
Thank you for your message(s) asking us to reconsider our decision on your manuscript.

We recognise that here you were able to provide evidence for the first time that mutations within the COX1 gene do not result in a defect in the assembly of complex IV, nor of supercomplex formation as one could imagine and expect, but rather in fully assembled, but intrinsically unstable respiratory chain complexes and supercomplexes that are degraded rapidly. Furthermore, I would like to apologise again for the oversight of the data with the second mutant. However, I am sorry to say that the editors are still concerned that at this stage the study remains too much at the level of an interesting phenomenon and that the quality control mechanism that leads to the recognition and degradation of defective/less active respiratory (super)complexes essentially remains unclear. This is particularly important as the general concept that the different respiratory (sub)complexes are interdependent has been put forward before. All in all we therefore still think that the depth of novel insight is not sufficient to meet our rather stringent conceptual requirements - at least at this stage of analysis.

I am sorry that I cannot transmit more positive news to you at this point.

Yours sincerely,

Editor
The EMBO Journal

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The EMBO Journal  Peer Review Process File - EMBO-2011-79568

Additional correspondence (editor)  12 October 2010

Resubmission  09 February 2011

2nd Editorial Decision  14 March 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while referee 1 is considerably more positive the other two referees do not think that the study is sufficiently developed at this point to justify publication here. I will not repeat all their individual points of criticism, but essentially all three referees think that the role and effect of AGF3L2 in this quality control process would need to be analysed further. Furthermore, referee 2 raises concerns about the conclusiveness of the data also at this stage of analysis. Taking together all issues raised, it becomes clear that an extensive amount of further experimentation with uncertain outcome will be required before there is sufficient support for publication. I am afraid to say that at this point, the paper is therefore not publishable here.

Still, given the interest expressed by the majority of referees in principle, we would not exclude the possibility to look at a new submission on the same topic at some time in the future should you be able to develop the study further along the lines suggested by the referees. I should add and specify that while a deeper analysis of the role and effect of AGF3L2 will be required as detailed in the specific suggestions by the referees, including referee 3, broader issues regarding a potential misfolded protein response mechanism (as mentioned by referee 3) would certainly go beyond the scope of this study and will not be required. To be completely clear, however, I would like to stress that if you wish to send a new manuscript this will be treated as a new submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh involving our original referees again, also with respect to the literature and the novelty of your findings at the time of resubmission.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments
helpful.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1

This manuscript investigates the assembly of respiratory complexes in cell lines that contain a mutation in CoxI. The manuscript is of particular interest because it investigates the specific pathomechanism for a decrease in respiratory function caused by mutations in CoxI and reports a role for the AAA protease AFG3L2 in turnover of CoxI. The experiments are of high technical quality and investigate the complex process of assembly/stability of the respiratory complex. AFG3L2 is shown to specifically bind CoxI and the Cox1 protein is stabilized when a dominant AFG3L2 is expressed.

Have the authors tested the extent with which this restores respiration? This would ultimately confirm that the complexes are functional and it is a competition between assembly and turnover.

Minor comments to address:

1. An explanation of how the mutation load is varied in the beginning of the results section would be useful to the general reader.
2. Commenting on the difference in the gel systems in Fig. 3 vs Fig. 4. In resolution of the respiratory complexes would be useful. In Fig. 3, supercomplex 1 and 2 can be resolved, but supercomplex III can be detected in Fig. 4. Also consistent labeling would be helpful (Fig. 3 it is referred to as SC1 and Fig. 4 as C1 +CIII2)
3. The mutation in the G6930 cybrid should be explained in the text.
4. The materials and methods should include details on how the mitochondrial DNA load is varied and additional antibodies such as LonP1, YME1L1 should be included.

Referee #2

Here, Hornig-Do et al. investigate the stability of respiratory chain supercomplexes in the presence of mitochondrial DNA-mutations in the COX1 gene. The A7339G mutation leads to a C-terminally truncated COX1, the central subunit of complex IV. The authors convincingly show that different mitochondrial mutation loads of generated cybrid cell lines lead to different degrees of respiratory defect. While cybrid cell lines seemed to tolerate up to 85% heteroplasmia in the CO1 gene, respiratory competence of cells dramatically dropped at higher levels, concomitantly leading to absence of complex IV. Interestingly, at 100% mutation load, other respiratory chain complexes that form higher oligomers with complex IV are also affected, as previously reported for other complex IV assembly defects. Based on their findings they suggest that truncated COX1 is incorporated into supercomplexes and causes breakdown of the assembled complexes. In elegant experiments they investigate the involvement of the AAA protease AFG3L2 of the mitochondrial quality control system in the degradation of the COX1 protein and suggest that overexpression of non-functional mutant AFG3L2 decreases the viability of cells expressing the truncated COX1 protein.

