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Coenzyme Q deficiency causes impairment of the sulfide oxidation pathway

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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.)

Editor: Roberto Buccione

1st Editorial Decision 14 April 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We are sorry that it has taken longer than usual to get back to you on your manuscript. In this case we experienced some difficulties in securing three appropriate expert reviewers, also due to the request to review two back-to-back submissions, and then obtaining their evaluations in a timely manner. Furthermore, one reviewer (#1) ultimately did not deliver.

As you will see the two Reviewers are globally positive, but do raise many issues. Reviewer 3, especially, raises an important and fundamental one. Although I will not dwell into much detail, I would like to highlight the main points.

Reviewer 2 raises a number of concerns that require your action. For instance s/he notes the lack of correlation between residual CoQ levels and sulfide oxidation and would like to better understand why GSH levels are decreased although both SQR and TST are diminished in the Pdss2 mutant kidneys. The reviewer also notes that causality between low SQR and up-regulation of downstream enzymes is not established. S/he also list additional points focused on improving precision and quality of controls

Reviewer 3 feels that, in addition to other items of concern, without improved mechanistic understanding and more conclusive demonstration of causal links, the manuscript would not be suited for publication. Specifically, s/he would like to understand how SQR activity is suppressed by decreased CoQ and also raises the same concern as Reviewer 2 on the relation between CoQ levels and sulfhydration. I fully agree that your work should thus be further developed in a mechanistic

sense. I should also mention that when deciding whether to send your manuscript out for review, I had sought counsel from an external advisor who agreed that the manuscript (s) was very interesting but noted that the potential shortcoming that the mechanisms were not clearly defined.

In conclusion, while publication of the manuscript cannot be considered at this stage, given the potential interest of your findings, we have decided to give you the opportunity to address the above concerns. We are thus prepared to consider a substantially revised submission, with the understanding that the Reviewers' concerns must be addressed with additional experimentation as appropriate and that acceptance of the manuscript will entail a second round of review.

I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #2 (Comments on Novelty/Model System):

Use of both patients' fibroblasts and organs from mutated mouse is appreciated

Referee #2 (Remarks):

The authors have investigated the effects of Coenzyme Q deficiency on sulfide oxidation that uses CoQ as acceptor of sulfide CoQ oxidoreductase (SQR), linking it to the respiratory chain. The study has been performed both in human fibroblasts from patients with different mutations in CoQ biosynthesis and in a mouse model with a mutation in Pdss2, a subunit of the first enzyme of the biosynthetic pathway of CoQ. In the fibroblasts as well as in the kidneys of the mutant mice the SQR activity and protein levels were decreased. On the other hand, the levels of downstream enzymes of the sulfide oxidation pathway generally increased in fibroblasts but decreased in mice. The kidneys of the mutant mice exhibited higher sulfide levels and decreased glutathione. In addition the mutant mice had high levels of short-chain acyl-carnitines probably caused by sulfide inhibition of their oxidation, although the sulfide levels were not enough to inhibit cytochrome oxidase.

This is a careful study considering how several aspects of sulfide metabolism may be affected by CoQ deficiency. The study has implications on the understanding some clinical features of CoQ deficiency in humans. Although the manuscript is clear and well organized, there are some points that need correction or clarification.

1. Introduction. Not all abbreviations are defined (for example ETHE1 and SUOX); the activities of the enzymes should be better defined, e.g. ETHE1 as a dioxygenase

2. Introduction and Fig. 1. The sulfide oxidation pathway is not defined clearly. For example in Fig. 1 TST and not SQR appears to be involved in formation of thiosulfate; on the other hand, in the text (line 118) it is stated that ETHE1 is involved in the conversion of sulfite to sulfate and not of thiosulfate to sulfite.

3. Results, oxygen consumption by sulfide. To better identify the level of CoQ deficiency, what are the corresponding activities for succinate oxidation in the different cells?

4. Table 1 and Fig. 3. There is no strict correspondence between residual CoQ levels and sulfide oxidation activity. For example P1 has the highest level among patients, but activity is lower than in P5; P2 has higher levels than P3, P4 ,P5 but has the lowest activity. Can this be explained?

5. Line 176. Can we state with certainty that it is the low SQR protein level to cause up-regulation of the downstream enzymes?

