Metabolic shift underlies recovery in reversible infantile respiratory chain deficiency

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Thank you for submitting your manuscript entitled "Metabolic shift underlies recovery in reversible infantile respiratory chain deficiency" [EMBOJ-2020-105364-T] to The EMBO Journal. The study has now been seen by three referees, whose comments are provided below.

As you can see, the referees concur with us on the overall interest of your findings. However, they also raise several critical points that need to be addressed before they can support publication in The EMBO Journal.

Given the overall interest of your study, I am pleased to invite submission of a revised manuscript as indicated in the referee's reports. I would like to point it out that addressing all of the referees' points in a conclusive manner, as well as a strong support from the referees, will be essential for publication in The EMBO Journal.

Please note that it is The EMBO Journal policy to allow only a single major round of revision and it is therefore important to resolve the main concerns at this stage.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File and therefore will be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process.

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Remember to provide a reviewer password if the datasets are not yet public.

We usually expect to receive revised manuscripts within three months of the first decision. We are aware that many laboratories cannot function at full capacity during the current COVID-19/SARS-CoV-2 pandemic and may relax this deadline. Also, we can extend our 'scooping protection policy' to cover the period required for a full revision to address all of the referees' points. Please inform us as soon as a paper with related content published elsewhere.

Thank you again for the opportunity to consider this work for publication, and please feel free to contact me with any questions about submission of the revised manuscript to The EMBO Journal. I look forward to your revision.

Referee #1:

The article of Horvath group is a continuation of their pioneering genetic and mechanistic research on a peculiar mitochondrial disease, previously called reversible COX-deficiency, now more appropriately termed reversible infantile respiratory chain deficiency, RIRCD. Mitochondrial diseases are typically progressive, but this disease, although causing severe muscle weakness in infants, shows spontaneous partial or total recovery around 1 years of age. The penetrance of the trait is incomplete, and according to the authors, only 1:100 of the gene variant carriers manifest a disease. Why so few manifest the severe disease has remained open.

The aim of the genetic study in this article was to identify whether a second gene mutation contributes to severe RIRCD manifestation. The authors succeeded to collect, together with a large international consortium, an exceptional material of 42 subjects with the variant m.14674T>C in mt-tRNA-Glu, which has been previously connected to RIRCD by these authors. The patient material contains disease manifesting subjects and carriers, from 8 different countries, all having the same homoplasmic mtDNA variant. Furthermore, muscle biopsy material was collected, giving an excellent opportunity for molecular pathology studies.

In addition to the mtDNA m.14674T>C variant, their NGS-exome analysis identified in 24 out of 27 affected patients mutations in EARS2 gene, encoding the mitochondrial glutamate tRNA synthetase, which mediates aminoacylation of mt-tRNA-Glu. The few remaining patients had variants in other genes, interestingly also related to glutamate or glutamine metabolism. The data of EARS2 is solid and exciting, and the other genes, even if present in only few patients, offer a surprisingly clear conclusion: nuclear gene variants that affect glutamate metabolism or mt-tRNA-Glu aminoacylation, contribute to manifestation of RIRCD, when co-occurring with the mtDNA m.14674T>C variant. The results indicate that variants in genes affecting mitochondrial metabolism may contribute to mtDNA tRNA-related disease manifestations, which are exceptionally heterogeneous in nature. Therefore, the findings have high relevance beyond RIRCD. Indeed, this is the first evidence of digenic mtDNA-nuclear gene variant contribution to disease.

Major comments:

1) The genetic findings are one of the most solid parts of the paper, but no figures are included in the actual article of these findings; all are in Suppl data files. Table 1 is missing from my article files; it is said to include description of the patients. Please include Table 1 and also shift Suppl table 3 to the actual article file, as it contains essential data of the contributing genes. Provide a figure that shows the mutation sites in the nuclear-encoded proteins (EARS2 and others), the conservation of those sites in species, and provide also in the same figure information in which functional domains the mutations reside. Include a schematic illustration of the mechanisms how the contributing glutamate metabolic genes are proposed to affect manifestation of RIRCD.

2) The authors then utilized their muscle biopsy materials, which they had from some manifesting patients and age-matched controls, and of one recovered patient. Such material is quite exceptional from children. How did the authors get muscle samples from healthy age-matched child controls? This should be described.

3) The muscle samples were subjected to a multiomics approach - proteomics and RNAseq, and the data are of high quality.Their study identifies in the child patients induction of the mitochondrial integrated stress response, ISRmt, previously reported in adult-onset mitochondrial myopathy, in a series of papers from Suomalainen group (Tyynismaa et al. 2010, Suomalainen et al. 2011, Nikkanen et al. 2016, Khan et al. 2017) and Larsson group (Kuhl et al. 2017) with upregulation of ATF5, GDF15 and FGF21, serine biosynthesis, activation of mTOR, as well as EIF2alpha phosphorylation. The authors propose a stage-wise activation of the pathway based on their one patient sample after recovery, similar to what was recently found to occur in ISRmt progression in mice by Forsström et al 2019. Even if from one patient, the data are intriguing concerning the recovery, and alleviation of ISRmt along the disease. This suggests that FGF21 and GDF15, biomarkers of mitochondrial diseases, could be used to follow treatment responses and disease progression/recovery. This could be commented in the discussion. Overall, ISRmt components in human muscle and its dynamic progression in mice have been previously described, but not in children, and not to follow recovery. The data are important.

