PI4P and BLOC-1 remodel endosomal membranes into tubules

Riddhi Jani, Aurélie Di Cicco, Tal Keren-Kaplan, Silvia Vale-Costa, Daniel Hamaoui, Ilse Hurbain, Feng-Ching Tsai, Mathilde Di Marco, Anne-Sophie Macé, Yueyao Zhu, Maria João Amorim, Patricia Bassereau, Juan Bonifacino, Agathe Subtil, Michael Marks, Daniel Lévy, Graça Raposo, and Cedric Delevoye

Corresponding Author(s): Cedric Delevoye, Institut Curie, CNRS-UMR144 and Daniel Lévy, Institute Curie

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Re: JCB manuscript #202110132

Dr. Cedric Delevoye
Institut Curie, CNRS-UMR144
Cell Biology and Cancer
26 Rue d'Ulm
Paris 75005
France

Dear Dr. Delevoye,

Thank you for submitting your manuscript entitled "PI4P and BLOC-1 remodel endosomal membranes into tubules". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

As you will see, the reviewers comment positively on the potential impact of your study. However, they both have concerns that additional data is required to link the in vitro and in vivo results from your study. We agree that significant new experimental evidence is required to this effect. In addition, a revised study must address all comments regarding your current data set, such as the need for conformation of your results in a system not reliant on overexpression, additional quantification and statistical analysis, and further assays examining lipid binding. Once you have had a chance to consider the reviewer comments, please prepare and submit a revision plan outlining both how you will address their specific points as well as the conceptual concerns, so that we may obtain feedback to ensure that a potential revision will be competitive.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

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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 them further once you've had a chance to consider the points raised. 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,

Harald Stenmark, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

SHORT SUMMARY OF THE MANUSCRIPT AND THE ADVANCE OFFERED TO THE FIELD
Tubular recycling endosomes transport cargoes from early endosomes to target membranes. However, the mechanism by which tubular endosomes are formed and stabilized is not well understood. Here, using HeLa cells and in vitro membrane systems, the authors show a requirement for phosphatidylinositol 4-phosphate (PI4P) and the BLOC-1 complex in the formation, stabilization, and transport functions of endosomal tubules. They first show that PI4P associates with RAB11+ recycling endosomal tubules produced by overexpression of KIF13A or KIF13B and they find that these tubules are in proximity to RAB5+ early endosomes. The authors further demonstrate that BLOC-1 and PI4P generate tubules from membranes in vitro. They exposed the purified BLOC-1 complex to membranes and showed that it could tubulate membranes containing PI4P, bind multiple species of PIPs immobilized on membranes and bind to tubular membranes 25-80 nm in diameter containing PI3P or PI4P. In HeLa cells, they show that simultaneous knockdown of PI4KIIalpha and PI4KIIbeta (siPI4KII) dramatically reduces the number and length of recycling endosome tubules, whereas knockdown of PI4KIIIbeta has no effect. They also showed that PI4K inhibition using low levels of PAO causes similar defects in KIF13+ tubule formation. They found that depletion of endosomal PI4P, but not Golgi PI4P, using the synthetic pseudojanin-Sac1 phosphatase dramatically reduced the number and length of KIF13+ endosomal tubules. Using electron microscopy, they found that PI4KIIs are needed for budding and elongation of endosomal tubules. They showed that siPI4KII led to reduced uptake and flux of transferrin through the endosomal pathway, indicating a recycling defect. In addition, the authors provide evidence that PI4KIIs are exploited by intracellular pathogens during their development. They show that siPI4KII in A549 lung carcinoma cells reduced the ability of these cells to support production of PR8 virus and that there were defects in association of RAB11+ puncta with influenza A virus inclusions in these cells. In addition, they found that the number and length of membrane tubules formed from the surface of Chalmydia inclusions in HeLa cells was significantly reduced by siPI4KII.

Overall, the authors show for the first time that BLOC-1 can bind and tubulate PIP-containing membranes and that simultaneous knockdown of PI4KIIalpha and PI4KIIbeta reduces the number, stability and function of endosomal tubules in cells. However, despite their best efforts at linking the two, they have not shown that BLOC-1 binds KIF13-induced endosomal tubules, nor that PI4KIIalpha and PI4KIIbeta affect BLOC-1 binding. In addition, they infer that BLOC-1 and PI4P are involved in all of the processes where they show PI4KII function, but this is not demonstrated. Thus, although the findings described here are potentially very exciting, concerns about aspects of the experimental approach in HeLa cells; lack of appropriate controls for the in vitro membrane tubulation assays; and absence of a link between their in vitro tubulation assays and BLOC-1 regulation in vivo will need to be addressed prior to publication.

MAIN POINTS THAT REQUIRE ADDITIONAL SUPPORT
1. The recycling endosomal tubules examined in this study are induced by overexpression of KIF13A or KIF13B in HeLa cells (p. 6). This is an artificial system and does not give confidence that endosomal tubules found endogenously in cells would behave similarly. It would be more convincing if these experiments could be repeated in another cell type that produces greater numbers

of endosomal tubules or if endosomal tubulation could be induced by some orthogonal method that does not require KIF13 overexpression. The use of Chlamydia inclusions, which form tubules decorated by RAB11, PI4KII and PI4P, lessens this concern to some extent, but is still not the same as examining what happens in cells that are unperturbed.

2. The claim that the KIF13B-positive tubules are "closely apposed to RAB5+ early endosomes" (p. 6) is not supported by any quantitation. The association shown in Fig. S1A appears random, and thus it is not clear whether it is meaningful. It would help to provide multiple insets to make this point, and statistical tests would be required to determine if the association is anything other than random (i.e., do RAB5+ early endosomes tend to associate with KIF13B-positive tubules more often than would be expected by random chance?).

3. Using PIP blots, the authors show that BLOC-1 can bind multiple PIP species, including binding strongly to PI4P, PI5P, PI(3,4)P2, and PI(4,5)P2, weakly to PI(3,5)P2 and PI(3,4,5)P3, and not detectably to PI3P (Fig. S2B). However, surprisingly, they show BLOC-1 binds similarly to membrane tubes containing PI3P or PI4P (Fig. S2E,F). This suggests that either their in vitro system lacks specificity or the PIP blot experiment produced inaccurate results. To address this, they would need to repeat their binding studies using lipid flotation assays to determine if BLOC-1 binds PI3P present on curved membranes. A positive result might explain why BLOC-1 decorates PI3P-positive tubes (Fig. S2E) even though their experiments reveals no affinity for PI3Pin the absence of PI3P binding on blots (Fig. S2B).

Along the same lines, the authors claim that BLOC-1 binds PI4P to tubulate membranes. However, their in vitro experiments lack important controls. For example, in Fig. 2 and Fig. S2H,I, BLOC-1 is added to swelled lipids or GUVs only in the presence of PI4P, so it is unclear whether it would have exhibited tubulating activity in the absence of this lipid. To address this, the authors need to repeat the experiments in Figs 2 and S2 with additional protein-only controls (i.e., liposome or GUV + BLOC-1 vs. liposome or GUV + BLOC-1 + PI4P). If a specific requirement for binding to PI4P (and not other PIPs) during membrane tubulation cannot be demonstrated, then the authors should tone down their interpretation of these experiments.

4. The authors use 300 nM PAO to inhibit type II PI4Ks. However, a previous study showed that 300 uM PAO is needed for 50% inhibition of type II PI4Ks in vitro and that PAO is much more potent inhibitor of PI4KIIIalpha, and to a lesser extent PI4KIIIbeta, than it is of PI4KII enzymes (PMID 11923287). Thus, it is unclear whether this treatment would actually inhibit PI4KII activity in cells, nor whether inhibition of PI4KIIIalpha (which is sensitive to PAO in the 300 nM range in vitro) could potentially account for the observed effects. To rule this out, the authors could use an inhibitor specific to PI4KIIIalpha (GSK-A1) to see if this produces similar results.

