Phosphatidylinositol and phosphatidylinositol-3-phosphate activate HOPS to catalyze SNARE assembly, allowing small headgroup lipids to support the terminal steps of membrane fusion

Thomas Torng and William Wickner

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Review Timeline:

| Event                  | Date       |
|------------------------|------------|
| Submission Date        | 2021-07-31 |
| Editorial Decision     | 2021-08-23 |
| Revision Received      | 2021-08-30 |
| Accepted               | 2021-08-31 |

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: Manuscript #E21-07-0373
TITLE: Phosphatidylinositol and phosphatidylinositol-3-phosphate activate HOPS to catalyze SNARE assembly, allowing small headgroup lipids to support the terminal steps of membrane fusion

Dear Bill,

Two experts have evaluated your manuscript, and I am happy to report that both of them are enthusiastic. They provided a number of specific suggestions, most or all of which can be addressed by revising the text and the data presentation.

Please submit a revised manuscript that addresses the comments. I will run it past one of the reviewers for a final evaluation.

I look forward to seeing the revised manuscript.

Sincerely,

Benjamin Glick
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Wickner,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.
In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Torng and Wickner present a study addressing the influence of lipid head groups on the HOPS-mediated SNARE-dependent fusion of reconstituted proteoliposomes mimicking the yeast vacuole. They are able to dissect the tethering and fusion process into two stages: They show that (i) the HOPS-dependent tethering relies on the presence of either PI or PI3P in the membrane and (ii) lipid bilayer mixing requires PE or PS, PA and DAG.

The presented results are based on assays previously introduced by the Wickner lab and are technically sound, and provide important novel insights of the lipid contribution to SNARE-mediated membrane fusion. The experiments are of very high standards, meet most criteria, and demonstrate the excellent experience of the lab. Unfortunately, the authors assume far too much insight even from the experienced reader, and there are several parts that would strongly profit from framing their questions and conclusions better. The detailed suggestions and issues are listed below:

1.) PI and PI3P activate the HOPS complex. In their discussion the authors state "that PI and PI3P are the crucial vacuolar lipids for this activation". I wonder if it is clear, if there is an activation event or if the effect is rather a recruitment of HOPS to the membrane surface? I would interpret the term "activation" as a conformational change in the HOPS-complex, allowing for a higher SNARE-assembly rate, whereas a recruiting effect of PI/PI3P would orient the HOPS on the membrane. I suspect both to lead to the same effect in the presented assays. In my opinion the authors should clearly state what effect they are referring here to and if they could distinguish between them. Especially since they also cite their own work, which showed the binding of HOPS to PI/PI3P. So, is this observed effect a novelty to the field?

2.) How do the authors then understand "activation" of HOPS then in terms of Ypt7? And how does one differ from the other?
3.) In Figure 5 and 6 the authors show experiments testing the effect of Sec17 and Sec18 on the fusion in the background of different lipid compositions. Whereas the initial experiments in panels (A) are very helpful to understand the influence of the lipid headgroups, the omission of the two chaperones did not lead to any new insights and could be moved to supplementary data.

4.) The overall presentation of the data is shifting between figures: Whereas Figure 1-3 use dot plots depicting results from single experiments, the mean between them and include error bars depicting standard deviation, Figures 4-9 show bar graphs. Why do the authors change in between the mode of representations? It would be better to depict the results in Figure 4-9 also as in the first ones to give the reader a better idea about the variation in experiments.

5.) Figure 9 is puzzling to me: why does the fluorescence signal after luminal mixing drops after around 7-8 minutes in the red and blue line? This also leads to a strange effect in the quantification where the bar graph indicates a "negative fusion". In addition, the yellow curve (excluding PE) shows a strange drop after 3 minutes. Taken together the data presented in Figure 9 seems not to reach the high-quality standard that the authors present in the other data sets.

