Rab5 regulates macropinocytosis by recruiting the inositol 5-phosphatases OCRL and Inpp5b that hydrolyze PtdIns(4,5)P_2

Michelle E. Maxson, Helen Sarantis, Allen Volchuk, John H. Brumell and Sergio Grinstein

DOI: 10.1242/jcs.252411 **Editor**: Daniel Billadeau

Review timeline
Original submission: 29 July 2020
Editorial decision: 1 September 2020
First revision received: 8 February 2021
Accepted: 25 February 2021

Original submission

First decision letter

MS ID#: JOCES/2020/252411

MS TITLE: Rab5 regulates macropinosome closure through recruitment of the inositol 5-phosphatases OCRL/Inpp5b and the hydrolysis of PtdIns(4,5)P2

AUTHORS: Michelle Maxson, Helen Sarantis, Allen Volchuk, John H Brumell, and Sergio Grinstein
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.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. In general, most all of the comments can be resolved through toning down the interpretation of the results or performing a few additional experiments to support the model and role of Rab5a in macropinosome closure. 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.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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

The manuscript by Maxson, et al. demonstrates roles for Rab5a and its effectors INPP5B, OCRL and APPL-1 in macropinosome closure. Morphological studies of living A431 cells, together with experimental manipulation of protein expression and effector recruitment to plasma membranes, indicated that Rab5a recruitment of catalytic 5Â'PI phosphatases to forming macropinocytic cups is required for cups to close into macropinosomes, and that experimentally inhibiting membrane depletion of PI(4,5)P2 inhibits macropinosome closure. Importantly, quantitative measurements of cell movements demonstrated that these Rab5a-dependent activities did not affect ruffling. This is an important advance in understanding the mechanism by which Rab5a contributes to macropinocytosis.

Major concerns:

1. It remains possible that macropinosome closure precedes Rab5a function.
The docking of GFP-Rab5a onto macropinocytic cups could be setting up for activities that follow closure (Fig. 2a, b). Also, because the Lyn11-RFP probe also labels fully closed macropinosomes, the timing of Rab5a association with Lyn11-RFP-positive structures relative to macropinosome closure remains ambiguous. An alternative possible mechanism is that Rab5a-dependent lipid phosphatase activities stabilize already closed macropinosomes and prevent them from recycling to the plasma membrane (and regurgitating TR-dextran).

2. Can the authors demonstrate that the early localization of GFP-Rab5a on forming macropinosomes is not an artifact of Rab5a overexpression? Can endogenous Rab5a be localized during macropinosome closure?

3. Figure 3 a-d fails to demonstrate the utility of the rapamycin-inducible system for manipulating macropinocytosis. Although the morphology indicates that rapamycin addition could displace FKBP-CFP-Rab5A to the plasma membrane (Fig. 3a), no evidence is provided to demonstrate that rapamycin addition ever altered macropinocytosis. FKBP-CFP-Rab5A(S34N) inhibited macropinocytosis with or without its recruitment to the plasma membrane (Fig. 3d). This contrasts with Figure 6, which does show an effect of rapamycin addition on macropinosome formation.

4. Can the inferred sequence of Rab5a Â– APPL-1 Â– OCRL/INPP5B be supported by experimental interference with known interactions between APPL-1 and Rab5a OCRL or INPP5B? If not, then the discussion should leave open the possibility that Rab5a can affect the activities of OCRL or INPP5B independent of APPL-1.

Minor concern:

5. Supplementary Movie figure legends. The term Â"acquisition rateÂ" is misplaced. The acquisition rate was one frame per 20 seconds. The playback rate was 6 fps.

Comments for the author

This is an important mechanistic study which should be published in JCS.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, the authors investigate the role of Rab5 in macropinosome closure. In the first 2 figures they nicely show that Rab5A is recruited to ruffles just before macropinosome formation,

and that expression of dominant negative (DN) Rab5A, or Rab5 knockdown is sufficient to inhibit macropinosome formation. This is consistent with previous studies, and clearly points to a role for Rab5 in macropinosome closure.