4) The authors find ISRmt in their omics analysis, but then go forward to study ER-stress-related proteins (figure 2). The reason for this is not completely clear to this reviewer. They try to make a point of ER-stress contribution, but the blots of PERK and EIF2alpha phosphorylation, or S6 or mTOR are not convincing. S2448 mTOR shows variability in signals and not convincing changes, and its target T421/S424 S6K as well. VDAC1 signal is not publication quality. No good antibodies against ATFs exists which is why the western analysis of ATF4 is not convincing - to make a clear point, they should replicate it with ATF4 siRNA or study ATF4 nuclear localization by immunofluorescence. ATF5 induction is, however, evident by RNAseq. To this reviewer, the westerns of GCN2 and SLC7A11 are solid - both being part of ISRmt. Taken together, all other signaling westerns except the two, are unconvincing, as is the conclusion of ER stress induction. ISRmt and ISRer are partially overlapping, so ER-stress does not need to be induced even if ISRmt is. Please either improve experimentation of ER-stress and analyze additionally ATF6 induction and XBP1 splicing related to ER-stress. I favor omission of the data; the signature of ISRmt is clear as such.

5) Stress response mechanism, Figure 4. Their own data beautifully show induction of ISRmt, but then in the mechanism figure, the authors emphasize the role of ATF4 and UPRmt, neither of which is backed up by their evidence. The role of ATF4 in muscle has not been verified in mammalian mitochondrial muscle disease - most evidence comes from cultured cells (Quiros et al. 2017). Previous evidence (Forsström et al) and also the current one points to ATF5 contribution (RNAseq) in the muscle. UPRmt response, mostly described in worms, includes induction of heat shock proteins, which are not changed in this articles results. Please revise to reflect the findings of this paper and previous relevant literature.

6) The authors state that de novo serine biosynthesis is induced, but show no data of it.

Minor comments:

7. Figure 2G. MtDNA copy number - always increases during the first year of life (articles by J Poulton). Is the increase related to age or recovery? Include patient ages to the figure.

8. If mtDNA depletion is disease-induced, what is the mechanism? ISRmt has been shown to cause nucleotide precursor changes in muscle of adult patients with mitochondrial myopathy (Nikkanen et al. 2016). Is RIRCD actually a mitochondrial DNA depletion syndrome, or how do the authors explain the mtDNA copy nr decrease?

9. One of the most induced proteins in ISRmt is MTHFD2 of the mitochondrial folate cycle. Induced or not?

Referee #2:

Hathazi et al describe 27 affected individuals with RIRCD and 15 unaffected controls. RIRCD patients carry homoplasmic m.14674T>C mt-tRNAGlu mutations, but only about a third of carriers develop symptoms. Almost all RIRCD patients recover completely from the disease after 6 mo of age. To explain the reduced penetrance of the disease and to define the molecular mechanism of the reversibility of the symptoms, the authors use genomic sequencing and analyse patients' muscle biopsies by transcriptomics and proteomics. WES of 6 RIRCD patients identified heterozygous mutations in various nuclear genes related to mt-tRNAGlu function such as EARS2 and TRMU, indicating that RIRCD is a digenic disease. Transcriptomic and proteomic approaches point to an activation of the integrated stress response pathway (ISR) in response to OXPHOS deficiency, mTORC1 activation and a metabolic shift affecting various pathways including fatty acid

oxidation and amino acid catabolism. These findings are reminiscent of previous observations in patients with mitochondrial dysfunction. As a novel finding, the authors observe reduced ISR in one patient that recovered from RIRCD. Based on these findings, the authors propose a model for the recovery, which includes three phases for recovery: ISR activation, mTORC1 activation and stimulation of fatty acid oxidation and amino acid metabolism, and finally increased mitochondrial biogenesis.

The characterization of RIRCD as a digenic disease is very interesting and could very well explain the limited penetrance of this disease, although as for other mitochondrial diseases the observed tissue specificity remains unexplained. However, insight into the molecular basis for the recovery of some of the patients remains very limited. The evidence provided using OMICS approaches does not establish causality nor does it sufficiently support the in part far-reaching conclusions of the authors. ISR activation is well established as a cellular response to mitochondrial OXPHOS deficiencies and is known to involve mTORC1 activation. Similarly, metabolic shifts in response to impaired OXPHOS have been reported. It therefore remains enigmatic why some RIRCD patients recover. The analysis of one recovered patient shows reduced ISR but whether this indeed contributes to recovery (as speculated by the authors) or reflects the reduced stress response as a result of recovery is unclear. Moreover, the authors propose a three-phase-model for the recovery in some patients, but the analysis of muscle biopsies only provides insight into the steady state situation.

Specific points:
1. The authors list metabolic enzymes that are significantly changed in the proteome or transcriptome in the text but often do not show these data. It would be informative to see the most significant changes in the transcriptomic and proteomic data set in an unbiased manner, rather than showing only selected pathways as in Figure 1C-D.
2. On page 11, the authors state that there is an increase in mTOR signal as well as increased PGC1a and mitochondrial biogenesis. However, the western blot data shows very little significant changes. Are these claims possibly supported by OMICS data?
3. Page 11: The authors refer to increased S2448mTOR in muscle in the text but show in Figure 2C-D that mTOR phosphorylation at S2448 and PGC1a levels are not significantly altered? They also refer to altered levels of DEPTOR, PIK3R3, PIK3CD and PIK3CG but supporting data are not shown.
4. There are a number of mistakes/inconsistencies in the manuscript, concerning data presentation and statistical evaluation of the data. p values are given although only two patients were analysed.

