Mdm1 maintains endoplasmic reticulum homeostasis by spatially regulating lipid droplet biogenesis

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September 13, 2018

Re: JCB manuscript #201808119

Dr. Mike Henne
UT Southwestern Medical Center
6000 Harry Hines Blvd NL6.120D
Dallas, Texas 75390

Dear Dr. Henne,

Thank you for submitting your manuscript entitled "Mdm1 maintains endoplasmic reticulum homeostasis by spatially regulating lipid droplet biogenesis". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that while all three reviewers are enthusiastic about your study, they have a number of concerns. The most important request, made by both reviewers 1 and 2, is for more functional insight into the relationship between Mdm1 and Faa1. How does the interaction of Mdm1 and Faa1 affect Faa1 activity and TAG production? The suggestion of review 1 (points 1 and 2) to focus on Faa1 activity and TAG synthesis, rather than on FAA accumulation, is a good idea. The other points raised by the reviewers should be addressed as well. While additional information about how various nutritional stresses affect LD clustering (rev 2, points 2 and 3) and whether fatty acid addition induces the UPR (rev 3, point 2) is welcome, addressing these point is not necessary.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

William Prinz, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Hariri et al. provide data to support the idea that the ER protein Mdm1 tethers the ER to lipid droplets via its N-terminal region at sites of droplet budding and that the PXA domain binds fatty acids and interacts with the acyl-CoA synthetase Faa1 at the bud sites. The experiments are convincing and generally support the conclusions, but several questions remain.

1. The authors focus on free fatty acids (FFA) as a marker of functional Faa1; FFA mark the lack of Faa1 activity. It would be more accurate to write about the actual function of Faa1, i.e. FA activation and acyl-CoA incorporation into TAG. Related to this point is the finding of "exacerbated" FA accumulation (page 9) in the ΔLDmdm1Δ strain and elsewhere. Would it not be more accurate to focus on the decrease in TAG levels in each case?
2. The authors imply that the association of Mdm1 and Faa1 is important and requires the PXA domain, but the functional outcome is unclear. By showing that free fatty acids increase when PXA is absent (page 6, Fig. 3), they infer a functional effect. If this were true - that Faa1 activity is impaired in the absence of the association, TAG synthesis would be impaired. Showing an increase in free fatty acids is not sufficient. They should examine TAG accumulation. Another possibility is that Faa1 activity requires binding by the PXA domain. To tesst this idea, the acyl-CoA synthetase activity could be measured when the mini-Mdm1ER-PM construct is the only Mdm1 protein present.
3. The authors describe the ER extensions as perturbed ER morphology as a result of lipotoxicity (page 8 and Fig. 5). These extensions have been observed previously in yeast deleted in the 4 acyltransferases and, consequently, blocked in TAG formation. Under similar conditions with oleate

in the media, fatty acids are incorporated into phospholipids as the cells deal with excess acyl-CoAs. The relevant papers should be referenced and discussed, as should the consequences of the oleate response in yeast: PMID:20571028; J Biol Chem. 2010 Aug 27;285(35):26832-41 and the references within this ms. The authors might also want to consider the effects of lipin phosphorylation and restoration of PA levels (papers by S. Siniossoglou).

Other points
1. Instead of writing, "FA processing" by Faa1, the authors should more accurately use the term "FA activation" or refer to the synthesis of an acyl-CoA, without which TAG formation will be impaired.
2. Why did the authors switch from oleate to palmitoleate? A note would be helpful.
3. Concentrations of fatty acid should be provided in mM, not as percent. On page 19, 1% oleic acid is noted - 35mM! Is this an error?
4. Each of the 2 BODIPY chemicals used should be described more accurately (source, chemical name, and catalog number) and authors should be consistent: BODIPY-C16 vs. BODIPY-PA.
5. Page 10 bottom. To be complete in describing the pathway of TAG synthesis, the acylation of glycerol-3-P should be noted as requiring an acyl-CoA.

Reviewer #2 (Comments to the Authors (Required)):

The paper by Hariri et al investigates the role of Mdm1 in the spatial regulation of lipid droplet (LD) formation. Mdm1 was previously shown to be involved in vacuole-ER tethering. Here the authors show that Mdm1 associates with LDs through its N-transmembrane region while its PXA domain binds fatty acids. Loss of Mdm1 disrupts proper TG formation and package in LDs with consequences on ER membrane organization and response to FA overload. As the vacuole is the terminal destination for LDs during nutrient depletion (but also a sink for lipids which could be recycled back to the cytoplasm), Mdm1 could coordinate FA processing with LD formation in the vicinity of the vacuole.

This is an interesting study focusing on LD interorganellar interactions, which is currently of great interest in the field. Overall the experiments are well executed and presented (but see below). Perhaps the way the FA link is discussed could be improved - although there is a lot of information I felt there is no clear hypothesis on what the activity of Mdm1 on Faa1 and/or FAs could be. For example it was not discussed how Mdm1 overexpression could increase FAs, or what the basis of the additive effect of the LD deficient strain with mdm1delta really could be.

Points to be addressed:

1. The observation that Mdm1 IMD alone targets the ER-associated LD domain is key for the "three-way junction" model proposed. Unfortunately, the micrographs shown in Fig. 1C are of poor quality; there is significant variability in the LD number/size per cell which complicates the interpretation of the targeting - for example compare LDs between the fourth and fifth row - better resolution, equally exposed and more cells must be shown. Co-labelling with an ER reporter would help to evaluate ER and NVJ association.

2. On a relevant note, it would be also important to check the Mdm1 IMD and other GFP fusions during nutritional stress, where LD clustering at the NVJ is strongly induced and is physiologically relevant (the authors appear to add oleate in the presence of glucose - but please specify this at

legends - which may not have the same effect on lipophagy).