5. The authors state that their results examining siPI4KII cells "were consistent with previous observations in cells expressing a catalytically inactive Sac2 mutant or Sac2 null cells" (p. 13). However, it is not clear what this means. Loss of Sac2 would be predicted to result in excess PI4P, not loss of PI4P, so the effect on cells might be expected to be quite different. Rather than including this statement in the Results, the authors could elaborate on the previously published Sac2 results and what these mean in the context of their PI4KII results in the Discussion.

ADDITIONAL ISSUES THAT NEED TO BE ADDRESSED
p. 4, line 9: should be "membrane", not "membranes"
p. 4, 7th line from bottom: "which and how" is awkward; could delete "which and"
p 6, 8th line from bottom: should be "1.4-fold"
p. 9, line 7: "hereafter" is one word
p. 9, line 12: "treated" is misspelled
p. 13, line 4: delete hyphen after "Control"
p. 16, 5th line from bottom: Burgess et al., 2012 showed that Drosophila PI4KIIalpha associates with tubules emerging from late endosomes, whereas Ma et al., 2020 showed that Drosophila PI4KIIalpha associates with tubules emerging from early endosomes; both references should be included
p. 17, line 17: PI4P does not induce endosomal tubule formation from maturing secretory granules; the tubules form at early endosomes and presumably deliver cargo to secretory granules (Ma et al., 2020)
p. 24, 6th line from bottom: should be "plasmid", not "plasmids"
Fig. 1, lower right panel: the inset appears to be behind the main panel, as the tubules marked by the arrowheads in the grayscale images are not evident here
Fig. 2H: scalebar would be easier to see if black
Fig. 6G, upper right: replace "Physio/patho-logical" with "Physio-/pathological"
Fig. 6G, 5: the meaning of "couple and scission of" is unclear; could delete "couple" for clarity
Fig. S2H: would help to have an inset showing the tubules at higher magnification

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

The manuscript of Jani et al. explores the role of PI4P in endosome membrane recycling and tubule formation. The authors show in their work that the biogenesis of PI4P via the PI4kinase II isoforms (identified in an accompanying manuscript) is required for tubule formation. The postulate that PI4P is needed for BLOC-1 association and provide data that purified BLOC-1 can tubulate membranes in vitro. PI4P is indeed required both for endosomal recycling and the association of the kinesin-3 proteins KIF13A and B with endosomes. If PI4P is depleted, both influenza virus infection and proliferation of the intracellular pathogen Clamydia is affected.

This is overall an impressive amount of data, where the authors show two apparently linked events: the PI4P-dependent function of KIF13A/B on endosomal membranes and the association of BLOC-1 with PI4P-containing membranes, which results in membrane tubulation. Both events are nicely shown, even though there are certain deficits in the analysis, however, I miss the link between the two. The authors demonstrate an important role of PI4P in KIF13A and Rab11 association, but is this a BLOC-1 dependent event? There is not a single image, where BLOC-1 is shown or where it is shown that BLOC-1 localization is lost if PI4P is missing from membranes. My main problem is thus that it does not become clear if all observed tubules generated by KIF13A overproduction are indeed BLOC-1 decorated or require BLOC-1 for their formation. This must be resolved in the context of their study. Alternatively, they need to reframe their entire study as BLOC-1 function is not addressed directly in any of their in vivo assays and incomplete for their in vitro studies.

Specific points:

1. The authors show in Figure 1 that KIF13A and B cause tubule formation and claim that such tubules are BLOC-1 dependent. I went back to the original Delevoye et al. 2016 manuscript, where KIF13A function was linked to BLOC-1 (based on siRNA depletion of BLOC-1), but did not find data that BLOC-1 indeed associates with KIF13A positive structures. If such tubules are formed (as also claimed in their 2016 model), are they BLOC-1 positive? I think this would be expected according to the authors' model.

2. Figure 2 is nice and problematic at the same time. It is very nice that the authors have a purified BLOC-1 in hand. But they need to demonstrate its specificity, and here in particular lipid specificity. Their dot-spotting (Figure S2) reveals that this is basically useless. An alternative method is liposome-dependent flotation. This is needed to show specificity to PI4P. They do not need to show all PIPs, but PI3P, PI4,5P2 and PI4P would be useful in comparison. They should then repeat the Cryo-EM and GUV assay using lipids with PI3P and PI4P on their surface and determine the relative activity of BLOC-1 to tubulate membrane. If the authors are right, then BLOC-1 would not work on PI3P decorated membranes, but only on PI4P positive membranes.

3. The remaining manuscript, the authors conclude that PI4P generation and depletion changes tubule formation and thus blocks function of BLOC-1. But these events are not linked here. For instance in Figure 3C, it BLOC-1 then also clustering with KIF13A or is it then cytosolic? Similarly, what happens if the SAC phosphatase is recruited (Figure 4)? Is BLOC-1 then falling off the endosome?

4. The authors ignore all knowledge on retromer and SNX proteins in endosomal recycling. I am aware that these have been mainly linked to PI3P, but it would make their data stronger if they would show that the observed recycling mechanism they observe here is independent of retromer and SNX proteins, but linked to BLOC-1.

5. The discussion and much of the text reads as if all PI4P dependent events are exclusively linked to BLOC-1 function. The authors need to be much more cautious in their writing. For instance, they did not show that BLOC-1 and PI4P are required for influenza virus proliferation or chlamydia proliferation. Their data shows that PI4KII depletion blocks this process and then conclude that this is due to a loss of BLOC-1 - which was not shown here.

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

SHORT SUMMARY OF THE MANUSCRIPT AND THE ADVANCE OFFERED TO THE FIELD

Tubular recycling endosomes transport cargoes from early endosomes to target membranes. However, the mechanism by which tubular endosomes are formed and stabilized is not well understood. Here, using HeLa cells and in vitro membrane systems, the authors show a requirement for phosphatidylinositol 4-phosphate (PI4P) and the BLOC-1 complex in the formation, stabilization, and transport functions of endosomal tubules. They first show that PI4P associates with RAB11+ recycling endosomal tubules produced by overexpression of KIF13A or KIF13B and they find that these tubules are in proximity to RAB5+ early endosomes. The authors further demonstrate that BLOC-1 and PI4P generate tubules from membranes in vitro. They exposed the purified BLOC-1 complex to membranes and showed that it could tubulate membranes containing PI4P, bind multiple species of PIPs immobilized on membranes and bind to tubular membranes 25-80 nm in diameter containing PI3P or PI4P.

In HeLa cells, they show that simultaneous knockdown of PI4KIIalpha and PI4KIIbeta (siPI4KII) dramatically reduces the number and length of recycling endosome tubules, whereas knockdown of PI4KIIIbeta has no effect. They also showed that PI4K inhibition using low levels of PAO causes similar defects in KIF13+ tubule formation. They found that depletion of endosomal PI4P, but not Golgi PI4P, using the synthetic pseudojanin-Sac1 phosphatase dramatically reduced the number and length of KIF13+ endosomal tubules. Using electron microscopy, they found that PI4KIIs are needed for budding and elongation of endosomal tubules. They showed that siPI4KII led to reduced uptake and flux of transferrin through the endosomal pathway, indicating a recycling defect. In addition, the authors provide evidence that PI4KIIs are exploited by intracellular pathogens during their development. They show that siPI4KII in A549 lung carcinoma cells reduced the ability of these cells to support production of PR8 virus and that there were defects in association of RAB11+ puncta with influenza A virus inclusions in these cells. In addition, they found that the number and length of membrane tubules formed from the surface of Chlamydia inclusions in HeLa cells was significantly reduced by siPI4KII.

Overall, the authors show for the first time that BLOC-1 can bind and tubulate PIP-containing membranes and that simultaneous knockdown of PI4KIIalpha and PI4KIIbeta reduces the number, stability and function of endosomal tubules in cells. However, despite their best efforts at linking the two, they have not shown that BLOC-1 binds KIF13-induced endosomal tubules, nor that PI4KIIalpha and PI4KIIbeta affect BLOC-1 binding. In addition, they infer that BLOC-1 and PI4P are involved in all of the processes where they show PI4KII function, but this is not demonstrated.

