EMBO Molecular Medicine

Alternative oxidase-mediated respiration prevents lethal mitochondrial cardiomyopathy

Jayasimman Rajendran, Janne Purhonen, Saara Tegelberg, Olli-Pekka Smolander, Matthias Mörgelin, Jan Rozman, Valerie Gailus-Durner, Helmut Fuchs, Martin Hrabe de Angelis, Petri Auvinen, Eero Mervaala, Howard T. Jacobs, Marten Szibor, Vineta Fellman, Jukka Kallijärvi

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Editor: Lise Roth

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 21 September 2018

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

As you will see from the reports below, the three referees are positive and support publication of the article in EMBO Molecular Medicine pending appropriate revisions. Addressing the reviewers concerns in full will be necessary for further considering the manuscript in our journal. Particular attention should be given to the discussion, which should address the organ-specificity of the effects and the potential application to human disease. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status. Please also contact us as soon as possible if similar work is published elsewhere. If other work is published, we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

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

Referee #1 (Comments on Novelty/Model System for Author):

mouse models sometime do not reflect human diseases but - there are no other relevant models

Referee #1 (Remarks for Author):

This interesting and extensive manuscript -shows the ability of ectopic AOX expression to alleviate mitochondrial dysfunction in a mouse model of mitochondrial complex III deficiency. The findings corroborate previous studies performed in Drosophila models with complex IV defects. The work is thorough, and the data are ample to support the conclusions. I have only minor comments mainly related to linking human disease to the present findings, as follows.

Introduction:
1) For the benefit of the reader-it would be nice to have a visual scheme/figure pointing out the location/function of CIII and AOX in electron flow/OXPHOS

2) The meaning of "different genetic background from previous " -page 5- is unclear
Does the GRAC mouse better reflect human disease than the other models?

Results:
1) Were plasma /urine metabolites measured? to compare metabolic GRAC results with patients with CIII and other mito/metabolic diseases (obviously in patients tissue metabolites are usually not measured) ;For example: Plasma -Proline and Alanine are often elevated in Lactic academia- could this be the reason for elevated Proline (inhibited proline oxidase by lactate- see also discussion) ;plasma Tyrosine and Methionine and plasma tyrosine-metabolites are often secondary elevated with liver dysfunction

2) What is - "CI and CII-linked OXPHOS-state 3 respiration"- fig 6 ? Explain)pyruvate+succinate substrates ?(

3) Normal wt or AOX values for comparison are lacking in some figures; 5g, 6b/c

4) Could elevated NO -inhibit CIV activity and thereby OXPHOS in situ ?

Discussion -
1) Re tissue specificity; compared wt with GROX- could there be some sort of threshold value that is crucial for liver function? i.e. liver has a priory low activities and is therefore more vulnerable ? What is presently known human/animal models?

2) Elaborate on the possibility if /how AOX could be delivered for example by a viral vector to patients?

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

This is a very interesting manuscript with relevant pre-clinical data.

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Rajendran et al describes the results of expression of Ciona intestinalis alternative oxidase (AOX) in a mouse model of Complex III deficiency. Specifically, this mouse model is a knock-in for the homozygous Bcs1lc.232A>G (Bcs1lp.S78G) mutation associated with the GRACILE syndrome and recapitulates many of the clinical manifestations, including growth failure, progressive hepatopathy, kidney tubulopathy, and short survival in a C57Bl/6NBomTac background. In this paper the Authors used another genetic background (C57BL/6JCrl) in which the homozygotes for the Bcs1l.S78G mutation, survive longer and develop additional later onset phenotypes to test the effects of AOX expression.
The manuscript is well written with interesting data. I have few comments:

-In Figure 1 d-i the histograms show different data obtained both in male and female. However, the body weight is reported only for males. Is there any difference in female?

-On page 8 this sentence: "AOX had only a minor effect on the hepatic metabolites (Fig. 4b)" refers to figure 4c and not on 4b as indicated.