6.) I recommend that the authors use small models in some of their figures (e.g. Figure 1 and 2), and a summary model to frame it all. This makes the work so much more readable. I am aware that they did this before in other studies, but then it is a lot to ask that readers also have all papers of the group available and in front of them if they read this work.

7.) Abstract: What do the authors mean that PI and PI3P "activate HOPS for cis-SNARE complex assembly"? Why should this be important for fusion? It would rather block fusion than promote it, and the statement is thus confusing. I recommend rewriting this.

8.) Intro: The authors introduce the entire lipid composition in the introduction. Is this really useful at this stage? Likewise, the discussion of the vertex ring and co-dependency of lipids is mentioned in the introduction, but not picked up elsewhere in the manuscript. Why then mention it?

9.) Is it fair to say that the minimal lipids contribute to fluidity and thus permit efficient fusion?

10.) Results: The authors dive right into the topic, but it is not clear what they want to understand here. There is neither a hypothesis nor a question in the beginning. It would be helpful to introduce the study better.

11.) When introducing the *Qb assay, it would be good if they would state what they want to determine by this and how this fits into the overall fusion analysis. This probably needs only a few refinements in their sentences.

12.) What do the authors understand under "bilayer rearrangement" in the last part of their manuscript?

Minor issues:

1.) The authors refer to the Chou et al. structure of HOPS. This is one of two published HOPS structures, and the Chou et al. study lacks any assignment of subunits. In addition, there has been another study showing that HOPS is a flexible structure on membranes (Fuellbrunn et al., elife 2021). It would make sense to cite these in context.
2.) The discussion narrows very much down on HOPS and the work done by the Wickner lab. It would be useful to frame this into the context of what has been found in the neuronal field or elsewhere to make the study discussion broader.

3.) It would have been nice if the authors would have labeled their figures by numbers as well as page numbers (or line numbers).

4.) "Modified RIPA buffer" comes out of nowhere. This should be explained in the context.

Reviewer #2 (Remarks to the Author):

In this study, Torng and Wickner studied the importance of membrane lipids in vacuole fusion. The authors made proteoliposomes using a previously optimized lipid composition and investigated the importance of individual lipids for homotypic vacuole fusion. Using FRET-based and sedimentation-based assays, the authors teased apart the importance of PI and PI3P (and other lipids to a lesser extent) in facilitating SNARE assembly and proteoliposome docking. Using a fluorescence-based lumen mixing assay, they also studied these lipids' role in HOPS activity and completing proteoliposome fusion.

Overall, the data are convincing and of high-quality, and the conclusions generated from this paper would be important for the field. The comments below are fairly modest but would help in improving the clarity of the paper.

1. This reviewer was confused about the arbitrary use of different timings selected for quantification (e.g. 50 mins in Fig. 1, 10 mins in Fig. 5, 8, 9, 30 mins in Fig. 7). Please comment on why these time points were selected and why they are most appropriate for these experiments. We recommend adding a graph similar to Fig. 3A in at least Fig. 1A to first show the change of the FRET intensity over time. This is to aid the readers to visualize how the experiment was done and from where the quantifications came from.

2. In Fig. 2, it is not very clear why soluble R-SNARE (sR), and not membrane-bound R-SNARE, was chosen in one of the controls. This information was mentioned in passing in the figure legend, but it would be helpful if this was commented on in the main text itself.

3. It is not clear to this reviewer the point of Fig. 2C when it has already been part of Fig. 2A. Please clarify.

4. Fig. 3C: Please label what protein was used as a bait and what protein was probed. Fig. 3D: If this reviewer understood correctly, the time points for this quantification are already mentioned at the bottom of the graph (i.e., 5 minutes, 30 minutes), and so "at 30 min" should be removed from the two y-axes labels.