3. Does Mdm1 redistribute LD sites when targeted to the cER-PM? The authors state that Mdm1 at the cER-PM region "relocalized" LD bud sites (p. 172 and p.318), however I think this is not clearly shown. Most of the ER-PM Mdm1 associate with cortical LDs, but how many perinuclear LDs are with or without ER-PM Mdm1 (the PM NVJ1 is not a great control as, unlike Mdm1, it appears to associate only with a small fraction of the PM). More importantly, during nutritional stress, when the majority of LDs cluster around NVJ, how does expression of Mdm1 ER-PM affect LDs?

4. Fig S6B shows that the deltaLD mdm1 mutant after 24 hours in galactose has no detectable TG; this clearly contradicts what is shown in Fig6A after 14 hours. Is the deltaLD lacking the inducible DGA in the Fig S6B experiment? In general, the nomenclature of the LD deficient strain is confusing.

Other points:

The authors state that "Mdm1 physically interacts with LDs" (p. 81); perhaps this statement could be modified as Mdm1 may be also associated with an ER domain in close proximity to LDs.

Protein levels of the Mdm1 lacking the IMD domain (Figure S1C) should be examined to show that the localization effects are not due to decreased expression/breakdown of GFP moiety; the same should be done for the Mdm1(Tcb2 ER-PM) fusion in Figure 3C.

Figure 4A: to establish specificity, the PXA domain in the BODIPY control sample should match the max concentration of that of the BODIPY C-16 sample (second lane).

Some figures legends lack information; for example what are the expression systems used in the various Mdm1-GFP imaging experiments? What means "associated LDs at EP" (Fig. 1B)? Red arrows in Fig. 4E?

The two papers that first documented lipophagy in yeast (Wang, 2014; van Zutphen 2014) could be also mentioned where the authors discuss microlipophagy (p. 355).

Reviewer #3 (Comments to the Authors (Required)):

Lipid droplets are induced in response to a variety of stressors, but their functions and mechanisms of crosstalk with other organelles under these conditions remain mostly unknown. Furthermore, the regulation and purpose of the specific spatial distribution of lipid droplets in cells also remains an open question. Recently, the authors discovered that the ER-vacuole tether Mdm1 plays an important role in clustering lipid droplets at nuclear-vacuolar junctions during periods of nutrient stress in yeast.

The current study builds on the authors' recent findings to understand the mechanism of Mdm1 in lipid droplet clustering and function. The authors find that Mdm1 demarcates sites of lipid droplet biogenesis through its N-terminal region and it binds to lipid droplets through its C-terminal region. Mdm1 reversibly binds fatty acids and recruits the acyl-CoA ligase Faa1 to these sites to promote lipid droplet biogenesis. Finally, they demonstrate that loss of Mdm1 impacts TAG synthesis and sensitizes cells to fatty acid-induced disruptions in ER morphology.

The data presented are of high quality and the findings are interesting. The tethering role of Mdm1 is convincing. The model presented in which Mdm1 coordinates local fatty acid channeling into TAG and forming lipid droplets, through its association with fatty acids and Faa1, is intriguing. Furthermore, the role for Mdm1 as a multidomain scaffold that coordinates the interaction of three organelles and the recruitment of lipid metabolism machinery is very interesting. Overall, this is a strong paper of interest to cell biologists. Minor comments below.

Comments:

1) The experiments using Mdm1ER-PM nicely demonstrate lipid droplet clustering at the PM with Mdm1ER-PM at the contact. Are these lipid droplets formed at this site or do they traffic to this site? Is Mdm1ER-PM sufficient to alter seipin localization to these sites or is seipin still present in the nuclear ER?

2) The alterations in ER morphology are quite dramatic. Is there an increase in UPR? Likely flux of fatty acids into ER lipids in the absence of lipid droplets / Mdm1.

3) The proposed model suggests that Mdm1 is ER localized and is present at sites of ER-lipid droplet contact. However, in several images, it looks like Mdm1 shows staining on the lipid droplet surface. It is clearly enriched at puncta at the ER-lipid droplet contact, but it still seems apparent as a ring around the neutral lipid core that is separate from the ER staining as well. Is there a pool of Mdm1 on the lipid droplet? (Fig 1C, Fig 2E/F, Fig S2A)

4) Some clarifications regarding the authors' thoughts on precisely how Mdm1 is associating with lipid droplets would be helpful. Mdm1 seems to have multiple areas that contact the lipid droplet. The PXA domain seems to be sufficient, but the authors also make a point that "the IMD is necessary and sufficient for LD association". Is this fragment actually making a physical interaction with the lipid droplet or is it present at the ER-lipid droplet contact independent of an interaction with the lipid droplet surface?

5) It may be appropriate to also cite the recent paper demonstrating that DGAT1-dependent lipid droplet prevent ER stress (in adipocytes) as an additional example connecting lipid droplets and lipotoxicity (Chitraju Cell Met 2017).

W. Mike Henne, Ph.D.
W.W. Caruth, Jr. Endowed Scholar in Biomedical Research
Assistant Professor, Depts. of Cell Biology & Biophysics

January 10, 2019

Andrea Marat, Ph.D.
Scientific Editor
The Journal of Cell Biology

Dear Andrea:

We have now completed the revisions for our manuscript #201808119 entitled **"Mdm1 maintains endoplasmic reticulum homeostasis by spatially regulating lipid droplet biogenesis"**. We were pleased that all three reviewers were enthusiastic about the study, and appreciated the suggested experiments that strengthened our work. We believe we have addressed all the concerns, and reformatted the manuscript to incorporate the new findings.