Referee #3:

In this interesting and exciting manuscript, Horvath et al. present data to suggest the mechanism by which infants born with Reversible infantile respiratory chain deficiency (RIRCD) can recover spontaneously after 6 months of age by changes in amino acid availability.
There is no doubt that the authors have put a lot of work and thinking into this study and provide evidence of the metabolic changes in RIRCD. The key strength of the manuscript is the use of genomic sequencing and database to investigate the markedly reduced penetrance of RIRCD. Through their investigation of potential genetic contributors, researchers found additional heterozygous mutations in nuclear genes interacting with mt-tRNAGlu including EARS2 and TRMU in the majority of affected individuals, but not in healthy carriers of m.14674T>C suggesting the additive effects of these variants underlie the phenotype.
The authors demonstrate that the digenic mutations in mitochondrial and nuclear DNA interact

with reduced nutritional intake of amino acids and activate several metabolic events which induce stress response signaling and FGF21 signaling, metabolic shift to TCA and fatty acid oxidation and enhanced mitochondrial biogenesis. Altogether, these pathways increase amino acids availability and enable the metabolic shift.

However, there are several shortcomings that addressing them would strengthen this manuscript. The authors do a great job measuring abundance of mitochondrial proteins and suggest a metabolic switch However, in order to claim that mitochondrial function has improved, mitochondrial protein abundance is not an adequate measure for overall metabolism. The study would benefit if the authors could provide additional measures of mitochondrial function such as respirometry. As an alternative to testing respiration authors can perform metabolic flux analysis. The authors suggest increase in amino acid metabolism and demonstrate changes in genes and proteins involved in amino acid metabolism however there is no functional data to demonstrate this. Adding any measure that would provide evidence for altered amino acid metabolism will also strengthen the claims of this study.

Specific comments:
In figure 2G authors show that recovery is associated with even further increase in mitochondrial DNA copy number. How can that be explained? Why do the control subjects have reduced mitochondrial DNA compared with both the affected and the control individuals?

In figure 2A-F authors present only a single recovered subject, which is being used to conclude changes in protein expression. Additional recovered subjects would strengthen the claim. With the current hypothesis of the paper the presentation of the recovered subject data is key to the conclusion of the study. In 2G authors present n=2 recovered subjects, why are these two subjects not included in 2A-2F.

In figure 3 authors present data on cysteine and glutamine depletion however it would be interesting to show as a reference data on other amino acids.

Authors describe how they showed previously that the respiratory chain defect in RIRCD myoblasts (Boczonadi et al., 2013) and in fibroblasts carrying mutations in TRMU, MTO1 or mt-tRNAs (Bartsakoulia, Mueller et al., 2016) can be rescued by cysteine supplementation by improving cysteine dependent thiourydilation, so why not perform this rescue experiment with patient fibroblasts? Can this be shown with the digenic mutation found in this study?

Minor Comments:
In the western blot images the authors might want to use abbreviations or small font for some of the titles, in order to better align the sample lanes with their appropriate group.

Graphs in figure 3B and 3D are too small compared to the western blot images

In figure 4B , can the statistical analysis of the strength of this correlation be presented on the graph, and not just in the text?

Figure 5 is beautiful and interesting for people that read the entire paper with enthusiasm, however most readers would look at this figure without reading the entire paper. Modifying then figure so that it is simpler to understand for a person who was browsing the figures would convey the message more clearly and increase the readership.

Point-by-point answers to the referees

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intention was to exclude its possible contribution to increasing ATF4 levels, as PERK activation was described to increase ATF4 synthesis (Kilberg et al. 2009 and Reich et al. 2020). We agree with the reviewer that ER stress is not a main pathway involved in this condition thus we omitted it from our data. We provide evidence that the ISRmt in RIRCD acts also via the GCN2-ATF4 axis, besides the canonical way via ATF5, leading to increased expression of proteins involved in amino-acid transport, lipid oxidation and TCA. We used the ATF4 D4B8 rabbit antibody from Cell Signalling to detect ATF4 via immunoblotting. We believe that this antibody works well as suggested by 252 publications (https://www.citeab.com/antibodies/654138-11815-atf-4-d4b8-rabbit-

mab?utm_campaign=Widget+All+Citations&utm_medium=Widget&utm_source=Cell+Signaling+Technology&utm_term=Cell+Signaling+Technology) including some recent papers (Shimizu et al. 2020 (https://pubmed.ncbi.nlm.nih.gov/32345659/); Bugallo et al. 2020 (https://pubmed.ncbi.nlm.nih.gov/32457286/); Jiang et al. 2020 (https://pubmed.ncbi.nlm.nih.gov/32439839/). As ATF4 is a key factor in linking ISRmt with amino acid metabolism (Quiros et al.2017) we think it may be useful to keep this blot in Figure 3D (previously 2E). We acknowledge that the activation of ATF4 is correlated with its cellular localization as during stress conditions ATF4 migrates to the nucleus where it binds to different DNAs and regulates their transcription (Han et al. 2013). However, immunofluorescence experiments were not possible as we do not have cryosections from the muscles of these patients.