5. Fig. 7A is mislabeled. The numbers in Fig. 7A do not correspond to Fig. 7B.

6. Fig. 8: It has been concluded that PE, PS, PA, and DAG were not important in the HOPS function. However, there seems to be a reduction in fusion efficiency when PE or PS/PA/DAG were removed vs. VML. Please comment on this discrepancy.
7. Fig. 9: For better clarity, please change your legends to "No tether" and "No tether + PEG"

8. PI3,5P2 is well-known to be important in vacuole fusion. In particular, salt-stressed yeast cells have fragmented vacuoles, and Fab1 mutants have gigantic vacuoles. In addition to PI and PI3P, it would be nice to know the importance of PI3,5P2 in the reconstituted vacuole fusion process. If you already have this data, it would be nice to include it in the paper. Otherwise, it could be an interesting area of future study.
"Rebuttal letter" (not a rebuttal at all- we've found each point very useful, and helpful for improving the manuscript. Thank You!). The reviews are copied below, and our responses are italicized.

Reviewer #1 (Remarks to the Author):

Torgn and Wickner present a study addressing the influence of lipid head groups on the HOPS-mediated SNARE-dependent fusion of reconstituted proteoliposomes mimicking the yeast vacuole. They are able to dissect the tethering and fusion process into two stages: They show that (i) the HOPS-dependent tethering relies on the presence of either PI or PI3P in the membrane and (ii) lipid bilayer mixing requires PE or PS, PA and DAG.

The presented results are based on assays previously introduced by the Wickner lab and are technically sound, and provide important novel insights of the lipid contribution to SNARE-mediated membrane fusion. The experiments are of very high standards, meet most criteria, and demonstrate the excellent experience of the lab. Unfortunately, the authors assume far too much insight even from the experienced reader, and there are several parts that would strongly profit from framing their questions and conclusions better. The detailed suggestions and issues are listed below:

1.) PI and PI3P activate the HOPS complex. In their discussion the authors state "that PI and PI3P are the crucial vacuolar lipids for this activation". I wonder if it is clear, if there is an activation event or if the effect is rather a recruitment of HOPS to the membrane surface? I would interpret the term "activation" as a conformational change in the HOPS-complex, allowing for a higher SNARE-assembly rate, whereas a recruiting effect of PI/PI3P would orient the HOPS on the membrane. I suspect both to lead to the same effect in the presented assays. In my opinion the authors should clearly state what effect they are referring here to and if they could distinguish between them. Especially since they also cite their own work, which showed the binding of HOPS to PI/PI3P. So, is this observed effect a novelty to the field?

To address this important point, we've now added “This is an allosteric activation of HOPS for catalysis of SNARE assembly rather than simply a co-localization of HOPS with its membrane-bound SNARE substrates, since either Ypt7 or the vacuolar lipids alone suffice for HOPS binding to membranes yet both are required for HOPS activation, and the activation of HOPS for catalysis of SNARE assembly is even seen when the four SNAREs are in aqueous solution without membrane
anchors (Torng and Wickner, 2020).” Additionally, we would like to reiterate that Figure 7 demonstrates that PI affects HOPS activity directly.

2.) How do the authors then understand "activation" of HOPS then in terms of Ypt7? And how does one differ from the other?

As noted in (1) above, this is an example of a catalyst (HOPS, which catalyzes SNARE assembly) being activated by binding critical ligands (Ypt7:GTP, PI, and PI3P).

3.) In Figure 5 and 6 the authors show experiments testing the effect of Sec17 and Sec18 on the fusion in the background of different lipid compositions. Whereas the initial experiments in panels (A) are very helpful to understand the influence of the lipid headgroups, the omission of the two chaperones did not lead to any new insights and could be moved to supplementary data.

We have now moved the entirety of Figure 5 to the supplementary data, while leaving behind the four important reactions with Sec17/18 as the new Figure 5. Panels C and D of Figure 6 have been moved to the supplementary data.