First, a major point raised by both reviewers 1 and 2 was to focus on the functional outcome of the interaction between Mdm1 and fatty acyl-CoA ligase Faa1. The question they raised was specifically how this interaction affects free fatty acid (FFA) activation and incorporation into TAG during lipid droplet production. We believe we have addressed this point in several ways. Specifically, we now show that:

1. Loss of either Mdm1 or Faa1 similarly impacts the activation of fluorescent FFAs and their subsequent incorporation into neutral lipids such as DAG and TAG (Fig. 4F-H, S3G-I). Interestingly, loss of Mdm1 does not affect the incorporation of already activated fatty acyl-CoAs into TAG; however, we observe a significant decrease in the incorporation of labeled FFAs into both DAG and TAG in both *mdm1*Δ and *faa1*Δ yeast (Fig. 4F-H). This suggests that the defect occurs at the FFA activation step where Faa1 functions.

2. We also show that Mdm1 and Faa1 co-localize at LD bud sites (Fig. 4A, B), and Mdm1's PXA domain binds to FFAs *in vitro* (Fig. 3A-E). Based on our collective data, we hypothesize that Mdm1 and Faa1 co-localize at the ER-LD interface during LD biogenesis to promote the local activation of FFAs into FA-CoAs for their incorporation into neutral lipids. We have now better clarified our hypothesis in the new revision as suggested by the reviewers, and we updated our model (Fig. 7) to reflect that as well.

3. Relatedly, we now directly show that ΔLD*mdm1*Δ yeast accumulate lipotoxic FFAs during times when they exhibit breakdown of ER integrity. Specifically, we used TLC to monitor lipid levels in yeast exposed to lipotoxic palmitoyltoleate (POA). We find that ΔLD*mdm1*Δ yeast accumulate the POA and have a defect in incorporating POA into DAG (TAG precursor). This is consistent with our model where loss of Mdm1 perturbs the activation of FFAs into FA-CoA and their subsequent incorporation into neutral lipids (Fig. S5A).

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www.utsouthwestern.edu/labs/henne/

4. Finally, we now show using confocal microscopy, that ΔLD*faa1*Δ yeast exhibit fragmented and deformed ER which morphologically resembles the defects observed in ΔLD*mdm1*Δ yeast upon POA treatment (Fig. S5C).

Second, to better understand how loss of Mdm1 perturbed ER morphology and caused lipotoxicity in the ΔLD*mdm1*Δ strain, we turned to a new methodology recently implemented in our department: focused ion beam (FIB) milling and cryogenic electron tomography (Cryo-ET). This work was done in collaboration with Dr. Daniela Nicastro (UTSW, Dept Cell Biology). These images provide, for the first time to our knowledge, high-resolution imaging of the morphological changes the ER network undergoes during FA-induced lipotoxic stress (Fig. 5D, SMovie 1). We observe significant accumulation of fat deposits in the ER bilayer, and several areas where ER bilayer integrity is compromised. Coupled with new biochemical and cell biological data, we believe this imaging adds new insights into the mechanism of FA-induced lipotoxicity in our yeast model system. Collectively we find that the absence of LD biogenesis and Mdm1 causes FFAs to accumulate in the ER network, thus compromising ER membrane integrity.

In addition, we have also conducted the suggested fluorescence microscopy experiments to examine how Mdm1 is able to re-localize LD bud sites using mCherry-tagged Seipin as a marker for LD buds, as well as thin-sectioning TEM to examine the topology of Mdm1-LD association at high resolution. Other minor adjustments requested by the reviewers are also done. Please see below our point-by-point response (in blue) to the reviewers' comments.

Thank you for your time and feedback. We look forward to your response.

Sincerely,
Mike Henne, Ph.D.

September 13, 2018

Re: JCB manuscript #201808119

Dr. Mike Henne
UT Southwestern Medical Center
6000 Harry Hines Blvd NL6.120D
Dallas, Texas 75390

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(rev 2, points 2 and 3) and whether fatty acid addition induces the UPR (rev 3, point 2) is welcome, addressing these point is not necessary.

We appreciate your time and thank you for handling our manuscript.

As suggested by the reviewers, we now have focused on dissecting the interaction between Mdm1 and Faa1 in regards to free fatty acid (FFA) activation and incorporation into DAG and TAG. Using an *in vitro* fluorescence assay (modified from McFie and Stone, J Lipid Resv.52 (9); 2011 SepPMC3151697), we now provide evidence that loss of either Mdm1 or Faa1 similarly perturbs the activation of FFAs and their incorporation into DAG and TAG (Fig. 4F-H, Fig. S3G-I). We also show that ΔLD*mdm1*Δ yeast accumulate palmitoleate (POA) and manifest defects in incorporating it into DAG (Fig. S5A). Additionally, we find that deleting *FAA1* in the ΔLD also caused similar ER extensions and morphological defects resembling those observed in ΔLD*mdm1*Δ yeast (Fig. S5C).

Collectively, our findings are consistent with a model where Mdm1 and Faa1 function together at LD bud sites to promote the activation of FFAs into fatty acyl-CoA and subsequent incorporation into neutral lipids. As such, Mdm1 may play a role in maintaining localized FA pools to facilitate this process. Consistent with this, the absence of either Mdm1 or Faa1 in the ΔLD strain leads to lipotoxic ER stress, membrane deformation, and reduced viability. Therefore, as we also mention later, assessing the levels of FFAs in our experiments was necessary to evaluate our hypothesis using ΔLD and ΔLDmdm1Δ yeast strains which cannot synthesize TAG. However, to satisfy the reviewers' concerns, we have now examined both TAG and DAG, as well as FFA levels in the presence and absence of Faa1 and Mdm1 in more detail.