Due to the limited amount of muscle material available for our western blots, we had to focus on protein steady state levels of the key factors and we could only perform limited repeats for each blot. We agree with the reviewer and omitted EIF2α S6K1 mTOR and their phosphorylated forms.

Furthermore, we highlighted ISRmt targets from our transcriptomic analysis that are significantly increased in affected patients, similar to the ISRmt proteins described in mice and humans by Forsström et al 2019 (Figure 3F).

5) Stress response mechanism, Figure 4. Their own data beautifully show induction of ISRmt, but then in the mechanism figure, the authors emphasize the role of ATF4 and UPRmt, neither of which is backed up by their evidence. The role of ATF4 in muscle has not been verified in mammalian mitochondrial muscle disease - most evidence comes from cultured cells (Quiros et al. 2017). Previous evidence

(Forsström et al) and also the current one points to ATF5 contribution (RNAseq) in the muscle. UPRmt response, mostly described in worms, includes induction of heat shock proteins, which are not changed in this articles results. Please revise to reflect the findings of this paper and previous relevant literature.

We agree with the reviewer and thank for highlighting the misleading labelling in Figure 6 (previously figure 5). We omitted ER chaperons and UPR^{mt} elements from Figure 6 and from the Discussion. While the ATF family is well conserved as mitochondrial stress sensors and drivers from worms to mammals (Forsström et al 2019), we agree with the reviewer that indeed the role of ATF4 in mammalian mitochondrial muscle diseases is still debated and not fully elucidated. Forsström et al (2019) show that indeed ATF3, 4 and 5 are progressively induced in mouse myoblasts upon decreasing mitochondrial translation and that ATF4 might also be regulated by an ISRmt independent mechanism. It is possible that the regulation of ATF4 during ISR is FGF21 controlled, similar to described in the Deletor mouse (Forsström et al 2019). We cannot exclude a possible contribution of ATF4 in RIRCD. However further studies would be necessary to confirm it.

We added to the Discussion (page 17):
"Our transcriptomic data in agreement with previous evidence points to a clear contribution of *ATF5* to RIRCD ISRmt in human skeletal muscle (Forsstrom *et al.*, 2019). The role of ATF4 in muscle during ISRmt has not been verified in human mitochondrial muscle disease, only in cultured cells (Quiros *et al.*, 2017) and in mouse muscle where ATF4 activation is FGF21 dependent (Forsstrom *et al.*, 2019). While ATF4 has been suggested to be regulated by an ISRmt independent manner in proliferating cells (Forsstrom *et al.*, 2019), we hypothesise that in skeletal muscle of RIRCD patients ATF4 is sensitive to the amino-acid status, which seems to be altered in our patients suggested by GCN2 increase **(Figure 3)**."

6) The authors state that *de novo* serine biosynthesis is induced, but show no data of it.

We thank the reviewer for this comment. We included that the components of the *de novo* serine biosynthesis pathway showed significant change in transcriptomic

analyses in RIRCD muscle in Figure 3, as we detected an increase in enzymes involved in the first 2 steps of *de novo* L-serine synthesis (*PSAT1, PHGDH* and *SDSL*).

Minor comments:

7. Figure 2G. MtDNA copy number - always increases during the first year of life (articles by J Poulton). Is the increase related to age or recovery? Include patient ages to the figure.

We are aware of the publication of Jo Poulton`s group demonstrating an age-related increase of liver and muscle mtDNA copy numbers in infants (Morten et al., 2007).

We added the following sentence to the Discussion on page 17.
"The mtDNA copy number in muscle of the RIRCD patients studied in the symptomatic phase was already >2 times higher than in similar-age infants and showed further 4-40-fold increase after recovery, thus this increase cannot be explained by a physiological age-related increase but rather represents a compensatory mechanism that contributes to the recovery of these patients (Morten *et al*, 2007)."

As requested by the reviewer we added the ages of the patients to Figure 3C (previously 2G) as well as the ages of the 4 control samples (3 months, 3, years 4 years and 7 years) to the legend of Figure 3C.

8. If mtDNA depletion is disease-induced, what is the mechanism? ISRmt has been shown to cause nucleotide precursor changes in muscle of adult patients with mitochondrial myopathy (Nikkanen et al. 2016). Is RIRCD actually a mitochondrial DNA depletion syndrome, or how do the authors explain the mtDNA copy nr decrease?

We appreciate the suggestion of the reviewer by referring to the paper by Nikkanen et al. 2016. We detected in part similar changes in RIRCD infants, however we

emphasize that muscle mtDNA copy number in RIRCD infants is not low, it is rather higher (>2 times) than in age matched control muscle (as the increase in mtDNA is compensating for the low steady state level of mt-tRNA^{Glu}), therefore RIRCD is not a mtDNA depletion syndrome.

We reference the Nikkanen paper at page 17 in the Discussion:
"The mitochondrial replication machinery communicates with cytoplasmic dNTP pools and the *de novo* serine biosynthesis, which are altered in RIRCD patients and may participate in the metabolic stress response which may offer rescue in primary or secondary mtDNA instability disorders (Nikkanen et al. 2016). This is accompanied by increased mitochondrial biogenesis via mTOR activation (increased mtDNA copy numbers) thus leading to mt-tRNA^{Glu} increase and improved mitochondrial translation."