They then perform a number of studies to attempt to eludicate a mechanistic pathway, and identify additional factors such as the inositol phosphatases OCRL and Inpp5B that cause a similar inhibition of macropinocytosis. They therefore propose a model by which local, Rab5-dependent depletion of PI(4,5)P2 drives cup closure and internalisation.

This is an interesting paper and model, which addresses an important but poorly understood process (i.e. macropinosome closure). Whilst their data are consistent with the model they propose, my major criticism is that much of the data they show is correlative and often does not always directly demonstrate causation in macropinosome formation as they state in the text. There are also important limitations in their data which should be better recognised and they need to tone down some of their conclusions and add important and appropriate caveats. Whilst some of these are acknowledged in the discussion they need to be more upfront in the results section. Nonetheless, even with limitations, this is a good piece of work and with a limited number of additional controls as detailed below, should be appropriate for publication.

Comments for the author

Whilst their data on the requirement for Rab5 are clear, evidence for a direct local role for Rab5 at the cup is less conclusive. In figure 3 they use rapamycin-driven heterodimerisation to recruit DN-Rab5a to the plasma membrane and show that this inhibits dextran uptake. However, as they have already shown in Figure 1 that untargeted DN-Rab5a is able to do this it is unclear what additional value this experiment gives. Are they arguing that in this system all the DN-Rab5A is recruited to the plasma membrane, and there are no effects elsewhere? This seems unlikely, so whilst there is nothing to indicate that it is not true, they should be more cautious and tone down their conclusion that Rab5 works locally at the site of closure (e.g. L172).

Similarly, in Figure 3E-G, they use a rapamycin-inducible system to acutely inhibit SNARE activity with dominant negative NSF. Whilst this again inhibited dextran uptake, it is a very blunt tool and will inhibit nearly every membrane fusion event in the cell causing widespread endocytic disruption (as they show in Supp Fig 1a). Their conclusion in L198, that “a SNARE-mediated fusion event likely that of Rab5-containing vesicles with the plasma membrane, is required for macropinocytosis” is only poorly supported. If they wish to make this conclusion they should directly show that Rab5-delivery is inhibited, but also add the caveat that they lack evidence of a direct role – perhaps the delivery of Rab5 from wherever it originates is disrupted instead?
In Figure 4b they show that cells expressing of DN-Rab5A retain PI(4,5)P2, and conclude that this suggests that active Rab5is normally required for PIP2 depletion. They further propose that this PIP2 depletion is required for closure itself (L210). Whilst they confirm previously published observations that PIP2 is lost after internalisation (Fig 4C-E), their data as is stands is insufficient to determine whether this happens before or after closure. If it only starts after closure the retention of PIP2 upon DN-Rab5A expression would simply be explained by the lack of closure, rather than being mechanistically involved in the closure process. Whilst it will be technically challenging to determine the precise timings, if they cannot do this, they need to discuss the limitations of their data.
To implicate plasma membrane PI(45)P2 levels in macropinosome formation, in figure 4F they overexpress PIP5K isoforms. Whilst this blocked dextran uptake in their images the morphology of the overexpressing cells appears significantly more round than the controls. As PIP2 is strongly implicated in cytoskeletal regulation, it is important that they also validate that the defect is not caused by lack of ruffling in these cells, as they have done with other manipulations.
Whilst the recruitment of Rab5 vesicles prior to closure is clear, their images of Inpp5B, OCRL, and APPL1 prior to closure are much less convincing and only based on the single images shown (Figure 5A and Supp Figure 3). The OCRL image in particular shows a large number of cytosolic puncta, so is impossible to say whether they are truly docked on the ruffles or not. As this is central to their narrative, and indeed the title of the paper, this needs to be shown more conclusively, perhaps with further examples of ideally convincing timelapse movies as shown for Rab5.

Minor points:
This is not the first time a role for OCRL in closure has been postulated. They should cite the previous work by Loovers et al. showing a role for OCRL in closure: Loovers, H. M. et al. (2007) 'Regulation of phagocytosis in Dictyostelium by the inositol 5-phosphatase OCRL homolog Dd5P4', Traffic, 8(5), pp. 618–628. doi: 10.1111/j.1600-0854.2007.00546.x.

