JCB

Arf1-PI4KIIIβ positive vesicles regulate PI(3)P signaling to facilitate lysosomal tubule fission

Maxime Boutry, Laura DiGiovanni, Nicholas Demers, Aaron Fountain, Sami Mamand, Roberto Botelho, and Peter Kim

Corresponding Author(s): Peter Kim, Hospital for Sick Children

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Re: JCB manuscript #202205128

Prof. Peter K Kim
Hospital for Sick Children
686 Bay St
Rm 19.9708
Toronto, Ontario M5G 1X8
Canada

Dear Prof. Kim,

Thank you for submitting your manuscript entitled "Golgi-derived vesicles regulate phosphatidylinositol-3-phosphate signalling to facilitate the fission of lysosomal tubules" to Journal of Cell Biology. Thank you also for your patience with the peer review process. 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.

You will see that while the reviewers find the premise of your work intriguing, they express varying degrees of enthusiasm regarding the suitability of the study for JCB in its current form. Reviewer #1 feels that the data does not support the conclusion that the non-lysosomal organelles are Golgi-derived due to reliance on overexpressed fluorescently tagged LAMP1 & Arf1 as the only lysosomal and Golgi markers. This reviewer also raises significant concerns regarding the usage of RAB7 to anchor proteins to lysosomes since RAB7 is typically localized at maturing late endosomes, overinterpretation of results and incomplete method descriptions, lack of important controls and quantifications. Reviewer #3 notes that it is not clear from the data whether Golgi-derived vesicles are actively enriched at sites of lysosomal tubule fission or that Golgi-derived PI4KIIIβ generates the PI(4)P that is required for tubule fission. Most importantly, both Reviewers #1 & 3 state that while SEC14L2 appears to be required for tubule fission, significantly more mechanistic insight into this process is necessary.

Although your manuscript is intriguing, we 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. Our journal office can transfer your reviewer comments to another journal upon request.

Given interest in the topic, we would be open to resubmission to JCB of a very significantly revised and extended manuscript that fully addresses the reviewers' concerns and would be subject to further peer-review. If you are interested in further consideration of this work at JCB we ask that you first provide us with a detailed revision plan that addresses all of the reviewer concerns since this will require a considerable amount of new experimental work on your part. It would be crucial to explain how you will obtain positive insight into the mechanism of SEC14L2 function and also important to fully address all of the other comments.

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. Of course, this decision does not imply any lack of interest in your work and we look forward to future submissions from your lab. 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
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

In this manuscript, Boutry et al suggest that phosphatidylinositol-4-phosphate (PtdIns4P) on Golgi-derived vesicles participates in the scission of tubules that emerge from lysosomes and phagolysosomes during lysosome reformation. Once lysosomes have

exhausted their hydrolytic contents following fusion with late endosomes, autophagosomes, or phagosomes, the release of membrane tubules plays an important role in maintaining the pool of functional lysosomes by recycling membrane components to reform competent lysosomes. Based on fluorescence microscopy experiments using overexpressed LAMP1 fluorescent fusion proteins to mark lysosomes and Arf1-GFP to mark the Golgi in several cell systems, Boutry et al implicate Golgi-derived vesicles in the scission of these tubules from lysosomes. Tubule formation is induced in several following induction of engorged endolysosomes, autolysosomes, or phagocytosed particles, and then visualized by live cell imaging. In such experiments, Arf1-GFP is frequently observed at sites of fission of LAMP1-containing tubules emerging from larger LAMP1-containing structures, defined here as lysosomes. Combining quantitative analysis of live cell imaging with a series of clever pharmacological treatments, siRNA knockdowns, labeling with fluorescent fusion protein probes for PtdIns4P and PtdIns3P, and inducible treatments with targeted PtdIns phosphatases and kinases, the authors propose a model in which PtdIns4P derived from PI4KIIIbeta (PI4KIIIb) on non-lysosomal sources is necessary for fission, perhaps to generate Golgi-derived vesicles, and that a Golgi vesicle-localized pool of PtdIns3P that is transferred to lysosomes by SEC14L2 also participates in fission.