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Monitoring Editor

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Journal of Cell Biology

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1. The authors focus on free fatty acids (FFA) as a marker of functional Faa1; FFA mark the lack of Faa1 activity. It would be more accurate to write about the actual function of Faa1, i.e. FA activation and acyl-CoA incorporation into TAG. Related to this point is the finding of "exacerbated" FA accumulation (page 9) in the ΔLDmdm1Δ strain and elsewhere. Would it not be more accurate to focus on the decrease in TAG levels in each case?

Thank you for this key question. Indeed, we present in this manuscript clear evidence that Mdm1 binds FFAs through its PXA domain (Fig. 3A-E). We hypothesize that this function is needed to facilitate FFA activation (in this case by Faa1, which co-IPs with Mdm1; Hariri et al., EMBO Reports, 2018, Fig. 5) and incorporation into TAG. TAG formation is known to be used by cells as a mechanism for detoxification of lipotoxic FAs (Brookheart et al., Cell Metabolism, 2009; Listenberger et al., Proc. Natl. Acad. Sci., 2003; Garbarino et al., JBC, 2009). Therefore, in this study we used ΔLD and ΔLD*mdm1*Δ yeast strains which cannot synthesize TAG to evaluate the effect of FFA accumulation on cellular homeostasis. Consistent with our model, we find that in absence of Mdm1 and LDs, FFA accumulate in the ER causing lipotoxicity (Fig. S4F-G, Fig. 5).

Assessing the levels of FFAs in our experiments was necessary to evaluate our hypothesis that Mdm1 maintains a localized pool of FAs and works with Faa1 (and possibly other enzymes) to channel these FAs to less toxic lipid molecules (in this case TAG). Therefore, we use FFA accumulation as a marker for a dsyregulation of this interplay between Mdm1 and Faa1. However, to satisfy the abovementioned concern, we have now examined both TAG and DAG, as well as FFA levels in the presence and absence of Faa1 and Mdm1 in more detail:

First, we find that steady-state TAG levels do not drastically change in the absence of Faa1. This was also published in our previous paper (Hariri, et al., EMBO Reports, 2018; Fig. 5). The TLC data from these past experiments indicate that loss of Faa1 does not abolish steady-state TAG levels. This is not surprising given that in yeast, there are four fatty acyl-CoA synthetases encoded by separate genes: Faa1, Faa2, Faa3, and Faa4. Deletions of each of the four FAA alone genes does not compromise cell viability, suggesting that *de novo* fatty acid synthesis provides sufficient acyl-CoA for essential cellular functions (Johnson et al., JCB,

1994). Faa1 and Faa4 are functionally interchangeable as the primary enzymes involved in activation of long-chain fatty acids (such as oleic acid). There was also no impairment in the incorporation of FAs into neutral lipids in strains lacking either *FAA1* or *FAA4*. A dramatic reduction is observed only in the absence of both *FAA1* and *FAA4* (Johnson et al., JCB, 1994; Fig. 3).

Faa1 deletion does however affect FA uptake into cells (Narita et al., Science Reports, 2016). To get around this, we used an *in vitro* assay to examine FFA activation and incorporation into DAG and TAG in yeast lysates (modified from McFie and Stone, J Lipid Resv.52 (9); 2011 SepPMC3151697). Indeed, we find that loss of Mdm1 or Faa1 cause a similar defect in FFA activation and incorporation into neutral lipids (Fig. 4F-H). This is consistent with the data presented in Fig. 6A where we show that loss of Mdm1 or Faa1 perturbs Dga1-dependent TAG production.

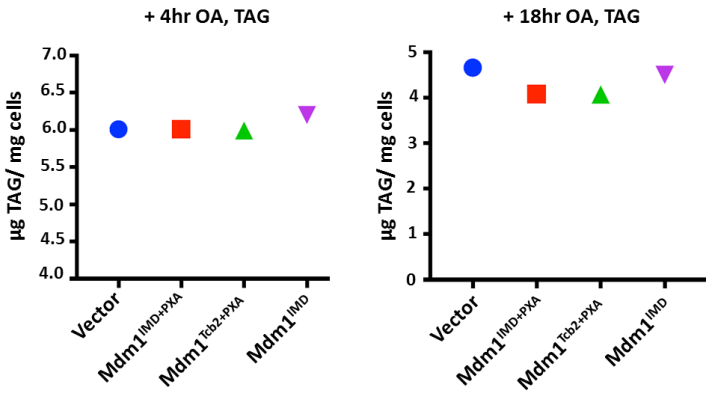
Finally, we now present evidence that ΔLD*mdm1*Δ yeast accumulate POA and have defects in the ability to incorporate them into neutral lipid DAG (Fig. S5A). We also provide new cryo-FIB data showing the morphological results of this FFA accumulation on ER bilayer integrity (Fig. 5D, SMovie).

2. The authors imply that the association of Mdm1 and Faa1 is important and requires the PXA domain, but the functional outcome is unclear. By showing that free fatty acids increase when PXA is absent (page 6, Fig. 3), they infer a functional effect. If this were true - that Faa1 activity is impaired in the absence of the association, TAG synthesis would be impaired. Showing an increase in free fatty acids is not sufficient. They should examine TAG accumulation. Another possibility is that Faa1 activity requires binding by the PXA domain. To test this idea, the acyl-CoA synthetase activity could be measured when the mini-Mdm1ER-PM construct is the only Mdm1 protein present.

We realize that our data presentation was confusing, and we have now rearranged Figures 3 and 4 (and added new data) accordingly to enhance clarity especially in explaining the functional relevance of Mdm1-Faa1 interaction. In brief, our current model states that Mdm1 coordinates local fatty acid flux into TAG at sites of LD formation. This is achieved through its association with fatty acids and Faa1.