Ensure that all abbreviations are properly introduced in full at first usage.

E.g L62 M-CSF and EGF.

Timelapses are referred to as movies in the text, but videos in the files

L117 "As shown in Figs 1e and f" should be Figs 1e and g

First revision

Author response to reviewers' comments

SickKids
THE HOSPITAL FOR SICK CHILDREN

UNIVERSITY of TORONTO

February 5, 2021

Dr. Daniel Billadeau
Editor
Journal of Cell Science

Dear Dan

Thank you very much for your letter of Sept 1st regarding our manuscript JOCES/2020/25241, entitled *"Rab5 regulates macropinosome closure through recruitment of the inositol 5-phosphatases OCRL/Inpp5b and the hydrolysis of PtdIns(4,5)P2"*. Thank you also for your patience and willingness to extend the deadline for resubmission. Addressing fully the reviewers' suggestions required substantial additional experimentation and this took an inordinately long time because access to the laboratory was limited by the pandemic.

As described in detail below, we performed a considerable number of additional experiments and made changes to the manuscript text and figures to describe the new results and to address other comments. As a result, we feel that the manuscript is much improved, and we thank the reviewers for their suggestions.
The changes and additions made in response to the requests by the reviewers are itemized below. They are listed individually after the specific comments made by the reviewers, shown in red for reference.

Reviewer #1
Major concerns:

1. It remains possible that macropinosome closure precedes Rab5a function. The docking of GFP-Rab5a onto macropinocytic cups could be setting up for activities that follow closure (Fig. 2a, b). Also, because the Lyn11-RFP probe also labels fully closed macropinosomes, the timing of Rab5a association with Lyn11-RFP-positive structures relative to macropinosome closure remains ambiguous. An alternative possible mechanism is that Rab5a-dependent lipid phosphatase activities stabilize already closed macropinosomes and prevent them from recycling to the plasma membrane (and regurgitating TR-dextran).

We agree with the reviewer that, with the methods currently available, it is impossible to ascertain unambiguously whether Rab5 fusion precedes closure. Our data show close apposition of Rab5 vesicles with the open macropinosome and also indicate that NSF activity is required for Rab5 to facilitate macropinosome formation. We feel that these combined findings are consistent with (but do not definitively prove) that fusion is required for proper macropinosome sealing. However, we agree with the reviewer that Rab5 may instead be preventing back-fusion of sealed macropinosomes with the plasma membrane, resulting in regurgitation of the TMR-dextran (a possibility we had not contemplated before). To alert the readers of this alternative explanation we have mentioned it specifically in several places in the manuscript, including the Abstract (p. 2), Results (pp. 6 and 11) and Discussion (pp. 15 and 16).

2. Can the authors demonstrate that the early localization of GFP-Rab5a on forming macropinosomes is not an artifact of Rab5a overexpression? Can endogenous Rab5a be localized during macropinosome closure?

Detection of endogenous Rab5 by immunostaining (as opposed to overexpression of tagged Rab5) would in principle address the reviewer's question directly. Unfortunately, the commercially available antibodies (at least the ones we have tested) lack the sensitivity and specificity to detect endogenous Rab5 with confidence. Instead, we chose to examine the localization of endogenous EEA1, a known Rab5 ligand and effector. The results of these experiments have been included in a new figure (Fig 2d), which shows the immunolocalization of endogenous EEA1 in vesicles apposed to membrane ruffles and circular ruffles, closely resembling the distribution of overexpressed Rab5A-GFP.

3. Figure 3 a-d fails to demonstrate the utility of the rapamycin-inducible system for manipulating macropinocytosis. Although the morphology indicates that rapamycin addition could displace FKBP-CFP-Rab5A to the plasma membrane (Fig. 3a), no evidence is provided to demonstrate that rapamycin addition ever altered macropinocytosis. FKBP-CFP-Rab5A(S34N) inhibited macropinocytosis with or without its recruitment to the plasma membrane (Fig. 3d). This contrasts with Figure 6, which does show an effect of rapamycin addition on macropinosome formation.