9. One of the most induced proteins in ISRmt is MTHFD2 of the mitochondrial folate cycle. Induced or not?

Previously a possible uncoupling of the regulation of mitochondrial folate cycle during ISRmt was suggested (Forsstrom *et al.*, 2019). *MTHFD2* transcript is slightly increased (not statistically significant) in skeletal muscle of the RIRCD patients which was shown to be FGF21 independent. In contrast, the FGF21 controlled *MTHFD1L* shows a significant increase in RIRCD muscle (Figure 3F).

Referee #2:

Hathazi et al describe 27 affected individuals with RIRCD and 15 unaffected controls. RIRCD patients carry homoplasmic m.14674T>C mt-tRNAGlu mutations, but only about a third of carriers develop symptoms. Almost all RIRCD patients recover completely from the disease after 6 mo of age. To explain the reduced penetrance of the disease and to define the molecular mechanism of the reversibility of the symptoms, the authors use genomic sequencing and analyse patients' muscle biopsies by transcriptomics and proteomics. WES of 6 RIRCD patients identified

heterozygous mutations in various nuclear genes related to mt-tRNAGlu function such as EARS2 and TRMU, indicating that RIRCD is a digenic disease. Transcriptomic and proteomic approaches point to an activation of the integrated stress response pathway (ISR) in response to OXPHOS deficiency, mTORC1 activation and a metabolic shift affecting various pathways including fatty acid oxidation and amino acid catabolism. These findings are reminiscent of previous observations in patients with mitochondrial dysfunction. As a novel finding, the authors observe reduced ISR in one patient that recovered from RIRCD. Based on these findings, the authors propose a model for the recovery, which includes three phases for recovery: ISR activation, mTORC1 activation and stimulation of fatty acid oxidation and amino acid metabolism, and finally increased mitochondrial biogenesis.

The characterization of RIRCD as a digenic disease is very interesting and could very well explain the limited penetrance of this disease, although as for other mitochondrial diseases the observed tissue specificity remains unexplained. However, insight into the molecular basis for the recovery of some of the patients remains very limited. The evidence provided using OMICS approaches does not establish causality nor does it sufficiently support the in part far-reaching conclusions of the authors.

We agree with the reviewer that our data do not fully explain the recovery, however we performed a multi-omics analysis from the limited amount of muscle tissue which was available from RIRCD infants. We agree that further experiments with targeting the key components in cell lines or other model systems would be valuable. Unfortunately, we did not have access to muscle cells, and there are no animal models of this condition. We studied fibroblasts, however these do not manifest the molecular defect without additional stressors (such as amino acid deprivation), making any of these targeted analyses very difficult.

While ISRmt and its dynamic progression has been previously described in mice (Forsström et al 2019), this is the first report describing elements of ISRmt in children, and following recovery. Despite having follow up data before and after recovery only from one patient, we show an alleviation of ISR^{mt} during the course of

the disease which gives us valuable biomarkers such as FGF21 and GDF15 that could be used to follow up treatment response and overall disease progression in patients with mitochondrial myopathies.

ISR activation is well established as a cellular response to mitochondrial OXPHOS deficiencies and is known to involve mTORC1 activation. Similarly, metabolic shifts in response to impaired OXPHOS have been reported. It therefore remains enigmatic why some RIRCD patients recover. The analysis of one recovered patient shows reduced ISR but whether this indeed contributes to recovery (as speculated by the authors) or reflects the reduced stress response as a result of recovery is unclear. Moreover, the authors propose a three-phase-model for the recovery in some patients, but the analysis of muscle biopsies only provides insight into the steady   state   situation.

We highlight that we performed transcriptomic and proteomics studies on 6 affected and 2 recovered patients, however a direct comparison was only available in one patient, where we collected muscle in the symptomatic phase and after recovery from the same individual.

We agree with the reviewer that it is unclear, why similar alterations rescue the disease in RIRCD and not in other mitochondrial diseases such as m.3243A>G and how ISR can be protective (reversible mitochondrial disease) or deleterious (progressive mitochondrial disease). We hypothesize that it is potentially depending on its duration (short term-beneficial by activating specific rescue mechanisms vs long term-pathogenic, leads to cell apoptosis). Future studies performed on suitable model systems, such as patient derived myoblasts/myotubes may be able to provide an answer for this question.

We added to the Discussion on page 18.
"We detected similar alterations of metabolic pathways in a clinical trial using bezafibrate in skeletal muscle of patients with m.3243A>G-related mitochondrial myopathy (Steele et al., 2020), where the number of complex IV deficient muscle fibres decreased and cardiac function improved, while FGF21 and GDF15 increased in all patients. The changes in treated patients were accompanied by alterations in

Specific points:

1. The authors list metabolic enzymes that are significantly changed in the proteome or transcriptome in the text but often do not show these data. It would be informative to see the most significant changes in the transcriptomic and proteomic data set in an unbiased manner, rather than showing only selected pathways as in Figure 1C-D.

As the number of genes/proteins showing significant changes is very high we only included some key factors in Figure 2 (previously figure 1) in the original submission. However, we agree with the reviewer that it is important to show changes of metabolic enzymes in an unbiased manner and added Figure EV4, showing a heatmap of the most significantly changed genes. Furthermore, we highlighted ISRmt targets from our transcriptomic analysis that are significantly increased in affected patients, similar to the ISRmt proteins described in mice by Forsström et al 2019 (Figure 3).

