EMBO reports

# Loss of mitochondrial protease ClpP protects mice from diet-induced obesity and insulin resistance

Shylesh Bhaskaran, Gavin Pharaoh, Rojina Ranjit, Ashley Murphy, Satoshi Matsuzaki, Binoj C. Nair, Brittany Forbes, Suzana Gispert, Georg Auburger, Kenneth M. Humphries, Michael Kinter, Timothy M. Griffin and Sathyaseelan S. Deepa

**Review timeline:**

| | |
|---|---|
| Submission date: | 15 August 2017 |
| Editorial Decision: | 12 September 2017 |
| Revision received: | 14 November 2017 |
| Editorial Decision: | 1 December 2017 |
| Revision received: | 8 December 2017 |
| Accepted: | 22 December 2017 |

Editor: Martina Rembold

## 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 12 September 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, they also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. Referee 2 indicates that the increased mitochondrial biogenesis data should be strengthened and referee 3 suggests to investigate the involvement of mitochondrial chaperones and the relationship between the UPRmt and OXPHOS in more detail. The signal that drives PGC1a upregulation would certainly be of interest but a conclusive answer might be beyond the scope of a revision.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main

 

HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

**********
REFEREE REPORTS

Referee #2:

Bhaskaran describe the metabolic phenotype of ClpP knockout mice. These mice have reduced body

 

weight and improved insulin sensitivity. The changes in body weight are in line with previous publications on the ClpP knockout (Gispert et al 2013), but the authors solidify the metabolic phenotype by additional measurements and they link the phenotype to adipose tissue mitochondrial biogenesis.

The experimental design and execution is solid. There are, however, a few discrepancies in the paper that make interpretation of the data difficult and it would be great if the authors address these issues experimentally or by modifying the conclusions of their manuscript.

(1) The increased mitochondrial biogenesis data should be strengthened, because PGC-1a and Tfam protein levels is not enough. Also the mitochondrial images in Fig 1G-H are of poor quality and difficult to judge. Measuring citrate synthase activity and/or mtDNA copy number are the golden standard and should be added. Having more mitochondria may also explain some of the respiratory phenotypes in Fig 6, and the authors should consider normalizing their Oroboros data for CS activity. Is OXPHOS protein expression changed in WAT and other tissues of the ClpP knockouts? And is mitochondrial respiration altered in other tissues as well?

(2) The authors primarily link the phenotypic changes to WAT mitochondrial biogenesis but at the same time there are some drastic molecular/metabolic changes in other tissues as well, even in the absence of changes in PGC-1a expression. For instance, Akt phosphorylation is markedly higher in muscle and liver following insulin stimulation. It is therefore unclear whether the WAT mitochondrial biogenesis really explains the clinical phenotype. Rather, these seems to be a multi-tissue contribution that is yet to be uncovered. The conclusions in the abstract should hence be less WAT-centric.

(3) The conclusions on metabolic phenotypes are much influenced by the fact that the mice are leaner. For instance, it is not surprising that ClpP knockouts have better insulin sensitivity given their lean phenotype. It is even more striking that they don't appear to have improved glucose tolerance (Fig 4A). These issues should be carefully discussed.

Minor issues:
(4) The increased UCP1 expression in sWAT suggests browning. Please show some other markers to bolster this phenotype.
(5) Page 9 typo: assosictaed associated

Referee #3:

The paper of Bhaskaran et al., describes an interesting new role of the mitochondrial protease ClpP to regulate whole body physiology. This work uncovers a novel link between a ClpP-deficiency induced compensatory response, most pronounced in WAT, to increase mitochondrial function which has beneficial systemic metabolic effects and protects from high-fat diet induced obesity. Although it lacks mechanistic insights about ClpP loss and the triggered mitochondrial induction, as well as no explanation of why it only/mainly occurs in WAT, the paper describes a novel and interesting finding, which I think is well suited for the readership of Embo reports. The novelty that ClpP is not only required for UPRmt (might even be dispensable), but also to regulate physiological hoemeostasis is an important point in the understanding of WAT mitochondria-whole body crosstalk.

However, a few clarifications are needed in my view, before publication.

Specific points:
1. The current view seems to be that UPRmt attenuates OXPHOS and shifts metabolism to glycolysis, which is in contrast to the observations here. Does the loss of ClpP impairs proper UPRmt, that allows the bypass of OXPHOS attenuation? Is Mitophagy activated, which would allow the recovery of healthy mitochondria?
2. The Tom20 staining in 1G should be quantified over several images/cells and presented with statistics.
3. The last author had a paper describing a mitochondrial dysfunction in muscle cells upon ClpP knockdown, whereas the knockout mouse muscle does not seem to be influenced by loss of ClpP. It

 

would be helpful to briefly discuss this.
4. It would be interesting to look for a potential signal that drives PGC1a upregulation. Are ROS levels changed upon ClpP loss? Can UPRmt signal to PGC1a?
5. Is it known if the mitochondrial chaperones are regulated by PGC1a?
6. In order to determine if changes in chaperone expression result from increased mitochondrial number, one could isolate mitochondria and blot/analyse expression normalised to mitochondria.
7. Fig7 shows a downregulation of ClpP in WT mice on HFD in muscle. Is there also in change in expression of Lon, Hsp60/40/10 and ClpX? Does HFD lead to UPRmt induction?

Minor points:
1. Fig. 2B, D, 4C
Were the seperated blots run on different gels? I understand that relative quantification to tubulin should make it comparable (if tubulin is equal between wt and ko), but it would be better to have them presented on the same blot.
2. The results for Figure 6C: "succinate to measure OXPHOS capacity through complex I and II" Since succinate is only substrate for Complex II, this setup would only measure CII activity. Please rephrase.

1st Revision - authors' response 14 November 2017

Reviewer's comments to authors:

Referee #2:

The study describe the metabolic phenotype of ClpP knockout mice. These mice have reduced body weight and improved insulin sensitivity. The changes in body weight are in line with previous publications on the ClpP knockout (Gispert et al 2013), but the authors solidify the metabolic phenotype by additional measurements and they link the phenotype to adipose tissue mitochondrial biogenesis. The experimental design and execution is solid. There are, however, a few discrepancies in the paper that make interpretation of the data difficult and it would be great if the authors address these issues experimentally or by modifying the conclusions of their manuscript.

(1) *The increased mitochondrial biogenesis data should be strengthened, because PGC-1a and Tfam protein levels is not enough. Also, the mitochondrial images in Fig 1G-H are of poor quality and difficult to judge. Measuring citrate synthase activity and/or mtDNA copy number are the golden standard and should be added. Having more mitochondria may also explain some of the respiratory phenotypes in Fig 6, and the authors should consider normalizing their Oroboros data for CS activity. Is OXPHOS protein expression changed in WAT and other tissues of the ClpP knockouts? And is mitochondrial respiration altered in other tissues as well?*

- As suggested by the reviewer, we included more markers of mitochondrial biogenesis in the revised manuscript: protein expression of electron transport chain (ETC) subunits (Figure 2B); mtDNA content (Figure 2C), and citrate synthase protein levels (Figure S2A) and all these measures showed increased expression in WAT of *ClpP*$^{-/-}$ mice compared to wild type mice (included in the result section, page 7).
- We agree with comment by the reviewer that 'more mitochondria may explain some of the respiratory phenotypes in Fig 6' and our data in the revised Figure 2 support increased mitochondrial mass in white adipose tissue of *ClpP*$^{-/-}$ mice. The reviewer has suggested normalizing the Oroboros data with CS activity. However, this is technically difficult because we are using only 40-60mg of adipose tissue for the experiments using Oroboros and to recover the tissue for CS activity after experiment is nearly impossible. Because of this reason, we normalized the data using amount of tissue used for the assay. However, based on the suggestion from the reviewer, we have revised the manuscript stating that increased respiration may be due to increased mitochondrial content (page 6).
- The reviewer asked whether OXPHOS protein expression changed in WAT and other tissues of the ClpP knockouts. We looked at the expression of ETC subunits in WAT in our proteomic analysis and found that ETC subunits are significantly elevated in *ClpP*$^{-/-}$ mice adipose tissue (Figure 2B). Gispert et al. (2013) looked at the protein expression of ETC

subunits in testis, heart, liver and brain and found that expression of none of these subunits are increased in these tissues, rather they found a decrease in complex I subunit (ND6) in testis and brain, and complex IV-1 subunit in testis and liver (Table 1 of Gispert et al., 2013). This information is included in the discussion (page 15).

- The reviewer also asked whether mitochondrial respiration is altered in other tissues as well. We have measured respiration only in adipose tissue. Previously, Gispert *et al* (2013) measured mitochondrial respiration in heart, skeletal muscle, and brain, and found that respiration is reduced in the heart of *ClpP*$^{-/-}$ mice, whereas respiration in muscle and brain are similar to wild type mice. Szczepanowska K *et al* (2016) also reported reduced mitochondrial respiration in heart of *ClpP*$^{-/-}$ mice compared to wild type mice. Thus, ClpP deficiency shows a differential effect on respiration in tissues. This information is included in the discussion of revised manuscript (page 15).
- We have also replaced the mitochondrial images in Figure 1, as suggested by the reviewer.

*(2) The authors primarily link the phenotypic changes to WAT mitochondrial biogenesis but at the same time there are some drastic molecular/metabolic changes in other tissues as well, even in the absence of changes in PGC-1a expression. For instance, Akt phosphorylation is markedly higher in muscle and liver following insulin stimulation. It is therefore unclear whether the WAT mitochondrial biogenesis really explains the clinical phenotype. Rather, these seems to be a multi-tissue contribution that is yet to be uncovered. The conclusions in the abstract should hence be less WAT-centric.*

- Based on the suggestion from the reviewer, we modified the conclusions in the abstract and we agree with the reviewer's suggestion that WAT mitochondrial biogenesis is not the only cause of the phenotype, rather one of several adaptations to ClpP deficiency in a tissue-specific manner.

*(3) The conclusions on metabolic phenotypes are much influenced by the fact that the mice are leaner. For instance, it is not surprising that ClpP knockouts have better insulin sensitivity given their lean phenotype. It is even more striking that they don't appear to have improved glucose tolerance (Fig 4A). These issues should be carefully discussed.*

- This is a very important question raised by the reviewer. *ClpP*$^{-/-}$ mice fed normal chow are insulin sensitive, however their glucose clearance in response to glucose tolerance test is similar to wild type mice. This would suggest that the *ClpP*$^{-/-}$ mice have a lower or slower insulin release in response to the glucose challenge and in support of this, insulin levels in *ClpP*$^{-/-}$ mice in fed state is lower than wild type mice (Figure 4D). Thus, a reduction in glucose-induced insulin secretion could be a potential reason why we don't see improved glucose clearance in chow fed animals. However, when fed HFD, wild type mice develop glucose intolerance and therefore the difference between wild type and *ClpP*$^{-/-}$ mice in glucose clearance become more obvious. This information is included in the discussion of the revised manuscript (page 19).
-

Minor issues:

*(4) The increased UCP1 expression in sWAT suggests browning. Please show some other markers to bolster this phenotype.*

- We looked at the transcript level of browning markers in sWAT of *ClpP*$^{-/-}$ mice and found that expression of PGC1a, CIDEA, Cox8b are higher in *ClpP*$^{-/-}$ mice, whereas expression of Prdm16 was similar in *ClpP*$^{-/-}$ mice and wild type mice. This information is included in the revised manuscript (Figure S3A) and also in results (page 10).

*(5) Page 9 typo: assosictaed associated*

- Typo is corrected in the revised manuscript (page 10).

Referee #3:

The paper describes an interesting new role of the mitochondrial protease ClpP to regulate whole body physiology. This work uncovers a novel link between a ClpP-deficiency induced

compensatory response, most pronounced in WAT, to increase mitochondrial function which has beneficial systemic metabolic effects and protects from high-fat diet induced obesity.
Although it lacks mechanistic insights about ClpP loss and the triggered mitochondrial induction, as well as no explanation of why it only/mainly occurs in WAT, the paper describes a novel and interesting finding, which I think is well suited for the readership of Embo reports. The novelty that ClpP is not only required for UPRmt (might even be dispensable), but also to regulate physiological homeostasis is an important point in the understanding of WAT mitochondria-whole body crosstalk. However, a few clarifications are needed in my view, before publication.

Specific points:

1. *The current view seems to be that UPRmt attenuates OXPHOS and shifts metabolism to glycolysis, which is in contrast to the observations here. Does the loss of ClpP impairs proper UPRmt, that allows the bypass of OXPHOS attenuation? Is Mitophagy activated, which would allow the recovery of healthy mitochondria?*

- The finding that ClpP is critical for the initiation of UPR$^{mt}$ and UPR$^{mt}$ initiation will shift cell metabolism from respiration to glycolysis was made in *C. elegans* (Haynes et al., 2007; Nargund et al., 2012). In contrast, many aspects of mammalian UPR$^{mt}$ are less well understood, even though loss of mitochondrial proteostasis is shown to increase the expression of Hsp60 and ClpP in mammalian cells (Zhao et al., 2002; Houtkooper et al., 2013). The role of ClpP in mammalian UPR$^{mt}$ was assumed to be similar to that C.elegans, based on the studies by Hayes et al. (2007) in *C.elegans* and Zhao et al. (2002) in mammalian cells. However, a definite role of ClpP in the initiation of mammalian UPR$^{mt}$ and how UPR$^{mt}$ affects metabolism in mammals is not known. Recent study by Seiferling et al. (2016) suggest that ClpP is neither required for, nor it regulates the UPR$^{mt}$ in mammals. Their study demonstrated that a strong mitochondrial cardiomyopathy and diminished respiration due to DARS2 deficiency can be alleviated by the loss of ClpP. Thus, further studies are needed to understand the role of ClpP in mammalian UPR$^{mt}$ and UPR$^{mt}$-associated metabolic shift. This information is included in the discussion of revised manuscript (page 18-19).
- To test whether mitophagy is activated, we measured the protein expression of PINK1 and Parkin as markers of mitophagy (Narendra et al., 2010) in WAT of *ClpP*$^{-/-}$ mice. Surprisingly, we found that expression of PINK1 and Parkin are decreased in WAT of *ClpP*$^{-/-}$ mice. This information is included in Figure S2C and results section of the revised manuscript (page 9).

*2. The Tom20 staining in 1G should be quantified over several images/cells and presented with statistics.*

- Quantification of the images is presented in revised Figure 1G and the methodology for quantification is given in page 27.

*3. The last author had a paper describing a mitochondrial dysfunction in muscle cells upon ClpP knockdown, whereas the knockout mouse muscle does not seem to be influenced by loss of ClpP. It would be helpful to briefly discuss this.*

- In our previous study in C2C12 muscle cells, we did an acute knock down and found that decline in ClpP (70% down-regulation) can cause mitochondrial dysfunction (*Deepa SS et al., Free Radic Biol Med., 2016*). However, in *ClpP*$^{-/-}$ mice, loss of ClpP is a chronic effect that is compensated by molecular adaptations. Thus, the lack of adaptations in acute knockdown could explain the differential outcome. This aspect is discussed in the revised manuscript (page 18).

*4. It would be interesting to look for a potential signal that drives PGC1a upregulation. Are ROS levels changed upon ClpP loss? Can UPRmt signal to PGC1a?*

- This is a very important suggestion by the reviewer. In literature, there is evidence that increased of ROS is linked to activation of PGC-1a expression and PGC-1a levels are increased in response to an oxidative stressor, $H_2O_2$, in skeletal muscle (Irrcher *et al*., 2009, Silveira *et al*., 2006, St-Pierre *et al*., 2006). In humans also, in response to oxidative stress induced by short-term exercise increases PGC1a expression in skeletal muscle (Ristow *et al.,* 2009). Based on these findings, we also tested whether ROS levels are increased in adipose tissue of *ClpP*$^{-/-}$ mice that showed an increase in PGC-1a levels. We measured levels of 4-Hydroxynonenal (4-HNE), a marker of oxidative stress in adipose tissue by western blotting and found that levels of 4-HNE are significantly increased in *ClpP*$^{-/-}$ mice

 

compared to wild type mice, suggesting increased oxidative stress in adipose tissue (Figure 2E). Elevated levels of $H_2O_2$ is shown to induce PGC-1a expression through activation of AMPK in skeletal muscle (Irrcher *et al*. 2009). Assessing AMPK activation in adipose tissue of *ClpP*$^{-/-}$ mice showed that the ratio of Phospho-AMPK/AMPK is increased in *ClpP*$^{-/-}$ mice compared to wild type mice, suggesting increased AMPK activation (Figure 2F). Thus, increase in ROS and AMPK activation could contribute to the increased expression of PGC-1a in WAT of *ClpP*$^{-/-}$ mice. These data are included in the revised manuscript (page 7).

- At present, there is no evidence to show that UPR$^{mt}$ can induce PGC-1a expression. Although this is an important question, answering this question is beyond the scope of this revision.

*5. Is it known if the mitochondrial chaperones are regulated by PGC1a?*

- The transcription factors CHOP and C/EBPβ are the proposed transcription factors for mitochondrial chaperones and they bind to the conserved regulatory element in promoters of the UPR$^{mt}$ related genes (e.g., Hsp60, Hsp10, Hsp40, ClpP etc.) (Aldridge et al., 2007). However, no information is available in the literature that shows that PGC-1a regulates mitochondrial chaperones. This information is included in the revised manuscript (page 17).

*6. In order to determine if changes in chaperone expression result from increased mitochondrial number, one could isolate mitochondria and blot/analyse expression normalised to mitochondria.*

- This is a great suggestion by the reviewer. However, adipose tissue contains very few mitochondria compared to skeletal muscle or heart and isolation of mitochondria from fat tissue require large amounts of fat tissue to begin with. Based on our data in Figure 2 it is possible that increase in mitochondrial chaperones in adipose tissue of *ClpP*$^{-/-}$ mice is a reflection of elevated mitochondrial number in adipose tissue. However, in other tissues that showed an increase in mitochondrial chaperones (testis, heart, liver, brain ), mitochondrial biogenesis marker Tfam or porin was not elevated suggesting that mitochondrial chaperones are induced in those tissues (Gispert et al., 2013). Similarly, in *ClpP*$^{+/-}$ mice adipose tissue, mitochondrial chaperones Hsp60 and HSp40 are elevated and *ClpP*$^{+/-}$ mice showed no change in mitochondrial biogenesis markers, suggesting mitochondrial chaperone induction (Figure 2G). This is discussed in the revised manuscript (page 15).

*7. Fig7 shows a downregulation of ClpP in WT mice on HFD in muscle. Is there also in change in expression of Lon, Hsp60/40/10 and ClpX? Does HFD lead to UPRmt induction?*

- We observed a small reduction in ClpP expression in skeletal muscle of WT mice fed HFD, however this difference was not statistically significant (Figure 7B). However, a significant reduction in ClpP protein was observed in white adipose tissue of WT mice fed HFD (Figure 7A). Our published study (Bhaskaran et al., 2017) shows that in response to HFD feeding, protein levels ClpP reduces, whereas expression of Hsp60, Hsp10 or Lon are unchanged in white adipose tissue. In contrast, in response to fish oil feeding (unsaturated diet), there was a significant increase in the protein levels of ClpP, Hsp60, but not Hsp10 or Lon, compared to mice fed LFD.
-

Minor points:

*1. Fig. 2B, D, 4C*
*Were the seperated blots run on different gels? I understand that relative quantification to tubulin should make it comparable (if tubulin is equal between wt and ko), but it would be better to have them presented on the same blot.*

- As suggested by the reviewer, blots in figures 2B, 2D and 4C are replaced in the revised manuscript.

*2. The results for Figure 6C: "succinate to measure OXPHOS capacity through complex I and II" Since succinate is only substrate for Complex II, this setup would only measure CII activity. Please rephrase.*

- "Succinate to measure OXPHOS capacity through complex I and II" is rephrased as "succinate to measure OXPHOS capacity through complex II" in the revised manuscript (page 13).

 

2nd Editorial Decision 1 December 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see both referees are now positive about the study and support publication in EMBO reports without further revision.

Browsing through the manuscript myself, I noticed a few things that we need before we can proceed with the official acceptance of your manuscript:

- Please add a running title (up to 40 characters incl. spaces) on the first page of the manuscript.

- Please provide up to five key words (on the first page of the manuscript)

- Please provide editable high-resolution TIFF or EPS-formatted figures that fit on one page each. (Please see also our figure guidelines on the technical requirements for figure in EMBO press: http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)

- Please reformat the references according to the numbered style of EMBO reports 'Scientific reports'. The respective EndNote style file can be downloaded from our Guide to Authors (https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view)

- Supplementary information: Please rename the file "Supplemental Data" as "Appendix". The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

- Please rename the Supplementary Data Table 1 as Table EV1. Please provide the table in Excel format and include a legend in the file (first row of Excel table) and then upload it as Expanded View file. The callout in the main text has to be updated accordingly.

- Methods:
Please provide a reference for the statement "ClpP-/- mice were generated as described previously..."
- and provide catalogue numbers for the antibodies, if available.

- Please add a scale bar to the following figure panels: Fig. 1D, G, E, and Fig. 5D, F.

- Statistics: Please review all figure legends and make sure that they describe the measurements and data accurately. I noticed, e.g., that the legend for Figure 3 states the following: "(A-H) Bars represent mean {plus minus} SEM". Yet, panels (A-F) show actually individual data points and (H) displays no bars at all. Also in Figure 4, panels A and B do not show bars and are thus not described properly in "Data information". Please review all legends for consistency and accuracy.

- Along these lines I noticed that the legend for Fig. 2D states that the sample size was 6-8 (n = 6-8), there are however only 4 lanes in the respective Western blot per genotype. The same is true for Fig 2F and 4C. If the quantification is based on more mice than samples shown in the Western blot, please indicate this in the legend (e.g. WB shows representative examples for n=X and quantification is based on n=X).

- Could you please review the graphs in Figure 3 A-D? For some reason the panels A and C and the data points in B and D look very similar.

- Please also review the proposed changes I made to the abstract (attached).

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the

 

final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

**********
REFEREE REPORTS

Referee #2:

I have no further comments on this manuscript

Referee #3:

Bhaskaran et al. have revised their manuscript on the role of ClpP in systemic metabolism. The authors fully addressed and satisfied my concerns raised in the original submission. I support publication of the manuscript in EMBO Reports.

With regard to the point-to-point:
1. Appropriate information was added to the discussion. Markers for autophagy were anaylsed, albeit results are difficult to interpret.
2. Quantification of the staining intensity was done and analysed statistically.
3. The aspect was discussed appropriately by the authors.
4. The 4-HNE and AMPK results are interesting and the data strengthen the manuscript.
5. Information was added appropriately
6. I understand the technical difficulty. The discussion and interpretation is sufficient.
7. The issue was clarified by the authors.
Minor points:
1. Western blots were changed in the revised version appropriately.
2. The issue was clarified by the authors.

2nd Revision - authors' response 8 December 2017

Please find the point-by-point response.

- **Please add a running title (up to 40 characters incl. spaces) on the first page of the manuscript.**

*Running title is added in the revised manuscript.*

- **Please provide up to five key words (on the first page of the manuscript)**

*Key words are included.*

- **Please provide editable high-resolution TIFF or EPS-formatted figures that fit on one page each.**
  *Figures in TIFF format are submitted.*
- **Please reformat the references according to the numbered style of EMBO reports 'Scientific reports'.**

*Reference reformatted as suggested.*

- **Supplementary information: Please rename the file "Supplemental Data" as "Appendix". The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.**

*Changes are made in the revised manuscript as suggested.*

- **Please rename the Supplementary Data Table 1 as Table EV1. Please provide the table in Excel format and include a legend in the file (first row of Excel table) and then upload it as Expanded View file. The callout in the main text has to be updated accordingly.**

*Table renamed and excel table uploaded.*

 

- **Methods: Please provide a reference for the statement "ClpP-/- mice were generated as described previously..." and provide catalogue numbers for the antibodies, if available.**

*Reference provided and catalogue number for antibodies included.*

- **Please add a scale bar to the following figure panels: Fig. 1D, G, E, and Fig. 5D, F.**

*Scale bars added*

- **Statistics: Please review all figure legends and make sure that they describe the measurements and data accurately.**

*Figure legends reformatted.*

- **Could you please review the graphs in Figure 3 A-D? For some reason the panels A and C and the data points in B and D look very similar.**

*Values in Figure 3C is obtained from Figure 3A and values for Figure 3D is obtained from Figure 3B. Therefore, the graphs look similar. However, the Y-axes are different.*

- **Please also review the proposed changes I made to the abstract (attached).**

*Proposed changed were made in the revised manuscript.*

- **Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.**

*Synopsis and graphical abstract added.*

 

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**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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

## B- Statistics and general methods

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

| | |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | We have not used any statistical method to to determine sample size in our experiments. Rather, selection of sample size was done based on our prvious experience of including multiple biological replicates analyzed in multiple independent experimental settings. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | All our experiments were performed using at least 6 biological samples . |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No samples were excluded from our studies. The samples would be excluded if the genotyping of th emice is not correct or we observe sample degradation. |
| 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. | We used an unbiased approch in in the selection of animals for our study. Different litters generated from different parents were used for all experiments |
| For animal studies, include a statement about randomization even if no randomization was used. | We used an unbiased approch in in the selection of animals for our study. Different litters generated from different parents were used for all experiments |
| 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. | For our study, we have used animals from different litters that were born to different breeding pairs. Animals were selcted for the study based on their genotype alone and other phenotyping criteria were not taken into account. Because the investiagters performed the genotyping, they were not blinded to the genotyping. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | Because the mice needed to be genotyped prior to the study, we did not use blinding approach in our study. |
| 5. For every figure, are statistical tests justified as appropriate? | Yes, we used ANOVA for statistical analysis. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | |
| Is there an estimate of variation within each group of data? | We have not performed any tests to estimate variation within each group. |
| Is the variance similar between the groups that are being statistically compared? | Yes, the variance was similar between groups, as we have not detected any outliers in the groups used for our study. |

## 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). | Ani-PGC-1 alpha (1:1000, ab54481, Abcam, rabbit polyclonal); Anti-VDAC1/Porin (1:1000, ab15895, Abcam, rabbit polyclonal); Anti-Tfam (1:1000, ab131607, Abcam, rabbit polyclonal); Anti-Hsp60 (1:1000, ab46798, Abcam, rabbit polyclonal); Anti-Hsp40 (1:1000, ab69402, Abcam, rabbit polyclonal); Anti-Hsp10 (Cpn10) (1:1000, ab53106, Abcam, rabbit polyclonal); Anti-OPA1 (1:1000, ab42364, Abcam, rabbit polyclonal); Anti-UCP1 (1:1000, ab23842, Abcam, rabbit polyclonal); Anti-Parkin (1:500, ab77924, Abcam, mouse monoclonal); Anti-PINK1 (1:500, ab75487, Abcam, mouse monoclonal); Phospho-AMPKα (Thr172) (1:1000, 2531, Cell Signaling Technology, rabbit polyclonal); AMPKα (1:1000, 2532, Cell Signaling Technology, rabbit polyclonal); Insulin Receptor β (1:1000, 3020, Cell Signaling Technology, mouse monoclonal); Glut4 (1:500, 2213, Cell Signaling Technology, mouse monoclonal); Phospho-Akt (Ser473) (1:1000, 9271, Cell Signaling Technology, rabbit polyclonal); Akt2 (1:1000, 2964, Cell Signaling Technology, rabbit monoclonal); β-Actin (1:1000, 4970, Cell Signaling Technology, rabbit monoclonal); Anti-CLPP (1:1000, WH0008192M1, Sigma, mouse monoclonal); Anti-β-Tubulin (1:2000, T5201, Sigma, mouse monoclonal), Anti-CLPX (1:1000, AP10767b, Abgent, rabbit polyclonal) |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | 3T3-L1 cell line was obtained from ATCC and ClpP knockdown was achieved , 3T3-L1 cells were infected with mission shRNA lentiviral transduction particles for ClpP (Sigma, St. Louis, MO) or shRNA control transduction particles and transduced cells were obtained by puromycin selection. |

* 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. | Wild type and ClpP-/- mice in C57Bl/6 background was used for the study. Both male and female mice were used. Generation of ClpP-/- mice is decribed by Gispert et al. (2013) and we obtained the breeding pair from Dr. Georg Auburger (Goethe University Medical School, Frankfurt am Main, Germany). The mice were group housed (five animals per cage) in ventilated cages 20 ± 2° C, 12 h/12 h dark/light cycle and were fed ad libitum. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | All animals protocols were in accoradance with the giullines for humane treatment and all experiments were approved by the Institutional Animal Care and Use Committee at the Oklahoma Medical Research Foundation. |
| 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 are confident that our manuscript and data are in coordance with ARRIVE guidelines. |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | N. A. |
| 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. | N. A. |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | N. A. |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | N. A. |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | N. A. |
| 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'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | N. A. |
| 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). | N. A. |
| 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). | N. A. |
| 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. | N. A. |

## 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. | N. A. |