The manuscript contributes new data that, in principle, extend the field in several ways. First, they provide additional details regarding the molecular mechanisms underlying the reformation of lysosomes, a critical physiological process in all cells that may be impaired in some heritable disorders or disrupted by infectious agents. Second, the data unequivocally support a role for non-lysosomal membranes in facilitating the fission event, which has been heretofore poorly characterized. Third, the manuscript corrects the literature in documenting that PtdIns4P and the PtdIns-4-kinase, PI4KIIIbeta (PI4KIIIb), that generates it play a positive role, rather than a negative one as previously proposed, in promoting fission of at least some of the tubules that emerge from endolysosomal organelles. It also implicates a phosphoinositide transfer protein, SEC14L2, in this process (although these data are not as strong). With some caveats discussed further below, these points are supported by an extensive body of high quality, quantitative data. Thus, a suitably revised manuscript would be of interest to readers of the JCB.

However, there are several major concerns with the way that the data are presented and interpreted. While the paper clearly documents contacts with non-lysosomal organelles in facilitating the fission of LAMP1-containing tubules from larger LAMP1-containing structures, the data in the paper do not prove that these non-lysosomal organelles are Golgi-derived, and quite frankly, it seems very unlikely that they are (see more below). Moreover, while in some cases (e.g. for phagolysosomes) the source of the LAMP1-containing tubules is clear, the extensive use of only overexpressed LAMP1 fluorescent fusion proteins to define lysosomes and the absence of other labels to validate the source organelle raises concerns that many of the data (e.g. in Figure 6) reflect tubule formation from non-lysosomal organelles such as early and late endosomes rather than from spent lysosomes.

These concerns are compounded by a pervasive problem throughout the manuscript in which results are reported as the interpretation of the data rather than the actual content of the data themselves. This appears to reflect the assumption that the methods and reagents used are more specific than they are. For example, in all of the experiments, structures labeled by an overexpressed GFP- or mCherry-tagged LAMP1 are interpreted to be lysosomes; however, it is well known that overexpressed LAMP1 labels structures throughout the endocytic system, and even endogenous LAMP1 localizes in part to late endosomes and to transport carriers en route to late endosomes and lysosomes. Similarly, the Results section never even mentions that the FRB fusion partner used to inducibly localize FKBP fusion proteins to "lysosomes" is RAB7, which targets to maturing late endosomes and not lysosomes in the MEFs and HeLa cells used in the manuscript (not to mention the confusion introduced into these experiments of using rapamycin as the dimerizing agent in a system where rapamycin-induced autophagy influences the results). Even more troubling is the use of Arf1-GFP as a "marker" of Golgi-derived vesicles. While it is true that the predominant accumulation of Arf1-GFP in cells is at the Golgi and few readers would question the identity of the massive pericentriolar pool of Arf1, in the periphery there is a substantial pool of endosomal Arf1-GFP that functions in the recruitment of AP-1, AP-3, COPI, and perhaps AP-4 to endosomes; thus, the peripheral punctate structures labeled by Arf1-GFP are not necessarily Golgi-derived and, frankly more likely, may be endosomal. The authors add one modestly quantified supplemental figure in which additional markers (overexpressed fluorescent fusion proteins to TGN46 and PI4KIIIb) are also used to mark LAMP1-mCherry fission sites, but these components also cycle through the endocytic pathway. This lack of specificity in terminology and overinterpretation/ over-presentation of the data detracts largely from an otherwise very interesting and data-filled study that generates (but does not absolutely prove, as the authors suggest) an interesting model in the control of lysosomal tubulation. In order to be a valuable contribution, the hyperbole must be toned down, the data and methods used to obtain them reported accurately and completely, and the nature of the Arf1-labeled structures better defined.

In addition to these overarching concerns, there are a number of additional specific concerns over the interpretation of specific data, validation of the structures shown as lysosomes, incompletely described methods of quantification, experiments that lack controls, and additional lack of clarity in the presentation that are detailed below. Additionally, the data implicating phosphoinositide exchange by SEC14L2 are preliminary and need to be bolstered by additional data.

