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OPA1 Deficiency Promotes Secretion of FGF21 from Muscle that Prevents Obesity and Insulin Resistance

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 04 January 2017

Thank you for the submission of your manuscript (EMBOJ-2016-96179) to The EMBO Journal. I apologise for the delay in getting back to you at this time of the year, which was due to delayed referees' correspondence due to travel. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, referee #1 points out that some conclusions are not sufficiently well supported by the current data (ref #1, pts. 1, 2, 3, 6). In particular, this referee states the need for you to provide further proof of the generality of the phenotype in different muscle types and cell lines and in addition asks you to consolidate your findings with complementary mitochondrial/metabolic assays. Referees #2 and #3 agree that consolidating work is needed and in addition ask you to explore the kinetics and detailed mechanistic level of the mitochondrial metabolic defects observed (see ref #2, pt. 2; ref #3, points 1 and 2). In addition, all referees list a number of technical issues and controls that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and we are the in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I agree that it would be essential to corroborate the broader relevance and consistency of your observations and

also to add refined insights into the mitochondrial functional level involved in these cellular phenotypes.

Please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

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

REFEREE REPORTS

Referee #1:

The manuscript by the Dale Abel group evaluates the whole body metabolism in mutant mice lacking the Opa1 mitochondrial fusion factor in skeletal muscles. Despite the observed defects in mitochondrial function, the authors find a surprising improvement in glucose tolerance, insulin sensitivity and the resistance to high fat diet. They trace these effects to a sharp up-regulation of Fgf21 expression in Opa1-deficient muscles. By genetic epistasis experiments they demonstrate that Fgf1 is the cause of this metabolic program and they attempt in finding how mitochondrial dysfunction signals to Fgf21 in muscles.
The data are convincing and the conclusions are well demonstrated. Additional information would improve the clarity of the study:
1)- The key observations should be reproduced in 2-3 muscle types. Some experiments are performed in oxidative soleus muscles while others in glycolytic gastrocnemius muscles. Mitochondrial dysfunction, FGF21 expression, and muscle atrophy should be replicated in both muscle types
2) The defects in mitochondria (Figure 1) should be strengthened by using more standard assays (mitochondrial DNA, Seahorse analysis, membrane potential)
3)- The effects on atrogene expression are mild (Figure 2) and are accompanied by no effect on Akt activity. What about mTORC1, myostatin and other growth regulators?
4) Is the genetic background of mice homogenous? The information in the Methods is confusing. Bl6 vs. other backgrounds respond differently to high fat diet.
5) The immunoblot data should be analyzed on the same membrane without cutting and pasting lanes as in Figures 5A, 6A, 6B, 6C, 6I, S1A, S1G, S4A, S5E.
6) Rather than using C2C12 cells, the authors should compare myotubes or isolated fibers from control and Opa1 mutant mice for Fgf21 expression, ER stress markers and PBA response. This should considerably improve the data in Figure 6.

Referee #2:

In this work, Pereira et al. explore the role of OPA1 in the skeletal muscle by generating a muscle inducible knock-out mouse of OPA1 (mOPA1). These mice developed a muscle atrophy and progressive mitochondrial dysfunction. However, at the same time, mOPA1 mice are resistant to age- and diet-induced obesity and insulin resistance. The underlying mechanism proposed and validated by the authors involves the secretion of the myokine FGF21, which increases the metabolic rate and improves whole body insulin sensitivity.
The study is interesting and provides new evidence about the hormetic response activate by mitochondrial dysfunction in vivo. However, although the results are clear, more extensive metabolic analysis is needed to reinforce these results and increase the novelty of the study.

Major points:
- Analysis of mitochondrial respiratory complexes is poor; immunoblot of individual subunits of OXPHOS complexes is not an indicative of the amount of the entire complexes. BN-PAGE is required to assess the status of OXPHOS complexes.
- How do authors explain the increased distance of mOPA1 KO during the exhaustion test? Are those differences already apparent at early time points as 12 or 20 weeks?
- No effect in liver is shown. Are there any changes in hepatic steatosis? And in blood parameters?

- The observed increase in EE in mOPA1 mice in both CD and HFD suggests an increase in BAT function. Analysis of BAT functionality (e.g. mitochondrial respiration) would increase the strength of the results. Also, are there any differences in cold tolerance and/or browning?
- Physiological parameters of mOPA1 KO under HFD were similar to WT mice under CD. This could suggest that HFD improves physiology of mOPA1 KO as happens for other models. Does HFD rescue any muscle related defect? (e.g. fiber size, Murf1 or atrogin-1 expression levels, ...)
- Cellular experiments with C2C12 should be performed with a knockdown model of OPA1. Mitochondrial stress can activate the integrated stress response in vitro by different mechanisms. Therefore, to specifically asses the role of ER stress and AMPK activation, an OPA1 deficient cellular model is required.