Most of the experiments are of sound quality, however, I have major concerns that the conclusions drawn are somewhat premature with regard to the presented data.

Major points:

(1) The data from which the authors conclude that the truncated COX1 protein is incorporated into
mature respiratory chain supercomplexes is not convincing. This conclusion is solely drawn on the basis of co-migration on native gels, which does not provide sufficient evidence for an interaction. Experimental evidence for physical association is required. In the worst case the authors may look at COX1 in an AAA-protease complex or an unknown complex. The same is true for the incorporation into "subassemblies", e.g. S2. Direct evidence for an association is required. Can the authors explain their observation that truncated COX1 should be present in S2, but not S1 or S3?

(2) Along the same lines, if the authors provide more convincing data that the complexes that contain truncated COX1 are part of respiratory chain complexes, it is still not evident that the COX1 protein is integrated into a completely assembled and functional complex IV (this is shortly discussed by the authors). Since this is not trivial to show, it would be necessary to consider a dynamic association of truncated COX1 to supercomplexes or to partially assembled complexes more seriously. This should be differentiated from an association into mature complex IV in both results and discussion.

(3) The title of the manuscript is misleading and should be changed. On the one hand only two mutations are analyzed. On the other hand, a number of previous studies demonstrate that COX1 can be degraded from assembly intermediates. Moreover, the assembly process of respiratory chain supercomplexes is hardly investigated in the presented data.

(4) In Figure 2A 100% cybrid cells show a strong reduction in synthesis of almost all mitochondrially-encoded gene products, which could explain the observed reduction in other complexes. The authors need to make sure that the observed defects on the complex level are not due to reduced synthesis, e.g. due to mtDNA depletion, in the first place.

(5) The assay used to analyze oxphos complex assembly generates an artificial situation by preloading mitochondria with nuclearly-encoded subunits and depleting mitochondrially-encoded ones. This is valid for analyzing mutant cells compared to controls, but conclusions drawn from such an approach have to be taken with care, and should be phrased accordingly. In the course of the experiments the overall signal intensities drop (most likely due to cell divisions). These changes in intensities need to be included in the interpretation of the data.

(6) The observation that complex IV levels in SC3 seemed less decreased compared to SC1 are not obvious. A partial dissociation of some CIV from CI+CII2+CIV1-4 could lead to CI+CII2+CIV1-3 and CI+CII2. Moreover, if monomeric CIV served as a pool to maintain supercomplexes, wouldn't it be depleted first?

(7) The conclusions about the potential assembly intermediate CI+CII2* are based mainly on subunit ND5. Again, co-migration is a week argument, and there should be at least a convincing number of subunits running at the same size. Unfortunately, the 2D gels from the control cells are not of sufficient quality to draw many conclusions about CI+CII2*, as it is not enlarged and indicated as for Figure 5B. Perhaps a different gel system could be used to get a better separation in the region of interest. The same applies to Figure 6.

(8) Multiple experiments corresponding to the one shown in Figure 7E should be quantified to strengthen the point that the degradation of truncated COX1 and COX2/3 is significantly slower when mutant AFG3L2 is transfected.

(9) The experiments performed in this study do not give any insight into folding states of proteins, the conclusion that AFG3L2 can interact with unassembled proteins should include that these unassembled proteins could also be misfolded. The finding that mutant AFG3L2 interacts with newly synthesized COX1 forms is interpreted as a function of AFG3L2 in COX1 degradation, which is only one possibility, since it could simply assist in the folding process.