Detailed concerns:

1. The conclusion from Figure 1 is highly premature.

a. As described above, the use of Arf1-GFP as a TGN/ Golgi marker is highly faulted, and the punctate labeling that abuts LAMP1-mCherry labeling may in fact represent Arf1 present on endosomal structures rather than Golgi-derived structures (in

fact, at this point in the manuscript - where the data with TGN46 or PI4KIIIb have not been shown - it could simply represent Arf1-GFP recruitment to late endosomes). Reference to supplementary data shown later and many additional controls will be necessary to conclude whether this reflects Golgi-derived material or endosomes. In addition to the data currently shown in Suppl. Fig. 2 for TGN46-mEmerald and GFP-PI4KIIIb labeling, TGN content that is less prone to recycling (such as galactosyl transferase, sialyl transferase, or sulfotransferase) should be tested, and negative controls should be included, such as RAB5, RAB11, and/or internalized Transferrin for early endosomes; these might label the early endosomal compartments from which Arf1 recruits AP-1 and AP-3 and through which TGN46 (and likely PI4KIIIb) cycle, and this reviewer suspects that they will identify the Arf1-labeled structures as early endosomes.

b. In addition, given that the cells are transiently transfected and thus likely overexpress the transgenes, it does not seem possible to define the LAMP1-mCherry-labeled structures as late endosomes/ lysosomes. Do the LAMP1-mCherry-labeled structures also label for internalized and chased dextran, DQ-BSA, or Magic Red, or at least Lysotracker?

2. Fission of LAMP1-mCherry containing structures is not obvious in Suppl. Movie 2 - it is more apparent in Suppl. Movie 3. Movie legends were not provided to the reviewers.

3. The conclusions drawn from Figure 2 suffer from the same caveats as for Figure 1 (point #1).

a. How are the LAMP1-mCherry-labeled structures defined as "autolysosomes"? Simply labeling with LAMP1-mCherry after starvation is not sufficient. Are these any different from the "endolysosomes" described in Figure 1? Do they contain autophagic materials such as degrading mitochondrial or peroxisomal proteins or lipids? Or an acid-resistant LC3b-fusion protein (such as LC3b-mCherry; not LC3b-EGFP)?

b. In panel b the term "expected percentage of Arf1-GFP at fission if due to chance" in the quantification is an interpretation of the data, not a description - it should be labeled for what it is, i.e. the percentage of LAMP1-mCherry labeled structures that also label for Arf1-GFP. Is this number derived from Figure 1 or from the starved cells used in this experiment?

c. The data on sucrosomes in Figure 2d and e are nice, but what is the percentage of resolving sucrosomes that co-label for Arf1-GFP? Is it similar to the "autolysosomes" and plain LAMP1-mCherry labeled structures in Fig. 1 and Fig. 2a-c? Same question for the phagolysosomes in Fig. 2g-h.

4. Fig. 3 contains a panel showing the absence of GFP-Rab5 from a single fission event, but (a) this needs to be done in the other systems and not just in YM201636 washout cells and (b) it needs to be quantified; 45-50% of the structures in cells expressing Arf1-GFP would look the same as this. In addition, some discussion should be devoted to the potential impact of YM201636 washout on PtdIns3P levels on lysosomes and how that might impact tubule fission.

5. The data in Sup. Fig. 3b and c that YM201636 treatment did not induce autophagy and that the LAMP1-mCherry structures in these cells are not LC3-containing autolyosomes are weak. In the blot in panel b, there is a clear increase in autophagic flux after YM treatment (and surprisingly not after rapamycin treatment - did the rapamycin actually work in this experiment?). LC3-I levels seem to be high in the YM-treated cells, and so it is possible that LC3 expression was also increased. If this is not representative, then a more representative blot should be shown. In panel c, GFP is a poor fusion partner for LC3 since it is sensitive to pH, and thus might be quenched in active autolysosomes. The authors should consider repeating the experiment using either LAMP1-EGFP and mCherry-LC3 or labeling for GFP-LC3 in the presence of bafilomycin A1. The appropriate experiment here should be done and quantified.