Minor points:
- Are there any changes in the number of fibers of mOPA1 KO mice?
- Western-blots from mice should be provided with more than n=1.
- Figures need to be revised. Proportions are not conserved and some plots and text are deformed.

Referee #3:

The role of FGF21 in total body metabolic adaptation following mitochondrial dysfunction is a timely and significant issue, but controversial. Recent data indicate that the type of mitochondrial dysfunction determines either whether FGF21 is induced or whether it contributes to increased glucose tolerance during high fat diet. In this respect the study by Pereira et al. presents potentially important findings in the mouse model of induced OPA1 depletion. While the descriptive data on the metabolic phenotype of the inducible OPA1 KO and OPA1/FGF21 DKO mice are solid, unfortunately the work does not give sufficient mechanistic insight to address the outstanding question: what differentiates their model from previously published negative results on the role of FGF21 in whole body metabolic adaptations to mitochondrial stress? My main concerns are the following:

1. It is not determined what is the exact mitochondrial metabolic defect in the OPA1 deficient muscle. Fig. 1. shows that ADP driven respiration in mice up to 20 weeks is basically maintained (Fig. 1D), while mitochondrial ATP production is drastically reduced (Fig. 1G). It is difficult to explain this finding. It cannot be the consequence of uncoupling, but rather a previously unseen effect on the efficiency on the ATP synthase or simply increased ATP consumption in the saponin-permeabilised fibers? How total ATP content differs between WT and KO mice in intact fibers? Is there glycolytic compensation in intact muscle? Is membrane potential reduced? These data would be required to conclude on relating the full body phenotype to muscle mitochondrial defect.
2. Along the same lines, it has been recently shown that in human disease and mouse models FGF21 induction is associated with mtDNA translation defects and mtDNA deletions, while no FGF21 response is observed in direct respiratory chain defects. OPA1 depletion has been associated with both loss of respiratory chain integrity and loss of mtDNA maintenance, thus the mtDNA phenotype of both the OPA1 KO and OPA1/FGF21 DKO muscle should be determined.
3. To conclude on the effect of FGF21 in the OPA1/FGF21 DKO, it is essential that OPA1 levels are reduced to the same level as in the OPA1 KOs. This does not seem to be the case, the authors report 70% reduction in OPA1 KO (Fig. 1) and about 50% reduction in OPA1/FGF21 DKOs (Fig. S4), and according to the representative blots the KO effect induction by tamoxifen is quite heterogeneous. The authors should determine how the primary OPA1 KO phenotype correlates with OPA1 levels in individual mice to resolve this issue.
4. Along the same lines, there is significant reduction of total body weight in OPA1 KOs (Fig. 2A), which the authors attribute to muscle atrophy and not to changes in fat mass (Fig. 2C). On the other hand, in OPA1/FGF21 DKOs loss of body weight did not occur (Fig. S4B). Does this mean that FGF21 causes the reduction in total lean mass? Or is it related to less severe OPA1 reduction? How is muscle fiber diameter in these mice? Overall, the mitochondrial and muscle phenotype should be characterised in the OPA1/FGF21 DKO mice to resolve this issue.

Minor comment:

MW markers on WBs should be shown. Some blots (e.g. Fig. 5A) are pieced together, and the CB loading standard is very faint, which is not up to standards.

1st Revision - authors' response 14 April 2017

Response to Reviewers

Referee #1: The manuscript by the Dale Abel group evaluates the whole-body metabolism in mutant mice lacking the Opa1 mitochondrial fusion factor in skeletal muscles. Despite the observed defects in mitochondrial function, the authors find a surprising improvement in glucose tolerance, insulin sensitivity and the resistance to high fat diet. They trace these effects to a sharp up-regulation of Fgf21 expression in Opa1-deficient muscles. By genetic epistasis experiments they demonstrate that Fgf1 is the cause of this metabolic program and they attempt in finding how mitochondrial dysfunction signals to Fgf21 in muscles. The data are convincing and the conclusions are well demonstrated. Additional information would improve the clarity of the study:
1)- The key observations should be reproduced in 2-3 muscle types. Some experiments are performed in oxidative soleus muscles while others in glycolytic gastrocnemius muscles. Mitochondrial dysfunction, FGF21 expression, and muscle atrophy should be replicated in both muscle types.