(10) The authors ignored a large number of important findings that have been made in yeast on the issue. There are several publications about the involvement of the mitochondrial quality control in the degradation of misassembled COX1 by the Winge group. COX1 stability during assembly has been addressed by several labs. Most importantly, a truncated COX1 lacking the C-terminus has been found to be stabilized by the group of Perez-Martinez. This finding contradicts the data presented here and should be discussed.
Minor points

- Does the COX1 antibody used in this study recognize the truncated COX1 forms? If not, some of the western blot results are rather trivial.
- The observed increase in Cytc at steady state in the mutant cells is very interesting, especially in the light of the work by Bestwick et al. 2010 who identify Cytc as suppressor of certain assembly defects in yeast. This finding could be discussed.
- The statement that 100% mutant cells show no assembly intermediates needs further evidence. The antibody does recognize bigger complex species that are labeled as "unspecific bands", which needs further data showing that they do not represent assembly intermediates. If the authors refer to reported assembly intermediates that are smaller than mature complex IV, these are also not recognized by the antibody in the wild type.
- Monomeric complex I cannot be seen in figure 4A, text or figure should be changed accordingly.
- In figure S5 the labels are too far away from the actual protein bands and seem to be misplaced. This should be corrected. Labels and panels should be appropriately aligned in figure S7.
- The COX1 signal from G6930A cells in figure 7A could be masked by ND2.
- The interpretation that the NADH dehydrogenase module may dissociate from a CI+CHI2 complex in the absence of CIV is lacking the possibility that it might have never been fully assembled.

Referee #3

The paper by Horning-do et al entitled "The stability, not the assembly of respiratory chain supercomplexes, is impaired in COXI mutants" studies nonsense mutations in COXI cybrid cells derived from patients suffering from mitochondrial diseases.

They find that activity and stability of respiratory chain complexes, not limited to Complex IV, but including complexes I, II, and III, are affected by the loss of COXI. They compare these results across two nonsense mutations in COXI, in one of which respiratory chain assembly defects had already been studied and published by other groups. They also show that inhibition of the AFGL2 protease stabilizes both wild type and mutant COXI protein, suggesting that this protease is involved in COX I turnover.

The study is overall well done. The experiments are of high quality and support the conclusions, especially in regards to the interdependence of the various complex stabilization mechanisms. However, the fact that the function of more than one respiratory complex is affected when one complex is disrupted, and the interdependence of the respiratory complex and supercomplex stability is not a novel concept. The reason why the two mutations have different levels of residual truncated COXI is not explained. The main potential novelty of this work is the role of the AFGL2 protease in COXI degradation. However this has not yet been sufficiently explored in this study, and will require much more extensive characterization and definition of its function.

Inhibition of AFGL2 promotes stabilization of both wild type and mutant COXI. Does it also increase the levels of assembled COX and its function? Does it stabilize the other complexes as well? If COX stability is what is impaired in these COXI mutants, as mentioned by the title of the manuscripts, inhibition of AFGL2 that leads to stabilization of COXI, should also lead to stabilization of the other respiratory complexes. Then why do mutant cells die more than wild type ones when AFGL2 is inhibited? It would be important to determine if there is accumulation of a truncated, dysfunctional, COXI subunit in both stop-mutant cell lines and if this accumulation is responsible for toxicity. It would be novel to identify and demonstrate the equivalent of a misfolded-protein response in the mitochondrial inner membrane.

Minor points:

- Page 8 of the manuscript; it should say see Fig S4 and no Fig S5
- In figure 1 that comprise the biochemical characterization of the COXI mutant cybrids, activity of all complexes but Complex I are shown. Since, based on the Western Blots of Fig 2, Complex I
seems to be more affected than Complex II or Complex III, it would be nice to see Complex I activity and its threshold.
- The paragraph entitled "The new SC2*...." should be part of the previous paragraph and the results moved to Supplementary

Referee #1

(1) Have the authors tested the extent with which this restores respiration? This would ultimately confirm that the complexes are functional and it is a competition between assembly and turnover.

Respiration could be restored in 90% mutant cells, but not in 100% mutant cells (Fig. 7B and C).

Minor comments to address:
1. An explanation of how the mutation load is varied in the beginning of the results section would be useful to the general reader.

We have added this information to supplementary material (Fig. S1).