6. Lines 189-192. The data are not shown. Is recovery of SQR complete?

7. Lines 236-238. Can the authors suggest some reason for the variable effect on transcript levels in the different samples? Can different levels of CoQ biosynthetic intermediates or different extents of

ROS be a reason?

8. Line 249. Quinzii et al 2012 does not seem to be the correct reference for the CoQ levels in mutant mice.

9. Lines 269-273. I do not understand the reasoning: if SQR and TST are both decreased in kidney of Pdss2 mutants, then there should be less GSH used; therefore it seems unclear why GSH levels are decreased. The reason might be a different one from that proposed by the authors.

10. Line 444. Could intermediates of CoQ10 biosynthesis be detected in the chromatograms of some patients? Have the levels found in previous studies of CoQ (CoQ9 and CoQ10) been confirmed in this study?

11. Line 537 and following. Succinate dehydrogenase activity is mentioned nowhere in the text.

Referee #3 (Comments on Novelty/Model System):

The manuscript presents novel findings, but is largely phenomenological, failing to address molecular mechanisms that provide an explanation for changing levels of sulfide oxidation pathway enzymes when CoQ10 is deficient.

Referee #3 (Remarks):

This manuscript reports that aberrantly low Coenzyme Q levels, arising from mutations in CoQ biosynthetic enzymes, is associated with impaired hydrogen sulfide oxidation and altered expression of mitochondrial sulfide oxidation pathway enzymes - including a suppressed level of first enzyme in this pathway, SQR. In a study of skin fibroblasts from patients with attenuated CoQ levels, Ziosi et al demonstrate an impaired oxidation of hydrogen sulfide that can be rescued by CoQ supplementation. Further, in a mouse genetic model associated with low CoQ levels, the authors show diminished SQR abundance, sulfide accumulation, glutathione depletion and kidney failure.

Major Points
1. Increased protein sulfydration, owing to failure of H2S oxidation, is presumed by the authors to be a major basis for pathology associated with CoQ-depleting gene mutations. Experiments should be performed to determine whether CoQ levels are indeed inversely correlated with the extent of protein sulfhydration. Thus, it is recommended that the authors quantify the extent of protein sulfhydration in genetically CoQ deficient patient cell lines (using any of several reported proteomic methods) and determine the extent to which CQ levels are negatively correlated with total protein sulfhydration and/or sulfydration of specific proteins.

2. The authors report that SQR protein levels are reduced in proportion to CoQ10 levels in patient fibroblasts. Some consideration of the mechanistic basis for this phenomenon is needed. Does SQR loss arise from decreased SQR gene transcription, SQR protein translation, or accelerated SQR protein turnover? Is SQR loss driven by accumulation of sulfide in cells, i.e., can the effect be mimicked by chronic sulfide exposure of cells possessing control levels of CoQ10?

3. The authors demonstrate that exogenous CoQ10 can restore the ability of CoQ-deficient mutant cells to oxidize sulfide (Fig 3B). Surprisingly, this finding is made after a one-week exposure to CoQ10. While it is inferred that the observed restoration of sulfide oxidation is due to CoQ10 repletion, another possibility is that SQR levels are restored by this long duration of CoQ10 treatment. It is recommended that SQR levels are quantified in CoQ10-treated cells to ascertain whether SQR abundance is restored by CoQ10 treatment. Notably, it's surprising that CoQ10 repletion would require one week of incubation. Is this long incubation period necessary to replete cellular levels of CoQ10?

4. Fig 4: While CoQ and SQR levels decrease to the same degree in both P3 and P4 cell lines, TST and ETHE1 protein levels are only significantly increased in the P4 cell line. Can the authors provide an explanation for this apparent inconsistency? Was P5 purposefully omitted in this figure.

5. Fig 7A: In both the Results and Discussion sections, the authors state that SQR was almost undetectable in brains of WT mice and the level highly increased in mutant animals. This is not apparent in the presented data. Indeed, SQR protein bands seem to be present with similar intensities. Also a p-value has not been indicated on the graph - is the observed difference statistically significant? What is the basis for normalization of western blot findings to vinculin.

6. The discrepancy between findings made with human fibroblasts and the mouse model of CoQ10 deficiency is not been adequately addressed in the Discussion section. Is it organism or tissue related? Please comment.

Minor Points
Table I: The CoQ level for P5 has been omitted from the table, presumably by accident. Please add this value.