2. On page 11, the authors state that there is an increase in mTOR signal as well as increased PGC1a and mitochondrial biogenesis. However, the western blot data shows very little significant changes. Are these claims possibly supported by OMICS data?

We agree with the reviewer that some of the changes seen in our western blotting analysis may not reach statistical significance, therefore we omitted some of these blots from Figure 3. Our OMICS data did not show any alterations in the levels of *mTOR* transcripts however *DEPTOR* transcript, a component of the mTORC1 and mTORC2 complexes shows a significant decrease in affected patients compared to controls, and increases with recovery (Figure 3). The decrease of *DEPTOR* was associated with the activation of mTOR and its downstream targets, promoting cell survival and growth (Peterson et all, 2009). Our transcriptomic data further suggests an activation of mTOR and its downstream targets such as *PGC1α* (mitochondria biogenesis), *RRAGC* (cellular response to amino-acid availability), *EIF4EBP1*

3. Page 11: The authors refer to increased S2448mTOR in muscle in the text but show in Figure 2C-D that mTOR phosphorylation at S2448 and PGC1a levels are not significantly altered? They also refer to altered levels of DEPTOR, PIK3R3, PIK3CD and PIK3CG but supporting data are not shown.

4. There are a number of mistakes/inconsistencies in the manuscript, concerning data presentation and statistical evaluation of the data. p values are given although only two patients were analysed.

Referee #3:

In this interesting and exciting manuscript, Horvath et al. present data to suggest the mechanism by which infants born with Reversible infantile respiratory chain deficiency (RIRCD) can recover spontaneously after 6 months of age by changes in amino   acid   availability.
There is no doubt that the authors have put a lot of work and thinking into this study and provide evidence of the metabolic changes in RIRCD. The key strength of the manuscript is the use of genomic sequencing and database to investigate the markedly reduced penetrance of RIRCD. Through their investigation of potential genetic contributors, researchers found additional heterozygous mutations in nuclear genes interacting with mt-tRNAGlu including EARS2 and TRMU in the majority of affected individuals, but not in healthy carriers of m.14674T>C suggesting the additive   effects   of   these   variants   underlie   the   phenotype.
The authors demonstrate that the digenic mutations in mitochondrial and nuclear DNA interact with reduced nutritional intake of amino acids and activate several metabolic events which induce stress response signaling and FGF21 signaling, metabolic shift to TCA and fatty acid oxidation and enhanced mitochondrial biogenesis. Altogether, these pathways increase amino acids availability and enable the   metabolic   shift.
However, there are several shortcomings that addressing them would strengthen this manuscript. The authors do a great job measuring abundance of mitochondrial

proteins and suggest a metabolic switch. However, in order to claim that mitochondrial function has improved, mitochondrial protein abundance is not an adequate measure for overall metabolism. The study would benefit if the authors could provide additional measures of mitochondrial function such as respirometry. As an alternative to testing respiration authors can perform metabolic flux analysis. The authors suggest increase in amino acid metabolism and demonstrate changes in genes and proteins involved in amino acid metabolism however there is no functional data to demonstrate this. Adding any measure that would provide evidence for altered amino acid metabolism will also strengthen the claims of this study.

We agree with the reviewer completely that measuring respiration, metabolic flux and amino acid concentrations would be additional useful methods that would support our findings. Unfortunately, we did not have the possibility to perform these studies in the limited amount of muscle available from these infants and fibroblasts do not manifest the molecular defect without additional triggering factors thus making flux analysis and metabolomics unreliable. Muscle cells (myoblasts, myotubes) may have been a better cell type to perform these experiments, but we did not have access to patients` myoblasts.

Specific comments:
In figure 2G authors show that recovery is associated with even further increase in mitochondrial DNA copy number. How can that be explained? Why do the control subjects have reduced mitochondrial DNA compared with both the affected and the control individuals?

It is correct that the mtDNA copy number in muscle of the RIRCD patients in the symptomatic phase was >2 times higher than in similar-age infants – already illustrating some compensation for abnormal mitochondrial translation, and this showed further significant 4-40-fold increase in parallel with recovery. We think that this is triggered by increased mitochondrial biogenesis as part of the rescue mechanism and leads to increase in mt-tRNA^{Glu} steady state and clinical recovery of these infants.

In figure 2A-F authors present only a single recovered subject, which is being used

to conclude changes in protein expression. Additional recovered subjects would strengthen the claim. With the current hypothesis of the paper the presentation of the recovered subject data is key to the conclusion of the study. In 2G authors present n=2 recovered subjects, why are these two subjects not included in 2A-2F.

We highlight that we performed transcriptomic and proteomics studies on 6 affected and 2 recovered patients, however a direct comparison was only available in one patient from which we collected muscle in the symptomatic phase and after recovery.

In figure 3 authors present data on cysteine and glutamine depletion however it would be interesting to show as a reference data on other amino acids.

We agree with the reviewer completely. In fact, we are planning to perform such experiments with depleting and supplementing different amino acids, however demonstrating that depletion of the most important amino acids in regard of this specific defect (cysteine, glutamine and glutamic acid) triggers the mitochondrial defect in fibroblasts is already supporting our hypothesis. Comprehensive studies sequentially depleting different amino-acids may be also more relevant in myoblasts as amino-acids have different roles in individual tissues (e.g. increased intramuscular glutamine is needed in muscle protein synthesis (Mittendorfer et al. 2001).

