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Mfn2 is critical for brown adipose tissue thermogenic function

Marie Boutant, Sameer S Kulkarni, Magali Joffraud, Joanna Ratajczak, Miriam Valera-Alberni, Roy Combe, Antonio Zorzano, Carles Cantó

Corresponding author: Carles Canto, Nestle Institute of Health Sciences SA

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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

08 July 2016

Thank you for submitting your manuscript to us. I have now received reports from two referees, which I enclose below.

As you will see, the referees appreciate your analyses. However, the role of BATs for the observed metabolic effects needs to be better analyzed (referee #1, point 1; referee #2, second paragraph). They also note some lacking controls and technical issues that need to be addressed experimentally (referee #1, point 2, 4; referee #2, third paragraph), and they propose changes to your interpretations/phrasing (referee #1, points 4-6).

Given the positive recommendation of the referees, I would like to invite you to provide a revised version of your manuscript addressing all comments from the referees and in particular the issues noted above. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Please contact me in case you would like to discuss the revision further.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the

conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

The paper by Boutant et al., investigates the role of mitochondrial dynamics for adipose tissue function by characterizing mice carrying a genetic deletion of Mitofusin-2 (Mfn2) in adipocytes using Cre-loxP technology with Cre recombinase expressed under control of the adiponectin promoter. In particular, as brown adipose tissue (BAT) has high levels of mitochondria and Mfn2, the authors hypothesized that mitochondrial fusion is an important process for BAT function. This is a well-written manuscript, presenting novel, interesting, valid, yet somewhat surprising and contradictory data. The authors show that mice lacking Mfn2 in adipocytes display BAT hypertrophy, are cold sensitive and, contrary to expectations, are more insulin-sensitive when placed on a high-fat diet (HFD). They provide evidence that the phenotypic abnormalities associated with Mfn2 deficiency in adipocytes can be partially explained by defective mitochondria oxidative capacity. In addition, the authors present preliminary evidence that Mfn2 interacts with the lipid droplet protein perilipin-1. There are a few suggestions for clarifying experimental and conceptual issues.

Major points:

1. The authors use several direct and indirect in vitro and in vivo experiments to demonstrate that without Mfn2 BAT function is impaired. While their results are in support of their conclusion, they are not showing BAT function directly, e.g. cold tolerance is not a BAT-specific outcome. The adequate measurement of non-shivering thermogenesis (BAT function) is whole body oxygen consumption in response to norepinephrine or a specific beta3-agonist like CL316,243 (for a detailed review and instructions see Cannon & Nedergaard J Exp Biol 2011). These experiments will clarify how much BAT function in these mice can be found. This is important as the authors show that the overall improved metabolic phenotype on HFD seems to be driven by increased glucose consumption by BAT, arguing for a gain-of-function or at least differential change in functionality in BAT in the absence of Mfn2 rather than a true dysfunction.
2. The authors demonstrate that Mfn2 deficiency is associated with mitochondrial dysfunction in BAT extracts. While these results are in line with previous work, the proposed mechanism calls for a more refined and accurate analysis. This particularly will help to clarify the apparent discrepancy between this study and the co-submitted study by Mahdaviani et al. that finds increased respiration in mitochondria isolated from Mfn2 deficient-BAT. This apparent contradiction needs to be resolved; measurement of respiration in isolated mitochondria according to Mahdaviani et al. is required.
3. The Mfn2 model of action that is being introduced by the authors argues that Mfn2 is an important facilitator of BAT activation through the interaction with lipid droplet for lipolysis. What is the phenotype of adipocyte-specific Mfn2 deficient mice at thermoneutrality, a housing temperature where there is no BAT stimulus in terms of sympathetic tone? This would help to strengthen the concept if the phenotype was attenuated at thermoneutrality compared to room temperature and along the way would rule out any developmental impact of Mfn2 on BAT with this model. It is suggested that the authors include these measurements if possible but at the same time it is worth to emphasize that these studies are not essential.
4. What is the percentage of the BAT mitochondria that is in direct contact with lipid droplets in BAT? Can the authors provide low magnification EM pictures with some quantification?

4. The authors interpret their data regarding Mfn2 levels and functionality in BAT vs. WAT almost exclusively based on the amount of Mfn2 or the effect size of the lack of Mfn2 - this is not valid as the authors show e.g. diminished lipolysis in WAT without Mfn2 just as in BAT. From the clamp studies one could conclude that there is only an effect in BAT but as explained above glucose utilization not necessarily stands for a normal function of the tissue. The authors also discuss improved WAT expandability on HFD in Mfn2 adipose deficiency. Thus, it is possible that there is significant impact of Mfn2 deficiency in WAT on metabolic homeostasis. This should be carefully re-phrased.

5. In the discussion, the authors propose that without Mfn2, there is a beneficial gain-of-function that is protective. All the data point to BAT dysfunction and if proven, the glucose uptake is futile for non-shivering thermogenesis - less BAT function is beneficial would be the conclusion rather than Mfn2 deficiency makes BAT works better.

6. The finding that Mfn2 interacts with perilipin-1 is interesting but descriptive. Hence, the authors cannot conclude that this is the driving force behind the phenotype and should therefore down-tune this statement throughout the manuscript, especially in the title. It could also be the mitochondrial dysfunction, or both or something unexplored by the authors. Of note, the authors only demonstrate interaction of Mfn2 with perilipin-1 but do not show the interaction with lipid droplets (e.g. by immunofluorescence microscopy) and the functional relevance for lipolysis of this interaction remains unclear even though in the text they claim otherwise. This needs to be crafted more carefully throughout the manuscript.