Fig 1. Labels for sulfide oxidation enzymes are provided as a black text on a dark background - barely legible. Please modify to improve legibility.

Fig 5: On line 201 the text refers to Fig 5C, D. This should be corrected as 5A, B.
References to supplementary figures between Lines 260-266 need to be corrected. (Figure S3 apparently refers to S4, S2 refers to S3 and S1 refers to S2)

1st Revision - authors' response 15 September 2016

Referee #2 (Remarks):
"This is a careful study considering how several aspects of sulfide metabolism may be affected by CoQ deficiency. The study has implications on the understanding some clinical feature of CoQ deficiency in humans. Although the manuscript is clear and well organized, there are some points that need corrections or clarification"

We are grateful to the reviewer for the positive comments on our study.

1. Introduction. Not all abbreviations are defined (for example ETHE1 and SUOX); the activities of the enzymes should be better defined, e.g. ETHE1 as a dioxygenase

We defined the abbreviations and the activities of these enzymes as follow:" Then, the sulfur dioxygenase ethylmalonic encephalopathy protein 1 (ETHE1 or persulfide dioxygenase), a mitochondrial matrix protein, participates at the conversion of thiosulfate to sulfite. The terminal component of this known pathway is the sulfide oxidase SUOX, which oxidizes sulfite to sulfate, which is subsequently secreted into the blood and eliminated through the urine (Muller et al. 2004, Hildebrandt and Grieshaber 2008)"

2. Introduction and Fig.1. The sulfide oxidation pathway is not defined clearly. For example in Fig. 1 TST and not SQR appears to be involved in formation of thiosulfate; on the other hand, in the text (line 118) it is stated that ETHE1 is involved in the conversion of sulfite to sulfate and not of thiosulfate to sulfite.

We apologize for the mistakes. In fact, the order of the enzymes of the sulfide oxidation pathway is controversial. We believe that SQR converts sulfite into thiosulfate by transferring a sulfur group from H_2S to thiosulfate. The reaction requires the reduction of ubiquinone (CoQ). Thiosulfate is then converted into sulfite by TST and ETHE1; this reaction requires a sulfur acceptor (glutathione, GSH). Excess sulfite is converted into sulfate by SUOX. We corrected Fig.1 to match the text.

3. Results, oxygen consumption by sulfide. To better identify the level of CoQ deficiency, what are the corresponding activities for succinate oxidation in the different cells?

Succinate oxidation in patient cells is reduced proportionally to their CoQ levels. The data have been added to the Results section and Appendix Fig. S1

4. Table 1 and Fig. 3. There is no strict correspondence between residual CoQ levels and sulfide oxidation activity. For example P1 has the highest level among patients, but activity is lower than in P5; P2 has higher levels than P3, P4, P5 but has the lowest activity. Can this be explained?

We agree that the correspondence between levels of CoQ and SQR driven respiration is not strict. We hypothesize that the impairment of respiration with NaHS as substrate correlates with a certain range of CoQ deficiency. Here we have shown that >50% residual CoQ is associated with milder defect of SQR driven respiration compared with <50% (14%-29%) residual CoQ, which is associated with severe defect of SQR driven respiration. This result is consistent with our previous observations in fibroblasts with different degree of CoQ deficiency: >50% (51%-69%) residual CoQ is not associated with defect in ATP synthesis, while <50% (12%-42%) residual CoQ is associated with decreased levels of ATP and ATP/ADP (Quinzii et al., 2010; Lopez et al., 2010; Quinzii et al., 2012).

5. Line 176. Can we state with certainty that it is the low SQR protein level to cause up-regulation of the downstream enzymes?

We postulate that SQR activity and/or levels determine the levels of the downstream pathway enzymes. Up-regulation of the downstream enzymes compensates for the low levels of SQR activity, or SQR protein levels, when this is >20% residual levels. However, severe reduction of SQR levels (<20% residual SQR) is associated with reduction of all the enzymes of the downstream pathway. These conclusions are based on the following results:

1) Patients fibroblasts with CoQ deficiency have reduced SQR activity and increased downstream enzymes levels, independently of SQR protein levels (Fig.4)
2) CoQ supplementation in fibroblasts increases SQR levels in patients cell lines, while the other enzymes levels are unchanged (Fig.5)
3) In Hela cells, depletion of the CoQ biosynthesis regulatory protein ADCK3 causes reduction of SQR (40% residual), and increase of the downstream enzymes levels (Fig.6).
4) Knock down of SQR (5% residual levels) in Hela cells (Fig.EV4 A, B, C) causes down-regulation of *TST*, *ETHE1*, and *SUOX* mRNA levels (Fig. EV4C).
5) Kidney of Pdss2 mice show 16% residual levels of SQR, and reduction of the levels of all the other enzymes (Fig. 8)

6. Lines 189-192. The data are not shown. Is recovery of SQR complete?

CoQ_{10} supplementation in the two patients cell lines with more severe CoQ deficiency significantly increased SQR protein levels in P3 and partially in P4 (Fig.5)

7. Lines 236-238. Can the authors suggest some reason for the variable effect on transcript levels in the different samples? Can different levels of CoQ biosynthetic intermediates or different extents of ROS be a reason?

We measured CoQ levels by HPLC and we did not observe CoQ biosynthetic intermediates in any samples. We also assessed ROS production by MitoSox, a fluorescent probe specific for mitochondrial O^2, in mutant fibroblasts and we did not find any differences among mutant cell lines or between mutant cell lines and controls (Appendix Fig S3).
Our data suggest a variable level of transcriptional up-regulation of the genes encoding enzymes of the H_2S oxidation pathway downstream of SQR in COQ mutant fibroblasts, possibly related to the genetic background. However, changes in CoQ levels, induced by CoQ synthesis inhibition, clearly affect H_2S oxidation enzymes gene expression, possibly through his antioxidant function. Indeed, a role of CoQ on several biological processes, such as lipid metabolism, inflammation, and cell signaling through regulation of genes expression has been previously proposed (Fisher 2015; Schmelzer 2008)

8. Line 249. Quinzii et al 2012 does not seem to be the correct reference for the CoQ levels in mutant mice.

We corrected the reference, which is Quinzii et al., 2013

9. Lines 269-273. I do not understand the reasoning: if SQR and TST are both decreased in kidney of Pdss2 mutants, then there should be less GSH used; therefore it seems unclear why GSH levels are decreased. The reason might be a different one from that proposed by the authors.

We agree that there are different possible explanations. To address these possibilities, we have added the following paragraph to the Discussion: "Therefore, it is tempting to speculate that tissue-specific abnormalities of H_2S metabolism may contribute to oxidative stress in CoQ deficiency through alteration of the glutathione system. For example, H_2S autoxidation could produce reactive sulfur and oxygen radical causing GSH depletion (Truong et al), or synthesis of GSH could be down- regulated to balance the increase of GSH caused by decrease of TST. However, the causes of GSH can be independent of H2S oxidation impairment. Since CoQ is an antioxidant, both via direct prevention of lipid peroxidation and indirect regeneration of other antioxidants such as vitamins C and E, as well as an electron carrier in the mitochondrial respiratory chain, lack of CoQ may cause an increase in ROS production and oxidative stress because antioxidant defenses are reduced and electron transport in the respiratory chain is impaired. Therefore, chronic oxidative stress due to lack of CoQ could be responsible for depletion of antioxidant defenses, including GSH. Importantly, Luna-Sanchez and colleagues showed reduced GSH levels in brain of *Coq9* mutant mice. However, they also observed that SQR depleted cells have GSH levels comparable to controls (Luna-Sanchez, co-submitted), supporting the hypothesis that reduction of GSH is independent of SQR levels or it is tissue-specific".

10. Line 444. Could intermediates of CoQ10 biosynthesis be detected in the chromatograms of some patients? Have the levels found in previous studies of CoQ (CoQ9 and CoQ10) been confirmed in this study?

We did not detect CoQ intermediates in any samples, by HPLC. In this study we confirmed the levels of CoQ found in previous studies in all cell lines but P5, which was used only in the oxygen consumption experiment, and was not available for other experiments.

11. Line 537 and following. Succinate dehydrogenase activity is mentioned nowhere in the text.

We apologized for not explaining that succinate dehydrogenase activity was used as a marker of mitochondrial mass. In the Results section the following sentence "We did not detect a COX deficiency in any tissue analyzed" was changed in to "COX activity, normalized to protein amount and CS activity or SDH activity, indices of mitochondrial mass, was not reduced in any tissue analyzed".