Authors describe how they showed previously that the respiratory chain defect in RIRCD myoblasts (Boczonadi et al., 2013) and in fibroblasts carrying mutations in TRMU, MTO1 or mt-tRNAs (Bartsakoulia, Mueller et al., 2016) can be rescued by cysteine supplementation by improving cysteine dependent thiourydilation, so why not perform this rescue experiment with patient fibroblasts? Can this be shown with the digenic mutation found in this study?

We would have liked to perform such studies in RIRCD fibroblasts, however the mitochondrial respiratory chain defect is not expressed in these cells without triggering it by amino acid depletion. Therefore, we did not have a readout for demonstrating rescue after amino acid supplementation.

Minor Comments:

In the western blot images the authors might want to use abbreviations or small font for some of the titles, in order to better align the sample lanes with their appropriate group.

We agree with the reviewer and adjusted font sizes.

Graphs in figure 3B and 3D are too small compared to the western blot images.

We increased the size of the graphs in Figure 4B and 4D (previously 3B and 3D).

In figure 4B, can the statistical analysis of the strength of this correlation be presented on the graph, and not just in the text?

We added p values demonstrating the strength of the correlation to Figure 5 (previously Figure 4).

Figure 5 is beautiful and interesting for people that read the entire paper with enthusiasm, however most readers would look at this figure without reading the entire paper. Modifying then figure so that it is simpler to understand for a person who was browsing the figures would convey the message more clearly and increase the readership.

We thank the reviewer for this suggestion. We simplified the text in Figure 6 (previously Figure 5) and we hope that this version is easier to understand for the readers.

Thank you for submitting your revised manuscript. Please accept my apologies for the delay in getting back to you with our decision due to summer holidays. The study has been re-reviewed by two of the original referees, whose comments are shown below.

As you can see, while the referees find that their criticisms have been sufficiently addressed, reviewer #2 points out that the pathophysiological relevance of the observed alterations remains unclear and invites you to tone down the abstract. In addition, there are a few editorial issues concerning the text and the figures that I need you to address before we can officially accept your manuscript.

Referee #1:

The authors have satisfactorily responded to my criticism. I have no further comments.

Referee #2:

The authors have carefully considered the points of criticisms when revising their manuscript and significantly improved the presentation and discussion of the data. The manuscript establishes beautifully RIRCD as a digenic disease and describes comprehensively associated metabolic changes. However, the limited availability of patient material does not allow to establish causality or a longitudinal analysis of metabolic changes during the recovery phase. I am still concerned that in some cases only one patient has been analysed. Therefore, it remains rather speculative which of the observed alterations are of pathophysiological relevance and whether different recovery phases can indeed be distinguished. The authors discuss their model and its limitations now more convincingly in the discussion but a more careful wording in the abstract still appears appropriate.

2nd Authors' Response to Reviewers
31st Aug 2020

We wish to thank you for having our revised manuscript entitled " **Metabolic shift underlies recovery in reversible infantile respiratory chain deficiency**" reviewed by two reviewers. We have addressed the comment made by reviewer 2 and corrected the issues pointed out by the editorial team and would now like to resubmit the revised manuscript. The changes are marked with yellow in the manuscript.

2nd Revision - Editorial Decision
9th Sep 2020

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

Congratulations!

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

USEFUL LINKS FOR COMPLETING THIS FORM

Corresponding Author Name: Dr. Rita Horvath	
Journal Submitted to: The EMBO Journal	
Manuscript Number: EMBOJ-2020-105364-T	

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 $\chi2$ 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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We studied as many patients as possible with this rare mitochondrial condition called reversible infantile respiratory chain deficiency. Skeletal muscle sampl,es of infants with this condition were included when available. All experiments were repeated at least 3 times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	no animal studies were performed
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We have not exclude any samples from 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.	no treatment included
For animal studies, include a statement about randomization even if no randomization was used.	no animal studies were performed
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.	The individuals studied were all homoplasmic carriers of m.14674T>C, and we analysed individuals who were 1. affected, 2. recovered or 3. healthy unaffected and used controls who did not carry m.14674T>C. We could not use blinding when studied these groups, because the affected or recovered status was the base of the comparison.
4.b. For animal studies, include a statement about blinding even if no blinding was done	no animal studies were performed
5. For every figure, are statistical tests justified as appropriate?	yes, we used justified statistical tests for every figure
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We compared the genetic variants detected in our m.14674T>C homoplasmic carriers with large control databases such as ExAc and gnomAD.
Is there an estimate of variation within each group of data?	We estimated variation and showed it in the figures for each group of data.
Is the variance similar between the groups that are being statistically compared?	The variance was similar in the groups which were statistically compared.