-The Authors demonstrated that AOX expression rescue pathological phenotype mainly in hearth and kidney but not liver. The data reported in figure 6a show that RISP in SC1 and CIII is higher is GRAC vs GROX in kidney and liver, but the enzymatic activity of Complex III is comparable between heart and kidney, but significantly reduced in liver. Is it possible that AOX can't rescue the phenotype of the liver because there is a partially assembled CIII, although not biochemically active? Moreover, western-blot analysis in supplementary figure 4 show that AOX expression is the lowest in liver and kidney. Could this also contribute to the lack of rescue in the liver?
-I would strongly recommend adding in the discussion the recently published paper by Dogan SA et al "Perturbed Redox Signaling Exacerbates a Mitochondrial Myopathy", Cell metabolism, in view of possible human therapeutic strategy based on AOX expression

Referee #3 (Remarks for Author):

A very well performed study proposing a natural method to replace defective mitochondrial respiration. The aothors propose defferend damage mechanisms between tissues (energetics vs. ROS damage) to explain different responses to AOX. I would like to see data on transgene expression in different tisues because this could be an alternative explanation.

1st Revision - authors' response 16 October 2018

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Introduction:
1) For the benefit of the reader-it would be nice to have a visual scheme/figure pointing out the location/function of CIII and AOX in electron flow/OXPHOS
Thank you for the suggestion. We have now prepared a cartoon depicting the respiratory chain complexes, AOX and changes in electron flow when cIII is compromised. **(Synopsis Figure)**.

2) The meaning of "different genetic background from previous" -page 5- is unclear
Does the GRAC mouse better reflect human disease than the other models?
The GRAC mice faithfully recapitulate most manifestations of the GRACILE syndrome, e.g. growth restriction, liver and kidney disease, and early death, in all three genetic backgrounds that have been studied over the years (mixed 129Sv:C57BL/6JBomTac, congenic C57BL/6JBomTac and congenic C57BL/6JCrl). There are differences mainly in the survival in the different colonies. In a C57BL/6JBomTac-derived colony, the GRAC mice have a short survival of 35 days due to a lethal metabolic crisis (Kotarsky et al, 2012; Leveen et al, 2011; Rajendran et al, 2016). For the current study, we transferred the mice to another facility and used a slightly different congenic background, C57BL/6JCrl. On this background, the disease progression is somewhat milder and the mice do not succumb to the early metabolic crisis. Their extended survival, to up to 200 days (Purhonen et al. 2017) brings additional phenotypes, most prominently the lethal late-onset cardiomyopathy, which has not been reported in the patients and in mice with short survival. Therefore, GRAC mice in all studied backgrounds reflect human cIII deficiency, but the main focus of this study is the late-onset phenotypes, mainly the cardiomyopathy. We have clarified this in the text **(PAGE 3, LINES 5)-62)**.

Results:
1) Were plasma /urine metabolites measured? to compare metabolic GRAC results with patients with CIII and other mito/metabolic diseases (obviously in patients tissue metabolites are usually not measured) ;For example: Plasma -Proline and Alanine are often elevated in Lactic academia- could this be the reason for elevated Proline (inhibited proline oxidase by lactate- see also discussion)

;plasma Tyrosine and Methionine and plasma tyrosine-metabolites are often secondary elevated with liver dysfunction.
For this study, we measured liver enzymes from plasma and albumin, creatinine and isoprostanes from urine. However, we reported changes of plasma metabolites in GRAC mice in our previous study (Purhonen et al, 2017), in which we show that plasma alanine and proline were, along with general elevation of plasma amino acids, significantly elevated in GRAC mice at P95. Here, we show that proline is highly increased in heart tissue. The referee is right that the elevated proline level may be linked to elevated lactate, which is a known inhibitor of proline oxidase, as shown first by Kowaloff et al. (1977). We have now cited this paper. **(PAGE 8, LINES 174-175)**

2) What is - "CI and CII-linked OXPHOS-state 3 respiration"- fig 6 ? Explain) pyruvate+succinate substrates ?
CI&cII-linked respiration is a respiratory state in which a combination of substrates produces a convergent electron flow to the ubiquinone pool via CI and CII. To this end, we first added malate, pyruvate and glutamate to generate NADH for the CI. Subsequently, we added the CII substrate, succinate, to obtain CI&CII-linked respiration. By OXPHOS and state 3 we refer to phosphorylating respiration (ATP-producing respiration) in intact mitochondria in presence of saturating ADP. We have revised the main text **(PAGE 9, LINES 209-210)** and figure legends **(Fig. 6I, J)** to clarify the terminology.

3) Normal wt or AOX values for comparison are lacking in some figures; 5g, 6b/c
In healthy wild-type mice, ectopic AOX has been shown to be inert (Szibor et al. 2017), and in line with this we observed very few changes in the "AOX" mouse group compared to WT. This is why we did not include the AOX group in all assays, e.g. the metabolomics. We have added a statement to clarify this in the Methods section **(PAGE 20, LINES 474-475)**.
In the BNGE analyses, the amount of RISP in free $CIII_2$ and supercomplexes was normal in AOX mice (**Fig 6A**). Therefore, we do not present the ratio of RISP/CORE2 for the AOX group in **Fig 6B**.