Referee #3 (Remarks):
The manuscript presents novel findings, but it is largely phenomenological, failing to address molecular mechanisms that provide an explanation for changing levels of sulfide oxidation pathway enzymes when CoQ10 is deficient

We thank the reviewer for recognizing the novelty of our results.
To explain the molecular mechanisms underlying the changes of the H_2S oxidation pathway enzymes observed in CoQ deficient patients fibroblasts and pdss2 mutant mice, we now investigated the H_2S oxidation pathway after 1) CoQ supplementation in patients fibroblasts, 2) pharmacological inhibition of CoQ biosynthesis in wild-type fibroblasts, 3) knock-down in Hela cells of ADCK3, a CoQ biosynthesis regulatory protein, 4) knock-down in Hela cells of SQR, 5) NaSH supplementation in wild-type fibroblasts.

Major Points

1. Increased protein sulfydration, owing to failure of H2S oxidation, is presumed by the authors to be a major basis for pathology associated with CoQ-depleting gene mutations. Experiments should be performed to determine whether CoQ levels are indeed inversely correlated with the extent of protein sulfhydration. Thus, it is recommended that the authors>quantify the extent of protein sulfhydration in genetically CoQ deficient patient cell lines (using any of several reported proteomic methods) and determine the extent to which CQ levels are negatively correlated with total protein sulfhydration and/or sulfydration of specific proteins.

We postulate that defects of H_2S oxidation cause H_2S binding to *heme* moieties in proteins thereby inhibiting their activity. Consistent with our hypothesis, CoQ deficient mice showed abnormal acylcarnitine profile, indicating that the enzymatic activity of short-chain acyl CoA dehydrogenase (SCAD) is inhibited.
However, we agree with the reviewer that alterations of protein sulfhydration may be another pathomechanism associated with CoQ deficiency. We therefore quantified total protein S-sulfhydration in two cell lines with different degrees of CoQ deficiency, P1 (~50% residual CoQ and normal SQR) and P4 (~15% residual CoQ and ~25% residual SQR). We noted that sulfhydration was increased in both cell lines compared with controls. We have now added a new figure (Fig 7) in the manuscript reporting data on protein sulfhydration.
This result is consistent with results of SQR-driven respiration studies, that showed that SQR activity is also reduced in cell lines with normal SQR protein levels, although there is not strict correlation between SQR-driven respiration defect and the magnitude of protein sulfhydration. This may be due to 1) experimental conditions, since SQR-driven respiration was measured adding excess of NaHS as substrate, while protein sulfhydration was measured under native conditions, or 2) presence of other compensatory mechanisms regulating H_2S levels, for example in the upstream H_2S biosynthesis pathway, depending on CoQ levels or genetic background of the fibroblasts.

2. The authors report that SQR protein levels are reduced in proportion to CoQ10 levels in patient fibroblasts. Some consideration of the mechanistic basis for this phenomenon is needed. Does SQR loss arise from decreased SQR gene transcription, SQR protein translation, or accelerated SQR protein turnover? Is SQR loss driven by accumulation of sulfide in cells, i.e., can the effect be mimicked by chronic sulfide exposure of cells possessing control levels of CoQ10?

Our hypothesis is not that SQR loss is driven by accumulation of sulfides in cells. We postulate that CoQ deficiency leads to reduced SQR activity and protein instability, which in turn causes accumulation of sulfide. However, CoQ biosynthesis inhibition affects *SQR* gene expression. Our hypothesis is based on the following results:

1) SQR protein levels are reduced in proportion to CoQ levels in patient fibroblasts (Fig. 4), while SQR mRNA was significantly reduced in P1, P3 and P4 (Fig. EV1).
2) Inhibition of CoQ biosynthesis in wild-type fibroblasts by a pharmacological approach, using 4-NB, causes the same level of CoQ deficiency of P2. In both cases SQR protein levels are normal, but mRNA levels are increased, suggesting a compensatory mechanism (Fig. EV3A, B).
3) CoQ supplementation in patient fibroblasts increased SQR protein levels (Fig. EV2A and Fig. 5A, B), indicating an effect of CoQ on protein stabilization.
4) In Hela cells, knock-down of ADCK3, causes CoQ deficiency (~50% residual), and consistently, significantly reduces SQR mRNA and protein levels (Fig. EV3C and Fig.6).
5) Exposure of wild-type fibroblasts to 0.5 mM NaHS for 24h (previously shown to be enough to have toxic effects, Di Meo I, 2011) does not cause reduction of SQR levels (Appendix Fig.S3).