In this study, we present evidence that Mdm1 binds free FAs via its PXA domain (Fig. 3A-C). Contrary to the statement above, our data in Figure 3 (now revised Fig. 3G) actually showed that free FAs increase by 2-folds in yeast *over-expressing* the Mdm1 upon oleate treatment (not in the absence of the PXA domain). We interpret this as Mdm1 binds to cellular FFAs via the PXA domain, thus increasing the cellular capacity to accommodate exogenous FFAs. To better understand the relationship between the PXA domain and FFA levels, we have conducted lipid analysis of yeast over-expressing only the ER-anchored PXA domain ($Mdm1^{IMD+PXA}$). Indeed, we find that over-expression of this constructs alone was sufficient to cause a similar increase in intracellular FFAs (Fig. 3G).

Regarding the effect of impaired Mdm1-Faa1 association on TAG synthesis, we have now added in vitro data showing that in the absence of Mdm1 we observe a delay in the incorporation of fluorescent-FFAs (BODIPY-C16) into DAG and TAG (Fig. 4F, G). It is worth noting, however, that deletion of Mdm1 increases steady state levels of TAG (previously published in Hariri et al., 2018), and causes LD accumulation on the nuclear envelope (Fig. S4A, B).

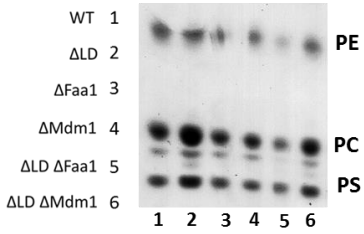


Moreover, as mentioned above, we find that steady-state TAG levels do not drastically change in the absence of Faa1. This was also published in our previous paper (Hariri, et al., EMBO Reports, 2018; Fig. 5). This is not surprising given that, in yeast, Faa1 and Faa4 are functionally redundant as the primary enzymes involved in activation of long-chain fatty acids (Johnson et al., JCB, 1994; Fig. 3). Therefore, in order to understasymand the full picture, we believe it is imperative that we assess levels of FFAs as well as TAG in our experiments.

Finally, per the reviewer suggestion we have also evaluated the effect of expressing the different Mdm1 chimera on TAG levels. Indeed, we find that ectopic expression of either the Mdm1$^{IMD+PXA}$, Mdm1$^{Tcb2+PXA}$, and Mdm1^{IMD} alone do not significantly alter TAG levels (see data below). We interpret this finding that the N-terminal region of Mdm1 functions to recruit Mdm1 to LD bud sites (via the IMD region) and to bind to FFAs (via the PXA domain), which promotes a localized FFA pool that can be activated by Faa1. These constructs however to not impair TAG production. Relatedly, we also know from our previous work (Hariri et al., 2018) that the complete deletion of Mdm1 does not impair the ability of Faa1 to target to LDs. As such, the association of Faa1 with LDs does not require the PXA domain.

3. The authors describe the ER extensions as perturbed ER morphology as a result of lipotoxicity (page 8 and Fig. 5). These extensions have been observed previously in yeast deleted in the 4 acyltransferases and, consequently, blocked in TAG formation. Under similar conditions with oleate in the media, fatty acids are incorporated into phospholipids as the cells deal with excess acyl-CoAs. The relevant papers should be referenced and discussed, as should the consequences of the oleate response in yeast: PMID:20571028; J Biol Chem. 2010 Aug 27;285(35):26832-41 and the references within this ms. The authors might also want to consider the effects of lipin phosphorylation and restoration of PA levels (papers by S. Siniossoglou).

We thank the reviewer for these comments, and for bringing this paper to our attention. Indeed, we do observe an increase in PLs in ΔLD yeast; however this increase is rescued upon deletion of Mdm1 supporting the model that FAs are accumulating instead in this deletion strain. Moreover, we now present direct evidence (using TLC) that the perturbed ER morphology is occurring when FFAs (POA) are accumulating in the ΔLD*mdm1*Δ strain (Fig. S5A).



We acknowledge in our manuscript that TAG synthesis (LD formation) is a major route to segregate toxic FA away from the ER and maintain homeostasis (Chitraju et al., 2017; Listenberger et al., 2003; Nguyen et al., 2017). We have added the suggested papers to the revised manuscript as well. Moreover, we now provide detailed assessment of these ER extensions using cryogenic electron tomography in cells. Our data provide, for the first time to our knowledge, high-resolution imaging of the morphological changes the ER network undergoes during FA-induced lipotoxic stress (Fig. 5D). We observe significant accumulation of fat deposits in the ER bilayer, and several areas where ER bilayer integrity is compromised.

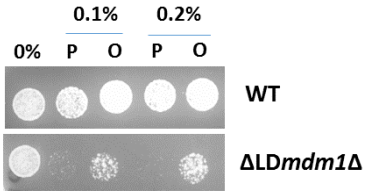
Finally, investigating the effects of Pah1 phosphorylation and restoration of PA levels on POA-induced lipotoxicity in our system is very interesting. However, we believe it is beyond the scope of the current study and will be pursued in future projects.

Other points
1. Instead of writing, "FA processing" by Faa1, the authors should more accurately use the term "FA activation" or refer to the synthesis of an acyl-CoA, without which TAG formation will be impaired.

Thank you. We have amended the text as suggested.

2. Why did the authors switch from oleate to palmitoleate? A note would be helpful.

The unsaturated FA palmitoleate (POA) has been associated with lipotoxicity in yeast strains that cannot generate TAG (Garbarino et al., JBC, 2009; Petschnigg et al., JBC, 2009; reviewed in Kohlwein1, JBC, 2010). Also, in our hands, POA displayed a more dominant effect in yeast plating experiments than OA (see right). Therefore, to test our hypothesis that Mdm1 helps maintain ER homeostasis and protects from lipotoxicity, we used POA. We have added a note about that in the text to clarify this point.