2. Commenting on the difference in the gel systems in Fig. 3 (now Fig. 5) vs. Fig. 4 (now Fig. 3) in resolution of the respiratory complexes would be useful. In Fig. 3 (now Fig. 5), supercomplex 1 and 2 can be resolved, but supercomplex III can be detected in Fig. 4 (now Fig. 3). Also consistent labeling would be helpful (Fig. 3 it is referred to as SC1 and Fig. 4 as C1 +CIII2)

The difference in the gel systems is explained now more in detailed in Material and Methods p.23. We also have consistently labelled the supercomplexes in both figures as suggested.

3. The mutation in the G6930 cybrid should be explained in the text.

The G6930A mutation is now better explained (see p.7).

4. Additional antibodies such as LonP1, YME1L1 should be included.

Antibodies against LonP1 and YME1L1 have now been used and information is now included in materials and methods (p. 23).
Referee #2

Major points:

1. The data from which the authors conclude that the truncated COX1 protein is incorporated into mature respiratory chain supercomplexes is not convincing. This conclusion is solely drawn on the basis of co-migration on native gels, which does not provide sufficient evidence for an interaction. Experimental evidence for physical association is required. In the worst case the authors may look at COX1 in an AAA-protease complex or an unknown complex. The same is true for the incorporation into "subassemblies", e.g. S2. Direct evidence for an association is required.

2. The authors explain their observation that truncated COX1 should be present in S2, but not S1 or S3?

3. The title of the manuscript is misleading and should be changed. On the one hand only two mutations are analyzed. On the other hand, a number of previous studies demonstrate that COX1 can be degraded from assembly intermediates. Moreover, the assembly process of respiratory chain supercomplexes is hardly investigated in the presented data.

We have changed the title into “Not the assembly, the stability of respiratory chain complexes is impaired by nonsense mutations in the COX1 subunit” and hope that this is more appropriate.
(4) In Figure 2A (now Fig. 4A) 100% cybrid cells show a strong reduction in synthesis of almost all mitochondrially-encoded gene products, which could explain the observed reduction in other complexes. The authors need to make sure that the observed defects on the complex level are not due to reduced synthesis, e.g. due to mtDNA depletion, in the first place.

*We apologise for the bad image, which also did not contain any loading control. Actually, we have not seen any significant differences in the synthesis of mitochondrially encoded proteins. Furthermore, we also did not observe any differences in synthesis rate (Fig. S5B). We have corrected this and now show another image with beta actin as a loading control (Fig. 4A).*

(5) The assay used to analyze oxphos complex assembly generates an artificial situation by preloading mitochondria with nuclearly-encoded subunits and depleting mitochondrially-encoded ones. This is valid for analyzing mutant cells compared to controls, but conclusions drawn from such an approach have to be taken with care, and should be phrased accordingly. In the course of the experiments the overall signal intensities drop (most likely due to cell divisions). These changes in intensities need to be included in the interpretation of the data.

*We agree with the reviewer and added this interpretation in the description of our results (p. 10, and p.12).*

(6) The conclusions about the potential assembly intermediate CI+CIII2* are based mainly on subunit ND5. Again, co-migration is a week argument, and there should be at least a convincing number of subunits running at the same size. Unfortunately, the 2D gels from the control cells are not of sufficient quality to draw many conclusions about CI+CIII2*, as it is not enlarged and indicated as for Figure 5B. Perhaps a different gel system could be used to get a better separation in the region of interest. The same applies to Figure 6.

*This part is taken out of the new version or has been moved to supplementary data.*

(7) Multiple experiments corresponding to the one shown in Figure 7E (now Fig. 7A, right panel) should be quantified to strengthen the point that the degradation of truncated COX1 and COX2/3 is significantly slower when mutant AFG3L2 is transfected.

*This is now done. Furthermore, we also have added more data about the stability of RC proteins after inhibition of AFG3L2 activity in A7339G WT cells (Fig. 7A, left panel).*

(8) The experiments performed in this study do not give any insight into folding states of proteins, the conclusion that AFG3L2 can interact with unassembled proteins should include that these unassembled proteins could also be misfolded. The finding that mutant AFG3L2 interacts with
newly synthesized COX1 forms is interpreted as a function of AFG3L2 in COX1 degradation, which is only one possibility, since it could simply assist in the folding process.

Indeed, we have not investigated the folding states of proteins as this out of the scope of the present study. However, we are very grateful for this idea and have included it in the discussion part (p. 18).