3. The authors demonstrate that exogenous CoQ10 can restore the ability of CoQ-deficient mutant cells to oxidize sulfide (Fig 3B). Surprisingly,

this finding is made after a one-week exposure to CoQ10. While it is inferred that the observed restoration of sulfide oxidation is due to CoQ10 repletion, another possibility is that SQR levels are restored by this long duration of CoQ10 treatment. It is recommended that SQR levels are quantified in CoQ10-treated cells to ascertain whether SQR abundance is restored by CoQ10 treatment.

We quantified SQR protein levels and mRNA in P3, P4, and control fibroblasts after CoQ supplementation and we observed that SQR protein levels were significantly increased in P3 and partially in P4 (Fig. 5 and Fig. EV2), indicating that restoration of sulfide oxidation is not entirely due to increased SQR.

Notably, it's surprising that CoQ10 repletion would require one week of incubation. Is this long incubation period necessary to replete cellular levels of CoQ10?

We supplemented fibroblasts with CoQ_{10} for one week because the H_2S oxidation pathway enzymes are localized to mitochondria and CoQ pharmacokinetic to reach the mitochondria is delayed by its poor bioavailability, thus a long period of incubation is required for effective results. We previously showed that CoQ_{10} cellular repletion happens after 24h of supplementation with 5 µM CoQ10; however, one week is necessary for CoQ_{10} to reach the mitochondria and to improve mitochondrial bioenergetics, as measured by ATP production (Lopez LC, 2010). Thus, the delayed normalization of SQR levels after initiation of CoQ supplementation is consistent with the timeline of normalization of the mitochondrial respiratory chain.

4. Fig 4: While CoQ and SQR levels decrease to the same degree in both P3 and P4 cell lines, TST and ETHE1 protein levels are only significantly increased in the P4 cell line. Can the authors provide an explanation for this apparent inconsistency? Was P5 purposefully omitted in this figure?

P5 was not purposefully omitted. It was used only for the oxygen consumption experiment (Fig. 1, Appendix Fig.S1 and Appendix Fig.S2). It was not available for the other experiments.
Our data suggest that CoQ regulates SQR levels, which triggers a response of the downstream pathway. In patients fibroblasts there is a trend toward up-regulation of the pathway. We can not account for the differences between cell lines, which might be due to genetic background. We excluded differences in ROS, or the presence of CoQ biosynthesis intermediates, as suggested by Reviewer 2.

5. Fig 7A: In both the Results and Discussion sections, the authors state that SQR was almost undetectable in brains of WT mice and the level highly increased in mutant animals. This is not apparent in the presented data. Indeed, SQR protein bands seem to be present with similar intensities. Also a p-value has not been indicated on the graph - is the>observed difference statistically significant?

We measured the intensity of the bands of 5 WT and 5 MUT extracts repeated in 3 independent experiments. The intensity of the SQR band in mutant animals was 154% ±72 SD compared to controls. We agree that the difference was not statistically significant. To clarify this point, we changed the text from “exhibited a considerable increase” to “exhibited a trend toward increase”.

What is the basis for normalization of western blot findings to vinculin?

We used vinculin because it is a housekeeping protein whose size does not overlap with any of the proteins we wanted to test. Western blot of mice tissue extracts were also normalized to TOM20, a mitochondrial outer membrane protein, and the results were the same of the normalization to vinculin, therefore were not included.

6. The discrepancy between findings made with human fibroblasts and the mouse model of CoQ10 deficiency is not been adequately addressed in the Discussion section. Is it organism or tissue related? Please comment.

We added this comment to the Discussion: “We hypothesize that the discrepancy between findings in human fibroblasts and mouse kidney is mostly tissue-related. Tissue-specificity is typical of human and murine mitochondrial disorders, and fibroblasts are not clinically affected. We previously observed that Pdss2 mutant fibroblasts do not show the detrimental effects of CoQ deficiency observed in Pdss2 mutant mice kidney. It is possible that the molecular and biochemical abnormalities observed in patients fibroblasts reflect more severe abnormalities or a selective vulnerability to the effects of CoQ deficiency of the affected organs, for example kidneys, which is often affected in CoQ deficiency, independently of the molecular defect.”