3. Concentrations of fatty acid should be provided in mM, not as percent. On page 19, 1% oleic acid is noted - 35mM! Is this an error?

Thank you. We now note the mM levels of the FAs in the methods section. 1% oleic acid noted on page 19 was a typing error, and we have corrected that. The concentration of oleic acid used in our experiments typically are no more than 0.2%.

"Oleate (Sigma; O1008) and palmitoleate POA (Sigma; P9417) were added to the culture media as indicated (0.2% oleate is equivalent to 6.32 mM, and 0.2% POA is equivalent to 7.04 mM)."

4. Each of the 2 BODIPY chemicals used should be described more accurately (source, chemical name, and catalog number) and authors should be consistent: BODIPY-C16 vs. BODIPY-PA.

Thank you, we have now added all the requested details to the methods section.

"BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene. Catalog # D3922) and BODIPY™ FL C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid. Catalog # D3821) were purchased from ThermoFisher."

5. Page 10 bottom. To be complete in describing the pathway of TAG synthesis, the acylation of glycerol-3-P should be noted as requiring an acyl-CoA.

Thank you, this is amended and added a diagram to reflect that (Fig 4E).

Reviewer #2 (Comments to the Authors (Required)):

The paper by Hariri et al investigates the role of Mdm1 in the spatial regulation of lipid droplet (LD) formation. Mdm1 was previously shown to be involved in vacuole-ER tethering. Here the authors show that Mdm1 associates with LDs through its N-transmembrane region while its PXA domain binds fatty acids. Loss of Mdm1 disrupts proper TG formation and package in LDs with consequences on ER membrane organization and response to FA overload. As the vacuole is the terminal destination for LDs during nutrient depletion (but also a sink for lipids which could be recycled back to the cytoplasm), Mdm1 could coordinate FA processing with LD formation in the vicinity of the vacuole.

This is an interesting study focusing on LD interorganellar interactions, which is currently of great interest in the field. Overall the experiments are well executed and presented (but see below). Perhaps the way the FA link is discussed could be improved - although there is a lot of information I felt there is no clear hypothesis on what the activity of Mdm1 on Faa1 and/or FAs could be. For example it was not discussed how Mdm1 overexpression could increase FAs, or what the basis of the additive effect of the LD deficient strain with mdm1delta really could be.

Points to be addressed:

1. The observation that Mdm1 IMD alone targets the ER-associated LD domain is key for the "three-way junction" model proposed. Unfortunately, the micrographs shown in Fig. 1C are of poor quality; there is significant variability in the LD number/size per cell which complicates the interpretation of the targeting - for example compare LDs between the fourth and fifth row - better resolution, equally exposed and more cells must be shown. Co-labelling with an ER reporter would help to evaluate ER and NVJ association.

Thank you. We have now conducted numerous new imaging experiments and added both ER and NVJ (Nvj1) co-labels to better assess the localization of Mdm1 and LDs within the cell (Figures 1C, D and Figures S1D, G). The images we added are of better quality and show the localization of Mdm1 fragments with respect to the ER and the vacuole.

Indeed, the experiments presented in Figure 1 show that Mdm1 associates with LDs through its N-terminus. We agree that there is variability LDs size and number. We have noticed that, specifically, LDs seem smaller and scarcer in "soluble" Mdm1 experiments which lacks the IMD domain ($Mdm1^{FL(-IMD)}$) and with $Mdm1^{Tcb2+PXA}$ (Fig. S1F, G). We reason that this could be due to the mis-targeting of these constructs; however, we think that pursuing this is beyond the scope of the current manuscript.

2. On a relevant note, it would be also important to check the Mdm1 IMD and other GFP fusions during nutritional stress, where LD clustering at the NVJ is strongly induced and is physiologically relevant (the authors appear to add oleate in the presence of glucose - but please specify this at legends - which may not have the same effect on lipophagy).

Thank you. The purpose of this experiment was to determine the structural components of Mdm1 that are required for LD association. Therefore, while checking the localization of Mdm1 chimera in nutritional stress is a great idea indeed, we believe it is beyond the scope of the current study. Similarly, investigating how different Mdm1 truncations affect lipophagy is definitely an interest that we are pursuing for future studies. Finally, as the reviewer pointed, we do supplement oleate directly to the SC-dextrose media. We have now more explicitly stated how the experiments were conducted in the text and methods.

3. Does Mdm1 redistribute LD sites when targeted to the cER-PM? The authors state that Mdm1 at the cER-PM region "relocalized" LD bud sites (p. 172 and p.318), however I think this is not clearly shown. Most of the ER-PM Mdm1 associate with cortical LDs, but how many perinuclear LDs are with or without ER-PM Mdm1 (the PM NVJ1 is not a great control as, unlike Mdm1, it appears to associate only with a small fraction of the PM). More importantly, during nutritional stress, when the majority of LDs cluster around NVJ, how does expression of Mdm1 ER-PM affect LDs?

Thank you for these questions. We find that $Mdm1^{ER-PM}$ forms foci in the presence of oleate that are decorated by LDs in the cortical ER. To better dissect this, we have now added images showing that $Mdm1^{ER-PM}$ co-localizes with Seipin-mCherry in the cortical ER (Fig. S2C, D). Indeed, $Mdm1^{ER-PM}$ is able to re-localize Seipin from the ER network in WT cells to specific cortical ER foci where these peripheral LDs are budding. We interpret these observations as $Mdm1^{ER-PM}$ being able to redistribute LD sites to the cortical ER. We are also intrigued by how $Mdm1^{ER-PM}$ will affect LD distribution under different nutrient stresses, and whether ER-PM LDs are accessible for lipophagy/turnover. This is currently an area of active investigation, but we believe is beyond the scope of the current study.