(9) The authors ignored a large number of important findings that have been made in yeast on the issue. There are several publications about the involvement of the mitochondrial quality control in the degradation of misassembled COX1 by the Winge group. COX1 stability during assembly has been addressed by several labs. Most importantly, a truncated COX1 lacking the C-terminus has been found to be stabilized by the group of Perez-Martinez. This finding contradicts the data presented here and should be discussed.

We are aware of the precious works of Winge’s and also by Barrientos’ group, particularly as they have contributed a lot to the understanding of the assembly of COX by discovering a huge numbers of important assembly factors and unravelled new mechanisms of the assembly translation feed back regulation. However, as far as we know these works are mostly done in yeasts which share homology, but do show some differences to the mammalian or human system.

For example, the hydrophilic C terminus of COX1 is less conserved overall and seemingly is crucial for COX activity in mammals, whereas it does not affect OXPHOS in yeast at all and is rather required for the assembly mediated regulation of COX1 translation. Alignment of the yeast and bovine Cox1 sequences revealed that S. cerevisiae has 23 additional residues in the C-terminal region. Thus, the truncated COX1 mutants lacking 11 or 15 residues at the C terminus described by Perez-Martinez’s group are not comparable with our truncated COX1 mutant cell lines lacking 35 AA and –180 AA at the C-terminus. Therefore, we cannot share the reviewer’s opinion that our findings contradicts the data obtained by Shingu-Vazquez et al. However, we are grateful for the motivation to include this point in the discussion part.

Minor points

- Does the COX1 antibody used in this study recognize the truncated COX1 forms? If not, some of the western blot results are rather trivial.

As shown in Fig. S2 we have tried to detect the truncated COX1 using different antibodies. Immunoprecipitations with different holo-Cox antisera and anti COX1 (directed against the N-terminus) followed LCMS- analysis only revealed the wildtype COX1 in WT cells, but no truncated COX1 mutant cells (data not shown). Even after inhibition of AFG3L2 activity we failed to detect the truncated COX1 proteins at steady state.
- Monomeric complex I cannot be seen in figure 4A (now Fig. 3A), text or figure should be changed accordingly.

_We admit that the signal for complex I on the western blot analysis is indeed weak, but still visible in right panel (probed with NDUFA9 and SDHA). The signal for complex I is further confirmed in IGA (Fig. 3B, former Fig. 4B), which shows the same sample run on a parallel gel._

- In figure S5 (now S8B) the labels are too far away from the actual protein bands and seem to be misplaced. This should be corrected. Labels and panels should be appropriately aligned in figure S7 (now S3).

_Thank you for pointing out the mislabelling. We have corrected this also._

- The COX1 signal from G6930A cells in figure 7A could be masked by ND2.

_The truncated COX1 signal is not detectable under normal conditions, but after inhibition of AFG3L2 it is visible (Fig. 6F, right panel)._ 

**Referee #3**

1. Inhibition of AFGL2 promotes stabilization of both wild type and mutant COXI. Does it also increase the levels of assembled COX and its function?

_We could show that inhibition of AFG3L2 activity and depletion of AFG3L2 promotes stabilization of COX and CI proteins (Fig. 6E and 7A). Inhibition of AFG3l2 could rescue the severe biochemical phenotype of the 90% mutant: COX activity and steady state levels were increased upon expression of the dominant negative AFG3L2 variant (Fig. 7B and C)._ 

2. If COX stability is what is impaired in these COXI mutants, as mentioned by the title of the manuscripts, inhibition of AFGL2 that leads to stabilization of COXI, should also lead to stabilization of the other respiratory complexes. Then why do mutant cells die more than wild type ones when AFGL2 is inhibited?
The severe phenotype in 100% mutant could not be rescued after inhibition of AFG3L2 activity (Fig. 7B and C) as the cells died (Fig. 7D and E). The stabilization of COX proteins does not lead to the stabilization of complexes and supercomplexes, as these still are instable (Fig. S9).

3. It would be important to determine if there is accumulation of a truncated, dysfunctional, COXI subunit in both stop-mutant cell lines and if this accumulation is responsible for toxicity. It would be novel to identify and demonstrate the equivalent of a misfolded-protein response in the mitochondrial inner membrane.