6. The experiment shown in Fig. 3g, h is described in the text as documenting autolysosome tubule fission, but there is no description in the figure legend of autophagy induction in this experiment; were these cells starved for 8 h prior to analysis? In Figure 3k-l, the legend indicates no time scale for image acquisition after SRBC phagocytosis. The length of treatments with various drugs is not indicated in the figure legend. In addition, it would be very helpful to indicate what is being analyzed (e.g. sucrosome, phagosome, autolysosome) on the figure itself without having to refer to the legend. Note, while the data are consistent with the conclusion drawn, they do not provide proof that Golgi-derived vesicles, as opposed to some process dependent on the intact functional Golgi or BFA-sensitive recruitment of Arf1 to endosomes, are the agents that support lysosomal tubule scission.

7. In Fig. 4g, h, how do tubule numbers in the cells expressing active or mutant Sac1 fusion protein compare in cells that have not been transfected with any LysoGFP-Sac1 construct? Is it the same as in the catalytically inactive transductants? Also, it is intriguing that the LysoGFP-Sac1 construct is more effective in reducing tubule formation (Fig. 4g, h; Suppl. Fig. 6e-h) than the GFP-ORPSAC1 construct (Suppl. Fig. 6c, d). Might this reflect the limited distribution of ORP1L to ER-lysosome contacts, and if so, how would the lower impact of GRP-ORPSAC1 be interpreted in the context of the ER contacts at the fission site shown in Suppl. Fig. 2C?

8. The data and interpretation of Figure 5 raise some concerns.

a. In Fig. 5a,b and Suppl. Fig. 7a, b, the conclusion that SEC14L2 localizes to Golgi-derived vesicles is premature. Again, the

authors use Arf1 instead of a more specific Golgi/ TGN marker to define Golgi, invalidating the conclusion. This is also a problem in concluding that "formation of Golgi vesicles is unaffected" in panels c and d; what is measured is the number of Arf1-containing puncta, only some of which would be expected to be Golgi-derived. Moreover, in panels a and b there is no quantification of the overlap. These conclusions should be softened or bolstered by additional data using Golgi/TGN-specific contents. The same problem applies to the interpretation of data in Fig. 7e and f and Fig. 6f that VPS34-IN1 does not block Golgi vesicle formation (I suspect this is true but the data do not show this) but increases "Golgi vesicle" contacts with lysosomes. Again, the graph should state what the data are, not their overinterpretation.

b. The impact of knocking down SEC14L2 expression in Figure 5 is clear and the quantification of the rescue experiment in panel f is nice, but these data alone (or in combination with the data in Figure 6) do not prove that SEC14L2 lipid transfer activity is required for LAMP1 tubule formation. Lipid binding residues in SEC14L2 have been predicted and tested for function (Gong et al., Nat. Cell Biol. 23: 782), and others could be predicted based on homology to the well-studied yeast Sec14. To affirm that SEC14L2 lipid transfer activity is required, it should be tested whether lipid binding mutants are able to rescue the SEC14L2 knockdown as in Figure 5f.

c. In Fig. 5i and j, remind us in the text of how the rate of lysosomal fission was measured. In Fig. 5k and l, how are "enlarged LAMP1 positive structures" defined? Were the structures measured? How? Arrows in the figures would help here, but some objective definition of what was measured needs to be provided.

d. Finally, in Fig. 5m, n, the observation of GFP signal from LC3-GFP associated with LAMP1-positive structures in SEC14L2-depleted cells suggests either that these compartments are not acidic - and perhaps not fused to lysosomes or de-acidified. How do the authors explain their presence in SEC14L2-depleted cells despite the conclusion that autophagy is not affected by tubule impairment? Does SEC14L2 play another role in this process besides tubule severing?

9. The approach to quantify labeling of "lysosomes" by PX-GFP in Fig. 6a, b is confusing. Most PtdIns(3)P in cells accumulates on early endosomal vacuoles, not on lysosomes (or the Golgi - see below). Is this again a problem of using overexpressed LAMP1-mCherry as way to mark "lysosomes", or was the lysosomal signal a small proportion of the punctate structures observed? It is confusing that the quantification was of signal overlapping lysosomes relative to cytosolic PX-GFP as opposed to early endosomal GFP, where most would be expected to accumulate. This concern extends into Fig. 6c-e, as the appearance of the enlarged LAMP1-mCherry-containing structures in the VPS34-IN1-treated cells in 6c look more like the enlarged early endosomal vacuoles that are well known to accumulate in cells with impaired PtdIns-3-kinase activity. Are these lysosomes or early endosomes? They should be labeled by cargo (e.g. chased dextran) and not just by LAMP1-mCherry.