Both reviewers were understandably concerned about these experiments. Therefore we sought an alternative, more straightforward and compelling approach to assess whether Rab5 exerts its effects at the plasma membrane. To this end we obtained from Dr. Alexander Sorkin (University of Pittsburgh) chimeras of WT Rab5A, Rab5A^{Q67L} (CA) and the DN Rab5A^{S34N} (DN) that were targeted to the plasma membrane by attachment of a C-terminal CAAX motif. As we hoped, expression of these constructs recapitulated the results seen using the rapamycin- inducible system. These new data are presented in the revised Fig. 3. The original, less compelling iRap data, which is now merely confirmatory, has been relegated to a supplementary figure (Fig. S1).

4. Can the inferred sequence of Rab5a - APPL-1 - OCRL/INPP5B be supported by experimental interference with known interactions between APPL-1 and Rab5a, OCRL or INPP5B? If not, then the discussion should leave open the possibility that Rab5a can affect the activities of OCRL or INPP5B independent of APPL-1.

Prompted by the reviewer's suggestion, we undertook new experiments to test the requirement for APPL1 for the recruitment of the 5-phosphatases, using siRNA-mediated silencing. Cells that were depleted of APPL1 with siRNA recruited markedly less OCRL to Rab5-containing endosomes. This was determined by quantifying the Manders' colocalization coefficient (M) of well over 1,000 endosomes from multiple cells and experiments for each condition: the colocalization coefficient dropped from M = 0.785 in cells treated with scrambled RNA to M = 0.280 in cells that were APPL1-depleted. This is consistent with previous findings in endocytosis and phagocytosis, where APPL1 contributes greatly to the recruitment of 5- phosphatases to early compartments. The new data are presented in the new panels **c** and **d** of the revised Fig. 5.
We thank the reviewer for suggesting these experiments.

Minor concern:
5. Supplementary Movie figure legends. The term "acquisition rate" is misplaced. The acquisition rate was one frame per 20 seconds. The playback rate was 6 fps.
We have fixed the misnomer. Thanks for pointing this out.

Reviewer 2
Major points:

1) Whilst their data on the requirement for Rab5 are clear, evidence for a direct local role for Rab5 at the cup is less conclusive. In figure 3 they use rapamycin-driven heterodimerisation to recruit DN-Rab5a to the plasma membrane and show that this inhibits dextran uptake. However, as they have already shown in Figure 1 that untargeted DN-Rab5a is able to do this it is unclear what additional value this experiment gives. Are they arguing that in this system all the DN- Rab5A is recruited to the plasma membrane, and there are no effects elsewhere? This seems unlikely, so whilst there is nothing to indicate that it is not true, they should be more cautious and tone down their conclusion that Rab5 works locally at the site of closure (e.g. L172).
Both reviewers were understandably concerned about the conclusions drawn using of the rapamycin-inducible system. Therefore we sought an alternative, more straightforward and compelling approach to assess whether Rab5 exerts its effects at the plasma membrane. To this end we obtained from Dr. Alexander Sorkin (University of Pittsburgh) chimeras of WT Rab5A, Rab5AQ67L (CA) and the DN Rab5AS34N (DN) that were targeted to the plasma membrane by attachment of a C-terminal CAAX motif. As we hoped, expression of these constructs recapitulated the results seen using the rapamycin-inducible system. These new data are presented in the revised Fig. 3. The original, less compelling iRap data, which is now merely confirmatory, has been relegated to a supplementary figure (Fig S1). In addition, as also detailed above in response to Reviewer #1, we now explicitly consider also the possibility that Rab5 may be actin after scission, preventing back-fusion of sealed phagosomes with the plasma membrane, possibly causing regurgitation of the internalized contents (please see above for details).