RESPONSE:
We appreciate the reviewer's comments and suggestions. We have performed additional analysis of mitochondrial respiratory capacity in red and white gastrocnemius muscle, which support the conclusion that mitochondrial dysfunction, activation of ER stress, induction of FGF21 and muscle mild muscle atrophy develop in oxidative and glycolytic muscles when OPA1 content is reduced (see Fig.1G,H,I,J, Fig. 2D and Fig. EV-1, Fig. EV-2, Fig.5A, Fig. EV-3D).

2) The defects in mitochondria (Figure 1) should be strengthened by using more standard assays (mitochondrial DNA, Seahorse analysis, membrane potential).

RESPONSE:
We have performed further analysis of mitochondrial respiratory capacity in vivo in red and white gastrocnemius permeabilized fiber bundles using O_2K Oroboros high resolution respirometry. These data confirm our initial observation that OPA1 deficiency impairs respiratory capacity and expand our initial findings in oxidative fibers to glycolytic muscles. We have also performed BN-PAGE to more rigorously quantify mitochondrial OXPHOS complexes and measured mtDNA content in gastrocnemius muscle (See Fig 1). We did not have sufficient mice to measure membrane potential in isolated mitochondria from OPA1 deficient mice. However, we investigated mitochondrial membrane potential in OPA1 deficient myotubes by performing live cell imaging of TMRM fluorescence over time in response to oxidative stress (Fig. 7E,F). In addition, seahorse analysis was performed in OPA1-deficient myotubes, confirming reduced oxygen consumption and increased glycolysis following OPA1 deletion (Fig. 7C,D).

3)- The effects on atrogene expression are mild (Figure 2) and are accompanied by no effect on Akt activity. What about mTORC1, myostatin and other growth regulators?

RESPONSE:
We have expanded our findings by examining basal and insulin stimulated S6 phosphorylation in gastrocnemius muscle (Fig. 6D,E). Interestingly, insulin-mediated activation of mTORC1 signaling was significantly increased in mOPA1 KO gastrocnemius muscle. These observations are consistent with increased insulin sensitivity in OPA1 deficient muscle. We also speculate that activation of mTORC1could potentially counteract the activation of atrophic pathways reported in our study, thereby explaining the mild atrophic phenotype observed.

4) Is the genetic background of mice homogenous? The information in the Methods is confusing. Bl6 vs. other backgrounds respond differently to high fat diet.

RESPONSE:
We appreciate you pointing this out. All mice utilized in this study are on the C57Bl6J background. This information has been clarified in the Methods section of the revised manuscript.

5) The immunoblot data should be analyzed on the same membrane without cutting and pasting lanes as in Figures 5A, 6A, 6B, 6C, 6I, S1A, S1G, S4A, S5E.

RESPONSE:
We appreciate this concern. All comparative analyses were originally performed on the same membranes, even though some images were cut and pasted together for ease (in our view) of data presentation. In the revised manuscript, we now show all the representative samples side by side on the same membranes (see Fig. 6A,F, Fig. 7A).

6) Rather than using C2C12 cells, the authors should compare myotubes or isolated fibers from control and Opa1 mutant mice for Fgf21 expression, ER stress markers and PBA response. This should considerably improve the data in Figure 6.

RESPONSE:
We appreciate your suggestions very much and have now performed in vivo and in vitro experiments in mOPA1 KO mice and in OPA1-deficient primary myotubes. We confirm induction of ER stress in OPA1 deficient myotubes (Fig. 7A), and show attenuation of ER stress and FGF21 induction and secretion following treatment with the ER stress inhibitor PBA. Moreover, we also treated mice with the ER stress inhibitor TUDCA (Fig. 6 F,G,H) revealing that inhibition of ER stress pathways in vivo reduced FGF21 levels in muscle and in the circulation in vivo. Taken together, these data suggest that ER stress in necessary for FGF21 induction in and secretion from muscle cells, and that ER stress inhibition, significantly decreases FGF21 levels in response to OPA1 deficiency. The original data in C2C12 are now moved to the supplementary data (Fig. EV-5).