Upon overexpression of the dominant negative AFG3L2 variant the truncated COXI seems not to accumulate to steady state levels. We failed to detect it after inhibition of AFG3L2 using immunoprecipitations with different holo-Cox antisera and anti COXI (directed against the N-terminus) followed LCMS- analysis. These experiments only revealed the wildtype COXI in WT cells, but no truncated COXI mutant cells.

We also share the editor’s opinion that the investigation of misfolded protein response is out of the scope of this study.

Minor points

- In figure 1 that comprise the biochemical characterization of the COXI mutant cybrids, activity of all complexes but Complex I are shown. Since, based on the Western Blots of Fig 2, Complex I seems to be more affected than Complex II or Complex III, it would be nice to see Complex I activity and its threshold.

  We measured the activity of CI and added that to Fig. 1.

- The paragraph entitled "The new SC2*...." should be part of the previous paragraph and the results moved to Supplementary

  We have followed this suggestion and moved this part to the supplementary part.
Thank you for sending us your revised manuscript (previously EMBOJ-2011-77256) as a new submission. Let me first of all apologise for the delay in getting back to you with a decision. This was caused by severe difficulties with the availability of our original referees at the time of resubmission. In fact, one of the referees has still not been able to return his/her report. To save you from further loss of time, I am taking the decision on your manuscript now, based on the two reports we received (please see below).

In general, referees 2 and 3 are now positive about publication of your paper, but put forward a number of minor issues that still need to be addressed (see below) before we can ultimately accept your manuscript.

In addition, there are a number of editorial issues that need further attention:

Please include an author contributions section as well as a conflict of interest statement into the main body of the manuscript text, just after the acknowledgements section.

Please reformat the references section to fit The EMBO Journal format (no numbering, alphabetical order, for details, please refer to our author instructions).

Please provide us with a merged PDF that contains the whole supplementary material section in one file, including all supplementary figures.

Please confirm that in the right panels of supplementary figure S3 (COX10) all lanes come from the same gel and include this information into the figure legend. We also need the primary scans for these panels as source data files.

We now more generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files" (a zip file would also be fine). The gels 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 will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

We are looking forward to receiving you amended manuscript in due course.

Yours sincerely,

Editor
The EMBO Journal

REFeree COMMENTS

Referee #2

In this manuscript Hornig-Do et al. have addressed the molecular pathology of a patient with a point mutation in the mitochondrial COX1 gene leading to a truncated protein. They show that the truncated COX1 protein is expressed in mitochondria and that it assembles into cytochrome c oxidase complexes. However, the COX1 protein is rapidly turned over leading to a loss of complex function. The authors show that the AFG3L2 protease is responsible for this quality control step and that inactivation of the protease can stabilize COX1 concomitantly rescuing cytochrome c oxidase function. Thus, this study provides insight into the mechanism of membrane protein complex quality...
control and how it leads to disease.

The manuscript has certainly improved in the resubmitted form. Many of the criticisms raised by the referees have been addressed. Overall the manuscript presents sound experiments that are state of the art. The findings presented here indicate that quality control mechanism do not just act on unassembled protein but also at the level of an assembled but destabilized enzyme.

Minor points:

1) Figure 2 B: Full Blue Native PAGE gels need to be shown in order to allow the reader to assess if subcomplexes or aberrant forms accumulate.

2) Despite the fact that several mitochondrial proteins appear to be unaffected in A7339G (100%) cells (Fig. 2A), I still believe that one can not exclude that the lower amounts of complexes I, II, III and IV, as seen in Fig 2B, are secondary effects of a lowered membrane potential and thus defective import of some subunits. Dependencies of individual protein on the membrane potential are known to vary significantly and moreover such a reduced import might also be worsened by a reduced half-life of a protein. Thus, the authors need to check the membrane potential and ideally assay protein import. Moreover, the quantifications of 2 B are not clear to me. I see less complex V on the blot and not more, as suggested by the quantification. I could not find information on the quantification methods, were films used or were the signals digitally acquired?

3) The authors need to check Figure legends carefully. E.g. what is "CS" in Fig. 1, what is "#" in 4 D.

4) In Fig. 6 the radioactive signals that are quantified need to be internally standardized by using ratios e.g. to an ATPase subunit of Cytochrome b. If one looks at the gels there are some variations between lanes that can be equalized this way.

5) Figure 4 C needs a loading control to demonstrate efficiency of the isolation.