4) Could elevated NO -inhibit CIV activity and thereby OXPHOS in situ?
In our model, the amount of RISP is decreased but it is not completely lost, which allows some electron transfer to cIV. Therefore, increased nitric oxide may theoretically further decrease cIV activity. However, we found no differences between the groups in cIV enzymatic activity in isolated mitochondria (Fig. 6G). This does not rule out an effect *in vivo* (or *in situ*), as the reviewer suggest. Even though our data speak against global ROS or nitrosative damage as an important pathomechanism, they do not rule out a more localized damage, such as to some RC complexes. Future studies should address this question more in detail. We have added a comment on cIV activity in Discussion. **(PAGE 14, LINE 332)**

Discussion -
1) Re tissue specificity; compared wt with GROX- could there be some sort of threshold value that is crucial for liver function? i.e. liver has a priory low activities and is therefore more vulnerable ? What is presently known human/animal models?
This is an important but difficult-to-answer theoretical question. In GRACILE syndrome patients and *Bcs1l*$^{p.S78G}$ mice, early histopathology and cIII deficiency are first seen in the liver, but the reason for this is unknown. In the mice, cIII activity is of similar magnitude in liver, kidney and heart (Fig. 6D), but cI&cII-linked phosphorylating respiration an order of magnitude lower in liver than in kidney and heart (**Fig 6I**), suggesting that the liver has relative low need to maximize ATP production by OXPHOS. This may explain why AOX does not rescue the liver manifestations efficiently. It is also possible that the numerous other anabolic and catabolic special tasks of hepatic mitochondria, including detoxification of numerous compounds, contribute to the pathogenesis in this tissue, but are not affected by the CIII bypass. We have elaborated the Discussion. **(PAGES 12-13, LINES 284-298)**

2) Elaborate on the possibility if /how AOX could be delivered for example by a viral vector to patients?
We have now added discussion on further preclinical studies and on prospects of translatability of our findings to human patients. **(PAGE 15, LINES 340-352)**

Referee #2 (Comments on Novelty/Model System for Author):
This is a very interesting manuscript with relevant pre-clinical data.

Referee #2 (Remarks for Author):
Rajendran et al describes the results of expression of Ciona intestinalis alternative oxidase (AOX) in a mouse model of Complex III deficiency. Specifically, this mouse model is a knock-in for the homozygous Bcs1lc.232A>G (Bcs1lp.S78G) mutation associated with the GRACILE syndrome and recapitulates many of the clinical manifestations, including growth failure, progressive hepatopathy, kidney tubulopathy, and short survival in a C57Bl/6NBomTac background. In this paper the Authors used another genetic background (C57BL/6JCrl) in which the homozygotes for the Bcs1l.S78G mutation, survive longer and develop additional later onset phenotypes to test the effects of AOX expression.

The manuscript is well written with interesting data. I have few comments:

-In Figure 1 d-i the histograms show different data obtained both in male and female. However, the body weight is reported only for males. Is there any difference in female?
We have now added weight data of female mice (**Figure 1C).**

-On page 8 this sentence: "AOX had only a minor effect on the hepatic metabolites (Fig. 4b)" refers to figure 4c and not on 4b as indicated.
The error has been corrected.