Referee #2: In this work, Pereira et al. explore the role of OPA1 in the skeletal muscle by generating a muscle inducible knock-out mouse of OPA1 (mOPA1). These mice developed a muscle atrophy and progressive mitochondrial dysfunction. However, at the same time, mOPA1 mice are resistant to age- and diet-induced obesity and insulin resistance. The underlying mechanism proposed and validated by the authors involves the secretion of the myokine FGF21, which increases the metabolic rate and improves whole body insulin sensitivity. The study is interesting and provides new evidence about the hormetic response activate by mitochondrial dysfunction in vivo. However, although the results are clear, more extensive metabolic analysis is needed to reinforce these results and increase the novelty of the study.

Major points:
- Analysis of mitochondrial respiratory complexes is poor; immunoblot of individual subunits of OXPHOS complexes is not an indicative of the amount of the entire complexes. BN-PAGE is required to assess the status of OXPHOS complexes.

RESPONSE:
We agree that BN-PAGE is the gold standard assay to investigate the status of mitochondrial OXPHOS complexes. We have now performed BN-PAGE analyses in gastrocnemius muscle of mOPA1 KO and DKO mice (Fig. 1C and EV-4B). By, BN-PAGE, we do not observe any major differences in OXPHOS complexes abundance between wildtype controls and mOPA1 KO and DKO mice. These data suggest that the impaired mitochondrial respirations that we observed might reflect the impact of OPA1 deficiency on mitochondria cristae integrity and density, which are regulated by OPA1, rather than loss of OXPHOS complexes per se.

- How do authors explain the increased distance of mOPA1 KO during the exhaustion test? Are those differences already apparent at early time points as 12 or 20 weeks?

RESPONSE:
At 40 weeks of age, mOPA1 KO mice are considerably leaner than WT mice. Our calculation of vertical work, which accounts for body mass differences, suggests that both genotypes performed the same amount of work. Therefore, we believe mOPA1 KO mice ran a longer distance simply because they weigh considerably less. Indeed, when we performed the same test in younger mice (12 weeks of age), when body weight differences are not as accentuated, mOPA1 KO mice ran the same distance as WT mice (Fig. EV-2C,D,E).

- No effect in liver is shown. Are there any changes in hepatic steatosis? And in blood parameters?

RESPONSE:
We have now analyzed liver and serum triglycerides levels in WT and mOPA1 mice fed either a control diet or a HFD for 12 weeks. Consistent with systemic metabolic findings, high-fat fed mOPA1 KO mice exhibit reduced hepatic steatosis and decreased circulating concentrations of triglycerides in the serum (Fig EV3A,B).

- The observed increase in EE in mOPA1 mice in both CD and HFD suggests an increase in BAT function. Analysis of BAT functionality (e.g. mitochondrial respiration) would increase the strength of the results. Also, are there any differences in cold tolerance and/or browning?

RESPONSE:
We appreciate the reviewer's point. Due to limitations in animal availability and the time constraints in submitting the revised manuscript we were unable to measure mitochondrial respiration in BAT or to examine the animals' response to cold exposure. However, we examined indices of brown fat activation in BAT and inguinal subcutaneous fat (scWAT) in samples previously collected from 20-week old mice. Although we observe no significant differences in PGC-1α or UCP1 in BAT of mOPA1 KO mice, importantly, we observed a significant increase in UPC1 and a trend towards increased PGC-1α in sc-WAT, consistent with browning of white adipose tissue (Fig. EV-3E,F).

- Physiological parameters of mOPA1 KO under HFD were similar to WT mice under CD. This could suggest that HFD improves physiology of mOPA1 KO as happens for other models. Does HFD rescue any muscle related defect? (e.g. fiber size, Murf1 or atrogin-1 expression levels, ...).

RESPONSE:
It does not appear that HFD improves physiology of mOPA1 KO muscle. Although we were not able to obtain data on atrophy markers or fiber diameter for these animals given constraints on time and animals, our NMR data demonstrates that the reduction in total lean mass observed in mOPA1 KO mice is not prevented when these mice are fed a HFD, suggesting that muscle atrophy persists in these animals (See Fig. 3B,C).

- Cellular experiments with C2C12 should be performed with a knockdown model of OPA1. Mitochondrial stress can activate the integrated stress response in vitro by different mechanisms. Therefore, to specifically assess the role of ER stress and AMPK activation, an OPA1 deficient cellular model is required.