4. Fig S6B shows that the deltaLD mdm1 mutant after 24 hours in galactose has no detectable TG; this clearly contradicts what is shown in Fig6A after 14 hours. Is the deltaLD lacking the inducible DGA in the Fig S6B experiment? In general, the nomenclature of the LD deficient strain is confusing.

We sincerely apologize for this error, and we thank your attention in assessing our figures. The Figure in S6B was mistakenly marked as 24 hours, but is actually at 14 hours (same conditions as Fig. 6A), and has

been amended. In fact, we do observe some TAG in this strain at 14 hours as also seen in Fig 6A. The TLC in Figure 6A had more total lipids loaded in each lane as evident by the more prominent sterol bands. To be accurate, we have added this information to the figure legends.

Other points:

The authors state that "Mdm1 physically interacts with LDs" (p. 81); perhaps this statement could be modified as Mdm1 may be also associated with an ER domain in close proximity to LDs.

We have re-phrased several sections in the revision text to essentially say that our data are consistent with a model where Mdm1 is ER-associated and localizes to ER-LD contacts. The C-terminal PX domain further localizes it to the vacuole surface, so that it enriches at ER-LD-vacuole tri-organelle junctions.

Protein levels of the Mdm1 lacking the IMD domain (Figure S1C) should be examined to show that the localization effects are not due to decreased expression/breakdown of GFP moiety; the same should be done for the Mdm1(Tcb2 ER-PM) fusion in Figure 3C.

We have now done this control experiment as suggested (see right). We don't observe major breakdown of either Mdm1 lacking the IMD ($Mdm1^{FL(-IMD)}$) nor $Mdm1^{Tcb2+PXA}$. Indeed, the GFP signal of $Mdm1^{FL(-IMD)}$ and $Mdm1^{Tcb2+PXA}$ chimera was somewhat dim compared to the rest of the constructs (we now explicitly state this in our manuscript for accuracy). However, this is not surprising as it is the case for chimeric constructs. Despite this, however, we believe that this should not affect our conclusion that Mdm1 associates with LDs via the N-terminal region. We have added more imaging (Fig. 1C, D, Fig. S1D, E) to further establish this point.

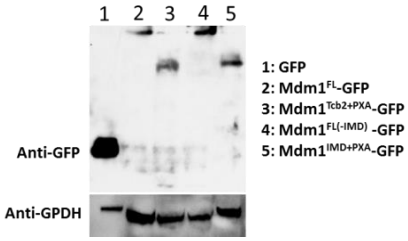
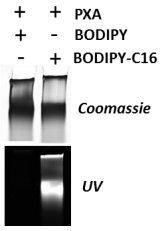


Figure 4A: to establish specificity, the PXA domain in the BODIPY control sample should match the max concentration of that of the BODIPY C-16 sample (second lane).

Thank you. Indeed, the fatty acid binding property of the PXA domain is clearly demonstrated in Figures 3A and B. Here is another native gel experiment which we believe satisfies the above mentioned remark (right). Due to space limitations, we did not add this figure to the manuscript. However, we can add it if necessary.



Some figures legends lack information; for example what are the expression systems used in the various Mdm1-GFP imaging experiments? What means "associated LDs at EP" (Fig. 1B)? Red arrows in Fig. 4E?

We have provided the information requested, and more thorough description of several figures in corresponding legends.

The two papers that first documented lipophagy in yeast (Wang, 2014; van Zutphen 2014) could be also mentioned where the authors discuss microlipophagy (p. 355).

Thank you. We have now added these references.

Reviewer #3 (Comments to the Authors (Required)):

Lipid droplets are induced in response to a variety of stressors, but their functions and mechanisms of crosstalk with other organelles under these conditions remain mostly unknown. Furthermore, the regulation

and purpose of the specific spatial distribution of lipid droplets in cells also remains an open question. Recently, the authors discovered that the ER-vacuole tether Mdm1 plays an important role in clustering lipid droplets at nuclear-vacuolar junctions during periods of nutrient stress in yeast.

The current study builds on the authors' recent findings to understand the mechanism of Mdm1 in lipid droplet clustering and function. The authors find that Mdm1 demarcates sites of lipid droplet biogenesis through its N-terminal region and it binds to lipid droplets through its C-terminal region. Mdm1 reversibly binds fatty acids and recruits the acyl-CoA ligase Faa1 to these sites to promote lipid droplet biogenesis. Finally, they demonstrate that loss of Mdm1 impacts TAG synthesis and sensitizes cells to fatty acid-induced disruptions in ER morphology.

The data presented are of high quality and the findings are interesting. The tethering role of Mdm1 is convincing. The model presented in which Mdm1 coordinates local fatty acid channeling into TAG and forming lipid droplets, through its association with fatty acids and Faa1, is intriguing. Furthermore, the role for Mdm1 as a multidomain scaffold that coordinates the interaction of three organelles and the recruitment of lipid metabolism machinery is very interesting. Overall, this is a strong paper of interest to cell biologists. Minor comments below.

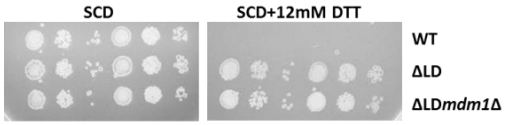
Comments:

1) The experiments using Mdm1ER-PM nicely demonstrate lipid droplet clustering at the PM with Mdm1ER-PM at the contact. Are these lipid droplets formed at this site or do they traffic to this site? Is Mdm1ER-PM sufficient to alter seipin localization to these sites or is seipin still present in the nuclear ER?