6) The immunoprecipitations shown in Fig 4 are very important to support complex association. Why are ATP8 and ATP6 enriched in the COX IPs? Again a total is essential to judge enrichment. In Fig 4 D why is there the same amount of Cox2 in both IPs? How can an antibody directed against the C-terminus be used to precipitate the truncated protein, please clarify.

Referee #3

This manuscript offers a very in depth analysis of the fate of truncated COX I in mtDNA mutant cells. The data convincingly indicate that the truncated proteins are briefly assembled into highly unstable complexes. They also show, using dominant negative mutants, that the m-AAA protease AFG3L2 is involved in the degradation of misassembled peptides of the respiratory chain. Interestingly, in heteroplasmic cells harboring a high proportion of mutant COX I gene, the dominant negative AFG3L2 induces an improvement of CIV assembly and function, while the homoplasmic mutant cells die in the presence of the dominant negative.

Overall, the experiments are excellent, and the data presented support the conclusion that instability of the complexes causes their decay in COX I mtDNA mutant cells and that AFG3L2 is involved in the turnover of unassembled proteins. In heteroplasmic cells, modulation of AFG3L2 could represent a therapeutic approach.

A relatively minor concern is that the abstract, title, and introduction tend to be directed to readers specialized in the field. The meaning of respiratory chain assembly and mtDNA mutations should be made more easily appreciated by the less specialized audience, at least in the introductory sections.
Answers to the reviewers

Referee #2

We like to express our thanks to the reviewer for his comments again helping to improve the quality of the manuscript. In particular, we appreciate the reviewer’s accuracy of observations very much.

Minor points:

1) Figure 2 B: Full Blue Native PAGE gels need to be shown in order to allow the reader to assess if subcomplexes or aberrant forms accumulate.

Fig. R1 shows no special subcomplexes or aberrant forms in 100% mutant cells. However, we will put a set of full blue native pages in the primary source data file.
Fig. R1: Full Blue native Pages
2) Dependencies of individual protein on the membrane potential are known to vary significantly and moreover such a reduced import might also be worsened by a reduced half-life of a protein. Thus, the authors need to check the membrane potential and ideally assay protein import.

As suggested by the reviewer we have measured the membrane potential using JC-1 in FACS analysis. As already reported for cells lacking mtDNA or with mitochondrial dysfunctions (von Kleist-Retzow, 2007; Kwong et al, 2007), membrane potential was also lower in A7339G 100% mutant cells (Fig. R2). However, it is still controversial whether such a lowered membrane potential would impair import of nuclear encoded proteins (Mercy et al., 2005). Although we cannot fully exclude a potential effect of the lower delta Psi on the mitochondrial import machinery, we do not think that it could markedly impair the import of nuclear encoded proteins in our mutant cells as

(i) protein levels of most nuclear encoded proteins HSP60, TFAM and ATP5B (Fig. 2A) were unchanged in A7339G 100% mutant cells, and

(ii) mRNA array revealed that mRNA levels of mitochondrial translocases of the outer membrane (TOMM20, TOMM22, TOMM34 and TOMM40) as well as translocases of the inner membrane (TIMM10, TIM13, TIMM22, TIMM23, TIMM40 and TIMM50) were found not be significantly altered in COX1 mutant cells (data not shown).

Fig. R2: Membrane potential in A7339G cells. A7339G Wt and 100% mutant cells were stained with JC-1 dye and subjected to FACS analysis. Results were obtained from 3 individual experiments, mean ± SDS, p= 0.0012
Also, an import assay, in this case accumulation of a nuclear encoded, processed protein resistant to protease over time, will be rather difficult to interpret since the rate of this accumulation will be determined not only by the rate of import, but also by the rate of degradation.

Finally, we would like to point out that the main point of our manuscript is the turnover of mitochondrially encoded subunits, particularly the truncated COXI protein, and not the fate of nuclear encoded proteins.

- Moreover, the quantifications of 2 B are not clear to me. I see less complex V on the blot and not more, as suggested by the quantification.