10. I find a great deal about the data presentation, conclusions, and documentation for Fig. 6 to be very frustrating.

a. The quantification data in Fig. 6 are presented with axes labeling the data interpretation rather than what was actually measured. This must be remedied. For example, the y-axis in panels b and d should read something like "PX-GFP overlapping with LAMP1-mCherry relative to cytosolic PX-GFP" and the y-axis in panel f should read "% of LAMP1-mCherry structures contacting Arf1-GFP structures".

b. Panel g (and the text of the Results - incredibly frustrating that the information is not there) should indicate that the cells are expressing RAB7-FRB - note this should target the FKBP fusions to late endosomes, not lysosomes, and thus the conclusions drawn need to be reconsidered.

c. It is not clear why in panel g the localization of the mRFP or mCherry fusion is shown, whereas the quantification in h documents PX-GFP association with lysosomes, which is not shown at all. How do the authors explain the accumulation of the FKBP-MTM1 fusion in aggregates that do not localize to the LAMP1-containing structures? Does this not alter the interpretation of the result and suggest that the fusion protein is not actually removing PtdIns3P from lysosomes per se?

d. In the experiments of 6j-l, it is not stated in the figure legend or the text how long the cells were treated with rapamycin - was this a brief treatment to induce FRB-FKBP interaction or was this long-term treatment to activate autophagy as in Fig. 2b, c? Because of this complication, this experiment would be much more clear with a non-functional rapalog dimerization agent.

e. The effect of the MTM1 recruitment to phagolysosomes has an extraordinarily modest effect on tubule number, and one wonders whether the statistical significance was assessed correctly. Was the ANOVA test done using each measured cell as a replicate, or was it done properly in which the mean from each time the experiment was done was used as a replicate (see Lord et al., J. Cell Biol. 219: e202001064)?

f. The use of Arf1 as a fusion partner to target MTM1 to the TGN is inappropriate, as discussed at length above. The data in no way suggest that PtdIns3P on the Golgi plays any role in the lysosomal tubulation process, and again in fact suggests that the Arf1 labeling observed in the periphery is largely on early endosomes rather than Golgi-derived vesicles. To test this more directly, why not anchor MTM1 to the TGN with an FRB fusion to galactyosyl transferase, sulfotransferase, or sialyl transferase?

11. The discussion lacks any reference to any of the caveats raised in this review regarding data interpretation, and starts with

assuming that their model is correct. This must be amended.

12. The summary cartoon in Figure 7 refers to PI(4)P being involved in tubule formation, which was not at all discussed in this manuscript, but omits the requirement shown in the manuscript that PI(4)P is required for tubule scission (by their interpretation, via conversion to PtdIns3P). The cartoon should be amended to focus on the main point of the paper.

Minor concerns.

13. General - as different cell types are used in different experiments, it would be helpful to have the cell type used indicated on the figures themselves.

14. The rationale for documenting the long-term association of Arf1-GFP-labeled structures with LAMP1-mCherry-containing structures in Figure 1 is not clear, given that the rest of the study focuses on the rapid association of Arf1 with tubules in the process of severing.

15. Regarding the quantification of the rate of lysosomal tubule fission, the authors should clarify how they define the fission (e.g. fission occurs at the neck or the middle of the tubule that is associate with a lysosome, or at a tubular structure).

16. In Suppl. Fig. 4e, it would be helpful to indicate the outline of the cells.

17. The quantification in Supp. Fig. 5c is unclear and needs to be clarified. Were 345 lysosomes counted in each sample (vehicle- and BFA-treated), and among those, the indicated number of tubulation and vesiculation events were counted? Over what time frame? This might be more clearly presented as a percentage of total lysosomes.

18. In the text describing Fig. 3g, h (lines 170-172), the authors should briefly indicate how the rate of tubule fission was quantified. The figure legend here is not very clear - were these manually counted over a given area? What area?