Thus, although the findings described here are potentially very exciting, concerns about aspects of the experimental approach in HeLa cells; lack of appropriate controls for the in vitro membrane tubulation assays; and absence of a link between their in vitro tubulation assays and BLOC-1 regulation in vivo will need to be addressed prior to publication.

As already detailed in the letter to the editors and the reviewers from last January, the current technical limitations in the field preclude our possibility to better link the *in vitro* and *in vivo* parts. There is no technical approach to quantitatively image BLOC-1 in cells. Therefore, we cannot formally demonstrate that BLOC-1 binds to KIF13-induced recycling endosomal tubules. However, the revised manuscript now includes new biochemical data showing that extinguishing the protein expression levels of PI4KIIα and PI4KIIβ in HeLa cells did not affect the overall binding of BLOC-1 to membranes. This result is consistent with that of our companion paper showing that depleting the expression of either PI4KIIα or PI4KIIβ did not impact BLOC-1 binding to cellular membranes in pigment cells (Zhu et al, manuscript accepted for publication in the Journal of Cell Biology). Zhu et al studied the impact of PI4KIIα and PI4KIIβ on melanosome maturation in pigment cells, which develop endogenously a more tubulated network of endosomal tubules relative to the network observed in HeLa cells. Their findings document that PI4KIIα and PI4KIIβ each contribute to the biogenesis and dynamics of recycling endosomal tubules in pigment cells, which we previously demonstrated to be dependent on

BLOC-1 (Delevoye et al., 2016). The revised submitted manuscript has now been modified in several places to refer more explicitly to this study by Zhu and colleagues. The revised manuscript has been also reinforced by additional in vitro experiments to better demonstrate the ability of BLOC-1 to bind and tubulate negatively charged membranes.

We believe that the additional requested data and the revised text completely address these concerns.

Delevoye, C., X. Heiligenstein, L. Ripoll, F. Gilles-Marsens, M.K. Dennis, R.A. Linares, L. Derman, A. Gokhale, E. Morel, V. Faundez, M.S. Marks, and G. Raposo. 2016. BLOC-1 Brings Together the Actin and Microtubule Cytoskeletons to Generate Recycling Endosomes. *Curr Biol*. 26:1–13. doi:10.1016/j.cub.2015.11.020.

Zhu, Y., S. Li, A. Jaume, R.A. Jani, C. Delevoye, G, Raposo and M.S. Marks. 2022. Type II phosphatidylinositol 4-kinases function sequentially in cargo delivery from early endosomes to melanosomes. *J. Cell Biol.* in press. doi: 10.1083/jcb.202110114.

MAIN POINTS THAT REQUIRE ADDITIONAL SUPPORT

1. The recycling endosomal tubules examined in this study are induced by overexpression of KIF13A or KIF13B in HeLa cells (p. 6). This is an artificial system and does not give confidence that endosomal tubules found endogenously in cells would behave similarly. It would be more convincing if these experiments could be repeated in another cell type that produces greater numbers of endosomal tubules or if endosomal tubulation could be induced by some orthogonal method that does not require KIF13 overexpression. The use of Chlamydia inclusions, which form tubules decorated by RAB11, PI4KII and PI4P, lessens this concern to some extent, but is still not the same as examining what happens in cells that are unperturbed.

We have previously documented that mouse and human pigment cells harbor a tubulated endogenous recycling endosomal network, decorated by the SNARE syntaxin-13 (STX13) (Delevoye et al., 2009, 2016; Dennis et al., 2015). The formation of this tubulated recycling endosomal network in pigment cells requires BLOC-1 and the kinesin KIF13A (Delevoye et al., 2009, 2016). In HeLa cells, depletion of KIF13A impairs the tubulation of RAB11-positive recycling tubules and overexpression of KIF13A-YFP results in the extreme elongation of these tubules (Delevoye et al., 2014; Shakya et al., 2018). Finally, BLOC-1 expression is required for the biogenesis of the KIF13A-positive tubules in HeLa cells and the STX13-positive tubules in melanocytes (Delevoye et al., 2016). Therefore, BLOC-1 is an ubiquitous complex required for the biogenesis of STX13 and/ or KIF13A-positive recycling tubules in pigment and non-pigment cells (Thankachan and Setty, 2022).

The recently accepted companion publication, Zhu et al, show that PI4KIIα and PI4KIIβ are both required for the dynamics and function of BLOC-1-dependent recycling endosomal tubules in pigment cells, in which these tubules are largely exploited for cargo delivery to melanosomes. These data are (i) generated, as proposed by the reviewer, in a cell type that produces endogenously a greater number of recycling endosomal tubules, and (ii) fully consistent with our submitted data generated in HeLa cells overexpressing KIF13. We have accordingly revised the text of this manuscript to explicitly refer to the companion paper where appropriate. Please, see the **revised text in p5, 8, 15,** and **18-21.**

Delevoye, C., X. Heiligenstein, L. Ripoll, F. Gilles-Marsens, M.K. Dennis, R.A. Linares, L. Derman, A. Gokhale, E. Morel, V. Faundez, M.S. Marks, and G. Raposo. 2016. BLOC-1 Brings Together the Actin and Microtubule Cytoskeletons to Generate Recycling Endosomes. *Curr Biol*. 26:1–13. doi:10.1016/j.cub.2015.11.020.

Delevoye, C., I. Hurbain, D. Tenza, J.B. Sibarita, S. Uzan-Gafsou, H. Ohno, W.J. Geerts, A.J. Verkleij, J. Salamero, M.S. Marks, and G. Raposo. 2009. AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. *J Cell Biol*. 187:247–64. doi:10.1083/jcb.200907122.

Delevoye, C., S. Miserey-Lenkei, G. Montagnac, F. Gilles-Marsens, P. Paul-Gilloteaux, F. Giordano, F. Waharte, M.S. Marks, B. Goud, and G. Raposo. 2014. Recycling endosome tubule morphogenesis from sorting endosomes requires the kinesin motor KIF13A. *Cell Rep*. 6:445–54. doi:10.1016/j.celrep.2014.01.002.

Dennis, M.K., A.R. Mantegazza, O.L. Snir, D. Tenza, A. Acosta-Ruiz, C. Delevoye, R. Zorger, A. Sitaram, W. de Jesus-Rojas, K. Ravichandran, J. Rux, E.V. Sviderskaya, D.C. Bennett, G. Raposo, M.S. Marks, and S.R. Setty. 2015. BLOC-2 targets

recycling endosomal tubules to melanosomes for cargo delivery. *J Cell Biol*. 209:563–577. doi:10.1083/jcb.201410026.

Shakya, S., P. Sharma, A.M. Bhatt, R.A. Jani, C. Delevoye, and S.R. Setty. 2018. Rab22A recruits BLOC-1 and BLOC-2 to promote the biogenesis of recycling endosomes. *EMBO Rep.* 19. doi:10.15252/embr.201845918.

Thankachan, J.M., and S.R.G. Setty. 2022. KIF13A-A Key Regulator of Recycling Endosome Dynamics. *Front Cell Dev Biol*. 10:877532. doi:10.3389/fcell.2022.877532.

Zhu, Y., S. Li, A. Jaume, R.A. Jani, C. Delevoye, G, Raposo and M.S. Marks. 2022. Type II phosphatidylinositol 4-kinases function sequentially in cargo delivery from early endosomes to melanosomes. *J. Cell Biol.* in press. doi: 10.1083/jcb.202110114.

2. The claim that the KIF13B-positive tubules are "closely apposed to RAB5+ early endosomes" (p. 6) is not supported by any quantitation. The association shown in Fig. S1A appears random, and thus it is not clear whether it is meaningful. It would help to provide multiple insets to make this point, and statistical tests would be required to determine if the association is anything other than random (i.e., do RAB5+ early endosomes tend to associate with KIF13B-positive tubules more often than would be expected by random chance?).

