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Cholesterol promotes clustering of PI(4,5)P2 driving unconventional secretion of FGF2

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August 25, 2021

Re: JCB manuscript #202106123

Prof. Walter Nickel
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Dear Prof. Nickel,

Thank you for submitting your manuscript entitled "Cholesterol promotes head group visibility and clustering of $PI(4,5)P_2$ driving unconventional secretion of FGF2". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

As you will see, the three reviewers find the role of membrane composition on FGF interaction with PIP2 interesting, but each of them had serious concerns about the conclusions drawn. Although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, I would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns with new experiments where requested and is subject to further peer-review. Specifically:

Reviewer 1 and 3 have commented on the simulations and bring up serious technical issues with the same. Reviewer 3 is also concerned with the ability to relate the same to the experiments, diluting the conclusions drawn.

Reviewers 2 and 3 request additional experimental controls, such as:
1) measuring the time course of FGF association with the membrane, a serious concern is the time taken for binding
2) include sphingolipids and PS in the in vitro binding experiments to reflect a more physiological composition of the bilayer that the protein would encounter; include addition PIP2 binding controls
3) additional methods to manipulate cholesterol and measure cholesterol at the cell surface
4) analyze colocalization of FGF2, cholesterol, and PIP2 in a cellular system. As a point of mention, it is essential to document the acyl chains being used in the in silico and in vitro systems, to ascertain if they reflect physiological membranes.

If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Satyajit Mayor, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

I focused on the simulations in the paper, because that's my area of expertise.

For the most part, the simulations appear to have been performed well, and in my opinion largely support the authors' conclusions. That said, I have some technical questions that I think should be answered, and clarified in the manuscript.

- I found the methods section description of the umbrella sampling calculations confusing. The authors mention a pulling rate of 0.1 nm/ns, which would imply a time-dependent restraint potential. If that's the case, the wham analysis would be inappropriate, since that method assumes equilibrium sampling. I suspect I misunderstood, and the pulling was just used to build the starting structures, but this needs to be clarified.

- I'm troubled by the addition of a second restraint, on the lipids, because this too violates the assumptions of wham (unless the authors performed a 2D wham calculation, then collapsed the resulting free energy curve back down to 1D. The only way this is ok is if the lipid restraint energy is nearly constant, and doesn't vary much with the main reaction coordinate, the membrane position. It wouldn't surprise me if this assumption holds, since the restraint is very strong, but the authors need to address it and show their verification in the supplemental information. I'm actually quite surprised the authors chose to apply the membrane restraint -- their patch is small enough that the membrane couldn't bend much anyway, and if I recall correctly gromacs/plumed has ways of restraining the distance between the centers of mass of 2 objects (in this case the protein and the membrane).

- If I understand correctly, the free energy calculations were performed with a single starting protein orientation. Which one was used, and how was it chosen?

- The caption for Fig 3 is missing a description of part B. Also, each panel should have its own letter, to make referring to it easier.

-The charge density plot was quite interesting, but I think there's a simple extra step that could make it more interpretable. The charge density could be integrated along z to give the electric field (see the work by Sachs and Woolf from the early 2000s for the correct way to handle periodicity), which would be interpretable in terms of the long-range effect on the protein.