4.) The overall presentation of the data is shifting between figures: Whereas Figure 1-3 use dot plots depicting results from single experiments, the mean between them and include error bars depicting standard deviation, Figures 4-9 show bar graphs. Why do the authors change in between the mode of representations? It would be better to depict the results in Figure 4-9 also as in the first ones to give the reader a better idea about the variation in experiments.

We’ve switched all figures to show the statistics with bar graphs.

5.) Figure 9 is puzzling to me: why does the fluorescence signal after luminal mixing drops after around 7-8 minutes in the red and blue line? This also leads to a strange effect in the quantification where the bar graph indicates a "negative fusion". In addition, the yellow curve (excluding PE) shows a strange drop after 3 minutes. Taken together the data presented in Figure 9 seems not to reach the high-quality standard that the authors present in the other data sets.

An inherent limitation of the assay, performed in 20 μL volumes in small wells with a fluorescence plate reader, is that tethered clusters of liposomes can drift out of the center of the beam, causing some loss of signal. We note this in the text now,
but it doesn't affect the basic conclusion of the experiment. We've added: "(though the signal after fusion drifts due to liposome cluster drift in the plate reader)."

6.) I recommend that the authors use small models in some of their figures (e.g. Figure 1 and 2), and a summary model to frame it all. This makes the work so much more readable. I am aware that they did this before in other studies, but then it is a lot to ask that readers also have all papers of the group available and in front of them if they read this work.

Small models and a summary figure have been added as recommended.

7.) Abstract: What do the authors mean that PI and PI3P "activate HOPS for cis-SNARE complex assembly"? Why should this be important for fusion? It would rather block fusion than promote it, and the statement is thus confusing. I recommend rewriting this.

The "cis" is indeed misleading, as the paper starts with cis, but progresses on to "trans", so we've removed the "cis" from the abstract and ensure that we're clear in the Results about which we're dealing with as we progress through the figures.

8.) Intro: The authors introduce the entire lipid composition in the introduction. Is this really useful at this stage? Likewise, the discussion of the vertex ring and co-dependency of lipids is mentioned in the introduction, but not picked up elsewhere in the manuscript. Why then mention it?

We feel that the lipid composition is very important in the introduction, as the whole paper is built around the effects of varying that composition. Also, while we don't illuminate how the lipids assemble with proteins into the vertex ring (Fratti et al., 2004), it would be a serious omission not to point this out. We've added "A more complete understanding of the roles of individual lipids will be a foundation for understanding the vertex ring assembly and functions."

9.) Is it fair to say that the minimal lipids contribute to fluidity and thus permit efficient fusion?

The minimal lipids (MIN) don't permit efficient fusion (Figure 4B). Our PC, PE, PI, and PA are all 18:2; it's unclear that fluidity is at the heart of the difference in fusion between MIN and VML lipids' support of fusion.
10.) Results: The authors dive right into the topic, but it is not clear what they want to understand here. There is neither a hypothesis nor a question in the beginning. It would be helpful to introduce the study better.

*Good point! We now start the Results with: “Since vacuolar lipids activate HOPS (Torng and Wickner, 2020), we asked which specific vacuolar lipids are required for this activation, whether this activation suffices for trans-SNARE complex assembly and for fusion, and whether other vacuolar lipids are important for fusion mediated by other tethers and SNAREs or even by a non-proteinaceous dehydrating agent.”*

11.) When introducing the *Qb assay, it would be good if they would state what they want to determine by this and how this fits into the overall fusion analysis. This probably needs only a few refinements in their sentences.

*We've attempted to clarify this.*

12.) What do the authors understand under "bilayer rearrangement" in the last part of their manuscript?

*We've added: ... suggesting that these lipids act at apposed bilayers to support the lipid rearrangements which are the essence of fusion, progressing from two tightly apposed bilayers through possible nonbilayer intermediates such as hemifusion to a fully fused bilayer.*

**Minor issues:**

1.) The authors refer to the Chou et al. structure of HOPS. This is one of two published HOPS structures, and the Chou et al. study lacks any assignment of subunits. In addition, there has been another study showing that HOPS is a flexible structure on membranes (Fuellbrunn et al., elife 2021). It would make sense to cite these in context.