We now provide a quantification of the average shortest distance of the mcherry-KIF13B+ tubule to the iRFP-RAB5+ early endosomes (**revised Fig. S1A, right panel**). The results showed that the mCherry-KIF13B signal was on average at 1.94 ± 0.15 µm from the nearest iRFP-RAB5 signal, indicating, as originally proposed, that the KIF13B+ tubules were close to the RAB5+ vesicular structures (i.e., apposed), but not co-localized. See also revised text in **p7, 19, 37**, and **57**.

Of note, the submitted **Fig. S1B** (bottom panels) showed that the PI3P sensor GFP-FYVE co-distributed as expected with iRFP-RAB5 (bottom panels, arrowheads) but not with mCherry-KIF13B (**Fig. 1C**, bottom panels). However, GFP-FYVE and mCherry-KIF13B-labeled structures were very often found in proximity as revealed by linescan analysis across multiple mCherry-KIF13B+ tubules (see insets in **Fig. 1C** and quantification in **Fig. 1D**, bottom panels).

3. Using PIP blots, the authors show that BLOC-1 can bind multiple PIP species, including binding strongly to PI4P, PI5P, PI(3,4)P2, and PI(4,5)P2, weakly to PI(3,5)P2 and PI(3,4,5)P3, and not detectably to PI3P (Fig. S2B). However, surprisingly, they show BLOC-1 binds similarly to membrane tubes containing PI3P or PI4P (Fig. S2E,F). This suggests that either their in vitro system lacks specificity or the PIP blot experiment produced inaccurate results. To address this, they would need to repeat their binding studies using lipid flotation assays to determine if BLOC-1 binds PI3P present on curved membranes. A positive result might explain why BLOC-1 decorates PI3P-positive tubes (Fig. S2E) even though their experiments reveals no affinity for PI3Pin the absence of PI3P binding on blots (Fig. S2B).

We realize that a mistake was introduced in the labeling of the lanes of phosphoinositides in the originally submitted Fig. S2B that has led to the confusion and the question raised by the reviewer. We sincerely apologize for this error.

In fact, the purified BLOC-1 binds to PI3P in the PIP blot experiments (see **revised Fig. S2B**) as it did on membrane nanotubes imaged by negative staining EM (**revised Fig. S2D**) or Cryo-EM (**revised Fig. S2F**). The label depicting the PI species in the originally submitted Figure S2B was unintentionally shifted when generating the figure, and now corrected. The data show that BLOC-1 binds efficiently to PI3P, PI4P, PI5P, PI(3,5)P2, and PI(4,5)P2. Therefore, the data presented in the PIP blot were consistent with the ones using nanotubes doped with PI3P.

As proposed by the reviewer, we have now performed lipid floatation assays using recombinant BLOC-1 incubated with swelled liposomes doped with PI3P, PI(4,5)P2, PI4P, phosphatidylserine (as non-PIP but negatively charged phospholipid) or phosphatidylcholine (as a phospholipid with neutral charge). Using SDS-PAGE and Coomassie blue staining to detect the BLOC-1 bound to the membranes

(see **p30**), we showed that BLOC-1 was more efficiently bound to vesicles that contained negatively charged lipids than to PC-doped vesicles or a control without vesicles (see **revised Fig. S2C** and **text p8-9**, and **57**).

To strengthen this result, we have also extended our original analysis of the binding of recombinant BLOC-1 to nanotubes by negative staining EM imaging of Galactocerebroside (GalCer)/EPC nanotubes doped with the negatively charged lipids tested on the lipid floatation assay. We consistently observed among 100 independent tubes per condition that BLOC-1 bind to negatively charged lipid nanotubes (**revised Fig. S2D**, and **text p9**, and **57**).

Along the same lines, the authors claim that BLOC-1 binds PI4P to tubulate membranes. However, their in vitro experiments lack important controls. For example, in Fig. 2 and Fig. S2H,I, BLOC-1 is added to swelled lipids or GUVs only in the presence of PI4P, so it is unclear whether it would have exhibited tubulating activity in the absence of this lipid. To address this, the authors need to repeat the experiments in Figs 2 and S2 with additional protein-only controls (i.e., liposome or GUV + BLOC-1 vs. liposome or GUV + BLOC-1 + PI4P). If a specific requirement for binding to PI4P (and not other PIPs) during membrane tubulation cannot be demonstrated, then the authors should tone down their interpretation of these experiments.

As requested by the reviewer, we have performed again the GUV experiments as presented in the original Fig. 2D but now including control EPC GUVs incubated for 30 min with recombinant BLOC-1. Using fluorescence microscopy, EPC GUVs lacking negatively charged phospholipids did not tubulate when incubated with BLOC-1 (**revised Fig. S2J**, top panel). This result is consistent with experiments using GalCer lipid nanotubes (**revised Fig. S2D-E**) that demonstrate the absence of binding of BLOC-1 on membranes containing only EPC.

In addition, we also performed experiments with EPC GUVs doped with PI3P (as proposed by Reviewer#2), which PI3P+ GUVs indeed generated tubules upon addition of recombinant BLOC-1 (**revised Fig. S2J**, bottom panel). Together with the originally submitted data showing that PI4P+ GUVs tubulated only in presence of BLOC-1 (**Fig. 2D**), the results show collectively that BLOC-1 binds to GUVs when doped with PI3P or PI4P and causes them to tubulate. The corresponding text has been modified in **p10.**

4. The authors use 300 nM PAO to inhibit type II PI4Ks. However, a previous study showed that 300 uM PAO is needed for 50% inhibition of type II PI4Ks in vitro and that PAO is much more potent inhibitor of PI4KIIIalpha, and to a lesser extent PI4KIIIbeta, than it is of PI4KII enzymes (PMID 11923287). Thus, it is unclear whether this treatment would actually inhibit PI4KII activity in cells, nor whether inhibition of PI4KIIIalpha (which is sensitive to PAO in the 300 nM range in vitro) could potentially account for the observed effects. To rule this out, the authors could use an inhibitor specific to PI4KIIIalpha (GSK-A1) to see if this produces similar results.

We thank the reviewer for this suggestion, and we have performed the proposed experiments (**revised Fig. S3D-E**). HeLa cells were transiently transfected with the plasmid encoding KIF13A-YFP to generate recycling endosomal tubules in cells treated for 15 to 30 min with either DMSO (vehicle) as a control or with GSK-A1 (100 nM) to specifically inhibit the enzymatic activity of PI4KIIIα. Cells were then imaged live to detect the tubules, and the percentage of cells displaying at least one KIF13A-YFP-positive tubules were quantified (n>100, N=3 independent experiments). Such an incubation time and concentration have been previously documented to significantly decrease the intracellular PI4P levels in platelets (Bura and Jurak Begonja, 2021), or the plasma membrane-associated pool of PI4P in Cos7 cells (Bojjireddy et al., 2014). Of note, the release of the PI4P pool from the plasma membrane upon GSK-A1 treatment was shown to be concomitantly associated with an increase of the PI4P level on endomembranes (Sengupta et al., 2019; Boutry and Kim, 2021).

The results, presented now in the **revised Fig. S3D-E** and **p12 of the revised manuscript,** showed that incubating KIF13A-YFP-expressing cells with GSK-A1 did not significantly affect the average percentage of cells with at least one KIF13A-YFP-positive tubule as compared to DMSO-treated cells. This indicates that the enzymatic activity of PI4KIIIα is not required for KIF13A-dependent endosomal tubulation. The GSK-A1 treated cells behaved similarly as the wortmannin-treated cells (**revised Fig. S3D-E**), or as cells treated with a siRNA directed against PI4KIIIβ (**Fig. 3F**, middle panel). Together with the results showing that KIF13A+ recycling tubules were specifically affected in cells co-depleted of PI4KIIα and PI4KIIβ (**Fig. 3C-E**), or in PAO-treated cells (**Fig. S3D-E**), or in cells for which the endosomal PI4P level were reduced (**Fig. 4**), we conclude that the formation and/ or stability of KIF13A+ recycling endosomal tubules specifically relies on the PI4P production by endosomal PI4KIIα and PI4KIIβ (see **revised text p12** and **58-59**).