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

Unconventional means of secretion has been elusive and an important field to study. The senior author has a established a field featured by type I secretion of FGF2, a key cytokine involved in cancer and other biological processes. How cellular lipids coordinate with PIP2 to regulate the release of FGF2 has been unclear. The authors found that cholesterol could regulates the membrane rigidity and microdomain partition of PIP2. Therefore it increases the avidity of PIP2-FGF2 interaction and type I secretion. The finding is important, deepens our view of type I secretion regulation and potentialy explains a corelation between cholesterol level and cancer considering FGF2 is cancer related. The data presented is convincing. I suggest a few additional control before it is ready for publication.
1. In Fig. 1A the authors claim a linear increase of FGF2 binding within 6 hrs. To fully support the claim, it is good to add another time point, e.g.3hr.
2. Under physiological conditions the plasma membrane also contains sphingolipids that work together with cholesterol to regulate the biophysical properties. It is good to add sphingolipids in the in vitro binding assay shown in Fig.1 to test the role of cholesterol as well as the collaboration of sphingolipids in FGF2 binding.
3. In Fig.1, it would be good to add controls of FGF2 mutant deficient of binding PIP2, as well as another positive control, e.g. a PIP2 binding PH domain to confirm the final effect is PIP2 exposure.
4. In Fig.5, it would be good to use different ways to manipulate cholesterol. e.g. cyclodextran extraction to decrease cholesterol, as well as physiological methods like LPPS treatment+ statin treatment etc.
5. Since cholesterol has muliple effect on the membrane, it would be good to check if conventional secretion, e.g. ssGFP, or type III unconventional secretion, e.g. TMED10-mediated IL1 secretion, are also affected or the effect is specific for FGF2.
6. Since cholesterol directly promote PIP2 exposure, it would be good to analyze the colocalization of FGF2 puncta, cholesterol and PIP2 using TURF to strengthen the idea.
7. In the abstract, I am a little confused by the statement"...in a fully reconstituted system." Usually this means that a reconstitution of functional process, e.g. the translocation of FGF2 across the GUV as shown by the group previously. I would suggest something like " in a liposome binding assay"

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

This is a potentially interesting but ultimately unconvincing paper. The authors use several approaches to suggest a role for membrane cholesterol in promoting the unconventional secretion of FGF2 by the phosphoinositide PIP2. However, there is no significant attempt to cross-reference/synthesize the results which derive from experimental work in liposomes and cells, measurements of tension in lipid droplets/films and MD simulations.

Some specific issues:

Figure 1 - Some technical points. (i) The assays are carried out with what appears to be a FRET pair (Alexa488-FGF2 and rhodamine-PE-liposomes) - how does that work? (ii) Binding appears to require an exceptionally long time, 6 hours as indicated in panel A - what is the explanation? (iii) How do the authors account for compositional heterogeneity in the multi-component liposomes that they are studying?

Figures 2, 3 - The calculations are done on a very simple system comprising PIP2, POPC and cholesterol. Similar data on cholesterol-induced changes in charge density and bilayer thickness have been previously reported for PS/PC mixtures in response to cholesterol (for example ref 53: Doktorova et al. 2017). What happens in a more realistic membrane which contains PS, in addition to PC and PIP2? And PI, for that matter? As PS (and PI) would be present in much higher amounts than PIP2, the PIP2 effects may be muted. Would clustering be expected in these circumstances? Can any of the outcomes from MD simulations be directly tested experimentally?

Figures S3, 5 - Cholesterol should be assessed at the plasma membrane, for example by using plasma membrane-derived blebs or other fractionation method. Filipin is a poor substitute, but still better than measuring relative cholesterol at the whole cell level (Fig S3C, F). How do the authors reconcile the observed 1.5-2-fold changes in cholesterol level that they report with the capacity of the plasma membrane to retain cholesterol, especially in the inner leaflet which is the site of action for PIP2-FGF2 interaction? Was any attempt made to test the effect of modifying PIP2 levels in cholesterol-loaded cells?

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JCB manuscript #202106123; Lolicato et al

Point-by-point response to the reviewer's suggestions

Reviewer #1:

Technical aspects regarding the molecular dynamics simulations

Regarding both the umbrella sampling calculations and additional constraints applied in our molecular dynamics simulations, the description of those in the Materials and Methods section apparently has not been clear. As assumed correctly by Reviewer 1, the described pulling procedure was used only to build the starting structures. Likewise, the restraints on the lipid head groups have been used only for the pulling simulations needed to build the structures. In the revised manuscript, the Methods section has been improved clearly stating that the umbrella sampling calculations have been performed without additional restraints on lipid molecules. In summary, all MDS models and the implementation of their simulations were performed in accordance with the field's best practice.