RESPONSE:
We appreciate your suggestions very much. To address these concerns, we isolated satellite cells from OPA1 $^{fl/fl}$ mice and deleted OPA1 in differentiated myotubes by infecting these cells with an adenovirus expressing Cre recombinase. In conjunction, we further investigated the role of ER stress on FGF21 induction in muscle in vivo and in vitro by treating OPA1-deficient animals and differentiated myotubes with ER stress inhibitors, TUDCA and PBA, respectively. In both paradigms, attenuation of ER stress significantly reduced FGF21 induction in muscle cells, and circulating concentrations of FGF21 or release into the media respectively (Fig. 6F,G,H and Fig. 7A,B). OPA1 KO myotubes also demonstrated impaired oxidative capacity, increased glycolytic compensation, and enhanced susceptibility to oxidative stress-mediated disruption in membrane potential relative to WT cells (Fig. 7C,D,E,F). Although AMPK phosphorylation was increased in OPA1 deficient myotubes, it was not reversed by PBA treatment, despite a significant reduction in FGF21 levels. Together with our data in C2C12 myotubes (Fig. EV-5D,E) showing that pharmacological activation of AMPK does not result in FGF21 induction, we conclude that it is unlikely that AMPK plays a role in FGF21 induction in response to OPA1 deficiency.

Minor points:
- Are there any changes in the number of fibers of mOPA1 KO mice?

RESPONSE:
We have observed an increased number of fibers/area in mOPA1 KO mice (Fig. 2F).

- Western-blots from mice should be provided with more than n=1.

RESPONSE:
We now show at least 2 samples per genotype and/or condition for all western blot images.

- Figures need to be revised. Proportions are not conserved and some plots and text are deformed.

RESPONSE:
All figures have been reformatted to conform with the Journal's requirements and plots and texts are no longer distorted.

Referee #3:The role of FGF21 in total body metabolic adaptation following mitochondrial dysfunction is a timely and significant issue, but controversial. Recent data indicate that the type of mitochondrial dysfunction determines either whether FGF21 is induced or whether it contributes to increased glucose tolerance during high fat diet. In this respect, the study by Pereira et al. presents potentially important findings in the mouse model of induced OPA1 depletion. While the descriptive data on the metabolic phenotype of the inducible OPA1 KO and OPA1/FGF21 DKO mice are solid, unfortunately the work does not give sufficient mechanistic insight to address the outstanding question: what differentiates their model from previously published negative results on the role of FGF21 in whole body metabolic adaptations to mitochondrial stress? My main concerns are the following:
1. It is not determined what is the exact mitochondrial metabolic defect in the OPA1 deficient muscle. Fig. 1. shows that ADP driven respiration in mice up to 20 weeks is basically maintained (Fig. 1D), while mitochondrial ATP production is drastically reduced (Fig. 1G). It is difficult to explain this finding. It cannot be the consequence of uncoupling, but rather a previously unseen effect on the efficiency on the ATP synthase or simply increased ATP consumption in the saponin-permeabilised fibers? How total ATP content differs between WT and KO mice in intact fibers? Is there glycolytic compensation in intact muscle? Is membrane potential reduced? These data would be required to conclude on relating the full body phenotype to muscle mitochondrial defect.

RESPONSE:
The reviewer raises a very good point. Although by BN-PAGE we do not observe major differences in the abundance of OXPHOS complexes (Fig. 1C), the possibility remains that complex V activity could be diminished. In new studies using Seahorse analysis in OPA1-deficient myotubes (Fig. 7C), we provide evidence for decreased ATP-linked (oligomycin-inhibitable) respirations, which could reflect reduced ATP Synthase activity in intact mitochondria. Because of limitations in animals and tissues we have not measured ATP content in permeabilized fibers to assess for the possibility of increased ATP consumption in these fibers. However, our Seahorse data in mOPA1 deficient primary myotubes showing reduced ATP-linked respirations coupled with increased glycolysis suggests a primary defect in mitochondrial bioenergetics. Moreover, the demonstration of AMPK activation in skeletal muscle (Fig. 6A) and in myotubes (Fig. 7A) is consistent with decreased ATP generation and availability in intact cells. We have also observed reduced membrane potential in OPA1-deficient myotubes, which in conjunction with reduced proton leak in Seahorse analyses indicate that the reduction in membrane potential is not likely secondary to mitochondrial uncoupling but to impaired electron chain function. Taken together, these data indicate that OPA1 deficiency impairs mitochondrial bioenergetics by a mechanism related to an inability to maintain membrane potential likely on the basis of reduced electron flow and potentially by decreased ATP synthase function/activity in intact mitochondria. Moreover, we also believe that our data suggests that an important basis for reduced mitochondrial respirations and ATP synthesis could be impaired OPA1-mediated crista integrity.