Minor points:

1. Page 9: Was the K109A mutant validated? Can the authors provide a reference?
2. Page 15: "... to a metabolic rewiring in BAT aimed to maximize HEAT production..." is what the authors want to state I believe - again to make such conclusion the true thermogenic capacity of BAT needs to be assessed by adequate measurements.
3. Page 16: "hypercholesterolemia" instead of "hypercholesteronemia"

Referee #2:

The study by Canto and colleagues examines the role of Mfn2 in adipose tissue. When Mfn2 is deleted using an adipose-specific Cre, there are severe defects in brown adipose tissue (BAT). The BAT has less respiratory capacity, and the mice show defective thermogenesis. They find a defect in mitochondria-lipid droplet association, and provide evidence that Mfn2 interacts with perilipin to mediate this contact.

This manuscript makes some interesting observations, but more definitive analysis is required. It would be helpful to compare the metabolic phenotype of this mouse to other BAT defective mice. Is increased insulin sensitivity a feature in other mice with a BAT defect, or is it specific to this mouse? This would help to answer whether Mfn2 has a specific function giving rise to the phenotype, or whether the insulin sensitivity is simply secondary to BAT dysfunction.

The most interesting suggestion is that Mfn2 works with PLIN1 to mediate mitochondria-lipid droplet interactions. However, the current data presented are inadequate. Fig. 3E, the initial finding, is not controlled properly. It would be more convincing to IP for Mfn2 and check for the presence of PLIN1 (the authors performed the reverse). If the interaction is not found in the Mfn2 KO tissue, it would indicate specificity. In Fig. 3F, more PLIN1 is immunoprecipitated with CL treatment, raising a concern about why there is more Mfn2 in the IP. The data in Fig. 3G are very weak and do not argue strongly for a dependence on GTPase activity. It should be possible to directly test their model in the primary brown adipocytes by RNAi studies.

Other issues

- 1) In Fig. 1 C, the authors say there is no compensation of Mfn1 expression, yet Mfn1 RNA is

increased in Mfn2 KO BAT. Also, these experiments should be done with measurement of protein, not RNA. Compensation can occur at the level of translation or protein stability and would be missed by RNA analysis.

2) The authors state that Mfn1-adKO do not have a BAT defect; therefore, they say that the phenotype is not linked to mitochondrial fission. They say that deficiency of Mfn1 "leads to a completely fissioned mitochondrial network." This statement was not supported by any data.

1st Revision - authors' response

14 November 2016

Reviewer 1:

"1. The authors use several direct and indirect in vitro and in vivo experiments to demonstrate that without Mfn2 BAT function is impaired. While their results are in support of their conclusion, they are not showing BAT function directly, e.g. cold tolerance is not a BAT-specific outcome. The adequate measurement of non-shivering thermogenesis (BAT function) is whole body oxygen consumption in response to norepinephrine or a specific beta3-agonist like CL316,243 (for a detailed review and instructions see Cannon & Nedergaard J Exp Biol 2011). These experiments will clarify how much BAT function in these mice can be found. This is important as the authors show that the overall improved metabolic phenotype on HFD seems to be driven by increased glucose consumption by BAT, arguing for a gain-of-function or at least differential change in functionality in BAT in the absence of Mfn2 rather than a true dysfunction."

We thank the referee for this excellent suggestion. We have now performed the requested experiment and the results can be found in Figures 1J-L and Figure S5B, for mice fed under a low-fat diet (LFD) or a high-fat diet (HFD), respectively. As the results illustrate, the response to CL316,243 (CL) is dramatically impaired in Mfn2-adKO when mice are housed at 22°C, but not at thermoneutrality. These results demonstrate that Mfn2 deficiency in the BAT leads to impaired non-shivering thermogenic capacity.

To further support the point that the BAT from Mfn2-adKO mice displays impaired oxidative capacity response to b3 adrenergic stimulation, we have complemented the above tests with *ex vivo* experiments. For such purpose, respirometry analyses were performed in isolated mature brown adipocytes from Mfn2-adKO mice and control littermates. As reported now in Figure 1M, CL-stimulated respiration is impaired by more than 60% in brown adipocytes from Mfn2-adKO mice. This testifies again for a failure in BAT oxidative capacity in Mfn2-adKO mice, leading to thermogenic dysfunction.

As the reviewer duly notes, there is some confusion in the text caused by the use of "gain- or loss-of function" terms. This has now been corrected to better illustrate that Mfn2 deficiency in the BAT causes a change in functionality, characterized by thermogenic dysfunction, but increased insulin-stimulated glucose uptake upon high-fat feeding.

"2. The authors demonstrate that Mfn2 deficiency is associated with mitochondrial dysfunction in BAT extracts. While these results are in line with previous work, the proposed mechanism calls for a more refined and accurate analysis. This particularly will help to clarify the apparent discrepancy between this study and the co-submitted study by Mahdaviani et al. that finds increased respiration in mitochondria isolated from Mfn2 deficient-BAT. This apparent contradiction needs to be resolved; measurement of respiration in isolated mitochondria according to Mahdaviani et al. is required."

We have now performed respirometry assays in isolated mitochondria from BAT of Mfn2-adKO mice and their respective control littermates. Contrary to the observations from Mahdaviani et al., our results illustrate a marked decrease in Complex I and Complex II driven respiration in the BAT of Mfn2-adKO. These results are now shown on Figure 2H. Our observations in isolated mitochondria are in line with the results obtained in tissue homogenates. Similarly, the impaired respiration in mitochondria from Mfn2-adKO BAT nicely fits with the observation that Mfn2 deficiency leads to a dramatic reduction (over 80%) in Complex I and III levels, as reported by both works.