-The Authors demonstrated that AOX expression rescue pathological phenotype mainly in hearth and kidney but not liver. The data reported in figure 6a show that RISP in SC1 and CIII is higher is GRAC vs GROX in kidney and liver, but the enzymatic activity of Complex III is comparable between heart and kidney, but significantly reduced in liver. Is it possible that AOX can't rescue the phenotype of the liver because there is a partially assembled CIII, although not biochemically active? Moreover, western-blot analysis in supplementary figure 4 show that AOX expression is the lowest in liver and kidney. Could this also contribute to the lack of rescue in the liver?
The GRAC mice are, indeed, likely to have a mixture of partially and fully assembled cIII dimers due to poor, but not completely blocked, RISP incorporation in all three tissues studied. A cIII monomer without RISP is unable to oxidize quinols because the first electron transfer (from Q_o quinol to RISP) does not take place. A heterodimer containing one fully assembled monomer (with RISP) may still be active. However, the main trigger for AOX activation is thought to be the high reduction status of the quinone pool (El-Khoury 2014), in our case due to loss of the cIII quinol oxidase activity. Therefore, the assembly status of cIII as such should not affect AOX activity and its consequences. The partial rescue of cIII assembly and activity by AOX we observed in cardiac mitochondria (**Fig. 6A, D**) is interesting but neither our data nor literature offer any clear explanation. It could be linked to the generally improved mitochondrial morphology (**Fig. B-F**), or indirect damage to cIII, (e.g. by ROS or RNS). Conversely, the amount of fully assembled cIII was lower in GROX than in GRAC liver and kidney but the enzyme activity was not significantly changed. In these tissues, the apparent further loss of RISP from cIII in the presence of AOX could simply reflect relaxation of the need to keep cIII assembled and running when AOX replaces its ubiquinol oxidase activity. See Discussion **(PAGES 12-13, LINES 273-275, 284-298)**
Our Western blot results from the plain AOX mice are in agreement with Szibor et al. (2017), indeed showing highest protein expression in heart and lower in kidney and liver. However, AOX protein level was robustly increased by the cIII defect (independently of mitochondrial mass), and this resulted in similar level in all three GROX tissues (**Fig. EV3H**). Therefore, the amount of AOX protein in the affected tissues did not correlate with the rescue effect. We have added a statement to the discussion on this topic. **(PAGE 13, LINES 294-298)**.

-I would strongly recommend adding in the discussion the recently published paper by Dogan SA et al "Perturbed Redox Signaling Exacerbates a Mitochondrial Myopathy", Cell metabolism, in view of possible human therapeutic strategy based on AOX expression
As recommended, we have now discussed the very recent findings of Dogan et al. from their cIV deficiency-associated myopathy model. **(PAGE 13-14, LINES 309-325)**

Referee #3 (Remarks for Author):
A very well performed study proposing a natural method to replace defective mitochondrial respiration. The authors propose differend damage mechanisms between tissues (energetics vs. ROS damage) to explain different responses to AOX. I would like to see data on transgene expression in different tissues because this could be an alternative explanation.
This is an important point when working with transgenic overexpression. The *AOX* transgene was expressed constitutively under the widely used chicken b-actin promoter (Szibor et al. 2017). We have now added data on AOX mRNA expression, extracted from the transcriptomics data. These data show similar mRNA expression in liver and heart and somewhat lower in kidney. (**Figure EV3H)**

2nd Editorial Decision 26 October 2018

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees. As you will see the reviewers are now supportive, and I am pleased to inform you that we will be able to accept your manuscript pending following final editorial amendments.

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

Referee #1 (Remarks for Author):

The revision is satisfactory and I have no further comments

Referee #2 (Remarks for Author):

The Authors answered to all the questions and the manuscript has been improved.

Referee #3 (Remarks for Author):

Please refer to AOX protein expression in tissues (FIG EV3) in the text. Could the preferential cardiac extression account for the preferential cardiac benefit?

2nd Revision - authors' response 8 November 2018

Referee #3 (Remarks for Author):

Please refer to AOX protein expression in tissues (FIG EV3) in the text. Could the preferential cardiac expression account for the preferential cardiac benefit?

We apologize that we did not clarify this sufficiently in the first revision. AOX mRNA expression was similar in the heart and in the liver (Fig. EV3H), i.e. AOX expression did not correlate at transcriptional level with the rescue. In total tissue lysates from the AOX mice (Fig. EV3H, blue bars), the amount of AOX protein was, indeed, considerably higher in heart than in liver or kidney. However, this difference was mainly due to the much higher mitochondrial mass in heart, as shown by the mitochondrial loading control VDAC1 (Fig. EV3I), and also by the higher amount of most respiratory chain subunits (Fig. EV3I). Independent of this, we observed that the amount of AOX protein was affected by the *Bcs1l* mutation (AOX vs. GROX mice). In GROX liver and kidney, AOX protein was increased whereas in GROX heart it was decreased, resulting in almost identical AOX protein level in these tissues (Fig. EV3I, green bars). In summary, it is very unlikely that the preferential cardiac rescue was be due to higher expression of AOX in the heart. We have now added a short paragraph in Results (page 8, lines 168-176) to clarify this.