2. Along the same lines, it has been recently shown that in human disease and mouse models, FGF21 induction is associated with mtDNA translation defects and mtDNA deletions, while no FGF21 response is observed in direct respiratory chain defects. OPA1 depletion has been associated with both loss of respiratory chain integrity and loss of mtDNA maintenance, thus the mtDNA phenotype of both the OPA1 KO and OPA1/FGF21 DKO muscle should be determined**.**

RESPONSE:
We measured mtDNA content in both mOPA1 KO and DKO mice, and observed no differences relative to their WT controls. BN-PAGE also did not reveal any obvious changes in mitochondrial OXPHOS complexes in these mice, despite significant reduction in oxidative capacity and ATP synthesis rates. Thus, in this model of reduced OPA1, FGF21 induction is secondary to impaired electron transport chain function that is independent of changes in mitochondrial DNA.

3. To conclude on the effect of FGF21 in the OPA1/FGF21 DKO, it is essential that OPA1 levels are reduced to the same level as in the OPA1 KOs. This does not seem to be the case, the authors report 70% reduction in OPA1 KO (Fig. 1) and about 50% reduction in OPA1/FGF21 DKOs (Fig. S4), and according to the representative blots the KO effect induction by tamoxifen is quite heterogeneous. The authors should determine how the primary OPA1 KO phenotype correlates with OPA1 levels in individual mice to resolve this issue.

RESPONSE:
We agree that OPA1 deletion is incomplete and heterogeneous in our mice and that our model is hypomorphic for OPA1 deletion. Although OPA1 levels are not equivalently reduced in mOPA1KO or DKO gastrocnemius muscles (60% versus 40% respectively, Fig. 1A, Fig. EV4A), OPA1 levels were reduced by 40% in soleus muscle from mOPA1 KO mice, which however revealed a 4-fold significant induction of FGF21 (Fig EV-3D). Moreover, other major phenotypes such as impaired mitochondrial respiratory capacity and decreased fiber diameter exist in different muscle types in mOPA1 KO and in DKO mice (Fig. 1, Fig. 2C, Fig. EV-4A,B,C,D,E) in which OPA1 levels were reduced by 40% to 60%. These data strongly suggest that hypomporphic OPA1 expression in the ranges observed in the models evaluated in this study significantly impact the extent of FGF21induction, rendering it relatively unlikely that the phenotype of DKO mice is secondary to a lower degree of reduction of OPA1/FGF21 DKO in gastrocnemius muscle relative to OPA1 KO animals.

4. Along the same lines, there is significant reduction of total body weight in OPA1 KOs (Fig. 2A), which the authors attribute to muscle atrophy and not to changes in fat mass (Fig. 2C). On the other hand, in OPA1/FGF21 DKOs loss of body weight did not occur (Fig. S4B). Does this mean that FGF21 causes the reduction in total lean mass? Or is it related to less severe OPA1 reduction? How is muscle fiber diameter in these mice? Overall, the mitochondrial difference between phenotypes) and muscle phenotype should be characterised in the OPA1/FGF21 DKO mice to resolve this issue**.**

RESPONSE:
Our data suggest that a reduction in lean mass and fat mass both contribute to the significant reduction in body weight observed in mOPA1 KO mice. In fact, given the relatively mild muscle atrophy observed in our mice, it is likely that reduced fat mass contributes predominantly to the changes in body weight (Fig. 3C). Nonetheless, we have taken the reviewer's concerns into consideration and performed additional experiments to better characterize overall muscle health in DKO mice, as suggested. Although body weight and total body mass were not reduced in DKO mice at the time points analyzed (Fig. 5C,D,E), fiber diameter was significantly reduced (Fig. EV4). Thus it is most likely that muscle atrophy represents the consequence of OPA1 deficiency and fat mass loss is secondary to FGF21.

Minor comment: MW markers on WBs should be shown. Some blots (e.g. Fig. 5A) are pieced together, and the CB loading standard is very faint, which is not up to standards.

RESPONSE:
In response to your concerns that of other reviewers we have considerably improved the representation of our western blot images.

2nd Editorial Decision 07 May 2017

Thank you for submitting the revised version of your manuscript. It has now been seen by the three original referees, whose comments are enclosed below.

As you will see, the third referee remains more critical on the study than the two others, however we

decided - in light of the strong support of referees #1 and #2- to give you the opportunity to revise your manuscript to address this referee's points. Thus, I would like to invite you to submit a final revised version of the manuscript using the link enclosed below, addressing the reviewer's comments.