Bojjireddy, N., J. Botyanszki, G. Hammond, D. Creech, R. Peterson, D.C. Kemp, M. Snead, R. Brown, A. Morrison, S. Wilson, S. Harrison, C. Moore, and T. Balla. 2014. Pharmacological and genetic targeting of the PI4KA enzyme reveals its important role in maintaining plasma membrane phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate levels. *J Biol Chem*. 289:6120–6132. doi:10.1074/jbc.M113.531426.

Boutry, M., and P.K. Kim. 2021. ORP1L mediated PI(4)P signaling at ER-lysosome-mitochondrion three-way contact contributes to mitochondrial division. *Nat Commun*. 12:5354. doi:10.1038/s41467-021-25621-4.

Bura, A., and A. Jurak Begonja. 2021. Imaging of Intracellular and Plasma Membrane Pools of PI(4,5)P2 and PI4P in Human Platelets. *Life (Basel)*. 11:1331. doi:10.3390/life11121331.

Sengupta, N., M. Jović, E. Barnaeva, D.W. Kim, X. Hu, N. Southall, M. Dejmek, I. Mejdrova, R. Nencka, A. Baumlova, D. Chalupska, E. Boura, M. Ferrer, J. Marugan, and T. Balla. 2019. A large scale high-throughput screen identifies chemical inhibitors of phosphatidylinositol 4-kinase type II alpha[S]. *Journal of Lipid Research*. 60:683–693. doi:10.1194/jlr.D090159.

5. The authors state that their results examining siPI4KII cells "were consistent with previous observations in cells expressing a catalytically inactive Sac2 mutant or Sac2 null cells" (p. 13). However, it is not clear what this means. Loss of Sac2 would be predicted to result in excess PI4P, not loss of PI4P, so the effect on cells might be expected to be quite different. Rather than including this statement in the Results, the authors could elaborate on the previously published Sac2 results and what these mean in the context of their PI4KII results in the Discussion.

We agree with the reviewer that this sentence provides more confusion than clarity. Originally, we proposed that an imbalance in PI4P metabolism on endosomal membrane would lead to global defects in endosomal cargo sorting and trafficking. Since this sentence sounded more like a "discussion", and because we have already elaborated in the discussion that Sac2 could be enriched at vacuolar endosomal domains to counteract PI4P production by PI4KIIα and PI4KIIβ (**p18-19**), we decided to delete this sentence from the revised result section as proposed by the reviewer.

ADDITIONAL ISSUES THAT NEED TO BE ADDRESSED

p. 4, line 9: should be "membrane", not "membranes"
p. 4, 7th line from bottom: "which and how" is awkward; could delete "which and"
p 6, 8th line from bottom: should be "1.4-fold"
p. 9, line 7: "hereafter" is one word
p. 9, line 12: "treated" is misspelled
p. 13, line 4: delete hyphen after "Control"
p. 16, 5th line from bottom: Burgess et al., 2012 showed that Drosophila PI4KIIalpha associates with tubules emerging from late endosomes, whereas Ma et al., 2020 showed that Drosophila PI4KIIalpha associates with tubules emerging from early endosomes; both references should be included
p. 17, line 17: PI4P does not induce endosomal tubule formation from maturing secretory granules; the tubules form at early endosomes and presumably deliver cargo to secretory granules (Ma et al., 2020)
p. 24, 6th line from bottom: should be "plasmid", not "plasmids"

Fig. 1, lower right panel: the inset appears to be behind the main panel, as the tubules marked by the arrowheads in the grayscale images are not evident here
Fig. 2H: scalebar would be easier to see if black
Fig. 6G, upper right: replace "Physio/patho-logical" with "Physio-/pathological"
Fig. 6G, 5: the meaning of "couple and scission of" is unclear; could delete "couple" for clarity
Fig. S2H: would help to have an inset showing the tubules at higher magnification

We thank the reviewer for the careful inspection of the manuscript, and we have corrected these issues accordingly.

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

The manuscript of Jani et al. explores the role of PI4P in endosome membrane recycling and tubule formation. The authors show in their work that the biogenesis of PI4P via the PI4kinase II isoforms (identified in an accompanying manuscript) is required for tubule formation. The postulate that PI4P is needed for BLOC-1 association and provide data that purified BLOC-1 can tubulate membranes in vitro. PI4P is indeed required both for endosomal recycling and the association of the kinesin-3 proteins KIF13A and B with endosomes. If PI4P is depleted, both influenza virus infection and proliferation of the intracellular pathogen Clamydia is affected.

We apologize if we were not explicit enough in the original manuscript, but we wish to clarify an observation. We have not shown that PI4P is required for the association of kinesin-3 KIF13A/B with endosomes. In fact, these kinesins are most likely associated with endosomal membranes in the absence of PI4KIIα and PI4KIIβ expression or endosomal PI4P, as reflected by the KIF13A-YFP co-distribution with mCherry-RAB11 or iRFP-RAB5 (see **Fig. S3A** and **Fig. 4B**).

This is overall an impressive amount of data, where the authors show two apparently linked events: the PI4P-dependent function of KIF13A/B on endosomal membranes and the association of BLOC-1 with PI4P-containing membranes, which results in membrane tubulation. Both events are nicely shown, even though there are certain deficits in the analysis, however, I miss the link between the two. The authors demonstrate an important role of PI4P in KIF13A and Rab11 association, but is this a BLOC-1 dependent event? There is not a single image, where BLOC-1 is shown or where it is shown that BLOC-1 localization is lost if PI4P is missing from membranes.

My main problem is thus that it does not become clear if all observed tubules generated by KIF13A overproduction are indeed BLOC-1 decorated or require BLOC-1 for their formation. This must be resolved in the context of their study. Alternatively, they need to reframe their entire study as BLOC-1 function is not addressed directly in any of their in vivo assays and incomplete for their in vitro studies.

First, we thank the reviewer for the time spent analyzing the submitted manuscript and for the positive comments.

Second, we apologize for our lack of clarity. We previously showed in HeLa cells that almost all KIF13A-YFP+ recycling tubules (if not all) were positive for mCherry-RAB11 (Delevoye et al., 2014), and that the KIF13A-YFP+ tubules required BLOC-1 for their generation (Delevoye et al., 2016; Shakya et al., 2018). Given the role of PI4P in the formation of KIF13+ tubules presented in the submitted manuscript, we therefore reason that BLOC-1 is involved in this same PI4P and KIF13-dependent process. However, the requirement for BLOC-1 in tubule formation does not necessarily mean that BLOC-1 decorates the entire tubule in vivo as it does in vitro. All that we can conclude at the moment is that it is required for tubule initiation.

The reviewer is correct that we have not shown any imaging of BLOC-1 localization in cells (either endogenously or exogenously). The reason, as we explained to the editors and the reviewer in our letter (from last January), is that the tools needed to image BLOC-1 in cells do not currently exist. We will return to this point below.

Delevoye, C., X. Heiligenstein, L. Ripoll, F. Gilles-Marsens, M.K. Dennis, R.A. Linares, L. Derman, A. Gokhale, E. Morel, V. Faundez, M.S. Marks, and G. Raposo. 2016. BLOC-1 Brings Together the Actin and Microtubule Cytoskeletons to Generate Recycling Endosomes. *Curr Biol*. 26:1–13. doi:10.1016/j.cub.2015.11.020.

Delevoye, C., S. Miserey-Lenkei, G. Montagnac, F. Gilles-Marsens, P. Paul-Gilloteaux, F. Giordano, F. Waharte, M.S. Marks, B. Goud, and G. Raposo. 2014. Recycling endosome tubule morphogenesis from sorting endosomes requires the kinesin motor KIF13A. *Cell Rep*. 6:445–54. doi:10.1016/j.celrep.2014.01.002.