Indeed, we find that Mdm1$^{ER\text{-}PM}$ associated LDs are attached to the cortical ER, suggesting they formed at these sites. Consistent with this, we now show that Seipin and Mdm1 co-localize at the ER-LD bridge of these peripheral LDs (Fig. S2C, D). Additionally, we conducted thin-sectioning TEM to better resolve the topology of Mdm1$^{ER\text{-}PM}$ on LDs. We find more ER wrapping around LDs in the periphery in cells expressing Mdm1$^{ER\text{-}PM}$, indicating the protein is promoting ER-LD contacts (Fig. 2G).

2) The alterations in ER morphology are quite dramatic. Is there an increase in UPR? Likely flux of fatty acids into ER lipids in the absence of lipid droplets / Mdm1.

Thank you for the question. Our data support the model where, in the absence of Mdm1 and lipid droplets, fatty acids accumulate in the ER compromising the ER integrity and causing lipotoxicity. We now provide TLC data supporting this statement (Fig. S5A). Furthermore, to characterize how FFA accumulation alters the ER morphology we conducted high resolution focused ion beam milling and cryogenic electron tomography (Fig. 5D). These data reveal that the ER membrane bilayer in ΔLD*mdm1*Δ yeast appears to be compromised and irregular, and is associated with dense fatty deposits that bulge from the ER surface.



We are also interested in understanding whether UPR is increased in our system. In a preliminary experiment, we plated WT yeast and yeast lacking LDs and Mdm1 on DTT which induces ER stress. While WT yeast were sensitive to growth in presence of DTT, surprisingly we find that ΔLD and ΔLD*mdm1*Δ were resistant, suggesting that these strains have activate UPR which may provide some resilience to growth on DTT. However, we believe this line of investigation is beyond the scope of the current manuscript, and we are further developing it as part on a follow-up study.

3) The proposed model suggests that Mdm1 is ER localized and is present at sites of ER-lipid droplet contact. However, in several images, it looks like Mdm1 shows staining on the lipid droplet surface. It is

clearly enriched at puncta at the ER-lipid droplet contact, but it still seems apparent as a ring around the neutral lipid core that is separate from the ER staining as well. Is there a pool of Mdm1 on the lipid droplet? (Fig 1C, Fig 2E/F, Fig S2A)

Great question. Indeed, we observe ring-like staining on the surface of LDs specifically in truncated Mdm1 constructs that lack vacuole binding, whereas full-length Mdm1 predominantly forms foci at the base of LDs or asymmetrical cups partially covering the LD. We interpret this as Mdm1 being ER-anchored and localizes to ER-LD contacts, and binding to the vacuole physically restricts the ability of the protein to completely wrap around LDs. As stated above, to better understand the topology we conducted thin-sectioning TEM on Mdm1^{ER-PM} expressing cells (Fig. 2G). Indeed, we find ER wrapping around many LDs in the periphery, consistent with a model where Mdm1^{ER-PM} is ER localized but promotes ER-LD contacts at these LDs. We have added a note on this in the results and discussion.

4) Some clarifications regarding the authors' thoughts on precisely how Mdm1 is associating with lipid droplets would be helpful. Mdm1 seems to have multiple areas that contact the lipid droplet. The PXA domain seems to be sufficient, but the authors also make a point that "the IMD is necessary and sufficient for LD association". Is this fragment actually making a physical interaction with the lipid droplet or is it present at the ER-lipid droplet contact independent of an interaction with the lipid droplet surface?

We have modified the text in places to attempt to make our Mdm1-LD interaction model as clear as possible. Collectively, we conclude that Mdm1 localizes primarily to ER-LD contact sites at the base of LDs budding from the ER surface. This is based on several observations:

1. Mdm1-GFP remains ER associated and forms “cups” partially surrounding LDs (Fig. S1A, C).
2. The Mdm1^{ER-PM} encoding only the IMD and PXA domain localizes to the cortical ER (Fig. 2A) without oleate, and enriches at ER-LD contacts with oleate (Fig. 2B, F).
3. TEM imaging shows that Mdm1^{ER-PM} expression increases ER wrapping of the LD surface, consistent with Mdm1^{ER-PM} remaining in the ER and binding the LD surface via its PXA domain (Fig. 2G).
4. We identify a putative helical region if the hydrophobic PXA domain that is sufficient to target to LDs (Fig. 3F).

5) It may be appropriate to also cite the recent paper demonstrating that DGAT1-dependent lipid droplet prevent ER stress (in adipocytes) as an additional example connecting lipid droplets and lipotoxicity (Chitraju Cell Met 2017).

We agree, and we have added this reference as suggested.

Thank you for your time and effort in reviewing our work. We look forward to your response.

Sincerely,

W. Mike Henne, Ph.D.
Assistant Professor
W.W. Caruth Jr., Endowed Scholar

January 16, 2019

RE: JCB Manuscript #201808119R

Dr. Mike Henne
UT Southwestern Medical Center
6000 Harry Hines Blvd NL6.120D
Dallas, Texas 75390

Dear Dr. Henne:

Thank you for submitting your revised manuscript entitled "Mdm1 maintains endoplasmic reticulum homeostasis by spatially regulating lipid droplet biogenesis". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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3) * Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications (e.g. 2G). Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters

including spaces. Make the title concise but accessible to a general readership.

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7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition method for Western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

William Prinz, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

THe authors have addressed my concerns and have improved the manuscript. In my view the paper will be an important addition to the field.

Reviewer #3 (Comments to the Authors (Required)):

The authors have added new data and clarifications in response to my previous comments. This is an interesting manuscript that is, in my opinion, acceptable for publication and of broad interest.