2) Similarly, in Figure 3E-G, they use a rapamycin-inducible system to acutely inhibit SNARE activity with dominant negative NSF. Whilst this again inhibited dextran uptake, it is a very blunt tool and will inhibit nearly every membrane fusion event in the cell causing widespread endocytic disruption (as they show in Supp Fig 1a). Their conclusion in L198, that "a SNARE- mediated fusion event likely that of Rab5-containing vesicles with the plasma membrane, is required for macropinocytosis" is only poorly supported. If they wish to make this conclusion they should directly show that Rab5-delivery is inhibited, but also add the caveat that they lack evidence of a direct role - perhaps the delivery of Rab5 from wherever it originates is disrupted instead?
We agree with the reviewer that dominant-negative NSF will inhibit fusion at multiple sites in the cells and is hence a rather blunt tool (though less blunt than the overnight expression protocols often used in the past, which can compromise cell survival). We attempted to minimize off-target effects by reducing the time of exposure to the inhibitory constructs using the rapamycin analog-releasable system. Under these conditions cell viability was unaffected and the cells displayed normal morphology, endosome distribution and ruffling in response to EGF.

As discussed above (see reply to Reviewer #1), we are unable to ascertain when fusion of individual vesicles with the apical ruffling surface occurs using currently available methods. Instead, we performed new experiments to more directly investigate whether the dominant- negative NSF interacts with –and could therefore potentially impact– the fusion of the Rab5 vesicles. We observed Rab5-positive endosomes coated with NSFE329Q localized to actin-rich circular ruffles during macropinocytosis. These observations have been added to the revised Figure 3g, and further discussion of experimental caveats added to the Discussion (page 15).

As requested by the reviewer, we have nevertheless modified our conclusions to introduce the suggested caveat. The revised statement (lines 220-224 of the enclosed text) now states: *"We therefore concluded that a SNARE-mediated fusion event, possibly that of Rab5-containing vesicles with the plasma membrane, is required for macropinocytosis.*

However, an indirect effect of NSFE329Q cannot be ruled out".

3) In Figure 4b they show that cells expressing of DN-Rab5A retain PI(4,5)P2, andconclude that this suggests that active Rab5 is normally required for PIP2 depletion. They further propose that this PIP2 depletion is required for closure itself (L210). Whilst they confirm previously published observations that PIP2 is lost after internalisation (Fig 4C-E), their data as is stands is insufficient

to determine whether this happens before or after closure. If it only starts after closure the retention of PIP2 upon DN-Rab5A expression would simply be explained by the lack of closure, rather than being mechanistically involved in the closure process. Whilst it will be technically challenging to determine the precise timings, if they cannot do this, they need to discuss the limitations of their data.

We agree with the reviewer that precise determination of the timing of PIP2 clearance vis a vis sealing of the macropinosome is not feasible at present. However, disappearance of PIP2 prior to sealing of membrane vacuoles has been clearly documented in the case of phagosomes, whether using large particles or in frustrated phagocytosis models, so that local disappearance of the inositide at the base of a forming vacuole is certainly possible.

Nevertheless, because definition of the moment when macropinosome sealing occurs is currently impossible, we agree that further discussion of the experimental limitations of our study is warranted. We have modified the text on page 11 of the revised text to this effect. The revised text now reads: "*Taken together, these data confirmed that PtdIns(4,5)P2 localized predominantly to open macropinocytic cups, and was depleted quickly at the time of macropinosome sealing or shortly thereafter.*

These observations raised the possibility that PtdIns(4,5)P2 depletion is in fact required for the completion of macropinocytosis or to prevent macropinosome back-fusion with the plasmalemma."

Also, the revised Discussion (p. 16) now indicates that: *"Our data suggest a correlation between PtdIns(4,5)P2 depletion and macropinosome sealing, although temporal and resolution limits of live-cell imaging warrant caution when assigning causation."*

4) To implicate plasma membrane PI(45)P2 levels in macropinosome formation, in figure 4F they overexpress PIP5K isoforms. Whilst this blocked dextran uptake, in their images the morphology of the overexpressing cells appears significantly more round than the controls. As PIP2 is strongly implicated in cytoskeletal regulation, it is important that they also validate that the defect is not caused by lack of ruffling in these cells, as they have done with other manipulations.