Shakya, S., P. Sharma, A.M. Bhatt, R.A. Jani, C. Delevoye, and S.R. Setty. 2018. Rab22A recruits BLOC-1 and BLOC-2 to promote the biogenesis of recycling endosomes. *EMBO Rep.* 19. doi:10.15252/embr.201845918.

Specific points:

1. The authors show in Figure 1 that KIF13A and B cause tubule formation and claim that such tubules are BLOC-1 dependent. I went back to the original Delevoye et al. 2016 manuscript, where KIF13A function was linked to BLOC-1 (based on siRNA depletion of BLOC-1), but did not find data that BLOC-1 indeed associates with KIF13A positive structures. If such tubules are formed (as also claimed in their 2016 model), are they BLOC-1 positive? I think this would be expected according to the authors' model.

We have previously documented that mouse and human pigment cells harbor a tubulated endogenous recycling endosomal network, decorated by the SNARE syntaxin-13 (STX13), that is more clearly visualized than in HeLa cells (Delevoye et al., 2009, 2016; Dennis et al., 2015). The formation of this tubulated recycling endosomal network in pigment cells requires BLOC-1 and the kinesin KIF13A (Delevoye et al., 2009, 2016). In HeLa cells, depletion of KIF13A impairs the tubulation of RAB11-positive recycling tubules and overexpression of KIF13A-YFP results in the extreme elongation of these tubules (Delevoye et al., 2014; Shakya et al., 2018). Finally, BLOC-1 expression is required for the biogenesis of the KIF13A-positive tubules in HeLa cells and of the STX13-positive recycling tubules in in melanocytes (Delevoye et al., 2016). Therefore, BLOC-1 is an ubiquitous complex required for the biogenesis of STX13 and/ or KIF13A-positive recycling tubules in pigment and non-pigment cells (Thankachan and Setty, 2022).

As already discussed in our letter from January, it is technically impossible to quantitatively immunolocalize BLOC-1 in cells, and even more so on recycling tubules that are sensitive to chemical fixation. Also, there is currently no version of a functionally intact, fluorescently-tagged BLOC-1 that is compatible with live cell imaging and that can be expressed by eukaryotic cells (the Marks laboratory is currently working on developing such a reagent). Previously published data, including those from our laboratory, demonstrated that: (i) BLOC-1 is required for the biogenesis of KIF13A-YFP-dependent recycling endosomal tubules in pigment and non-pigment cells (Delevoye et al., 2016; Shakya et al., 2018); (ii) pigment cells or HeLa cells depleted of BLOC-1 or KIF13A by knockout or knock down, respectively, show a similar delay in transferrin recycling (Setty et al., 2007; Delevoye et al., 2014); (iii) KIF13A-YFP interacts with BLOC-1 subunits by co-immunoprecipitation (Delevoye et al., 2016); and (iv) the dysbindin subunit of BLOC-1 was localized to tubulo-vesicular structures associated with endosomes, by immunolabeling EM on ultrathin cryo-sections and on whole-mounted cells in which endosomes were selectively cross-linked (Di Pietro et al., 2006); however, the labeling for BLOC-1 in the latter experiments was too low to allow for quantitative evaluation.

In view of these data and technical limitations, we decided to exploit the KIF13A- and BLOC-1-dependent endosomal tubulation process as a proxy to study the biogenesis of recycling endosomal tubules in cells, and to explore the contribution of phosphoinositides and associated kinases to this process. The aforementioned technical limitations have led us to test the ability of BLOC-1 to bind and remodel membranes by other approaches, which are presented in the submitted manuscript, i.e., using purified BLOC-1 incubated with membrane systems and cryo-immobilized for visualization by cryo-EM imaging. Although these experiments were performed *in vitro*, they are the best and only approach currently available to define the role of BLOC-1 on membranes. *In vitro*, our results demonstrate that purified BLOC-1 can bind negatively charged membranes and tubulate those containing PI3P or PI4P (**revised Fig. 2** and **S2**). In cells, we demonstrate that the KIF13A-dependent tubulation of recycling endosomes requires the production of a pool of endosomal PI4P by PI4KIIα and PI4KIIβ (**revised Fig. 3, S3, 4, 5** and **S4**).

Thus, we hope that the reviewer understands the limitations of the field and will consider our revised manuscript in that light.

Delevoye, C., X. Heiligenstein, L. Ripoll, F. Gilles-Marsens, M.K. Dennis, R.A. Linares, L. Derman, A. Gokhale, E. Morel, V. Faundez, M.S. Marks, and G. Raposo. 2016. BLOC-1 Brings Together the Actin and Microtubule Cytoskeletons to Generate Recycling Endosomes. *Curr Biol*. 26:1–13. doi:10.1016/j.cub.2015.11.020.

Delevoye, C., I. Hurbain, D. Tenza, J.B. Sibarita, S. Uzan-Gafsou, H. Ohno, W.J. Geerts, A.J. Verkleij, J. Salamero, M.S. Marks, and G. Raposo. 2009. AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. *J Cell Biol*. 187:247–64. doi:10.1083/jcb.200907122.

Delevoye, C., S. Miserey-Lenkei, G. Montagnac, F. Gilles-Marsens, P. Paul-Gilloteaux, F. Giordano, F. Waharte, M.S. Marks, B. Goud, and G. Raposo. 2014. Recycling endosome tubule morphogenesis from sorting endosomes requires the kinesin motor KIF13A. *Cell Rep*. 6:445–54. doi:10.1016/j.celrep.2014.01.002.

Dennis, M.K., A.R. Mantegazza, O.L. Snir, D. Tenza, A. Acosta-Ruiz, C. Delevoye, R. Zorger, A. Sitaram, W. de Jesus-Rojas, K. Ravichandran, J. Rux, E.V. Sviderskaya, D.C. Bennett, G. Raposo, M.S. Marks, and S.R. Setty. 2015. BLOC-2 targets recycling endosomal tubules to melanosomes for cargo delivery. *J Cell Biol*. 209:563–577. doi:10.1083/jcb.201410026.

Di Pietro, S.M., J.M. Falcon-Perez, D. Tenza, S.R. Setty, M.S. Marks, G. Raposo, and E.C. Dell'Angelica. 2006. BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. *Mol Biol Cell*. 17:4027–38. doi:10.1091/mbc.E06-05-0379.

Setty, S.R., D. Tenza, S.T. Truschel, E. Chou, E.V. Sviderskaya, A.C. Theos, M.L. Lamoreux, S.M. Di Pietro, M. Starcevic, D.C. Bennett, E.C. Dell'Angelica, G. Raposo, and M.S. Marks. 2007. BLOC-1 is required for cargo-specific sorting from vacuolar early endosomes toward lysosome-related organelles. *Mol Biol Cell*. 18:768–80. doi:10.1091/mbc.E06-12-1066.

Shakya, S., P. Sharma, A.M. Bhatt, R.A. Jani, C. Delevoye, and S.R. Setty. 2018. Rab22A recruits BLOC-1 and BLOC-2 to promote the biogenesis of recycling endosomes. *EMBO Rep.* 19. doi:10.15252/embr.201845918.

Thankachan, J.M., and S.R.G. Setty. 2022. KIF13A-A Key Regulator of Recycling Endosome Dynamics. *Front Cell Dev Biol*. 10:877532. doi:10.3389/fcell.2022.877532.

2. Figure 2 is nice and problematic at the same time. It is very nice that the authors have a purified BLOC-1 in hand. But they need to demonstrate its specificity, and here in particular lipid specificity. Their dot-spotting (Figure S2) reveals that this is basically useless. An alternative method is liposome-dependent flotation. This is needed to show specificity to PI4P. They do not need to show all PIPs, but PI3P, PI4,5P2 and PI4P would be useful in comparison. They should then repeat the Cryo-EM and GUV assay using lipids with PI3P and PI4P on their surface and determine the relative activity of BLOC-1 to tubulate membrane. If the authors are right, then BLOC-1 would not work on PI3P decorated membranes, but only on PI4P positive membranes.

We realize that a mistake was introduced in the labeling of the lanes of phosphoinositides in the originally submitted Fig. S2B that has led to the confusion and the question raised by the reviewer. We sincerely apologize for this error.