Another point raised by Reviewer 1 was related to the starting orientation of FGF2 molecules used in our MD simulations. This choice was based on previous work in which we had identified a high-affinity FGF2 orientation characterized by strong binding to the membrane surface mediated by $PI(4,5)P_2$ (Steringer et al 2017, eLife; Fig. 13). In this orientation, the experimentally known $PI(4,5)P_2$ binding site in FGF2 (K127, R128, K133; Temmerman et al 2008, Traffic) is facing the membrane surface.

Regarding the charge density plot, we believe the suggestion from Reviewer 1 to determine the electric field is an excellent one. We followed this advice and calculated the electric field with the results being shown in the new Fig. S2A. A detailed description of the methodology being used is given in the Methods section of the revised manuscript.

Reviewer #2

1.) Linear range observed in the binding kinetics of PI(4,5)P_2-dependent membrane recruitment of FGF2-Halo

Reviewer 2 is correct in pointing out that the number of data points presented in Fig. 1 do not allow for a precise definition of the time interval in which PI(4,5)P_2-dependent membrane recruitment of FGF2-Halo increases in a strictly linear manner. Therefore, in the revised manuscript, we added another measurement at 3 hours as part of the new Fig. 1A. In addition, we have softened the corresponding text elements avoiding an overinterpretation in this matter.

2.) Plasma membrane like lipid compositions of liposomes used in FGF2 recruitment assays

As suggested by Reviewer 2, in order to further challenge our hypothesis, we performed new experiments increasing the complexity of the lipid compositions used in this study. Of note, all lipids were purified from natural extracts containing the full spectrum of fatty acid chains occurring in endogenous membrane lipids. Using plasma-membrane-like liposomes containing phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM), we tested whether omitting cholesterol has an impact on PI(4,5)P_2-dependent FGF2 membrane recruitment (see new table S2 for a detailed description of lipid compositions). These experiments are shown in the new Fig. 1C and demonstrate that, in the continued presence of other charged lipids, omission of cholesterol reduces PI(4,5)P_2-dependent membrane recruitment of FGF2-Halo-AF488 in a highly significant manner.

3.) Additional controls in FGF2 recruitment assays including a PI(4,5)P_2 binding mutant of FGF2 and a PH domain containing protein

As suggested by Reviewer 2, we included additional negative and positive controls in the FGF2 membrane recruitment assays contained in this study. As shown in the new Fig. S1, we used an FGF2-GFP variant form (K127Q, R128Q) that is known to be impaired in physical interactions with PI(4,5)P_2 (Temmerman et al 2008, Traffic). Combined with liposomes used in the current study consisting either of a simple PC/Cholesterol/PI(4,5)P_2 lipid composition or a more complex plasma membrane-like composition, we found this variant form to be incapable of interacting with membrane surfaces (new Fig. S1A and S1B).

In addition, as a positive control, we added new experiments analyzing another protein that binds to membranes in a PI(4,5)P_2-dependent manner, the Pleckstrin Homology domain of phospholipase C δ1 (PH-PLC-δ1). Again, using a PH-PLC-δ1-Halo-AF488 fusion protein, we analyzed membrane binding with different lipid compositions consisting either of a simple PC/Cholesterol/PI(4,5)P_2 mixture (new Fig. 1D) or a more complex plasma membrane-like composition (Fig. 1E). While PH-PLC-δ1-HaloAF488 did bind efficiently to both types of liposomes in a PI(4,5)P_2 dependent manner, the omission of cholesterol caused a highly significant decrease in binding efficiencies (Fig. 1D and 1E). These experiments demonstrate that the observed phenomenon extends beyond FGF2 with cholesterol having a general impact on the physico-chemical properties of lipid bilayers resulting in profound changes on how PI(4,5)P_2 is presented to phosphoinositide-binding proteins.