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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: Jukka Kallijärvi
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2018-09456-V3

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 χ2 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	A priori power analysis (80% power, 0.05 significance) indicated that to be able to detect a 20% increase in survival of Bcs1l mutant mice we would need 5 mice per group, both genders included. In the end, we observed a nearly 300% increase in the survival (n=18-21/genotype).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For measurements where no pre-specified effect size was known, we aimed at using a minimum of 5 mice per genotype.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The pre-established criterium for exclusion was error in genotyping. No genotyping errors resulting in exclusion of mice/samples were found.
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.	The mice were allocated from the litters to treatment/assessment groups based on genotype and gender only, without seeing the animals.
For animal studies, include a statement about randomization even if no randomization was used.	The genotypes were randomized by the Mendelian rules. The order of sample collection and fresh sample analyzes was dictate by the birth date of the mice.
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.	All stored samples were analyzed in computationally randomized order. The data were collected and analyzed without awareness to group allocation, though no strict blinding was applied.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The personnel evaluating the mice were blinded to the genotype information. However, due to the striking size difference, Bcs1l mutant mice can be easily distinguished from wild-type littermates.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We inspected normality of the data by plotting scatter plots, box plots and histograms. We also compared mean and median values. We also assessed normality of ANOVA residuals by D'Agostino & Pearson omnibus normality test and by inspecting histograms. When data was not compatible with parametric tests, we used non-parametric alternatives (i.e., Mann-Whitney U tests) as stated in the figure legends.
Is there an estimate of variation within each group of data?	Every bar graph shows standard deviation as an estimate of variation. Box plots show median, quartiles, and minimum and maximum value. Scatter plots show every data point and within-group standard deviations.
Is the variance similar between the groups that are being statistically compared?	For some parameters, variances and group sizes differed between the groups. We tested equality of variances with Bartlett's test. In case of unequal variance and/or group sizes, we followed ANOVA with unpaired t-test with Welch's correction.

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C- Reagents

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).	1. IHC, GFAP, Z0334, rabbit, DAKO; 2. IHC, 4-hydroxynonenal, ab46545, rabbit, Abcam; 3. IHC, Ki67, ab16667, rabbit, Abcam; 4. WB, Total OXPHOS Antibody Cocktail, MS603, mouse, Abcam; 5. BNPAGE/WB, UQCRFS1 (CIII subunit, Risp), ab14746, mouse, Abcam; 6. WB, VDAC1/Porin, ab154856, rabbit, Abcam; 7. WB, AOX, custom made, rabbit, 21st Century Biochemicals; 8. WB
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	Heterozygous knock-in (Bcs1lc.232A>G) mice (Mus musculus) in the C57BL/6JCrl (RRID:IMSR_JAX:000664) background and AOX transgenic mice were maintained in the animal facilities of University of Helsinki, Finland. All mice were housed in individually ventilated cages with 12 h light / 12 h dark cycle at a temperature of 22 to 23oC, and water and food (2018 Teklad global 18% protein rodent diet, Envigo) available ad libitum. The two mouse strains were crossed to generate double heterozygous mice which were backcrossed to the C57BL/6JCrl background for several generations. Wild-type or Bcs1l heterozygous animals, littermates whenever possible, were used as wild-type (WT) controls. Mice carrying a single copy of the AOX transgene (AOX mice), littermates whenever possible, were used as a second control group. Both genders were used in the experiments and were not separated unless separate analysis for males and females is indicated. Mouse health was monitored by manual behavioral scoring and weighing. The time points for assessments were chosen to verify early and late-onset manifestations: growth and survival data from weaning on, whole body metabolism and DEXA data between 10-14 weeks of age (at German Mouse Clinic), presymptomatic cardiac data at 5 months (P150), end-stage disease for GRAC mice at 6-7 months (P200), and survival and tissue histology of surviving GROX mice at up to 22 months (P680) (Supplementary Table. 4). The total number of experimental mice used in this study was 301.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The animal studies were approved by the animal ethics committee of the State Provincial Office of Southern Finland (ESAVI/6142/04.10.07/2014 and ESAVI/6365/04.10.07/2017) and were performed according to FELASA (Federation of Laboratory Animal Science Associations) guidelines.
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.	Yes.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	Transcriptomics data has been deposited to ArrayExpress database at EMBL-EBI (accession number: E-MTAB-7416).
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 have provided supplementary data in the form of appendix documents and extendard view figures. Additional raw data and detailed protocols are available from the authors upon request.
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).	NA
21. 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.	NA

G- Dual use research of concern

22. 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.	NA