We do not have a clear reason explaining the differences between our observations with those of the Shirihai lab. However, at least three potential issues might contribute to this discrepancy.

1/ The mitochondrial enriched fractions tested by the Shirihai lab were mostly composed of cytosolic mitochondria, and did not account for those in the fat cake, likely corresponding to peri-droplet mitochondria. It might be that cytosolic mitochondria compensate for deficits in the respiratory capacity of peri-droplet mitochondria.

2/ Our conditional model suffered deletions in the white and brown adipose tissues, while the model from the Shirihai lab is restricted to BAT. The Mfn2 deficiency in WAT allows for a higher lipid storage capacity in the WAT of the Mfn2-adKO mice, which does not take place upon specific ablation in the BAT. This might force the mitochondria from the BAT of BAT-Mfn2 KO to enhance their respiratory capacity in order to better cope with fat overflow than in the case of Mfn2-adKO mice, where lipids are largely stored also in WAT depots.

3/ Mfn2-adKO mice are also characterized by higher circulation levels of adiponectin, which can be inhibitory to BAT mitochondrial function (Qiao et al, 2014).

“3. The Mfn2 model of action that is being introduced by the authors argues that Mfn2 is an important facilitator of BAT activation through the interaction with lipid droplet for lipolysis. What is the phenotype of adipocyte-specific Mfn2 deficient mice at thermoneutrality, a housing temperature where there is no BAT stimulus in terms of sympathetic tone? This would help to strengthen the concept if the phenotype was attenuated at thermoneutrality compared to room temperature and along the way would rule out any developmental impact of Mfn2 on BAT with this model. It is suggested that the authors include these measurements if possible but at the same time it is worth to emphasize that these studies are not essential. “

This is an interesting suggestion by the referee. We have also evaluated the phenotype of Control and Mfn2-adKO at thermoneutrality. The results, now constituting Figure S6, show that thermoneutrality prevented the glycolytic rewiring in the BAT of HFD-fed Mfn2-adKO mice (Figure S6A). Consequently, HFD-fed Mfn2-adKO mice were no longer more insulin sensitive than their control littermates (Figure S6B).

The above results suggest that the glycolytic rewiring in the BAT of HFD-fed Mfn2-adKO mice is an adaptation to ensure the procurement of carbohydrate energy substrates for non-shivering thermogenesis, given their marked defects in fatty acid oxidation (FAO) capacity. If this were true, one would predict that in the absence of this metabolic rewiring, as happens when housed at thermoneutrality, Mfn2-adKO mice would be even less capable of sustaining non-shivering thermogenesis and, therefore, be more cold-sensitive. Indeed, Mfn2-adKO housed at thermoneutrality displayed an exacerbated cold-sensitivity (Figure S6C). In fact, 4 out of the 6 Mfn2-adKO mice used in the study, but none of the control mice, had to be removed 4 hrs after the initiation of the test, as their body temperature dropped below 30°C. For the referee's information, the vast majority of Mfn2-adKO mice can withstand this test up to 5 hrs when acclimated at regular housing temperatures (22°C).

“4. What is the percentage of the BAT mitochondria that is in direct contact with lipid droplets in BAT? Can the authors provide low magnification EM pictures with some quantification? “

As shown in Figure 3C, around 60% of the mitochondria are in direct contact with lipid droplets in the BAT of control mice in the regular housing conditions of our facility. All mice used for EM studies were perfused between 10 am and 11 am in the morning. The number of mitochondria in direct contact with the lipid droplet is reduced by almost 50% in the BAT of Mfn2-adKO mice. Low magnification images of the EM pictures are provided now as Figure S3A. Quantifications were already provided as Figure 3C.

“5. The authors interpret their data regarding Mfn2 levels and functionality in BAT vs. WAT almost exclusively based on the amount of Mfn2 or the effect size of the lack of Mfn2 - this is not valid as the authors show e.g. diminished lipolysis in WAT without Mfn2 just as in BAT. From the clamp studies one could conclude that there is only an effect in BAT but as explained above glucose utilization not necessarily stands for a normal function of the tissue. The authors also discuss improved WAT expandability on HFD in Mfn2 adipose deficiency. Thus, it is possible that there is

significant impact of Mfn2 deficiency in WAT on metabolic homeostasis. This should be carefully re-phrased.”

We fully agree with the interpretation of the reviewer and we apologize if this point was not clear in the previous version of our manuscript. Following our discussion on the differences between the model by the Shirihai lab and ours, we have added a sentence to clarify that “the WAT has an important non-negligible role in the metabolic phenotypes of Mfn2-adKO mice, not only through increasing adipose tissue expandability, but also by exacerbating the negative effects of the Mfn2 ablation in BAT at the levels of lipolytic and respiratory capacity“ (see Pages 19-20)

“6. In the discussion, the authors propose that without Mfn2, there is a beneficial gain-of-function that is protective. All the data point to BAT dysfunction and if proven, the glucose uptake is futile for non-shivering thermogenesis - less BAT function is beneficial would be the conclusion rather than Mfn2 deficiency makes BAT works better. “

We thank the referee for pointing out this possible source of confusion. We agree with his/her conclusion and that the higher glucose uptake might be an adaptation to cope with the defective oxidative capacity of the tissue. Nevertheless this effort remains insufficient to sustain non-shivering thermogenesis when mice are exposed to cold, a point now clarified in Page 18. As discussed above, we now modified the text so that the use of gain- or loss-of-function terminologies are attributed to particular features of BAT (e.g.: thermogenesis, glucose uptake, etc.).