*Thank you!! We have included this reference and now write: “HOPS has been proposed to have an elongated and somewhat unstructured conformation (Chou et al, 2016), though recent studies have shown that its association with Ypt7 can induce a major conformational change (Fuellbrunn et al., 2021) which is likely related to the functional activation reported here.”*
2.) The discussion narrows very much down on HOPS and the work done by the Wickner lab. It would be useful to frame this into the context of what has been found in the neuronal field or elsewhere to make the study discussion broader.

*Thanks for pointing this out! We’ve added the following: “Synaptic fusion also shows specific lipid requirements. Cholesterol, a major lipid in synaptic vesicles, is important for fusion mediated by synaptic SNAREs (Tong et al., 2009), and acidic lipids promote fusion with synaptotagmin and calcium as well (Lai and Shin, 2012). Fusion reconstituted with neuronal SNAREs, Munc18-1, and Munc 13-1 is stimulated by DAG and phosphoinositides (Liu et al., 2016). While synaptic fusion and vacuole fusion share requirements for SNAREs and an SM protein, synaptic fusion has not required a clear homolog to HOPS subunits other than Vps33, the SM subunit, and vacuolar fusion does not need homologs of Munc13, synaptotagmin, or complexin. Shared mechanistic lipid requirements between synaptic fusion and vacuolar fusion are not yet evident.”*

3.) It would have been nice if the authors would have labeled their figures by numbers as well as page numbers (or line numbers).

*We were under the mistaken impression that MBoC did not allow including Figure numbers in the PDFs when submitting articles to the journal. They are now included.*

4.) "Modified RIPA buffer" comes out of nowhere. This should be explained in the context.

*Thank you. We now cite its composition and purpose: “Aliquots of fusion incubations were removed after 5 min and 30 min of incubation and mixed with modified RIPA buffer (Song et al., 2020), a non-denaturing detergent mixture.”*

Reviewer #2 (Remarks to the Author):

In this study, Torg and Wickner studied the importance of membrane lipids in vacuole fusion. The authors made proteoliposomes using a previously optimized lipid composition and investigated the importance of individual lipids for homotypic vacuole fusion. Using FRET-based and sedimentation-based assays, the authors teased apart the importance of PI and PI3P (and other lipids to a lesser extent) in facilitating SNARE assembly and proteoliposome docking. Using a fluorescence-based lumen mixing assay, they also studied these lipids' role in HOPS activity and completing proteoliposome fusion.
Overall, the data are convincing and of high-quality, and the conclusions generated from this paper would be important for the field. The comments below are fairly modest but would help in improving the clarity of the paper.

1. This reviewer was confused about the arbitrary use of different timings selected for quantification (e.g. 50 mins in Fig. 1, 10 mins in Fig. 5, 8, 9, 30 mins in Fig. 7). Please comment on why these time points were selected and why they are most appropriate for these experiments. We recommend adding a graph similar to Fig. 3A in at least Fig. 1A to first show the change of the FRET intensity over time. This is to aid the readers to visualize how the experiment was done and from where the quantifications came from.

_The times are somewhat arbitrary, but were selected to allow the reactions to run as long as needed to test each point in question._

2. In Fig. 2, it is not very clear why soluble R-SNARE (sR), and not membrane-bound R-SNARE, was chosen in one of the controls. This information was mentioned in passing in the figure legend, but it would be helpful if this was commented on in the main text itself.