4.) Alternative approaches to manipulate cellular cholesterol amounts measuring FGF2 secretion under reduced levels of plasma membrane cholesterol

In the revised manuscript, in addition to loading cells with cholesterol using methyl-β-cyclodextrin (revised versions of Figs. 6 and S3), we added new experiments in which cellular cholesterol levels were lowered by a combinatorial treatment with mevastatin and mevalonate, compounds that inhibit cholesterol biosynthesis. The results are shown in the new Fig. 7 along with the corresponding analytical analyses determining cellular cholesterol levels under various conditions (new Fig. S4). Importantly, as also suggested by Reviewer 3 (see below), changes in cholesterol levels were not only determined by filipin staining (total cellular cholesterol) but also by mass spectrometry of subcellular fractions highly enriched in plasma membrane vesicles (new Figs. S3 and S4). As discussed in detail in the revised manuscript, lowering cellular cholesterol levels impairs both FGF2 recruitment at the inner plasma membrane leaflet (new Fig. 7A) and FGF2 membrane translocation to cell surfaces (new Fig. 7B). Thus, the combined results of the experiments shown in Figs. 6, 7, S3 and S4 demonstrate that plasma membrane cholesterol tunes $PI(4,5)P_2$-dependent membrane recruitment and translocation to cell surfaces, the core finding of this study. These data are consistent with both the biochemical *in vitro* experiments shown in Fig. 1 and the molecular dynamics simulations data shown in Fig. 2, demonstrating cholesterol to affect $PI(4,5)P_2$-dependent membrane recruitment of proteins.

5.) Potential pleiotropic effects cholesterol manipulation may exert on other pathways of membrane trafficking

As proposed, we tested whether manipulation of cellular cholesterol levels exerts pleiotropic effects on other membrane trafficking processes. Following the suggestion from Reviewer 2 we analyzed intracellular trafficking of CD4 (used as a GFP fusion protein), an integral membrane protein that is inserted into the ER and transported to the plasma membrane. As shown in the new Fig. 8, using the same TIRF setup and protocol with doxycycline-dependent induction of CD4-GFP expression that was also employed to quantify FGF2-GFP cell surface localization, neither increased nor decreased levels of plasma membrane cholesterol did have a significant impact on intracellular CD4 transport. These experiments demonstrate the effects of cholesterol on $PI(4,5)P_2$-dependent FGF2 membrane recruitment and translocation to be highly specific, excluding pleiotropic effects on other cellular processes such as protein transport along the ER/Golgi-dependent secretory pathway.

6.) Colocalization of FGF2, $PI(4,5)P_2$ and cholesterol at the inner plasma membrane leaflet

As suggested by Reviewer 2, we aimed at colocalizing FGF2, $PI(4,5)P_2$ and cholesterol in the vicinity of the plasma membrane to further corroborate the conclusions from the biochemical, computational and cell-biological analyses contained in this study. To resolve individual pairs of FGF2 and $PI(4,5)P_2$ as well as to visualize cholesterol at the plasma membrane, we used three-color STED super-resolution microscopy. While FGF2 and $PI(4,5)P_2$ were detected by antibodies, we used GRAM1b-G187L-GFP as a sensor for membrane regions with elevated levels (≥ 30 mol%) of accessible cholesterol. As shown in the new Fig. 5, individual pairs of FGF2 and $PI(4,5)P_2$ were found to be abundantly present in plasma membrane regions characterized by high levels of cholesterol.

7.) Use of the term 'reconstitution' in the context of FGF2 membrane recruitment assays

We agree with Reviewer 2 and modified the abstract accordingly.

Reviewer #3:

General comments from Reviewer #3

We provide robust biochemical *in vitro* data and cell-based experiments demonstrating that plasma membrane cholesterol levels have a direct impact on the efficiency of PI(4,5)P_2-dependent membrane recruitment of FGF2. We further demonstrate that this translates into higher rates of FGF2 membrane translocation to cell surfaces. In the revised manuscript, we have added additional experimental conditions corroborating these conclusions (see responses to Reviewers 1 and 2 above). Furthermore, as shown in the new Fig. 1, we provide evidence that cholesterol mediated tuning of protein binding to phosphoinositides is not restricted to FGF2, indicating a broader relevance of our findings for other types of proteins interacting with acidic membrane lipids. The other part of the manuscript deals with the mechanism by which cholesterol may affect PI(4,5)P_2-dependent membrane recruitment of FGF2. Using extensive molecular dynamics simulations, we propose cholesterol to affect both head group visibility and clustering of PI(4,5)P_2 molecules, resulting in faster binding kinetics and stronger interactions of FGF2 with PI(4,5)P_2. We also demonstrated experimentally that membrane tension increases at high levels of cholesterol, a condition that is well known to facilitate the formation of lipidic membrane pores, the key intermediates in unconventional secretion of FGF2. While it is true that we cannot judge on the individual contributions of these three parameters and can also not exclude additional effects cholesterol may exert on PI(4,5)P_2-dependent membrane recruitment, our computational studies deliver a compelling mechanistic explanation on how plasma membrane cholesterol levels tune unconventional secretion of FGF2. Our study is of high interest to this research field and will prompt follow-up studies by us and other laboratories working towards a comprehensive understanding on how the complex lipid compositions of biological membranes can modulate highly specific protein-lipid interactions.

Specific comments from Reviewer #3

1.) Principle of FGF2 membrane recruitment assays based on flow cytometry

The assay we are using is not based on FRET. Rather, liposomes are identified one by one by the light scattering unit of the flow cytometer. FGF2-Halo binding to single liposomes is quantified by Alexa Fluor 488 fluorescence derived from the Halo ligand associated with the FGF2-Halo fusion protein. Rhodamine-PE is added to the lipid mixture in order to be able to detect potential liposome clustering events. Therefore, FGF2-Halo membrane recruitment as measured by Alexa Fluor 488 fluorescence intensity can be normalized to rhodamine fluorescence intensity to obtain FGF2-Halo binding efficiencies relative to the amount of total membrane lipids present in liposomes or liposome clusters identified by light scattering. Compositional heterogeneities of liposome populations are possible, however, for each replicate and experimental condition, we measure 30,000 liposomes. Based on proper statistical analyses, an impact of small liposome subpopulations can be excluded. A detailed description of this assay has been published previously (Temmerman et al 2008, Traffic; Temmerman et al 2009, J Lipid Res).

Irrespective of the underpinnings of this assay, Reviewer 3 is right in pointing out that AF488 and rhodamine are a FRET pair. However, this is taken into account during data processing. In the context of the current study, if FRET were to occur under the experimental conditions used, it would actually reduce the absolute differences between liposomes with and without cholesterol. Therefore, the data set presented in this study is based on a highly reliable assay used to quantify relative differences in PI(4,5)P_2-dependent membrane recruitment of FGF2 under the experimental conditions described.

2.) Slow kinetics of FGF2-Halo membrane recruitment

It is true that the binding kinetics in the experiments shown in Fig. 1A are relatively slow. This is due to the Halo-Alexa Fluor 488 tag we are using. The Halo tag is a highly acidic protein domain that is repelled from membrane surfaces. Thus, by using a Halo-tagged version, we make it hard for FGF2 to interact with membrane surfaces in a PI(4,5)P_2-dependent manner, explaining the relatively slow binding kinetics. Nevertheless, as shown in Fig. 1 and in previous studies, the lipid specificity of FGF2 binding to membranes was shown not to differ between non-tagged FGF2 and fluorescent forms such as FGF2-GFP and FGF2-Halo-Alexa Fluor 488. Using FGF2-Halo fusion proteins with slow binding kinetics might actually be in general beneficial for studies aiming at revealing subtle differences in protein-lipid interactions in biochemical in vitro experiments.

3.) Lipid composition used to study PI(4,5)P_2-dependent membrane recruitment of FGF2

As also mentioned in our response to Reviewer 2, beyond using simple lipid compositions consisting of just PC, cholesterol and PI(4,5)P_2, we have added new experiments systematically comparing the results from the original manuscript with new data generated with more complex lipid compositions resembling plasma membranes (new Fig. 1C and 1E). In addition, in all *in vitro* experiments contained in this study, we are using membrane lipids such as PC, PE, PS, PI, SM and PI(4,5)P_2 that were purified from natural extracts containing the naturally occurring spectrum of fatty acid chains (see Materials and Methods for details). As shown in the new Fig. 1C, similar to simple lipid compositions (Fig. 1B), the omission of cholesterol from plasma-membrane-like lipid compositions causes a highly significant drop in PI(4,5)P_2-dependent FGF2 membrane binding efficiencies.