“7. The finding that Mfn2 interacts with perilipin-1 is interesting but descriptive. Hence, the authors cannot conclude that this is the driving force behind the phenotype and should therefore down-tune this statement throughout the manuscript, especially in the title. It could also be the mitochondrial dysfunction, or both or something unexplored by the authors. Of note, the authors only demonstrate interaction of Mfn2 with perilipin-1 but do not show the interaction with lipid droplets (e.g. by immunofluorescence microscopy) and the functional relevance for lipolysis of this interaction remains unclear even though in the text they claim otherwise. This needs to be crafted more carefully throughout the manuscript.”

This is an excellent, but challenging point. The interaction of mitochondria with the lipid droplets has been documented by multiple laboratories (see, for example, ([Jagerstrom et al, 2009](#); [Pidoux et al, 2011](#); [Pu et al, 2011](#))). Our EM analyses demonstrate that the interactions between mitochondria and lipid droplet are disrupted. Similarly, our new results on whole body and *ex vivo* O₂ consumption show that lipid oxidation is blunted in Mfn2 deficient brown adipocytes upon CL treatment. However, as the reviewer notes, whether the functional interaction between Mfn2 and PLIN1 could be at the root of lipolytic problems remained an open question. Importantly, Mfn2-adKO adipose tissues also show defective mitochondrial function, which could per se determine impaired lipolysis.

In order to shed light into this issue, we performed a number of experiments in MEF cells, where Mfn2 deficiency does not lead to impaired mitochondrial respiration or to decreased Complex I/III levels (Fig.S4A). In addition, MEFs do not express PLIN-1 (Fig.S4A). Given the absence of β_3 adrenergic receptors, we treated MEF cells with Forskolin (Fsk) in order to stimulate PKA signaling and lipolysis when cells were incubated in non-supplemented minimal essential medium (MEM). As now shown in Figure S4C, Fsk only increased O₂ consumption in wild-type (WT) MEFs when PLIN1 was introduced into the cells. This result illustrates that cAMP-triggered lipid oxidation relies on PLIN1 expression. Importantly, when similar experiments were performed in Mfn2KO MEFs, the cells were unresponsive to Fsk irrespective of PLIN1 expression or phosphorylation. Further, reintroduction of a wild-type form of Mfn2, but not the K109A (GTPase dead) mutant, was enough to rescue the responsiveness of Mfn2KO MEFs to Fsk. These observations indicate that both Mfn2 and PLIN1 are required for efficient lipolysis in response to elevations in cAMP levels.

The reviewer has to note, however, that the absence of Mfn2 does not fully prevent mitochondria to lipid droplet contacts, at least in the basal state. Therefore, our results suggest that multiple molecular layers might regulate these interactions, and that Mfn2 might be involved in those modulated via adrenergic stimulation.

Finally, we have also slightly changed the title of the manuscript in order to tone down the statement that the mitochondria-LD interaction is the sole root of thermogenic defects.

Minor points:

“- Page 9: Was the K109A mutant validated? Can the authors provide a reference?”

The K109A mutation has been described before ([Chen et al, 2003](#)), and this is now properly cited (Page 10). Our experiments on Fig.S4C further validate that wild type Mfn2, but not the K109A mutant, recovers the responsiveness of Mfn2KO MEFs to lipolytic stimuli.

“- Page 15: "... to a metabolic rewiring in BAT aimed to maximize HEAT production..." is what the authors want to state I believe - again to make such conclusion the true thermogenic capacity of BAT needs to be assessed by adequate measurements.”

We agree with the referee on this possible confusion. For this reason we have rephrased it as "... to a metabolic rewiring in BAT aimed to maximize glycolytic capacity" (now Page 17)

“- Page 16: "hypercholesterolemia" instead of "hypercholesteronemia"”

Thank you very much for noticing this mistake. It has now been corrected.

Referee #2:

“This manuscript makes some interesting observations, but more definitive analysis is required. It would be helpful to compare the metabolic phenotype of this mouse to other BAT defective mice. Is increased insulin sensitivity a feature in other mice with a BAT defect, or is it specific to this mouse? This would help to answer whether Mfn2 has a specific function giving rise to the phenotype, or whether the insulin sensitivity is simply secondary to BAT dysfunction.”

This is an excellent point raised by the referee. In the last 3 years several reports have tried to elucidate the role of mitochondrial function in adipose tissues through the use of the Adiponectin-Cre and UCP1-Cre drivers. While most of them converge at illustrating the key role of mitochondria for thermogenic function, the impact on whole body energy metabolism is not as clear. For example, while impairing mitochondrial function through the ablation of TFAM in adipose tissues leads to lipodystrophic syndrome with insulin resistance ([Vernochet et al, 2014](#)), the specific deletion of the mitochondria TCA cycle enzyme fumarate hydratase leads to a diametrically opposite phenotype ([Yang et al, 2016](#)). This said, our model is in line with the concept that impaired thermogenic function does not necessarily lead to glucose intolerance, but might actually prevent against diet-induced insulin resistance and hepatic steatosis, as recently reported for completely independent models ([Duteil et al, 2016](#); [Schoiswohl et al, 2015](#); [Yang et al, 2016](#)). The divergences between different models give strength to two basic ideas: 1/ that mitochondrial functions adipose tissue expand far beyond thermogenesis and respiration and 2/ that the outcomes of impairing mitochondrial respiration in BAT might depend on the ability of the organism to healthily expand their fat mass. These references and concepts are now discussed in page 17.