_We now present several controls, including the one we’d failed to mention, as follows: “*Qb does not sediment, alone (Figure 2A, lane 4), with protein-free liposomes (lane 3), or with all the SNAREs but without liposomes (lane 2).”_

3. It is not clear to this reviewer the point of Fig. 2C when it has already been part of Fig. 2A. Please clarify.

_The experiment in Figure 2A did not include the *Qb sedimented when there was omission of both HOPS and sQa. In Fig 2C, this is contrasted with the sedimentation when only one of HOPS or sQa is omitted. As discussed in the text: “This assay therefore measures both HOPS-dependent binding, which occurs when SNARE assembly is blocked by the absence of sQa (Figure 2C, lanes 3 vs. 4), and SNARE assembly, which occurs at these high SNARE concentrations in the absence of HOPS (Figure 2C, lanes 2 vs. 4), in accord with fusion studies (Mima et al., 2008).”_

4. Fig. 3C: Please label what protein was used as a bait and what protein was probed.
Figure 4C now states that the R SNARE is being probed after coimmunoprecipitation with Qa.

Fig. 3D: If this reviewer understood correctly, the time points for this quantification are already mentioned at the bottom of the graph (i.e., 5 minutes, 30 minutes), and so "at 30 min" should be removed from the two y-axes labels.

Thanks, we've done so for Figure 4D.

5. Fig. 7A is mislabeled. The numbers in Fig. 7A do not correspond to Fig. 7B.

Thank you, the numbers are now fixed. In addition, we now write:
“Ypt7/R and Ypt7/QaQbQc proteoliposomes of VML lipids will fuse with either HOPS or dimeric GST-PX as tether (Figure 7A, red, filled vs. open circles; Figure 7B, lanes 1 vs. 2).”

6. Fig. 8: It has been concluded that PE, PS, PA, and DAG were not important in the HOPS function. However, there seems to be a reduction in fusion efficiency when PE or PS/PA/DAG were removed vs. VML. Please comment on this discrepancy.

Thank you! Since the effects of removing PE, or removing PS/PA/DAG, or both are quite parallel with HOPS and with GST-PX (Figure 8B), their role is not limited to HOPS. We've now clarified this, writing:
“... we examined the fusion of Ypt7/R and Ypt7/QaQbQc proteoliposomes of either VML, MIN + PE, MIN + PS/PA/DAG, or MIN lipids with either HOPS or dimeric GST-PX. There was no fusion without a tether (Figure 8, gray symbols), and little or no fusion with MIN lipids and either tether (Figure 8A, diamonds, Figure 8B, lanes 10-12). Either PE or PS/PA/DAG sufficed to support fusion with either tether (squares and triangles), and optimal fusion was seen with MIN plus all four, i.e., VML (Figure 8, circles). Since PE, and PS/PA/DAG, had comparable effects with either tether, these four lipids are not simply needed for some specific HOPS function which might follow trans-SNARE assembly.”

7. Fig. 9: For better clarity, please change your legends to "No tether" and "No tether + PEG"

Done.
8. PI3,5P2 is well-known to be important in vacuole fusion. In particular, salt-stressed yeast cells have fragmented vacuoles, and Fab1 mutants have gigantic vacuoles. In addition to PI and PI3P, it would be nice to know the importance of PI3,5P2 in the reconstituted vacuole fusion process. If you already have this data, it would be nice to include it in the paper. Otherwise, it could be an interesting area of future study.

*Thank you – we don’t have such data, and it will indeed be a fascinating area for further study.*
RE: Manuscript #E21-07-0373R
TITLE: "Phosphatidylinositol and phosphatidylinositol-3-phosphate activate HOPS to catalyze SNARE assembly, allowing small headgroup lipids to support the terminal steps of membrane fusion"

Dear Bill,

I asked Reviewer #1 to take another look, and your changes were judged to be satisfactory. Thanks for your careful attention to the comments on the original submission.

The revised manuscript is accepted for publication. We are pleased that you sent this nice story to MBoC.

Sincerely,
Benjamin Glick
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Wickner:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,
Reviewer #1 (Remarks to the Author):

The authors addressed my concerns sufficiently. I have no further requests.