Both referee #1 and referee #2 find that their concerns have been sufficiently addressed and are broadly in favour of publication. Referee #3 states that your claims on OPA1-KO-induced mitochondrial impairment by diminished complex V activity and inability to maintain membrane potential are not sufficiently supported by the data at this stage. This referee points to alternative hypotheses to explain the phenotype observed with altered O2 consumption/superoxide production and asks you to consider these. Thus, I ask you to revise your manuscript regarding the point raised by referee #3 and evaluate, whether you would be able to add supportive complementary data as indicated, or, alternatively, relativise your statements and introduce caveats where appropriate. Please note, that while these points are well taken, taking into account the positive comments of referee #1and referee #2, we have decided that pending a satisfactory revision, we would go ahead with acceptance of this manuscript as soon as possible.

Please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your final revision.

REFEREE REPORTS

Referee #1:

The authors addressed the issues previously raised.

Referee #2:

The authors have addressed all my previous comments and criticisms and the manuscript has substantially improved.

Referee #3:

The paper has been significantly enhanced with new data and interpretations improved. The only concern remaining is the response to my previous question 1, which is not fully satisfactory. The authors state that 'the possibility remains that complex V activity could be diminished'. Reduced overall activity of the ATP synthase cannot explain reduced ATP production to the same amount of ADP driven oxygen consumption in 12 wks old mice (Fig. 1G-H in the revised version). Apart from the remote possibility of changing H+/ATP coupling efficiency in the ATP synthase, another hypothesis to explain these findings is that O2 consumption is linked to increased superoxide production on complex III (since succinate/rotenone has been used as substrates in these experiments). Were O2 consumption data normalised to e.g. Antimycin-A or to complex II inhibited respiration, in order to test this possibility?
Also, the units of O2 consumtion in Fig. 1G and I,J are different which is slightly confusing.

2nd Revision - authors' response 08 May 2017

Response to Reviewers

Referee # 3:

The paper has been significantly enhanced with new data and interpretations improved. The only concern remaining is the response to my previous question 1, which is not fully satisfactory. The authors state that 'the possibility remains that complex V activity could be diminished'. Reduced overall activity of the ATP synthase cannot explain reduced ATP production to the same amount of ADP driven oxygen consumption in 12 wks. old mice (Fig. 1G-H in the revised version). Apart from the remote possibility of changing H+/ATP coupling efficiency in the ATP synthase, another hypothesis to explain these findings is that O2 consumption is linked to increased superoxide production on complex III (since succinate/rotenone has been used as substrates in these experiments). Were O2 consumption data normalised to e.g. Antimycin-A or to complex II inhibited respiration, in order to test this possibility?
Also, the units of O2 consumption in Fig. 1G and I,J are different which is slightly confusing.

RESPONSE:
We thank the reviewer for this comment and agree that the reduction in ATP synthesis exceeds the level of reduction in ADP driven oxygen consumption in 12-week-old mice. Indeed, when we calculated the ATP/O ratio in these animals, this ratio was significantly lower at the age of 12-weeks. However, ATP/O ratios were not different in older mice. As such we believe that mitochondrial uncoupling could be present in young animals. We have added this statement to the results on page 4 of the revised manuscript (see *highlighted* sections below).

"Oxygen consumption was impaired starting at 20 weeks of age (Fig 1G) and was further reduced at 40 weeks of age (Fig EV1C) in mOPA1 KO mice relative to WT mice. ATP synthesis rates were reduced as early as 12 weeks, and persisted at later time points (Fig 1H and Fig EV1D). *ATP/O ratios were decreased at the age of 12-weeks (Data not shown), which could reflect mitochondrial uncoupling, but differences in ATP/O were not seen in older animals."*

In addition, we have modified the discussion on page 10 of the revised manuscript to acknowledge this possibility (see highlighted sections below).

"Of note, our data challenge a recent study, suggesting that FGF21 plays a negligible role on the whole body metabolic adaptations reported in mice with decreased muscle mitochondrial efficiency due to increased respiratory uncoupling (Ost, Coleman et al., 2016). In contrast, we were able to completely reverse the whole body metabolic adaptations observed in OPA1-deficient mice, by simultaneously deleting FGF21 from skeletal muscle, suggesting a clear role for muscle-derived FGF21 on the metabolic improvements observed in our model. Differences in the source of mitochondrial stress, the temporal nature of our inducible OPA1 deletion, and the tissue specificity of FGF21 deletion in our model could underlie the differences between our data and that of the aforementioned study (Ost et al., 2016). *Decreased ATP/O ratios in young mice suggest that mitochondrial uncoupling could exist in the OPA1 deficient mice examined in this study and we did not directly determine if increased complex III generated superoxide could contribute to this. However, our in vivo and in vitro models suggest that additional mechanisms contribute to the bioenergetic failure following OPA1 reduction. These include impaired electron transport chain function, decreased ATP generation and potentially impaired mitochondrial membrane potential, likely related to disrupted crista structure. These defects in aggregate, may activate mechanisms that lead to induction of FGF21, in contrast to the scenario when mitochondrial uncoupling is the primary basis for mitochondrial energetic compromise.*