“The most interesting suggestion is that Mfn2 works with PLIN1 to mediate mitochondria-lipid droplet interactions. However, the current data presented are inadequate. Fig. 3E, the initial finding, is not controlled properly. It would be more convincing to IP for Mfn2 and check for the presence of PLIN1 (the authors performed the reverse). If the interaction is not found in the Mfn2 KO tissue, it would indicate specificity.”

We thank the reviewer for his/her suggestion. Due to tissue amount limitations, we now provide data on differentiated brown adipocyte cultures showing the reverse IP strategy (IP for Mfn2 and evaluation of PLIN1 in the immunoprecipitated material) in Fig.S3C.

“In Fig. 3F, more PLIN1 is immunoprecipitated with CL treatment, raising a concern about why there is more Mfn2 in the IP. “

We understand the reviewer’s concern, but we would like to clarify that the higher PLIN1 levels after CL treatment are just apparent. In reality, the PLIN1 band is wider as the PLIN1 antibody detects both the phosphorylated and non-phosphorylated forms. This renders, at least, a duplet and provides the illusion of a higher amount of PLIN1. As we didn’t have a lower exposure of the experiment in Fig.3C, we show another experiment for the referee (figure below) to illustrate our point:

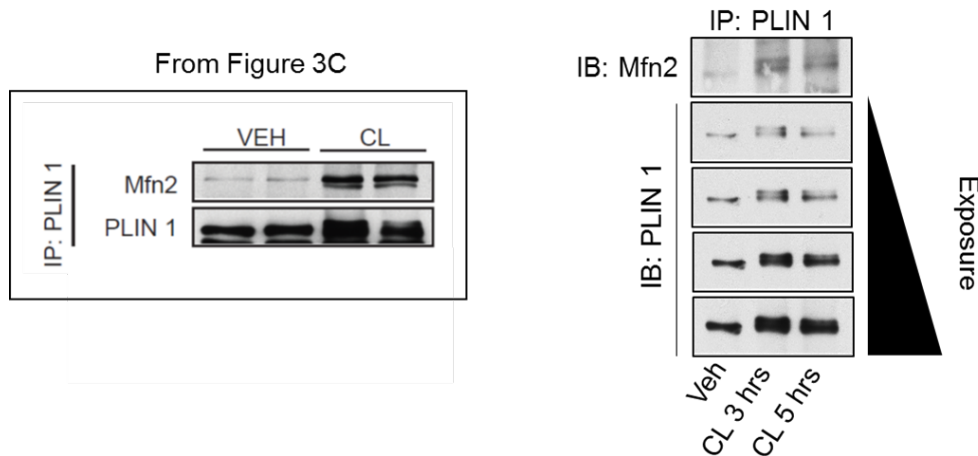


Figure for the referees 1. Differentiated brown adipocytes were treated with Vehicle (3 hrs) or CL (1 mM, for 3 or 5 hrs). Then, total protein homogenates were obtained and PLIN1 was immunoprecipitated. The right panel shows increasing exposures of the immunoblots against PLIN1

“The data in Fig. 3G are very weak and do not argue strongly for a dependence on GTPase activity. It should be possible to directly test their model in the primary brown adipocytes by RNAi studies.” The reviewer has to consider that these adipocytes still expressed endogenous Mfn2, so a total blockage of the interaction would not be expected. Nevertheless, experiments using siRNAs, as the reviewer suggests, would not clarify the role of the GTPase activity of Mfn2, as this would deplete the whole protein.

Now we provide novel evidence demonstrating that only the introduction of wild-type Mfn2, but not the Mfn2 K109A mutant, rescues the responsiveness to forskolin-stimulated O_2 consumption – as a readout for fatty acid oxidation - in Mfn2 deficient MEFs (Figure S4B).

“Other issues

In Fig. 1 C, the authors say there is no compensation of Mfn1 expression, yet Mfn1 RNA is increased in Mfn2 KO BAT. Also, these experiments should be done with measurement of protein, not RNA. Compensation can occur at the level of translation or protein stability and would be missed by RNA analysis.”

This is a great suggestion by the reviewer. It has turned out to be a very relevant point as, in line with the referee’s prediction, Mfn1 protein levels are differently regulated at the protein and mRNA levels. Contrary to mRNA levels, Mfn1 protein levels in adipose tissues were ~30% lower in Mfn2-adKO mice than in control littermates. These results are shown as Figure S1A. With this in mind, however, we would like to remind the reviewer that the phenotypes characteristic of the Mfn2-adKO mice are not present in the Mfn1-adKO mice. Therefore, the decrease in Mfn1 in the adipose tissues of the Mfn2-adKO mice is not the underlying cause of the phenotypes observed.

“The authors state that Mfn1-adKO do not have a BAT defect; therefore, they say that the phenotype is not linked to mitochondrial fission. They say that deficiency of Mfn1 “leads to a completely fissioned mitochondrial network.” This statement was not supported by any data.”

We apologize for this confusion, as the sentence did not mean to refer to phenotypes specifically related to adipose tissues. In all cell models and tissues evaluated to date, Mfn1 deletion has led to a completely fissioned mitochondrial network. Since we have not performed EM analyses in our Mfn1-adKO mice, we clarify now this point by rephrasing the statement: “Mfn1 is an essential protein for mitochondria fusion events. Accordingly, decreased Mfn1 function led to a profound shift in the fusion/fission balance, towards a dramatically fragmented mitochondrial network in most, if not all, cells and tissues tested to date ([Chen et al, 2003](#); [Dietrich et al, 2013](#); [Kulkarni et al, 2016](#); [Papanicolaou et al, 2012](#); [Park et al, 2008](#))”

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2nd Editorial Decision

08 December 2016

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by the two original referees whose comments are enclosed.

As you will see, the referees appreciate the work that went into the revision, but they are still not entirely convinced about the connection between Mfn2 and lipid droplets.

I would thus like to invite you to submit a revised version of the manuscript, addressing the

remaining concerns of both reviewers. Please let me know in case you would like to discuss the revision further.

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

REFEREE REPORTS

Referee #1:

The paper is well written, concise and the main findings are well supported by the data, which has been much improved in this revised version. The association between Mfn2 and lipid droplet is now better explored but still not entirely convincing. Maybe the title can still be modified to decrease the emphasis of the paper on the connection between lipid droplet and mitofusin2.

Otherwise the paper is very nice and brings novelties to the field.

Referee #2:

In general, the revisions have not done much to address my original concerns.

The response to the concern about the Mfn2/PLIN1 interaction is inadequate and therefore the concern about antibody specificity remains. The reason to IP Mfn2 is to test the specificity of the purported interaction. In Mfn2 depleted tissue or cells, the PLIN1 should no longer be immunoprecipitated. This is an important control to test the specificity of their antibody. However, the authors only perform the Mfn2 IP in WT cells (Fig. S3C), which does not address the concern. Their present data (Fig. 3E and S3C) do not provide any evidence of specificity and could be an artifact due to a nonspecific antibody.

In addition, in Fig. S3C, the increase in phosphorylated PLIN1 is taken as evidence that Mfn2 binds to phosphorylated PLIN1. However, there is no evidence for this conclusion, and in fact the opposite conclusion can be drawn--PLIN1 is co-immunoprecipitated regardless of whether it is phosphorylated or not. The statements in the Discussion that Mfn2's interaction with PLIN1 are regulated by phosphorylation, and that this might explain why PLIN3 does not bind (p.17), should be removed.

Concerning Fig. 3F: the data provided reinforces the original concern that more PLIN1 is immunoprecipitated with CL treatment.

Concerning Fig. 3G: Regardless of the explanation, the experimental result is quite weak. The experiment was done with Flag-tagged Mfn2, which may or may not negate the effect of endogenous Mfn2. Given the marginal nature of the result, I am not sure what the result reveals. The new experiment on O2 consumption is not relevant this experiment on the Mfn2-PLIN1 interaction.

2nd Revision - authors' response

16 February 2017

Referee #1:

“The paper is well written, concise and the main findings are well supported by the data, which has been much improved in this revised version. The association between Mfn2 and lipid droplet is now better explored but still not entirely convincing. Maybe the title can still be modified to decrease the emphasis of the paper on the connection between lipid droplet and mitofusin2. Otherwise the paper is very nice and brings novelties to the field.”

We thank the referee for his/her kind words. Given that both referees are aligned that the on toning down the emphasis on the relation between Mfn2 and the lipid droplet, manuscript, we now rephrased the title to: "Mfn2 is critical for brown adipose tissue thermogenic function"

Referee #2:

“The response to the concern about the Mfn2/PLIN1 interaction is inadequate and therefore the concern about antibody specificity remains. The reason to IP Mfn2 is to test the specificity of the purported interaction. In Mfn2 depleted tissue or cells, the PLIN1 should no longer be immunoprecipitated. This is an important control to test the specificity of their antibody. However, the authors only perform the Mfn2 IP in WT cells (Fig. S3C), which does not address the concern. Their present data (Fig. 3E and S3C) do not provide any evidence of specificity and could be an artifact due to a nonspecific antibody.”

We believe that the specificity of our antibody is clearly proven in multiple figures, including Fig.1B, Fig.3D, Fig.3E, Fig.S3B, Fig.S4A, Fig.S4D and Fig.S6A. In all these figures, no Mfn2 is detected in Mfn2 deficient cells or tissues upon protein analyses via western blot or immunoprecipitation. However, in order to fully satisfy the referee's concern, we have now performed the suggested immunoprecipitation experiments. In an unexpected way, they proved to be important as they unveiled a critical aspect on PLIN1.

We performed IPs against Mfn2 in BAT from WT and Mfn2-adKO mice. Much to our surprise, a band for PLIN1 appeared in the immunoprecipitate of both control and Mfn2-adKO mice (Figure for the Referees (FigR) 1A). As the referee mentions, this could be indicative of an unspecific action of our antibody. Indeed, a band ~60–65 kDa appeared in the input blot for Mfn2, which matches the molecular weight of PLIN1. To test if our Mfn2 antibody non-specifically detects PLIN1, we blotted total homogenates from 3T3 cells, where endogenous PLIN1 is virtually undetectable, transfected with either empty vector (3T3-EV) or with PLIN1 (3T3-PLIN1). BAT was loaded next to these samples as a positive control. The blots were run and the membranes blotted against Mfn2 and PLIN1 were placed in parallel. A low (top) and high (bottom) exposure are provided (FigR.1B). The results illustrate that our Mfn2 antibody does not recognize PLIN1. First, because the band detected at ~60-65 kDa is at a slightly lower MW than PLIN1. A dashed red line is included in order to facilitate the visualization of this difference. Second, the band recognized by the Mfn2 antibody appears in 3T3-EV, where PLIN1 is not expressed. Third, because the band does not linearly follow PLIN1 levels and appears even in EV-transfected 3T3s. As a whole, these observations proof that our antibody does not detect PLIN1. The images also show that PLIN1 does not cross-react with Mfn2. Therefore, the detection of Mfn2 in PLIN1 IPs (Figure 3) cannot be attributed to cross-reaction with the PLIN1 antibodies.

There could still be the possibility that Mfn2 antibody could bind a protein that interacted with PLIN1. In order to evaluate if this is the case, we blocked the immunoreactivity of the Mfn2 antibody by adding to the lysate 1.5 mg of the peptide against the recognition motif of the antibody. This way, this excess of peptide displaces the specific binding of proteins to the antibody. Testifying for this, the incubation with the blocking peptide reduced the ability of the antibody to IP Mfn2 by more than 90% (FigR.1C). However, the presence of PLIN1 in the IPd material was not affected by the blocking peptide (FigR.1C). This result indicate that the presence of PLIN1 in Mfn2 IPs does not respond to the specific detection of a protein by the Mfn2 antibody. Considering the high lipid context of the BAT homogenates, this made us wonder whether PLIN1 could non-specifically bind to the IP beads. To test this we evaluated the IP material in beads without BAT lysate or without antibody. As FigR.1D shows, the PLIN1 appeared only in IPs including lysate. However, it appeared irrespective of having the Mfn2 antibody present during the IP. Hence, PLIN1 from BAT samples appears in the IPs against Mfn2 due to an unspecific interaction with the IP beads.

Figure for the Referee 1

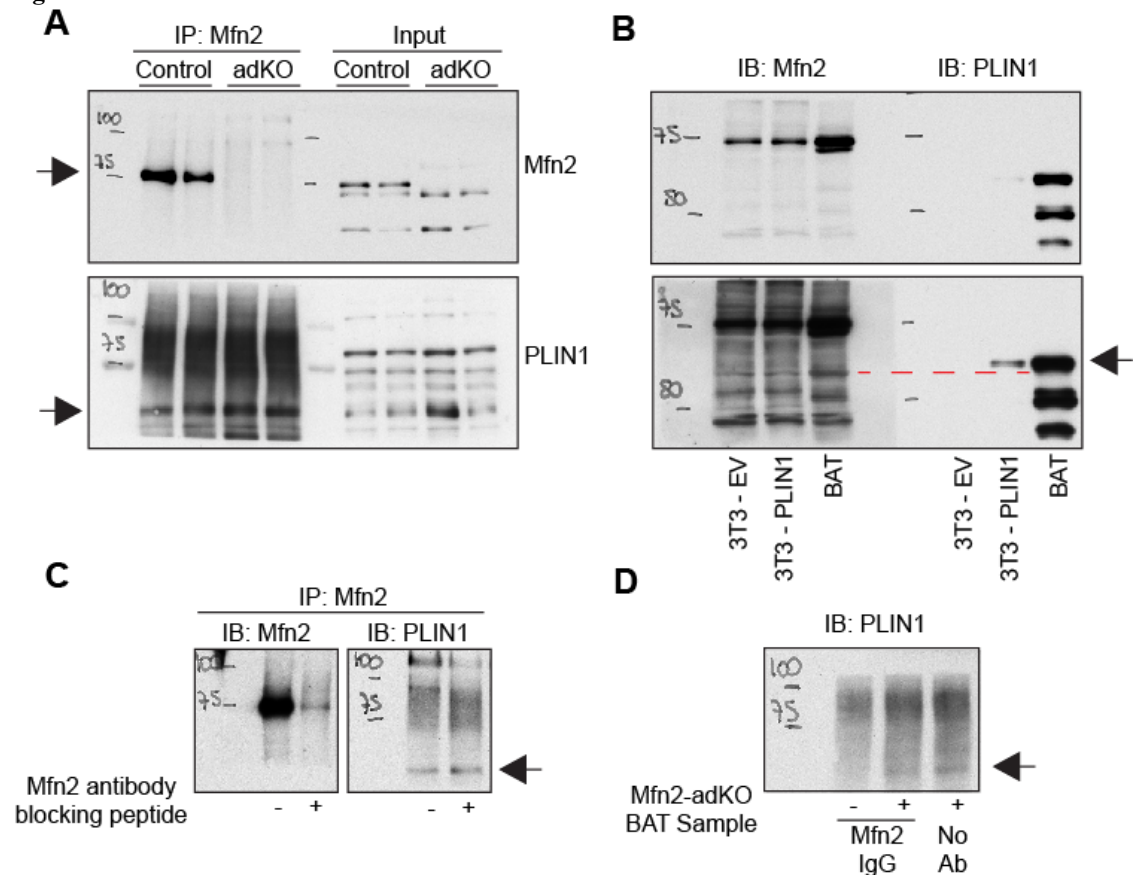


Figure for the Referees 1. (A) 1 mg of BAT protein was immunoprecipitated (IPd) against Mfn2. Then, Mfn2 and PLIN1 levels were assessed in the IPd material and in the input. The arrows indicate the bands for Mfn2 and PLIN1. (B) Total homogenates from 3T3-NIH fibroblasts transfected either with empty vector (3T3-EV) or mouse PLIN1 (3T3-PLIN1), as well as homogenates from BAT, were used for western blots against Mfn2 and PLIN1 and the membranes were placed in parallel. A low exposure (top) and a high exposure (bottom) are provided. A dashed red line is used to better visualize how the band detected on the Mfn2 blots is different from that of PLIN1 (arrow). (C) IPs on BAT homogenates against Mfn2 were carried in the presence or absence of 1.5 mg of blocking antibody against the binding site of the Mfn2 antibody. Then, the IP material was used to blot against Mfn2 and PLIN1. The arrow signals the PLIN1 band. (D) PLIN1 blots were performed in the material recovered from performing the IP protocol by combining as follows: left lane: beads + Mfn2 antibody, without loading any sample; middle lane: beads + Mfn2 antibody + BAT sample from Mfn2-adKO mice; right lane: beads + BAT sample from Mfn2-adKO mice, without antibody. The results suggest that PLIN1 from BAT samples unspecifically binds to the IP beads.