In fact, as now shown in the revised **Fig. S2B**, the purified BLOC-1 binds to PI3P in the PIP blot experiments as it did on membrane nanotubes imaged by negative staining EM (**revised Fig. S2D**) or Cryo-EM (**revised Fig. S2F**). The labels depicting the PI species in the originally submitted Figure S2B was unintentionally shifted when generating the figure, and are now corrected. The data show that BLOC-1 binds efficiently to PI3P, PI4P, PI5P, PI(3,5)P2, and PI(4,5)P2. Therefore, the data presented in the PIP blot were consistent with the data using nanotubes doped with PI3P.

We would also like to stress that our submitted data showed that the binding of BLOC-1 to PI4P is specific but not exclusive. Indeed, the original **Figure S2** showed that BLOC-1 bound to GalCer/ EPC nanotubes when doped with PI4P or PI3P (**revised Fig. S2D**, and **S2F-H**).

Nevertheless, as proposed by the reviewer, we have now performed assays in which recombinant BLOC-1 was incubated with swelled liposomes doped with PI3P, PI(4,5)P2, PI4P, phosphatidylserine (PS; as a non-PIP but negatively charged phospholipid) or phosphatidylcholine (PC; as a phospholipid with neutral charge) and then analyzed by lipid floatation and SDS-PAGE of the buoyant fraction (see **p30**). As shown in **revised Fig. S2C** and **text p8-9** and **57**, BLOC-1 was more efficiently bound to vesicles that contained negatively charged lipid as compared to PC-doped vesicles or a control reaction without vesicles. To strengthen this result, we have also extended our original cryo-EM analysis of the binding of recombinant BLOC-1 to nanotubes by negative staining EM imaging of Galactocerebroside (GalCer)/ EPC nanotubes doped with the negatively charged lipids tested in the

lipid floatation assay. We show that 100% of the nanotubes that contain negative phospholipids (100 independent tubes per condition), including those containing PI3P or PI4P, were decorated by BLOC-1 (**revised Fig. S2D,** and **text p9** and **57**). Together, these data confirm the previous observations by cryo-EM of BLOC-1 bound to PI3P+ or PI4P+ nanotubes (**revised Fig. S2F-H**).

Finally, in accordance with these results and as suggested by Reviewer #1, we have repeated the GUV experiments shown in the original **Fig 2D**, in which EPC GUVs doped with PI4P bound recombinant BLOC-1, but now including control reactions in which EPC GUVs doped with PI3P or lacking any negatively charged phospholipid were incubated with BLOC-1. Using fluorescence microscopy, we showed that the addition of BLOC-1 generated tubules from PI3P+ GUVs but not GUVs lacking a negatively charged phospholipid (**revised Fig. S2J**, bottom and top panels, respectively). These results are consistent with experiments using GalCer lipid nanotubes that demonstrate the absence of binding of BLOC-1 on membranes containing EPC alone (**Revised Fig. S2D-E**).

Collectively, the results show that BLOC-1 binds and tubulates GUVs when doped with PI3P or PI4P. The corresponding text has been modified on **p8-10**.

3. The remaining manuscript, the authors conclude that PI4P generation and depletion changes tubule formation and thus blocks function of BLOC-1. But these events are not linked here. For instance in Figure 3C, it BLOC-1 then also clustering with KIF13A or is it then cytosolic? Similarly, what happens if the SAC phosphatase is recruited (Figure 4)? Is BLOC-1 then falling off the endosome?

In **Fig. 3C**, we observed in cells co-depleted of the two PI4KII that KIF13A-YFP distributed as large vesicular structures, positive for mCherry-RAB11 (see **Fig. S3A**), rather than the elongated tubules captured in si-Control cells. Of note, similar KIF13A-YFP vesicular structures were observed co-distributing with iRFP-RAB5 in cells for which the PJ-SAC was recruited to early endosomes (**Fig. 4B**). Therefore, these vesicular structures most likely correspond to early endosomal membranes. Whether BLOC-1 co-distributes with KIF13A-YFP on these vesicular structures cannot be tested for the technical limitations previously mentioned.

However, we now provide additional biochemical information regarding the BLOC-1 binding to whole cellular membranes in cells depleted of PI4KIIα and PI4KIIβ. In **revised Figure S3C** (and **p11, 34-35**, and **58**), we use biochemical fractionation of homogenates of HeLa cells treated with control siRNAs or co-depleted of PI4KIIα and PI4KIIβ to generate membrane and cytosolic fractions, and then assessed the distribution of BLOC-1 among these fractions by immunoblotting with an antibody to the pallidin subunit; antibodies to a resident membrane protein of the endoplasmic reticulum (calnexin) and to actin served as controls to validate the integrity of the membrane and cytosolic fractions, respectively. The new data show that BLOC-1 is equally distributed among membrane and cytosolic fractions in PI4KII-depleted cells as in controls. Of note, similar experiments described in the companion paper by Zhu et al. and performed in mouse melanocytes depleted of either PI4KIIα or PI4KIIβ showed that the binding of BLOC-1 subunits to whole cell membranes was also not significantly impaired by loss of either PI4KII variant alone. Therefore, our additional data in HeLa cells are consistent with those in pigment cells. Note that these data do not address whether the membranes to which BLOC-1 associates are the endosomal membrane tubules.

We decided not to perform a similar experiment in cells expressing the Sac system for technical reasons. To biochemically exploit this system, cells should be co-transfected with plasmids encoding the FKBP-PJ-Sac and the iRFP-FRB-RAB5, and treated with rapamycin to induce the recruitment of PJ-Sac to early endosomes. We reasoned that the fraction of transfected cells expressing both plasmids and responding to rapamycin might be too low to allow for a solid interpretation of the results.

We hope that our approach and new data, in addition to our explanation and the results presented in pigment cells by Zhu et al will satisfy the reviewer.

Zhu, Y., S. Li, A. Jaume, R.A. Jani, C. Delevoye, G, Raposo and M.S. Marks. 2022. Type II phosphatidylinositol 4-kinases function sequentially in cargo delivery from early endosomes to melanosomes. *J. Cell Biol.* in press. doi: 10.1083/jcb.202110114.

4. The authors ignore all knowledge on retromer and SNX proteins in endosomal recycling. I am aware that these have been mainly linked to PI3P, but it would make their data stronger if they would show that the observed recycling mechanism they observe here is independent of retromer and SNX proteins, but linked to BLOC-1.

In our previous 2016 study (Delevoye et al., 2016), we showed in Supplementary Figure 4 that the KIF13A-YFP-dependent tubulation of recycling endosomes in HeLa cells did not involve retromer. Specifically: (i) no overlap was detected between KIF13A-YFP and the endogenous VPS35 subunit of retromer by immunofluorescence microscopy; and (ii) depletion of VPS35 using siRNA did not affect endosomal tubulation driven by KIF13A-YFP. These data together indicate that the recycling mechanism under study in this paper is independent of the retromer. Consistently, in mouse melanocytes, the EGFP-labeled VPS29 retromer subunit is observed on short tubules emanating from early endosomes but not on the long STX13-labeled recycling endosomal tubules that contact melanosomes (M. Dennis and M.S. Marks, unpublished data).

To further satisfy the reviewer, we provide additional data by using siRNA depletion of VPS35 (i.e., retromer) or of both sorting nexins 1 and -2 (SNX1+2) in HeLa cells expressing KIF13A-YFP. We decided to test the potential role of the SNX1/2 in addition to retromer in the KIF13A-YFP-dependent tubulation process because these sorting nexins are known membrane remodeling factors that bear a PX domain with preferential affinity for PI3P, which promotes their recruitment to endosomal membranes. We quantified the number of KIF13A-YFP-positive cells in depleted cells or controls observed by live cell imaging to generate at least one recycling tubule, as previously done in **Figs. 3D, 3H** and **S3E**. The result showed that the depletion of either VPS35 or SNX1/2 did not affect the number of KIF13A-YFP-expressing cells generating at least one recycling tubule (**revised Fig. S3G-H**). Together with the data presented in **Fig. S3D-E**, which shows that treatment with the PI3K inhibitor wortmannin did not alter KIF13A-YFP tubule generation, we conclude that the formation of KIF13A-dependent recycling tubules did not rely on PI3P or PI3P-binding proteins (see revised text **p12, 27-28,** and **59**).