The methods used to measure oxygen consumption in permeabilized fibers in Figure 1G used a ruthenium red oxygen sensor probe (Ocean Optics, Dunedin, FL) and is different from the method used to measure oxygen consumption in the data presented in Figure 1G-H, which was obtained using the Oroboros O_2K Oxygraph system that we just acquired. Both methods are described in the methods section. This is the basis for the different units.

3rd Editorial Decision 09 May 2017

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and discussed your response to the remaining concerns of referee #3 in the editorial team. From these considerations we have concluded that the concerns have been sufficiently addressed.

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dale E. Abel
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-96179

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For well-stablished techniques in the lab, adequate sample size has been previously determined by power analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was estimated based on previous studies performed in the laboratory. In general, for well-stablished techniques routinly performed in the lab, power analysis has been previously performed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Outliers were eliminated by the "Find outliers" function in the GraphPad Prism software. Technical replicates were eliminated if a technical problem was identified (low quality of RNA or stain on a immunoblot, for example). After euthanasia, animals in which knockdown of OPA1 wasn't confirmed by immunoblot were also removed from the study.
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.	All mice were injected with tamoxifen. After genotyping, wild type and KO mice were randomly assigned to various experiments. Whenever possible, investigators were blinded to the animals'/ samples' genotype and/or treatment.
For animal studies, include a statement about randomization even if no randomization was used.	Mice from either genotype were randomly distributed to each experiment and treatment.
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.	Every cohort of mice was composed of a mixture of different genotypes and treatments. Whenever possible, investigators were blinded to the animals'/ samples' genotype and/or treatment. Some experiments such as, mRNA expression, mt-DNA copy number, sehorse analysis, histological analysis and measurements of triglycerides were performed in a double-blinded fashion.
4.b. For animal studies, include a statement about blinding even if no blinding was done	To assure that an appropriate number of mice/genotype/treatment would be achieved, intial genotyping was performed and the proper number of animals from each genotype was randomly assigned to different treatments. Whenever possible, investigators were blinded to the animals'/ samples' genotype and/or treatment during the experiments and/or data analysis
5. For every figure, are statistical tests justified as appropriate?	We believe the statistical tests utilized are appropriate for each figure
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Whenever the "n" was large enough, normality distribution was assessed by the D'Augustino-Perason omnibus normality test.
Is there an estimate of variation within each group of data?	Yes. Especially with animal studies
Is the variance similar between the groups that are being statistically compared?	Generally, yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used have been previously validated and company and catalog numbers are provided in the main text of the manuscript. References are provided in the respective Company's webpages.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	C2C12 cells utilized in these studies were acquired from ATCC. No STR profiling was performed and cells were not assayed for mycoplasma contamination.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All experiments were performed in C57Bl6J males between 8-40 weeks of age. To generate muscle-specific OPA1 KO mice (mOPA1 KO) we crossed OPA1fl/fl mice with tamoxifen-inducible HSA-CreERT2 mice. OPA1fl/fl mice lacking the Cre recombinase were utilized as WT controls. To generate muscle-specific OPA1/FGF21 double knockout mice (DKO), OPA1fl/fl harboring the tamoxifen-inducible Cre recombinase (Cre-ERt2) under the expressional control of the human skeletal actin (HSA) promoter were crossed with FGF21fl/fl mice. Heterozygous mice for OPA1 and FGF21 genes containing the Cre-ERT2 were crossed with heterozygous mice lacking the Cre recombinase to generate OPA1/FGF21 homozygous mice. Once mice homozygous for both genes containing the Cre-ERT2 were generated, those were then crossed with homozygous mice lacking the Cre recombinase, which were also utilized as WT controls. Animals were housed at 22ºC with a 12-hour light, 12-hour dark cycle with free access to water and standard chow (2029X Harlan Teklad, Indianapolis, IN). All animals were injected with tamoxifen.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal work was performed in accordance with protocols approved by the University of Iowa Animal Care and Use Committee (IACUC).
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.	We confirm complience with the ARRIVE guidelines

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 accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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).	NA
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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA