Smaug1 membrane-less organelles respond to AMPK/mTOR and affect mitochondrial function

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Significant new data would be expected to support your conclusions, particularly around colocalization analysis including the use of further controls. The suggestions around further analysis of metabolism and of mitochondrial function are also good suggestions that I encourage you to consider. I would also like you to include the data indicated as "not shown" in the original submission.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Fernandez-Alvares et al. investigate in this study the role of Smaug1 and Smaug2 RNA-binding proteins in post-transcriptional regulation of two mRNAs encoding mitochondrial proteins and how they impact general mitochondrial function. The manuscript conveys three main messages:

1) Smaug1 and 2 downregulation affects mitochondrial respiratory function and mitochondrial morphology.
2) Smaug interacts with mRNAs encoding SDHB and UQCRC1 in membraneless organelles (MLOs).
3) Treatments that affects respiratory complex I lead to the dissolution of these MLOs and reduce binding of Smaug to the target mRNAs.

Although the findings are intriguing and the topic worth of investigation, several mechanistic questions remain open, and not all the conclusions of the authors appear fully supported by the data. In the present form, this study therefore appears rather preliminary.

Comments for the author

Major comments
1) Smaug and mitochondrial function: the data show that downregulation of Smaug 1 and 2 leads to a general reduction of the mitochondrial respiratory capacity and to mitochondrial fragmentation. Since in this study only two mRNAs encoding mitochondrial proteins are investigated, the reason for this phenotype remains unclear. How exactly are Smaug proteins affecting the expression of mitochondrial protein? The authors could easily test if the steady state levels of components of the respiratory chain (including but not limited to SDHB and UQCRC1) are reduced. Blue-native gels could also inform on the assembly of the respiratory chain complexes.
2) Smaug bodies and co-localization with SDHB and UQCRC1 mRNAs: The way how this co-localization was analyzed is not convincing. The authors mention in the methods that this was assessed manually. What does this mean exactly? The authors should use Manders' or Pearson's methods for co-localization. In addition, the fact that the authors see less percentage of co-localization in random images does not prove anything until a statistical test is applied (for example Fisher exact test) to make sure that this difference is significant. The authors state that more than 400 MLOs were analyzed. From how many cells? In how many experiments?
3) The role of MLOs: the authors try to dissect the region of the Smaug protein responsible for MLO formation and to understand what is the role of these membraneless organelles, however this last question remains unanswered. They propose that mRNAs are released upon dissolution of the MLOs. There is no strong data supporting this notion. In addition, although it is interesting to observe that rotenone and metformin lead to the progressive reduction of MLOs, it remains to be determined why this is the case and what this means for the translation of mRNAs encoding mitochondrial proteins. These treatments could for example induce other type of granules, such as stress granules, or simply induce cell suffering, leading to increased autophagy.

4) Quantification of many experiments is not convincing (see again point 2). This applies to the quantification of the contacts between mitochondria and MLOs; quantification of Drp1 puncta (western blot of Drp1 in mitochondria or cytosol could be a more convincing method). In addition, in several cases, experiments have been performed only once with a technical duplicate. The authors should be very cautious to draw conclusions from these data. Showing a SD and applying a t-test these data is meaningless.
5) In many instances, the authors mention data not shown. These data are important controls that should be present in the manuscript.

Reviewer 2

Advance summary and potential significance to field

This study by Fernández-Alvarez/Thomas et al. examines the relationship between the RNA binding proteins Smaug1/2 as a regulator of mitochondrial function and morphology through the repression of two mRNAs encoding subunits of Complex I and II. Known to bind specific elements in RNA, Smaug was initially shown to mediate RNA decay through deadenylation of polyA tails and act as a repressor of translation. More recent studies in different systems has revealed it can also repress mRNA translation without causing decay. There are many messages that bind Smaug but a number of studies have shown an enrichment in mitochondrial mRNAs, consistent with studies in yeast and mouse mutant models showing significant effects on metabolism.

This study looks at Smaug1/2 in cultured cells and makes a number of exciting observations. Perhaps the most striking is the dissolution of Smaug from membraneless organelles (MLO) upon inhibition of mitochondrial complex I, but not upon complete uncoupling with CCCP. This is the first time these kinds of MLOs have been seen to respond to mitochondrial metabolism. In addition, they observed that loss of Smaug leads to a reduction in complex I and II activities, along with increased mitochondrial fragmentation, presumably through the activation of Drp1 mediated fission (as opposed to inhibition of fusion). The binding of the two mitochondrial mRNAs to Smaug after MLO dissolution was reduced after 1 hour metformin treatment, but any changes in the translation of these specific messages was not confirmed. Metformin treatment led to a reduced incorporation of puromycin globally, as expected. Overall there are a number of important observations made within this study, and it is clearly a starting point for a great deal of future investigation. I have a few specific comments, questions and suggestions that the authors may take into consideration.

Comments for the author

1. The colocalization of Smaug MLOs with mitochondria is not very convincing, although I appreciate the authors used software to determine whether the colocalization was random or not. It could still be argued that it was only a very mild significance. The authors have showed videos of labelled mitochondria and Smaug in supp Vid2, but this is also not convincing. Do they move together? How would this kind of colocalization look if you labelled ER or endosomes as control? Looking a little more closely at the behaviour (image every 1-2 seconds) could be informative and help solidify this point. I'm not convinced that the MLOs are actively recruited to mitochondrial membranes, and it may not be important in a cultured cell like this. Perhaps in neurons, but even so there are many ways for RNA granules to transport.
2. The data shown in figure 2 reveal a reduction in complex I and II activity upon silencing the Smaugs, but it would be highly informative to run some blue-native page experiments to examine whether these changes reflect a loss of complex assemblies.
3. Some additional comments in the introduction on the additional mitochondrial mRNAs seen to bind Smaug in other studies would be helpful to the reader in interpreting the results. The citations used would take a lot of digging in the supplemental figures to understand the scope here. What level of "rewiring" would you expect from the loss of Smaug? Do other targets include additional OXPHOS messages, mitochondrial ribosomes, TCA cycle complexes, transporters? I agree that the dataset here with the 2 mRNAs is very important, but I fear that the focus on two could be misleading. They are excellent reporters, particularly in the FISH and pull-down qRT-PCR experiments, but its important to consider the spectrum of mRNAs that may be differentially regulated by Smaug.

4. How global is the metabolic effect of Smaug silencing? Do you see activation of AMPK (could drive Mff phosphorylation and explain the Drp1 recruitment PMID: 26816379)? Do you see mTOR inhibition with markers like S6K or others?
5. The choice of inhibitors used to visualize Smaug dissolution includes 2 complex I inhibitors, and CCCP. This led to the conclusion that inhibition of mitochondrial metabolism (defined broadly) signaled mRNA release and, likely, new translation to rescue the inhibition. As an extension of point 4 above, it would be interesting to add activators of AMPK, and potentially even active site inhibitors of TOR, to explore a little further what kind of metabolic stress Smaug is sensing.
6. Conceptually I wonder about the meaning of the subset of mitochondrial mRNAs controlled by Smaug relative to CLUH, Pum3 (discussed by the authors here) and perhaps also the observed regulation of mRNA translation by mTORC1/4EBP (PMID: 24206664). Smaug was linked to mTORC regulation in the supermodel mice carrying Smaug mutations, with greatly increased 4EBP phosphorylation, which they linked to the binding of Smaug to 14-3-3s (PMID: 24799716). In the study by Sonenberg it was suggested that it was a uniquely short 5' end of these messages encoding mitochondrial proteins that rendered them sensitive to mTOR regulated translation - which is distinct from the 3' polyA tails of Smaug binding. Smaug and mTORC1 are both nutrient sensing pathways and already shown to be linked. Indeed, metformin also regulates translation through mTOR/4EBP (PMID: 22611195 and others, although metformin targets are a bit of a mess), so the effects seen here on the dissolution of Smaug MLOs could also be linked to translation than directly on complex I inhibition. While the data shown here might also be consistent with the loss of Smaug somehow inhibiting mTORC1 (?), the fact that the mitochondria fragment upon Smaug silencing would really suggest AMPK activation and Mff phosphorylation, rather than the hyperfusion observed upon drug mediated TOR inhibition. Or what I'm saying is entirely wrong! I find it very fascinating and am convinced that there is something extremely important embedded in these data that will emerge in future studies. I suppose I'm only pushing for some mention of the bigger picture in the discussion outlining how the choice of Smaug binding mRNAs encoding mitochondrial proteins may complement those messages that are translated in a tightly regulated manner through the TOR pathway. I very much appreciate the discussion of the recent findings showing hedgehog regulation of Smaug, and those insights into neuronal regulation are also fascinating. I look forward to the future work from this group.

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
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Although the findings are intriguing and the topic worth of investigation, several mechanistic questions remain open, and not all the conclusions of the authors appear fully supported by the data. In the present form, this study therefore appears rather preliminary.

REVIEWER 1 Comments for the author
MAJOR COMMENTS
REVIEWER 1 -Point 1) Smaug and mitochondrial function: the data show that downregulation of Smaug 1 and 2 leads to a general reduction of the mitochondrial respiratory capacity and to mitochondrial fragmentation. Since in this study only two mRNAs encoding mitochondrial proteins are investigated, the reason for this phenotype remains unclear. How exactly are Smaug proteins affecting the expression of mitochondrial protein? The authors could easily test if the steady state

levels of components of the respiratory chain (including but not limited to SDHB and UQCRC1) are reduced. Blue-native gels could also inform on the assembly of the respiratory chain complexes.

OUR RESPONSE:
We have analyzed Uqcrc1 protein levels by immunofluorescence and found that Smaug1/2 KD correlates with higher UQCRC1 levels, which is compatible with translational repression by Smaug proteins. UQCRC1 protein levels were significantly upregulated in a fraction of cells, averaging 1.5 X (New FIGURE 2B and page 6, first paragraph). In accordance with this imaging data, the upregulation was less dramatic when analyzed by western blot of both whole cell extract or mitochondrial extracts prepared with specific purification kits (new Supplementary Figure S2A).
We have also investigated by western blot the levels of the following components of the respiratory chain: CI subunit NDUFB8, CII subunit SDHB, CIII-UQCRC2, CIV subunit MTOC1, and CV subunit VATP5A, and found no major changes (New Supplementary Figure S2A)
We invested great efforts in blue gels, which didn´t result successful in U2OS cells, while blue gels of mouse liver extracts performed in parallel were of excellent quality. We will be happy to provide these images upon request. Relevantly, blue gels of U2OS cells are not frequent in the literature, likely in connection with their scarce utility.

Finally, regarding the observation that “only two mRNAs encoding mitochondrial proteins are investigated”:
The definition or validation of the RNA regulon affected by Smaug is beyond the scope of the present work.
As mentioned in the MS, Smaug has been linked to mitochondrial mRNAs and mitochondrial phenotypes before (See supplementary Table I, Chen et al 2014; Chartier et al, 2015; Schatton and Rugarli 2018). In the present work, we are aimed to investigate the biological significance of Smaug1-MLO condensation and its regulation by mitochondrial respiration and cellular energetics. In this context, we used UQCRC1 and SDHB mRNAs as model mRNAs to analyze:

a) whether mRNAs bound by Smaug are indeed present in Smaug MLOs (FIGURE 1E, 1F)

b) whether defective Smaug1 MLO formation affects mRNA binding (Figure 6C; Supplementary Figure 4SB,C)

c) whether Smaug1 MLO dissolution upon specific cellular clues affects the interaction with target mRNAs (Figure 8C; Supplementary Figure 4SE,F).

None of these issues have been assessed before and we choose two example mRNAs to investigate these three important questions. As described in the initial submission, we fully agree that the reason of the phenotype include but are not limited to the dysregulation of SDHB and UQCRC1, as many other mitochondrial mRNAs have been shown to be affected by Smaug in both Drosophila and mammals (Aviv et al 2003, Chartier, et al. 2015, Chen, et al. 2014, SUPPLEMENTARY TABLE I). We have modified the text to further stress the point (Introduction, page 3, 2nd paragraph, page 4, 2nd paragraph; Results, page 5 3rd paragraph; page 7, 3rd paragraph; Discussion page 13, 3rd paragraph)

REVIEWER 1 -Point 2) Smaug bodies and co-localization with SDHB and UQCRC1 mRNAs: The way how this co-localization was analyzed is not convincing. The authors mention in the methods that this was assessed manually. What does this mean exactly? The authors should use Manders’ or Pearson’s methods for co-localization. In addition, the fact that the authors see less percentage of co-localization in random images does not prove anything until a statistical test is applied (for example Fisher exact test) to make sure that this difference is significant. The authors state that more than 400 MLOs were analyzed. From how many cells? In how many experiments?

OUR RESPONSE.
a) The criteria used for the manual categorization of “contacting” or “no contacting” bodies are depicted in panel 1F. Comparison with randomized images is a standard strategy to evaluate this type of spatial relationship between mRNAs and cellular organelles (Denes et al, bioRxiv 2021.02.26.433059). In the revised version, the statistical analysis for the presence of only SDHB mRNA, only UQCRC1 mRNA, of both mRNAs has been performed by paired t-test (New Figure 1F). In all cases the experimental value was significantly higher than the obtained in randomized images.

b) As requested, we have additionally applied Manders' analysis. Again, values resulted statistically lower in the randomized images (Paired t test) (right panel in new Figure 1F). However, Manders' and Pearson are mostly used to evaluate overlapping, which is not strictly the case here. SDHB and UQCRC1 mRNA molecules were detected inside as well as in the periphery of the Smaug1-bodies, and manual assessment is a more suitable analysis in this case. This spatial relationship is frequent in MLOs containing mRNAs, such as PBs and SGs. In both cases a number of mRNAs were shown to be tethered to MLOs, rather than inside MLOs (Moon SL, et al Nat Cell Biol. 2019).

c) In both strategies, manual assessment or Manders' analysis, we have analyzed additional images from the two independent experiments previously submitted. In the present version, a total of 751 bodies, from 13 cells from 4 coverslips were analyzed. Values remain virtually the same:
Uqcrc1: 24% (previously 23%)
SDHB: 34% (previously 33%)
Double: 15 % (previously 13%)

REVIEWER 1 -Point 3) The role of MLOs: the authors try to dissect the region of the Smaug protein responsible for MLO formation and to understand what is the role of these membraneless organelles, however this last question remains unanswered. They propose that mRNAs are released upon dissolution of the MLOs. There is no strong data supporting this notion. In addition, although it is interesting to observe that rotenone and metformin lead to the progressive reduction of MLOs, it remains to be determined why this is the case and what this means for the translation of mRNAs encoding mitochondrial proteins. These treatments could for example induce other type of granules, such as stress granules, or simply induce cell suffering, leading to increased autophagy.

OUR RESPONSE
a) We have performed new experiments that inform on the fate of mRNAs during Smaug1-body dissolution upon exposure to rotenone, metformin or rapamycin (NEW FIGURE 8D). A strategy commonly used in the specialized literature is to compare the effect of translation inhibitors that either "freeze" or disrupt polysomes. Cycloheximide vs puromycin is a common pair, and they have been used to demonstrate that PB dissolution correlates with the translation of released mRNAs (reviewed in Buchan JR, Parker R. Mol Cell. 2009). We have used this strategy previously in neurons, where the Smaug1-bodies respond to specific neurotransmitters by releasing transcripts that enter translation (Baez et al. J Cell Biol 2011).
Here we found that the presence of puromycin impairs the dissolution of Smaug1-bodies triggered by metformin, rotenone or rapamycin (NEW FIGURE 8D). These observations strongly suggest that Smaug MLOs dissolution is linked to the translational activation of bound mRNAs.

b) Regarding the observation that "these treatments could for example induce other type of granules, such as stress granules, or simply induce cell suffering, leading to increased autophagy":

i) First of all, the dynamics of other RNA granules is beyond the scope of the present work. Regarding the potential induction of SGs, is the reviewer asking whether mRNAs might be transferred from dissolving Smaug1-bodies to SGs?
First, metformin does not induce SGs, as expected for a drug used in the clinics, which shouldn't induce serious cellular stress. New Figure 7C shows no SG formation upon exposure to rotenone or metformin, while as expected arsenite strongly induced SGs. Smaug1-bodies showed an opposite behavior and do not respond to arsenite, but dissolve upon rotenone or metformin, which do not induce SGs. In other words, Smaug1-body dissolution does not correlate with SG formation and thus, it's seems largely unlikely that mRNAs are transferred from Smaug1-bodies to SGs.

ii) Regarding cell suffering and autophagy:
In the previous submission we have shown that CCCP, which is known to induce serious damage does not induce Smaug1-body dissolution. Similarly, the new data show that arsenite did not induce the dissolution of the Smaug1-bodies. Collectively, these observations indicate that Smaug1-body dissolution is not the consequence of a loss of cellular fitness. Rather, cell damage do not correlates with Smaug1-body dissolution, likely reflecting that Smaug1-body dissolution is part of a regulated cellular response that requires translational competence and likely additional cellular capabilities.

Regarding autophagy:
The time-lapse confocal analysis previously submitted shows that Smaug1EYFP puncta “shrinks” and do so gradually, starting immediately after exposure to metformin.
This pattern was clearly described in the figures and in the text in the previous submission and is quite incompatible with autophagy-mediated clearance (previous Figure 8C and D, current Figure 8A and 8B, video 3). In addition, it’s worthy to mention that metformin is not expected to induce autophagy in the conditions used.
Furthermore, new data (New panel 8D) shows that puromycin blocks metformin-induced Smaug-body dissolution. Puromycin does not block autophagy (it rather promotes it), thus adding evidence against a role for autophagy in Smaug1-body dissolution and supporting the incorporation of bound mRNAs to polysomes.
Finally, real-time PCR indicates that SDHB mRNA and UQCRC1 mRNA levels remained unchanged upon treatments that induce Smaug1-body dissolution (previously data not shown, now new panel S4F). Again, this is against their autophagy-mediated clearance.

All this fits with current literature that supports a model where the assembly and disassembly of MLOs can be conceptualized as phase transitions or demixing and mixing processes. Autophagy may drive a processes termed “granulophagy”, as for example during SG clearance under certain cellular conditions, or even mRNA clearance upon starvation (work by Roy Parker and others: Buchan et al, Cell. 2013; Frankel et al., Autophagy 2016). However, the above evidence indicates that granulophagy does not seem to be the case of the effect elicited by metformin, rotenone or rapamycin.

REVIEWER 1 -Point 4) Quantification of many experiments is not convincing (see again point 2). This applies to the quantification of the contacts between mitochondria and MLOs; quantification of Drp1 puncta (western blot of Drp1 in mitochondria or cytosol could be a more convincing method). In addition, in several cases, experiments have been performed only once with a technical duplicate. The authors should be very cautious to draw conclusions from these data. Showing a SD and applying a t-test these data is meaningless.

OUR RESPONSE:
a) Contacts with mitochondria: the quantification by manual assessment was replaced by an automatized analysis that calculate the euclidean distance between each Smaug1 body and the nearest mitochondria. Distances to mitochondria were calculated in original and randomized images obtained by randomization of Smaug1-bodies’ coordinates, according to current strategies (Denes, et al 2021; bioRxiv 2021.02.26.433059). Smaug1 bodies were classified as "in contact" when the distance was 0 (new panel 1G). The average values were 57% (original images) vs 49% (randomized images) ($p<0.001$, new panel 1G), similar to the previously submitted numbers obtained by manual assessment (58% vs 43%), which are not included in the present version.
Interestingly, these values are similar to those reported for the contact of PBs with mitochondria by Dominique Weil and coworkers (66% vs 58-60 %; Huang et al. J Biol Chem. 2011).
In addition, we have analyzed by western blot the presence of both Smaug1 and Smaug 2 in purified mitochondria obtained with a specific purification kit. In accordance with imaging data showing proximity but no colocalization, we found that Smaug proteins do not co-purify with mitochondria (new panel 1I)
b) Quantification of DRP1 recruitment by imaging is a common practice and was performed in duplicate experiments, as previoulsy indicated. In addition, we have further analyzed DRP1 recruitment by western blot as requested (new panel 3G). In agreement with the previously submitted data, we found a significant increase of DRP1 levels in the mitochondrial fraction upon KD of Smaug1 and Smaug2.

c) Replicates: Key experiments were repeated at least 3 times, some of them 5 or 6 times. Stainings were repeated at least twice, very often 5-6 times.
We apologize for omitting this important information in a few figure panels not related to the most important findings.
The number of independent replicate experiments is now indicated in each figure legend. In a few cases additional replicates and/or more cells or Smaug1 MLOs were analyzed from previously submitted experiments. Details follows:

Molecular studies:
-mRNA co-pulldown: as previously indicated, three independent experiments were performed in both fig 1D and 8C; and five independent experiments in figure 6C.
-tethering assays (fig 6B): six independent replicates, as previously indicated.
-Smaug1/2 KD (Fig 2A), two independent experiments were used for RT-PCR quantification (fig 2A). Validation by western blot was performed routinely.

Imaging:
-smFISH (Figure 1F): two independent stainings were used. We increased the number of images analyzed, with a total of 751 bodies from 13 cells from 4 coverslips.
-Smaug1 MLOs' contacts with mitochondria. A representative experiment out of three assessed by the new script is depicted (Figure 1G).
-Effect of Cycloheximide and puromycin on Smaug1 MLOs (Figure 1B and 8D). 5 times for CHM, three times for Pur
-Ubiquitin and 18S FISH stainings (Current Supplementary Figure 1SA): two independent stainings were performed, as indicated previously.

-Live cell imaging, three independent experiments with several movies each were performed for untreated cells (current fig 1C) and three experiments including both control and treated cells (current figure 8A and 8B).

Mitochondrial function:
-Respirometry: as previously indicated, three independent experiments performed in duplicate (current 2C)
-Mitochondrial membrane potential by JC1: FACS (current 2D), three independent experiments. Imaging (Current 2E) – two independent experiments, as previously indicated.
-mitochondrial network fragmentation upon Smaug1+Smaug2 KD: a total of eight independent experiments (figures 3A, figure 4; current Figure 6).
-Mitochondrial fusion/fission upon Smaug1+Smaug2 KD: two independent replicates for SIMH, western blot analysis of Opa1 fragments and Mfn2; and DRP1 recruitment (current 3D, 3E and 3F)
-phenotype rescue: three independent experiments (Current Fig 4 and 6A)

Smaug 1-body dissolution and related studies
-Rotenone effect on Smaug1 MLOs: three independent experiments for endogenous Smaug1; five independent experiments for transfected Smaug1-EYFP (figure 7A, B, C, D)
-metformin effect on Smaug1 MLOs: three independent experiments for endogenous Smaug1; eight independent experiments for transfected Smaug1-EYFP (figure 7A, B, C, D; supplementary figure S4D)
-rapamycin effect on Smaug1 MLOs: four independent experiments (figure 7B and D)
-Compound C effect on Smaug1 MLO dissolution: three independent replicates (new 7D).
-Puromycylation: two independent replicates (7E)
-mitochondrial fragmentation upon rotenone and CCCP: three independent experiments for imaging; two for western blot analysis. (The effect of these inhibitors is well known)

REVIEWER 1 -Point 5) In many instances, the authors mention data not shown. These data are important controls that should be present in the manuscript.

OUR RESPONSE:
The three cases of data not shown are now depicted in new figure panels, as follows:
i) Images of HeLa and Cos7 cells depicting a uniform distribution of ΔSSR1/2 are included in new supplementary Figure S4A.
ii) Controls linked to figure 6C: Protein expression levels and pull-down recovery of the V5-SBP-tagged constructs were assessed by western blot shown in new supplementary figure S4B. In addition, mRNA levels in the input samples are included as well in a new supplementary figure (S4C).
iii) Controls linked to Figure 1D: Protein expression levels and recovery of V5-SBP-tagged constructs were assessed by western blot, which are depicted in new Supplementary Figure S1B.

Finally, additional modifications introduced in this resubmission include:
a) An improved scheme that integrates the new data replaces the model in Figure 8E.

b) The Discussion section has been shortened and overall didactics has been improved. These minor changes are not highlighted unless they are directly connected with the reviewers' concerns.
c) Nine references were added to support new methodology and concepts.
d) A number of figure panels has been rearranged. Major changes follows:
-plot in previous 7A: quantifications (% of cells with Smaug1 bodies) are indicated in the microscopy images, which include the effect of metformin (previously depicted in 8A).
-plot in previous 7B: quantifications (% of cells with Smaug1-EYFP bodies) are included in the microscopy images, which include the effect of metformin and rapamycin
-Previous Figure 8A is included in 7A in the current version
-Zoomed insets in Sup Figure 4A are included in current Figure 8A
-The dose-response plot previously depicted in Figure 8B is included in current Fig S4D.

Reviewer 2 Advance summary and potential significance to field
This study by Fernández-Alvarez/Thomas et al. examines the relationship between the RNA binding proteins Smaug1/2 as a regulator of mitochondrial function and morphology through the repression of two mRNAs encoding subunits of Complex I and II. Known to bind specific elements in RNA, Smaug was initially shown to mediate RNA decay through deadenylation of polyA tails and act as a repressor of translation. More recent studies in different systems has revealed it can also repress mRNA translation without causing decay. There are many messages that bind Smaug but a number of studies have shown an enrichment in mitochondrial mRNAs, consistent with studies in yeast and mouse mutant models showing significant effects on metabolism.

This study looks at Smaug1/2 in cultured cells and makes a number of exciting observations. Perhaps the most striking is the dissolution of Smaug from membraneless organelles (MLO) upon inhibition of mitochondrial complex I, but not upon complete uncoupling with CCCP. This is the first time these kinds of MLOs have been seen to respond to mitochondrial metabolism. In addition, they observed that loss of Smaug leads to a reduction in complex I and II activities, along with increased mitochondrial fragmentation, presumably through the activation of Drp1 mediated fission (as opposed to inhibition of fusion). The binding of the two mitochondrial mRNAs to Smaug after MLO dissolution was reduced after 1 hour metformin treatment, but any changes in the translation of these specific messages was not confirmed. Metformin treatment led to a reduced incorporation of puromycin globally, as expected. Overall there are a number of important observations made within this study, and it is clearly a starting point for a great deal of future investigation. I have a few specific comments, questions and suggestions that the authors may take into consideration.

REVIEWER 2 Comments for the author
POINT 1.The colocalization of Smaug MLOs with mitochondria is not very convincing, although I appreciate the authors used software to determine whether the colocalization was random or not. It could still be argued that it was only a very mild significance. The authors have showed videos of labelled mitochondria and Smaug in supp Vid2, but this is also not convincing. Do they move together? How would this kind of colocalization look if you labelled ER or endosomes as control? Looking a little more closely at the behaviour (image every 1-2 seconds) could be informative and help solidify this point. I'm not convinced that the MLOs are actively recruited to mitochondrial membranes, and it may not be important in a cultured cell like this. Perhaps in neurons, but even so there are many ways for RNA granules to transport.

OUR RESPONSE:
We fully agree that although statistically significant, the proximity of Smaug1 MLOs to mitochondria is only modestly above random values (57% (original images) vs 49% (randomized images), ($p<0.001$). Interestingly, the proximity between Smaug1 MLOs and mitochondria is comparable to that of PBs and mitochondria, described by Dominique Weil and coworkers (66% vs 58-60 %; Huang et al. J Biol Chem. 2011).

Videos accompanying this manuscript are intended to show Smaug MLO dynamics (i.e, fusion and dissolution) and their temporal resolution is not the best to finely track the movement of Smaug1MLO relative to mitochondria. On spite of this limitation, we were able to preliminary found examples of Smaug1-EYFP MLOs that remained associated to mitochondria during at least 30minutes and examples of Smaug1-EYFP MLOs that briefly contact the mitochondrial surface, as reported previously for PBs. Additional studies, including movies with higher time-resolution and imaging of additional organelles as suggested by the reviewer will help to better describe the docking, its

duration and any potential regulation by mitochondrial activity. Unfortunately, due to the pandemic our confocal facility has been working with reduced timetables and time-lapse microscopy has not being available.
We also fully agree that these observations may result more significant in neurons or muscle cells, where mitochondrial activity is highly relevant. Indeed, our immediate plan is to investigate S-body dynamics in primary cells.

However, given the bidirectional interplay between Smaug MLOs and mitochondrial function, we think that at least a partial answer should be provided to the question of whether Smaug1 MLOs contact mitochondria. The manual assessment previously submitted was replaced by an automatized analyisis. Briefly, according to current strategies (Denes, et al 2021; bioRxiv 2021.02.26.433059), the euclidean distance between each Smaug1 body and the nearest mitochondria was calculated in original and randomized images (obtained by randomization of Smaug1-bodies' coordinates). Smaug1-bodies were classified as "in contact" when the distance was 0 (new panel 1G). The average values were 57% (original images) vs 49% (randomized images) ($p<0.001$), similar to the previously submitted numbers obtained by manual assessment (58% vs 43%), which are not included in the present version.

In addition, we analyzed by western blot the presence of Smaug 1 and Smaug 2 in mitochondrial fractions isolated with a specific purification kit. In accordance with imaging data showing a certain degree of proximity but not colocalization, we found that Smaug proteins do not co-purify with mitochondria, further suggesting that Smaug MLOs are not strongly tethered to the mitochondrial surface (new Figure 1I).
The text and model in panel 8E were modified accordingly.

REVIEWER #2
POINT 2.The data shown in figure 2 reveal a reduction in complex I and II activity upon silencing the Smaugs, but it would be highly informative to run some blue-native page experiments to examine whether these changes reflect a loss of complex assemblies.

OUR RESPONSE:
Unfortunately, we were unable to obtain reliable blue gels with U2OS extracts. We invested great effort in this technique and blue gels of mouse liver extracts performed in parallel were of excellent quality. We will be happy to provide these images upon request. Relevantly, blue gels of U2OS cells are not that frequent in the literature, likely in connection with their scarce utility.

To further describe the mitochondrial phenotype caused by Smaug loss of function, we have analyzed UQCRC1 protein levels by immunofluorescence and found that Smaug1/2 KD correlates with higher UQCRC1 levels, which is compatible with translational repression by Smaug proteins. UQCRC1 protein levels were significantly upregulated in a fraction of cells, averaging 1.5 X (New Figure 2B and page 6, first paragraph). In accordance with this imaging data, UQCRC1 upregulation was less dramatic when analyzed by western blot of whole cell extracts or mitochondrial extracts prepared with specific purification kits (new Supplementary Figure S2A).
We have also investigated by western blot the levels of the following components of the respiratory chain: CI subunit NDUFB8, CII subunit SDHB, CIII-UQCRC2, CIV subunit MTOC1, and CV subunit VATP5A, and found no major changes (New Supplementary Figure S2A)

Collectively, these observations suggest that the mitochondrial phenotype correlates with an upregulation of UQCRC1 and no major changes in other ETC enzymes. While future work will provide additional mechanistic insights -which might involve defective assembly of ETC supercomplexes-, I would like to stress that Smaug has been linked to mitochondrial function before and this work focuses on the relevance of the condensation of Smaug MLOs. Importantly, single-cell imaging is a suitable read-out in rescue experiments where condensation-defective Smaug mutants are transfected, and approaches that depend on "bulk" analysis such as blue gels are less useful.

REVIEWER #2
POINT 3.Some additional comments in the introduction on the additional mitochondrial mRNAs seen to bind Smaug in other studies would be helpful to the reader in interpreting the results. The

citations used would take a lot of digging in the supplemental figures to understand the scope here. What level of “rewiring” would you expect from the loss of Smaug? Do other targets include additional OXPHOS messages, mitochondrial ribosomes, TCA cycle complexes, transporters? I agree that the dataset here with the 2 mRNAs is very important, but I fear that the focus on two could be misleading. They are excellent reporters, particularly in the FISH and pull-down qRT-PCR experiments, but its important to consider the spectrum of mRNAs that may be differentially regulated by Smaug.

OUR RESPONSE:
We fully agree with this concept: Smaug was shown to bind numerous mRNAs and the observed phenotype is undoubtedly a consequence of the dysregulation of many of them, likely including mRNAs encoding additional mitochondrial as well as non-mitochondrial proteins (Chen, Dumelie et al. 2014).
We have modified the Introduction, Results and Discussion sections to further strength the concept (see highlighted paragraphs in Introduction, page 3, 2nd paragraph, page 4, 2nd paragraph; Results, page 5, 3rd paragraph; page 7, 3rd paragraph; Discussion page 13, 3rd paragraph).
In addition, a detailed list of fly messengers encoding mitochondrial proteins previously reported as Smaug targets and potentially linked to the phenotype described in this work is provided in Supplementary Table I. As the reviewer anticipated, these mRNAs code for TCE enzymes and transporters, or are linked to several functions including mitochondrial translation (ribosomal proteins, tRNA modifying enzymes and additional factors) as well as protein import and folding. In addition, Chen et al(2014) reported that mRNAs for several enzymes linked to glycolysis and related processes are bound and/or degraded and/or translationally repressed by Smaug in Drosophila embryos, including hexokinase A; phosphoglucose isomerase; glyceraldehyde 3 phosphate dehydrogenase 1, glyceraldehyde 3 phosphate dehydrogenase 2; phosphoglycerate kinase, transaldolase, phosphogluconate dehydrogenase, piruvate carboxylase, alcohol dehydrogenase and likely a few more (Supplementary Table 22 in Chen et al 2014). Glycolytic function is not assessed in the present work and these transcripts are not included in the Sup Table 1.

REVIEWER 2:
POINT 4.How global is the metabolic effect of Smaug silencing? Do you see activation of AMPK (could drive Mff phosphorylation and explain the Drp1 recruitment PMID: 26816379)? Do you see mTOR inhibition with markers like S6K or others?

OUR RESPONSE:
We agree with the reviewer that AMPK is an immediate candidate pathway in connection with increased DRP1 recruitment.
We have performed western blot analysis of AMPK, Phospho AMPK and its target ACC and found no evidence that AMPK is activated upon Smaug1/2 KD (SUPPLEMENTARY FIGURE S3B). In parallel, we tested the effect of metformin, rotenone and the AMPK activator AICAR, and as expected all them efficiently induced AMPK phosphorylation (SUPPLEMENTARY FIGURE S3B).
Thus, AMPK does not seem to be activated upon Smaug1/2 silencing and thus, DRP1 recruitment is unlikely to involve MFF. Interestingly, in addition to Mff, three additional DRP1 receptors were described (Fis1; MiD49 and MiD51). MiD 51 requires ADP as cofactor, thus providing another potential link between mitochondrial fission and energy metabolism (PMC3926961; PMID: 24508339). In summary, at this stage of the work, which signaling pathway(s) are activated downstream of Smaug KD and which DRP1 receptors are involved remains unclear.

REVIEWER 2:
POINT 5.The choice of inhibitors used to visualize Smaug dissolution includes 2 complex I inhibitors, and CCCP. This led to the conclusion that inhibition of mitochondrial metabolism (defined broadly) signaled mRNA release and, likely, new translation to rescue the inhibition. As an extension of point 4 above, it would be interesting to add activators of AMPK, and potentially even active site inhibitors of TOR, to explore a little further what kind of metabolic stress Smaug is sensing.

OUR RESPONSE:
This is another relevant point raised by the reviewer.
We have added the analysis of rapamycin, a known mTOR inhibitor, and found that rapamycin elicited a similar effect (new panel in figure 7B). Importantly we have challenged the responses elicited by metformin, rotenone or rapamycin with Compound C, a known AMPK inhibitor. We found

that Compound C dramatically inhibits the dissolution of Smaug1-body triggered by any of these stimuli (new panel 7D). Collectively, these observations directly implicate AMPK/mTOR in the control of Smaug1 MLOs.

In addition, we have tenaciously investigated the effect of the AMPK activator AICAR. As expected AICAR strongly induced AMPK phosphorylation (Fig S3B) and mitochondrial fragmentation, as described in PMID:26816379 (quoted by the reviewer in point 1). However, no effect on the Smaug1-bodies was observed. We speculate that the strong AMPK activation by AICAR is not fully comparable with its indirect (and more moderate) activation by rapamycin or rotenone (supplementary Figure S3B). Among other factors, strong AMPK activation is known to induce mitophagy (Egan et al.,Science 2011-PMCID: PMC3030664) and this might signal against Smaug1-body dissolution. Remarkably, the uncoupler CCCP strongly fragments mitochondria and does not affect Smaug bodies. These observations are compatible with a response where Smaug1 bodies react to physiological changes of the AMPK/mTOR balance but are not responsive when mitochondrial integrity is seriously compromised. Relevantly, exposure to arsenite did not affect Smaug1 bodies (new panel 7C).
For simplicity, the experiments with AICAR are not included in this revised manuscript and we will be happy to share them with the reviewer upon request.

REVIEWER 2
POINT 6.Conceptually I wonder about the meaning of the subset of mitochondrial mRNAs controlled by Smaug relative to CLUH, Pum3 (discussed by the authors here) and perhaps also the observed regulation of mRNA translation by mTORC1/4EBP (PMID: 24206664). Smaug was linked to mTORC regulation in the supermodel mice carrying Smaug mutations, with greatly increased 4EBP phosphorylation, which they linked to the binding of Smaug to 14-3-3s (PMID: 24799716). In the study by Sonenberg it was suggested that it was a uniquely short 5' end of these messages encoding mitochondrial proteins that rendered them sensitive to mTOR regulated translation - which is distinct from the 3' polyA tails of Smaug binding. Smaug and mTORC1 are both nutrient sensing pathways and already shown to be linked. Indeed, metformin also regulates translation through mTOR/4EBP (PMID: 22611195 and others, although metformin targets are a bit of a mess), so the effects seen here on the dissolution of Smaug MLOs could also be linked to translation than directly on complex I inhibition. While the data shown here might also be consistent with the loss of Smaug somehow inhibiting mTORC1 (?), the fact that the mitochondria fragment upon Smaug silencing would really suggest AMPK activation and Mff phosphorylation, rather than the hyperfusion observed upon drug mediated TOR inhibition. Or what I'm saying is entirely wrong! I find it very fascinating and am convinced that there is something extremely important embedded in these data that will emerge in future studies. I suppose I'm only pushing for some mention of the bigger picture in the discussion outlining how the choice of Smaug binding mRNAs encoding mitochondrial proteins may complement those messages that are translated in a tightly regulated manner through the TOR pathway. I very much appreciate the discussion of the recent findings showing hedgehog regulation of Smaug, and those insights into neuronal regulation are also fascinating. I look forward to the future work from this group.

OUR RESPONSE:
We share the reviewer's enthusiasm as we think that the submitted observations are the beginning of a fascinating story. Let´s go point by point as this paragraph is full of wonderful questions and ideas.
a) First, is there an overlapping or rather mutual exclusion of the "RNA regulons" defined by Smaug, CLUH and Pumilio? It will be great to have clear answer, but current knowledge is not enough to define the point. The lists of target mRNAs in each case were obtained by different strategies, and are from different cell types and organisms. Thus, their comparison is not truly informative. However, a number of interesting observations emerges. For example, the ATPsyntase subunit ATP5a1 mRNA is regulated by CLUH, whereas the beta subunit mRNA is under Drosophila Smaug control. This suggests that the translational regulation of this particular multimeric enzyme depends on both CLUH and Smaug.
In addition, mammalian CLUH granules and Smaug MLOs appears to behave quite differently. Mammalian CLUH granules are enhanced by nutrient starvation, and active translation appears to occur on mammalian CLUH granules (Pla-Martin et al EMBOJ 2020). (However the Drosophila ortholog Clueless shows a somehow different behavior and form granules that dissolve upon

starvation (Sheard et al., Dev Biol 2020)). Thus, if common target mRNAs do exist, the regulatory consequences might be different, likely providing response diversity.
Similar speculations can be done for Pumilio, and whether Smaug and Pumilio share target mRNAs and whether their activities are redundant, synergic, or opposing remains open. As substantial work is still required, we elaborate very shortly on these complex issues.
b) Regarding Smaug and mTOR in Supermodel paper (PMID: 24799716). Supermodel point mutation H86P affects S254 and S658 phosphorylation by AKT (and binding to 14-3-3). These observations add to the regulation of Smaug by metabolism-linked pathways. In addition, mTOR is inhibited in supermodel animals and whether the effect is direct or indirect is unknown. Didactics of previous paragraph describing Supermodel mice has been improved (Introduction, highlighted text, page 3 bottom).

c) Finally, and very important: Regarding mRNAs affected by metformin and mTOR inhibitors (all papers quoted by the reviewer are relevant and included –although not deeply discussed- in our manuscript). Sonenberg's lab (quoted PMID: 24206664 and PMID: 22611195) has shown that metformin (as well as rapamycin and PP242-another mTOR inhibitor-) affects the translatome involving 4EBP phosphorylation. These studies were performed with long treatments and high concentrations of metformin (12 hs 10 mM). This largely differs from the acute treatment shown here (1 h pulse with 1mM Metformin), which dissolves Smaug MLOs and releases mRNAs that are incorporated into polysomes (previous Fig 8A-D, current 7A-D and new figure 8D).
Adding to the different output of these different treatments, mitochondrial blocking by rotenone or metformin reduces global translation (PMID: 24206664 and PMID: 22611195, Howell et al., Cell Metabolism 2016), although moderately in the present conditions (previous Figure 7C, current 7E), and transiently in some other examples (15-30 min as in Kalender et al Cell Metabolism 2010). However, altogether these observations collectively suggest that changes in mitochondrial respiration triggers a translational reprogramming, with some transcripts being silenced (those strongly depending on mTOR) and others presumably being activated (those released from dissolving Smaug1-bodies). We have elaborated this point briefly in the revised Discussion (page 15, top).

Finally, additional modifications introduced in this resubmission include:
a) An improved scheme that integrates the new data replaces the model in Figure 8E.
b) The Discussion section has been shortened and overall didactics has been improved. These minor changes are not highlighted unless they are directly connected with the reviewer' concerns.
c) Nine references were added to support new methodology and concepts.
d) A number of figure panels has been rearranged. Major changes follows:
-plot in previous 7A: quantifications (% of cells with Smaug1 bodies) are indicated in the microscopy images, which include the effect of metformin (previously depicted in 8A).
-plot in previous 7B: quantifications (% of cells with Smaug1-EYFP bodies) are included in the microscopy images, which now include the effect of metformin and rapamycin
-Previous Figure 8A is included in 7A in the current version.
-Zoomed insets in previous Sup Figure 4A are included in current Figure 8A
-The dose-response plot previously depicted in Figure 8B is included in current Fig S4D.

Second decision letter

MS ID#: JOCES/2020/253591

MS TITLE: Smaug1 membrane-less organelles respond to AMPK/mTOR and affect mitochondrial function

AUTHORS: Ana Julia Fernández-Alvarez, María Gabriela Thomas, Malena Lucia Pascual, Martín Habif, Jerónimo Pimentel, Agustín Andres Corbat, João Pedro Pessoa, Pablo Ezequiel La Spina, Lara Boscaglia, Anne Plessis, Maria Carmo-Fonseca, Hernan Edgardo Grecco, Marta Casado, and Graciela Lidia Boccaccio
ARTICLE TYPE: Research Article
We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

There is only one minor amendment relating to data described in Lines 307-310 which I hope that you will be able to carry these out because I would like to be able to accept your paper. I would not intend to send it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This is an interesting paper that brings forward the knowledge on the role of Smaug bodies and the relevance for mitochondrial function.

Comments for the author

We really appreciate the efforts made by the authors to answer the criticisms of the Reviewers. They have added new experiments, important information on independent biological repetitions and statistical evaluation, and rephrased the manuscript to make their message more clear. The manuscript is now significantly improved.

Minor comments:
Line 88: "bind" not "bound"
Lines 307-310 describe an experiment, but there is no Figure showing it. This should be added, at least as Supplementary figure.

Reviewer 2

Advance summary and potential significance to field

I was already very excited about this study and offered a few suggestions, mainly conceptual, to be discussed and/or addressed with some experiments where relevant. The authors have responded in an incredibly thoughtful way to my remarks and I think it has helped me place the observed effects of Smaug shown here in context of my understanding of mTOR, CLUH, PUM3, etc to link the various aspects of metabolic control of mitochondrial function. I think this is a very impressive study and I am fully satisfied with the reviewers response to my questions, so I have no further concerns. This has made an important new contribution to the field and I congratulate all authors.

Comments for the author

no revisions.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
This is an interesting paper that brings forward the knowledge on the role of Smaug bodies and the relevance for mitochondrial function.

Reviewer 1 Comments for the author
We really appreciate the efforts made by the authors to answer the criticisms of the Reviewers. They have added new experiments, important information on independent biological repetitions and statistical evaluation, and rephrased the manuscript to make their message more clear. The manuscript is now significantly improved.

Minor comments:
Line 88: "bind" not "bound"

Lines 307-310 describe an experiment, but there is no Figure showing it. This should be added, at least as Supplementary figure.

OUR RESPONSE:
We have corrected the grammar mistake in lane 88.
We also added the figure corresponding to the experiment described in lines 307-310 as a new panel in the Supplementary Figure S4

Third decision letter

MS ID#: JOCES/2020/253591

MS TITLE: Smaug1 membrane-less organelles respond to AMPK/mTOR and affect mitochondrial function

AUTHORS: Ana Julia Fernández-Alvarez, María Gabriela Thomas, Malena Lucia Pascual, Martín Habif, Jerónimo Pimentel, Agustín Andres Corbat, João Pedro Pessoa, Pablo Ezequiel La Spina, Lara Boscaglia, Anne Plessis, Maria Carmo-Fonseca, Hernan Edgardo Grecco, Marta Casado, and Graciela Lidia Boccaccio
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Thank you for those final revisions. I did not consider it necessary to return this to the reviewers.