In the context of MD simulations, for the simple systems, we used POPC and PI(4,5)P_2 with a 20:4/18:0 fatty acid composition. This was necessary to keep the system simple to make atomistic simulations feasible. Nevertheless, PI(4,5)P_2 (20:4/18:0) is the most abundant species in brain extracts used for our biochemical in vitro experiments. Similarly, for the plasma-membrane-like composition, we used PE 18:0/20:4, SOPS 18:0/18:1, SAPI 18:0/20:4, and SM 18:1/16:0 to mimic the most abundant fatty acid distributions found in natural extracts that were used in the *in vitro* binding assays.

In this way, in the revised manuscript, we made every effort to run both the experimental projects and the MD simulations under the most realistic conditions possible.

4.) Potential effects of PS and PI on cholesterol-dependent PI(4,5)P_2 clustering

As suggested by Reviewer 3, we studied potential effects of other lipids on cholesterol-induced PI(4,5)P_2 clustering. The revised manuscript contains new MD simulations with model membranes containing phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and Sphingomyelin (SM), matching the lipid compositions used in the experiments shown in Fig. 1C and 1E with the details given in Table S2 (PM-like). As shown in the new Fig. S2B, even in the presence of other charged lipids such as PS and PI, PI(4,5)P_2 molecules cluster in a cholesterol-dependent manner. This effect is even stronger in a plasma-membrane-like lipid composition compared to what is observed with the simple lipid composition containing just PC, PI(4,5)P_2 and cholesterol. This observation suggests that FGF2 binding to PI(4,5)P_2-containing membranes should be stronger which is in direct agreement with previous work (Temmerman et al. 2008, Traffic; Temmerman et al. 2009, J Lipid Res) and the experimental part of the current study (Fig. 1). Furthermore, these findings are in line with new data in the revised manuscript demonstrating the observed phenomenon not to be restricted to FGF2 but also extending to PH-PLC-δ1, another phosphoinositide-binding protein (Fig. 1D and 1E).

These findings suggest cholesterol to have a general impact on the physico-chemical properties of the lipid bilayer increasing PI(4,5)P_2 head group visibility and clustering.

5.) Determination of cholesterol levels in plasma membrane fractions

We believe this comment of Reviewer 3 is important since the changes in cholesterol levels relevant for FGF2 membrane translocation are linked to the plasma membrane. In the revised manuscript, in addition to filipin staining analyzing global cellular cholesterol levels, we now produced plasma membrane enriched fractions for all conditions either increasing or lowering cholesterol levels. These fractions were analyzed for cholesterol using mass spectrometry (new Figs. S3 and S4). We found significant changes in plasma membrane cholesterol levels under the experimental conditions used for FGF2 recruitment and translocation assays in intact cells (new Figs. 6, 7 and 8).

August 21, 2022

RE: JCB Manuscript #202106123R-A

Prof. Walter Nickel
Heidelberg University
Heidelberg University Biochemistry Center
Im Neuenheimer Feld 328
Heidelberg 69120
Germany

Dear Prof. Nickel:

Thank you for submitting your revised manuscript entitled "Cholesterol promotes clustering of PI(4,5)P2 driving unconventional secretion of FGF2". 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.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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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.

6) * Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts, for example the methods referred to in (Steringer et al., 2017), (Ozbalci et al., 2013) should be briefly described here.*

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 and quantification methods for immunoblotting/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
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10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. 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.

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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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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Satyajit Mayor, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The authors have done enough.

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

As I have mentioned in my previos comments, the work is important to the community of UPS. In the revised manuscript, the authors have very well addressed my concerns. In addition, via extra experiments, their data can provide implications for studies regarding PI(4,5)P2 binding and membrane trafficking. Therefore, I endorse for publication.