Delevoye, C., X. Heiligenstein, L. Ripoll, F. Gilles-Marsens, M.K. Dennis, R.A. Linares, L. Derman, A. Gokhale, E. Morel, V. Faundez, M.S. Marks, and G. Raposo. 2016. BLOC-1 Brings Together the Actin and Microtubule Cytoskeletons to Generate Recycling Endosomes. *Curr. Biol. CB*. 26:1–13. doi:10.1016/j.cub.2015.11.020.

5. The discussion and much of the text reads as if all PI4P dependent events are exclusively linked to BLOC-1 function. The authors need to be much more cautious in their writing. For instance, they did not show that BLOC-1 and PI4P are required for influenza virus proliferation or chlamydia proliferation. Their data shows that PI4KII depletion blocks this process and then conclude that this is due to a loss of BLOC-1 - which was not shown here.

We have considered the comment of the reviewer and have revised the text accordingly (see text **p2, 5-6, 15-16,** and **21-22**).

Overall, we hope that the provided explanations and the additional experiments and data are sufficient to resolve the different points raised by the reviewer.

August 15, 2022

RE: JCB Manuscript #202110132R

Dr. Cedric Delevoye
Institut Curie, CNRS-UMR144
Cell Biology and Cancer
26 Rue d'Ulm
Paris 75005
France

Dear Dr. Delevoye:

Thank you for submitting your revised manuscript entitled "PI4P and BLOC-1 remodel endosomal membranes into tubules". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision please also carefully address all of the text edits requested by the reviewers.

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.

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

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: * Scale bars must be present on all microscopy images, including inset magnifications (you may alternatively indicate the diameter of the inset). 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.

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. *

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
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 figures, therefore please consider moving or combining some of your SI data and be sure to correct the callouts in the text to reflect any changes. 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.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

**The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to

publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.**

Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit https://rupress.org/jcb/pages/submission-guidelines#videoSummaries.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

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,

Harald Stenmark, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

SHORT SUMMARY OF THE MANUSCRIPT AND THE ADVANCE OFFERED TO THE FIELD
As described in my previous review, the authors show for the first time that BLOC-1 can bind and tubulate PIP-containing membranes and that simultaneous knockdown of PI4KIIalpha and PI4KIIbeta reduces the number, stability and function of endosomal tubules in HeLa cells and affects the life cycle of two intracellular pathogens. I feel the authors have done a nice job of addressing most of the reviewers' previous comments. However, there are a couple of sentences in the text (on pp 2 and 19) that are inconsistent with what they actually showed. These and other minor issues in the text and figures are listed below.

MAIN POINTS THAT REQUIRE ADDITIONAL SUPPORT
There are no main points that require additional support.

ADDITIONAL ISSUES THAT NEED TO BE ADDRESSED
General comments on the writing and figures:
1. Rather than using "PI4KII" (referring to PI4KIIalpha and beta) as a plural noun coupled with "are" (p. 5, last line, and elsewhere), it would be less awkward to use "PI4KIIs are" throughout.
2. Consider converting two-color images (currently shown in red and green) to magenta and green so the merged images will be accessible to colorblind readers.
3. For graphs with many individual datapoints (Figs 3E, 5C-D, 6E-F, S4B-D, S6G-H), the asterisks indicating statistical significance are not obvious at first glance. Making these asterisks either significantly larger or a different color (for example, red) would make them stand out.

Specific comments:
p. 2, line 8 of Abstract: Since the authors were unable to show that BLOC-1 binds tubules in cells, it is an overstatement to say "Endosomal PI4P production by type II PI4-kinases is needed to form nascent tubules through binding of BLOC-1...". The words "binding of" should be deleted.
p. 3, line 2: replace "membrane" with "membranes"
p. 4, 2nd paragraph, line 5: replace "regulate" with "regulates"
p. 5, line 3: replace "expression" with "overexpression"
p. 5, line 5, replace "stabilize" with "stabilizing"
p. 5, 2nd paragraph, line 6: delete "thus"
p. 5, 3rd line from bottom: change "remodel" and "stabilize" to "remodeling" and "stabilizing"
p. 7, 6th line from bottom: It is confusing to refer to this sensor as SidC-GFP, as this implies that the whole SidC protein is being used, but in reality it is just a short peptide. This could be addressed by inserting "derived from SidC" after "sensor" and putting

"SidC-GPF" in parentheses, preferably with a superscript indicating the amino acids that are present (609-776).
p. 7, lines 3-4 from bottom: For consistency, it would help to include the information following "TGN" through the end of the sentence in square brackets, as was done for "[here stained...]." In the next two lines
p. 9, 2nd paragraph, line 4: "GalCer/EggPC" needs to be defined here in case the reader is not familiar with what this is.
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p. 16, 6th line from bottom: delete "Marta"
p. 18, lines 7-15: It would help to number the different steps of the working model to correspond to the labels in Fig. 6G. For example, "1)" could be inserted after "we propose that" (line 7), and numbers 2) to 5) could be inserted before the descriptions of the corresponding steps in this section, as appropriate.
p. 19, line 4: replace "to which" with "where"
p. 19, lines 6-7: The data in the graph in Fig. S1A is not consistent with the statement, "the PI4P+/KIF13+ RE tubules are apposed to PI3P+/RAB5+ sorting endosomes". The distances vary, but the average is around 2 microns, which does not suggest apposition, which suggests two things are touching. This phrase should be deleted.
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p. 21, 7th line from bottom: replace "in" with "during"
p. 26, 1st line: change to "by recombination using" (delete "by")
p. 58, line 5: replace "fluorescent" with "fluorescence"
p. 58, lines 6-7 from bottom: delete "of the three fractions" (there are only two fractions; the other samples are unfractionated)
Fig. 3H: change "tubules" to "tubule" in the Y-axis label

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

The authors addressed all my concerns. I accept that visualization of BLOC-1 does not yet work in vivo, and understand that multiple experiments have demonstrated BLOC-1 function in tubule formation. The work is overall much improved and now contains the necessary controls for their in vitro analysis. I assume that some language issues will be resolved during type setting (e.g. "swollen" instead of "swelled"). It is an exciting step forward to see that BLOC-1 is now also analyzed in functional assays.

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PI4KII was replaced by PI4KIIs when appropriate.

2. Consider converting two-color images (currently shown in red and green) to magenta and green so the merged images will be accessible to colorblind readers.

The red color images have been converted to magenta in Fig. 1/ S1/ S3, as proposed by the reviewer.

3. For graphs with many individual datapoints (Figs 3E, 5C-D, 6E-F, S4B-D, S6G-H), the asterisks indicating statistical significance are not obvious at first glance. Making these asterisks either significantly larger or a different color (for example, red) would make them stand out.

The size of the asterisks has been increased.

Specific comments:

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The abstract has been slightly modified to take into account the comment of the reviewer and to better illustrate the in vitro data.

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Fig. 3H: change "tubules" to "tubule" in the Y-axis label.
All proposed modifications have been corrected.

We sincerely thank the reviewer for the careful inspection of the revised manuscript.

Reviewer #2 (Comments to the Authors (Required)):
The authors addressed all my concerns. I accept that visualization of BLOC-1 does not yet work in vivo, and understand that multiple experiments have demonstrated BLOC-1 function in tubule formation. The work is overall much improved and now contains the necessary controls for their in vitro analysis. I assume that some language issues will be resolved during type setting (e.g. "swollen" instead of "swelled"). It is an exciting step forward to see that BLOC-1 is now also analyzed in functional assays.
We greatly appreciate the reviewer's detailed inspection of the manuscript. Several language issues have been corrected in the revised manuscript.