Minor Points

Table I: The CoQ level for P5 has been omitted from the table, presumably by accident. Please add this value.

We added the value, which was omitted by accident.

Fig 1. Labels for sulfide oxidation enzymes are provided as a black text on a dark background - barely legible. Please modify to improve legibility.

We changed the background to improve legibility.

Fig 5: On line 201 the text refers to Fig 5C, D. This should be corrected as 5A, B.

We apologize for the mistake. Figures were re-numbered in the revised manuscript.

References to supplementary figures between Lines 260-266 need to be corrected. (Figure S3 apparently refers to S4, S2 refers to S3 and S1 refers to S2).

We apologize for the mistake. Figures were re-numbered in the revised manuscript.

2nd Editorial Decision 19 October 2016

Please find enclosed the final reports on your manuscript. We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

***** Reviewer's comments *****

Referee #2 (Remarks):

The Authors have performed an excellent revision of the original manuscript by performing new experiments and answering in detail in a satisfactory way to all queries by this reviewer. The manuscript represents an important and novel contribution to the field. I recommend acceptance.

Referee #3 (Remarks):

The authors have satisfactorily addressed all prior reviewer's concerns with new findings and revised text. The manuscript now makes a compelling case for CoQ levels as a physiological determinant of the sulfide oxidation pathway activity and enzyme expression.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Catarina M Quinzii
Journal Submitted to:	Embo Mol Med
Manuscript Number:	EMM-2016-06356

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For in vitro studies, to minimize the effect of the genetic background we chose to use at least three different control lines in fibroblast in each experiment.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We chose to study 10 animals/group to detect~60% changes (based upon alpha=0.05 and power of 1-b=0.8, and sigma=31) using STATA software
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All the animals were included in the analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	not applicable
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomly assigned in experimenatl groups by personnel different from the investigators involved in the study
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Human cell lines were coded and decoded after obtaining the results
4.b. For animal studies, include a statement about blinding even if no blinding was done	The person in charge of genotyping and euthanizing the animals was not the same one who performed the experiments. Samples were coded and experiments performed in blind. Samples were decoded after obtaining results
5. For every figure, are statistical tests justified as appropriate?	Yes they are
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The non parametric test (Mann-Whitney) that we used does not assume normal distribution and equal variance. The test is based on the assumption that the two group are independent. For the t test we assumed normal distribution and we chosen between homodeastic or heterodeastic based on the results of a F test.
Is there an estimate of variation within each group of data?	F test was used to assess variation within each group of data
Is the variance similar between the groups that are being statistically compared?	The Mann-Whitney U statistic that we employed is non parametric and irrespective of whether the two groups are similar in shape. Due to the assumption that the Mann-Whitney U statistic can underestimate a difference when two distributions are nothing alike, we improved the interpretation of our results performing also a F Test.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following antibodies were used: rabbit anti-SQR (1:1000, ab118772 Abcam for mouse; 1:1000, 17256-1-AP proteintech for human); rabbit anti-TST, (1:1000, ab155320, Abcam for mouse; 1:1000, 16311-1-AP proteintech for human); mouse anti-SUOX (1:1000, ab57852, Abcam); rabbit anti-ETHE1 (1:1000, Abcam ab154041); mouse anti-Vinculin (1:2000, Abcam ab18058) for both mouse and human. Secondary rabbit and mouse hrp (1:2000, Sigma A9044, and A0545).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	COQ2, PDSS2, COQ4, ADCK3 mutant fibroblasts were derived from human skin biopsies, and sent to us from clinicians. We generated ADCK3 and SQR depleted Hela cells. All the cell lines were authenticated and periodically tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Breeding colonies of B6/Pdss2 mutant mice have been established and maintained at Columbia University Medical Center in the facility for the care and use of laboratory animals. The animal care and use program at the Columbia University Medical Center is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and has an assurance with the Public Health Service (assurance number A3007-01).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We have a protocol with the proposed experiments approved by Columbia University IACUC (AC-AAAG8753)
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All Institutional and National (NIH) guidelines for the care and use of laboratory animals were followed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The study was conducted under Columbia University IRB protocol AAAB0483
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained for all the patients included in the study
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	not applicable

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We included an Appendix Table S2 with protein sulfhydration data in patients fibroblasts
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	no