C- Reagents

Question	Answer
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).	KEY RESOURCES TABLE Reagent or resource Source Identifier Antibodies mentioned in the Methods: mouse anti-SDHA Abcam ab14715 mouse anti-NDUFA9 Abcam ab14713 mouse anti-MTCO1 Abcam ab14705 mouse anti-MTCO2 Abcam ab110258 mouse anti-VDAC1 Abcam ab15895 rabbit anti-VNC Thermo Fisher 42H9BL44 rabbit anti-mTOR Cell Signalling 2983 rabbit anti-p-mTOR Cell Signalling 5536 rabbit anti-PGC1alpha Abcam ab54481 rabbit anti-P70S6K Cell Signalling 2708 rabbit anti-p-P70S6K Cell Signalling 9204 rabbit anti-GCN2 Cell Signalling 404575 rabbit anti-PERK Cell Signalling 5683T rabbit anti-p-PERK Cell Signalling 3179S rabbit anti-SLC7A11 Abcam ab37185 rabbit anti-ATF4 Cell Signalling 11815S rabbit anti-eIF2alpha Cell Signalling 5324T rabbit anti-p-eIF2alpha Cell Signalling 3398T rabbit anti-TOMM20 Abcam ab186735 Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP Thermo Fisher Scientific 31460 Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP Thermo Fisher Scientific 31430 Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 Thermo Fisher Scientific A-11008

	Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 Thermo Fisher Scientific A-11005
	Chemicals, peptides and Recombinant proteins
	L-Cysteine,from non-animal source, BioReagent, suitable for cell culture, ≥98% Sigma-Aldrich C7352-25G
	TaqMan master mix BioRad 4369510
	Trizol Life technologies 15596018
	Iodoacetamide Sigma-Aldrich I1149-25G
	Sequencing grade modified trypsin was from Promega Promega V5117
	Benzonase® Nuclease Novagen 70746
	Formic acid Biosolve 69141
	Trifluoroacetic acid Biosolve 213141
	Acetonitrile Biosolve 12041
	Orbitrap Fusion Lumos mass spectrometer Thermo Fisher Scientific NA
	Ultimate 3000 nano RSLC syste Thermo Fisher Scientific NA
	Thermo Scientific™ Acclaim™ PepMap™ Thermo Fisher Scientific 164567
	Amersham Typhoon Scanner GE Healthcare Life Sciences NA
	Critical Commercial Assays
	mirVana miRNA isolation kit Thermo Fisher Scientific AM1560
	Ribo-Zero Human ki Epicentre an Illumina Company MRZH11124
	TruSeq Stranded mRNA Illumina 20020594
	Pierce™ BCA Protein Assay Kit Thermo Fisher Scientific 23227
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The human primary fibroblasts were obtained from the Newcastle Biobank from donors via skin biopsy utilizing routine protocols. The samples were obtained from the following patients: Patient (F7/7F-healthy carrier, F7/2M-RIRCD, F7/5F-healthy carrier, F7/3F-RIRCD without a clear second mutation. For more details relating the patients see Table 1.
HEK293T cells (+/+) and PDE12 knock-out cells (-/-) and -/- cells expressing WT PDE12, p.Glu351Ala (catalytic mutant), Δ16 (coding for PDE12 lacking 16 first aa), Δ23 mutants (coding for PDE12 lacking 23 first aa), and p.Arg23Trp PDE12 Cdna and Human 143B osteosarcoma (HOS) were obtained from Michal Minczuk. We tested all cell lines for mycoplasma contamination and they were negative.

* 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.	no animal models were used
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	no animal models were used
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.	no animal models were used

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The human fibroblasts were obtained from the Biobank of the MRC Neuromuscular Translational Research Centre (London-Cambridge-Newcastle). All the necessary ethical approvals are available in these facilities (REC reference 08/H0906/28+5). Human skeletal muscle was obtained by biopsy. Patients and their family members have given consent to the study "Genotype and phenotype in inherited neurological diseases" study (REC: 13/YH/0310, end date 30/09/2023, IRAS ID: 2042290) which includes all relevant permission for this work. The consent form contains also the consent for any omics analysis.
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.	Infomred consent was obgtained from all subjects and experiments conformed to the principles set out in the WMA Declaration of Helsinki ad the Department of Healthand Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	no patient photos are included
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	We had a limited number of skeletal muscle biopsies from patients with RIRCD, as it is a very rare disease.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	no clinical trial has been done
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.	N/A
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.	N/A

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 | The exome sequencing data is made available also via RD-Connect while the RNAseq and Proteomics data is currently being deposited. Accession numbers and codes for the genetic data in RD-Connect is allocated within the Solve-RD project with the follwoing ID numbers: RD-Connect ID Local ID PhenoTips ID
E584491 PFC_0132 P0003849
E116005 PFC_0133 P0003850
E471427 PFC_0134 P0003851
E164998 PFC_0228 P0003870
E929927 PFC_0229 P0003871
E265637 PFC_0230 P0003872
E644260 PFC_0231 P0003873
E313533 PFC_0232 P0003874
E193704 PFC_0233 P0003875
E353393 PFC_0235 P0003876
E638771 PFC_0239 P0003877
E297999 PFC_0240 P0003879
E413805 PFC_0240_143 P0003878
E111703 PFC_433 P0003893
E169783 PFC_434 P0003894
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E991204 RH_500 P0006711
E625711 RH_504 P0006715
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E167325 RH_507 P0006718
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E681116 RH_510 P0006721 |
| | E802575 RH_551b P0006734
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E160701 RH_555 P0006738
E716071 RH_556 P0006740
E783248 RH_557 P0006741
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E066171 RH_559 P0006743
E393858 RH_560 P0006744 |
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).	Deposition of RNAseq and Proteomics datasets are in progress.
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).	genomic data of our patients is depositied in EGA and RD-CONNECT
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.	no special computational models were used

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. | Our study does not fall under dual use research restrictions. |