We thank the reviewer for his/her astute observation. As suggested, we performed additional experiments to validate whether the PIP5K-expressing cells have normal actin dynamics when stimulated with EGF. We have added a new Supplementary Fig. 4 displaying temporal projections of EGF-treated A431 cells co-expressing Lyn11-RFP and PIP5Kβ-GFP or PIP5Kβ$_{KD}$-GFP. Their ruffling was indistinguishable from that of EGF-stimulated cells co- expressing GFP; therefore we concluded that expression of the kinases at the levels used in our experiments do not largely affect actin dynamics. To avoid misrepresenting the behavior of most of the cells in our experiments we have replaced the images in Fig. 4f with more representative images of cells expressing average levels of the transfected PIP5K isoforms.

5) Whilst the recruitment of Rab5 vesicles prior to closure is clear, their images of Inpp5B, OCRL, and APPL1 prior to closure are much less convincing and only based on the single images shown (Figure 5A and Supp Figure 3). The OCRL image in particular shows a large number of cytosolic puncta, so is impossible to say whether they are truly docked on the ruffles or not. As this is central to their narrative, and indeed the title of the paper, this needs to be shown more conclusively, perhaps with further examples of ideally convincing timelapse movies as shown for Rab5.

The reviewer raises a valid point. To address this concern, and as suggested by the reviewer, we have bolstered our observations by including in Supplementary Fig 5a-c an extended montage of time-lapse experiments that cover all the frames acquired during the recruitment of OCRL, INPP5b and APPL1 to PIP2-positive circular ruffles; these include the time-points prior to and after PIP2 loss. Additionally, we have added what we hope are more convincing depictions of the Inpp5B, OCRL, and APPL1 vesicles apposed to the open circular ruffle, presenting XY, XZ and YZ slices of such vesicles along PIP2-containing circular ruffles (Supplementary Fig 5d-f). Their recruitment is consistent with that observed for Rab5- and endogenous EEA1- containing endosomes (Fig. 2a and c).

Minor points:

6) This is not the first time a role for OCRL in closure has been postulated. They should cite the previous work by Loovers et al. showing a role for OCRL in closure: Loovers, H. M. et al. (2007) 'Regulation of phagocytosis in Dictyostelium by the inositol 5-phosphatase OCRL homolog Dd5P4',

Traffic, 8(5), pp. 618-628. doi: 10.1111/j.1600-0854.2007.00546.x.
We apologize for the oversight, and have added this reference (see revised page 12)

7) Ensure that all abbreviations are properly introduced in full at first usage. E.g L62 M-CSF and EGF.
We have now introduced all abbreviations in full at first usage. Thanks for pointing this out.

8) Timelapses are referred to as movies in the text, but videos in the files
We have now ensured the labels are consistent.

9) L117 "As shown in Figs 1e and f" should be Figs 1e and g
This has been fixed.

Please thank the reviewers on our behalf for their efforts and valuable comments, which contributed greatly to improve this manuscript. Once again we would like to thank you for handling the paper and for inviting us to resubmit this revised version.

We look forward to hearing from you soon.

Sergio Grinstein Senior Scientist
Program in Cell Biology
The Hospital for Sick Children and Professor of Biochemistry University of Toronto

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Second decision letter

MS ID#: JOCES/2020/252411

MS TITLE: Rab5 regulates macropinocytosis by recruiting the inositol 5-phosphatases OCRL/Inpp5b that hydrolyze PtdIns(4,5)P2

AUTHORS: Michelle Maxson, Helen Sarantis, Allen Volchuk, John H Brumell, and Sergio Grinstein
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.

Reviewer 1

Advance summary and potential significance to field

The manuscript reports a significant advance in understanding of the role of Rab5 in promoting macropinosome closure through activation of PtdIns(4,5)2 phosphatases.

Comments for the author

In this revised manuscript, the authors have added new experimental evidence and re-wording of interpretations that adequately address my earlier concerns.

Reviewer 2

Advance summary and potential significance to field

I than the authors for their efforts to respond to my comments, which have all been fully addressed this new version. I congratulate them on a very nice piece of work.

Comments for the author

Nothing further