We agree with the reviewer that the amount of fully assembled complex V was slightly reduced in A7339G 100% mutant cells, and we often also see subcomplexes of CV, suggesting that CV might be also affected in the mutant cells. However, the amount of fully assembled CV and its subcomplexes in total was not significantly altered. As complex II and complex V seemed to be affected in the A7739G cells, we could not use them as a proper loading control in BN-PAGE. Thus, the same amount of solubilized mitochondria was run in a SDS-PAGE in parallel, transferred to PVDF and probed with antibody against VDAC, which was then used as a loading control. Steady state levels of complexes were then normalized to the signal of VDAC. The quantification diagram in Fig. 2B shows the steady state levels of the individual complexes in 90% mutant and 100% mutant cells, normalized to VDAC and in relation to the WT cells. This explanation is now included in the figure legend (p.25).

- I could not find information on the quantification methods, were films used or were the signals digitally acquired?

We used films, signals were not digitally acquired; this is now stated in the methods section (p.23-24).

3) The authors need to check Figure legends carefully. E.g. what is "CS" in Fig. 1, what is "#" in 4 D.

The mistakes in the figure legend have now been corrected (p.25 and p.26).

4) In Fig. 6 the radioactive signals that are quantified need to be internally standardized by using ratios e.g. to an ATPase subunit of Cytochrome b. If one looks at the gels there are some variations between lanes that can be equalized this way.

We are sorry that we have not clearly indicated that we have transferred proteins to PVDF membrane. After the exposition for autoradiography, we used the same membrane for probing with VDAC (Fig. 6A) or HSP60 (Fig. D, E and F). We think that these proteins are better internal loading control since complex V and complex III subunits (and particularly Cyt b, see Fig. 6A) could be affected in mutant cells.

5) Figure 4 C needs a loading control to demonstrate efficiency of the isolation.
We apologise that we did not pay more attention to equal loading in these previous experiments as the main aim of the experiment was to show that the truncated COXI is incorporated in higher molecular associations. We have repeated the experiment and also have added the loading control in the new Fig. 4c.

6) - Why are ATP8 and ATP6 enriched in the COX IPs?
Signals from ATP6 and ATP8 can be regarded as less specific, as they could be already seen in the preclearing fractions, and ATP8 was bound at relatively low amounts.

- Again a total is essential to judge enrichment.
The efficiency of the immunoprecipitation can also now be better seen in the new Fig. 4D showing that COX proteins and Core 2 are highly enriched in the immunoprecipitation fraction.

- In Fig 4 D why is there the same amount of Cox2 in both IPs?
COX2 was not enriched to the same amount in both IP as can be seen in Fig. 4B in both immunoprecipitations. In the repeated experiment we also can confirm that the amount of COX2 is much lower in the mutant cells than wild type cells (new Fig. 4D).

- How can an antibody directed against the C-terminus be used to precipitate the truncated protein, please clarify.
NOT the Cox1 antibody was used, but the anti holo COX antiserum was used to precipitate the truncated protein. In Fig. 4B, the immunoprecipitation with the COX1 antibody was done as an additional control.

**Referee #3**

A relatively minor concern is that the abstract, title, and introduction tend to be directed to readers specialized in the field. The meaning of respiratory chain assembly and mtDNA mutations should be made more easily appreciated by the less specialized audience, at least in the introductory sections.

We thank the reviewer for the positive response.

After reading the introductory part of the manuscript again, we have to admit that these sections are seemingly directed for a more specialized audience, which was not our original intention. In general, it is not an easy task to write a good introduction without losing the precision and depth of the subject, but also containing all the breadth of knowledge. Given that only 55,000 characters with spaces are allowed, we apologise that we could not have made it more accessible for the general reader. However, we think that our findings are highly interesting to those working in the
mitochondrial field in general and to those working on the extremely variable pathomechanisms caused by point-mutations in mitochondrial DNA in particular, but also to a more general audience interested in assembly and degradation of proteins and large protein complexes. We hope that the reviewer may forgive our literary inexperience and can share this opinion.

4th Editorial Decision 30 November 2011

Thank you for sending us the final revised version of your manuscript. I have now had a chance to go through it again, and you will be pleased to learn that you have now addressed all criticisms in a satisfactory manner and that the paper will be publishable in The EMBO Journal.

There is one point that needs clarification. From the source data for figure S3 it does not become clear which lane was used as the control in each of the subpanels and why. Also, it would be good, if you could clarify what patient A, C, D, E and control means specifically.

As providing the full set of source data is voluntary, we will go ahead and transfer the manuscript without them in this case to avoid further delays.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor

The EMBO Journal