Given the above results, four points need to be clarified.

- 1) Sample pre-clearing did not prevent PLIN1 binding. This might not be surprising given the high expression of PLIN1 in the BAT and the high lipid content, which might easily saturate the unspecific binding to the column. Testifying for this, we have never observed the unspecific binding of PLIN1 to the IP matrix when using cultured brown adipocytes, which have a very significant lower amount of intracellular lipids.
- 2) The relative amount of PLIN1 bound to Mfn2 might be too little to manifest over the unspecifically unbound. This might be due to the fact that even if the whole lipid droplet is coated with mitochondria, a great part of the outer mitochondrial membrane of these mitochondria does not directly contact with the lipid droplet. In addition, ~40% of the BAT mitochondria in the BAT are not interacting with lipid droplets. Therefore, only a very small fraction of Mfn2 might be bound to PLIN1, while most PLIN1 might be bound to Mfn2. This would explain why the IP always works better when PLIN1 is the one pulled down.

- 3) Unspecific binding of PLIN1 to the immunoprecipitation matrix is insignificant compared to the yields of PLIN1 obtained through specific PLIN1 homogenization (FigR.2)

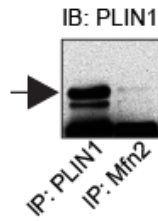


Figure for the Referees 2. 1 mg of BAT homogenates were used to immunoprecipitate (IP) PLIN1 and Mfn2 and then blot for the yield of PLIN1 on the IPd material. The result illustrate that the unspecific binding of PLIN1 in the IP matrix is residual compared to PLIN1 IPd through specific antibodies.

In order to certify the strength of our results and the specificity of our interactions, we obtained a second commercial antibody against PLIN1, albeit recognizing the N-terminal domain (instead of the C-terminal, as used previously in the manuscript). The results, now shown as Fig.S3B, indicate that the interaction of Mfn2 with PLIN1 is also detectable with this second, independent antibody. Overall, the fact that 2 independent PLIN1 antibodies, but not a PLIN3 antibody, lead to the Co-IP of Mfn2 constitutes robust evidence for the co-immunoprecipitation of these two proteins.

“In addition, in Fig. S3C, the increase in phosphorylated PLIN1 is taken as evidence that Mfn2 binds to phosphorylated PLIN1. However, there is no evidence for this conclusion, and in fact the opposite conclusion can be drawn--PLIN1 is co-immunoprecipitated regardless of whether it is phosphorylated or not. The statements in the Discussion that Mfn2's interaction with PLIN1 are regulated by phosphorylation, and that this might explain why PLIN3 does not bind (p.17), should be removed.”

We will follow the referee's advice and eliminating this sentence and old Fig.S3C in order to avoid any possible confusion around the interpretation of those data.

“Concerning Fig. 3F: the data provided reinforces the original concern that more PLIN1 is immunoprecipitated with CL treatment.”

We respectfully disagree with this comment, as the data provided in the previous revision clearly demonstrates that the overexposure of a duplet (due to protein phosphorylation) leads to an apparent magnified band. However, and to leave any trace of doubt aside, we now provide quantified data for several crucial blots in the manuscript, including Fig.3F and Fig.3G.

“Concerning Fig. 3G: Regardless of the explanation, the experimental result is quite weak. The experiment was done with Flag-tagged Mfn2, which may or may not negate the effect of endogenous Mfn2. Given the marginal nature of the result, I am not sure what the result reveals. The new experiment on O^2 consumption is not relevant this experiment on the Mfn2-PLIN1 interaction.”

We now present quantified data for the “marginal” nature of the results. While we agree that part of the effect might be weakened by endogenous Mfn2, the expression of a GTPase dead Mfn2 significantly impairs the interaction between PLIN1 and Mfn2 by 50%.

Accepted

28 February 2017

Thank you for sending the revised version of your manuscript to us. As you will see, I decided to involve referee #2 once again, please find this referee's comments below.

Given the input from this referee, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Congratulations!

REFeree REPORT

Referee #2:

The rebuttal letter contains a long explanation of the Mfn2/PLIN1 interaction. The bottom line appears to be that the IP results using Mfn2 antibodies are artifactual. Under that IP condition, PLIN1 seems to bind nonspecifically to the beads. Unless the authors can resolve this issue, they will not be able to produce a perfectly controlled experiment.

In spite of this, the authors argue that non-specific binding to beads is not an issue when IPs with anti-PLIN1 antibodies are used.

This is obviously not ideal. However, the authors show a IP results with a second PLIN1 antibody that show the presence of Mfn2. This helps to reduce the concern somewhat.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Carles Cantó

Journal Submitted to: EMBO Journal

Manuscript Number: EMBOJ-2016-94914

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 χ^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;
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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).

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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?	Page 25
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Page 25
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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.	Page 21
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes; Page 25
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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).	Pages 24-25
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page 24

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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.	Page 21
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 21
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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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
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