19. In Figure 4e, it is not clear what is actually being quantified and how the experiment was done. Were both LAMP1-GFP and LysoGFP-PI4KB cotransduced with LAMP1-mCherry, and the number of LAMP1-mCherry tubules quantified? Or something else? How did the number of

20. In Fig. 4h and Suppl. Fig. 6j and l, the blue and green colors are difficult to distinguish.

21. In Suppl. Fig. 6j stats should be shown between all 0, 4, and 8 h groupings.

22. Quantification of fission rate is needed for Figure 6c, l, k, and o.

23. In general, arrows and arrowheads should be used liberally to point to structures of interest in the fluorescence micrographs, particularly to illustrate examples of tubules or of marker overlap. One particular site of importance for the latter is Suppl. Fig. 7h, in which an overlap image of the inset is not provided.

24. Line 281 - reference 38 does not mention PX-GFP as a PtdIns3P probe.

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

The manuscript by Boutry et al describes how vesicles derived from the Golgi generated by the action of PI4KB/PI4P regulate PI3P signalling to control formation and fission of tubules from lysosomes. The authors carried out extensive high resolution cell microscopy following the formation of endolysosomes, autolysosomes, and phagolysosomes.

While I am not an expert in the microscopy approaches utilised, the methods are clearly described and the conclusions with one exception appear to be well justified.

Major concerns.
1. My major concern is the PI4KB inhibitor utilised. As the authors note, PI3P plays a very important role in the processes under study. The authors utilised a non-specific PI4KB inhibitor for a variety of their tests (see Fig 3C, 3G, etc). As PIK93 equally inhibits vps34 (the main source of PI3P), PI4KB, as well as some class I PI3Ks, this complicates the analysis of these experiments. While there are knockout experiments of PI4KB, and experiments showing the critical role of PI4KB localisation, to fully prove their underlying hypothesis would require testing a more PI4KB selective inhibitor (many commercially available, see https://www.medchemexpress.com/Targets/PI4K.html as a reference point).

I would suggest the authors repeat the PI4K inhibitor experiment in Fig 3C+G with one of the more selective PI4Kb inhibitors to

validate that this also leads to increased number of lysosomal tubules and decreased rate of tubule fission.

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

The manuscript builds on the burgeoning line of evidence that Golgi-derived vesicles assist in organelle fission reactions via membrane contact sites, in conjunction with the ER. Arf1 positive vesicles are shown to make frequent (~1/5 lysosomes) and stable (> 30s) contacts, and to be present at more than half of observed tubule fission events from autolysosomes, phagolysosomes and endolysosomes. Chemical inhibition of Arf1 or its effector, PI4KIIIbeta, increases lysosomal tubules and decreases their fission rate, suggesting a role in the process. Mechanistically, the lipid product of PI4KIIIbeta is shown to be present on the tubules. Depletion of this lipid by enzymatic removal causes loss of the tubules. The effects of Arf1 or PI4KIIIbeta inhibition are phenocopied by knockdown of the PI transfer protein, SEC14L2, which is shown to localize in part to Arf1-positive vesicles. Intriguingly, SEC14L2 knockdown prevents PI3P enrichment at lysosomes (as does inhibition of VPS34), which also increase lysosomal tubules. Depletion of PI3P by a Arf1-targeted PI3P phosphatase also increases tubulation, indicating aberrant fission. The paper concludes that "Golgi-derived vesicles contribute to the fission of lysosomal tubules of a wide range of lysosomal organelles by promoting a SEC14L2 dependent PI(3)P signaling at the site of fission."

Overall, the manuscript is clearly written, the experiments are thorough and analyzed with appropriate, statistically rigorous approaches, and the data are overall clear and convincing. The linking of Golgi-dervied vesicles to lysosomal tubules formation and membrane recycling is novel and adds to to the field. That said, the mechanistic model is not clearly defined or compelling. Specific areas needing clarification to support this model include:

(1) Enrichment of Golgi-derived vesicles at fission sites. Controls are performed to show that this occurs more frequently than expected by chance for random distributions. However, given that (as shown in fig S2), the ER is present at most fission sites, and that many organelles, including lysosomes, maintain tight contact with the ER, can the authors rule out that Arf1-vesicle and lysosomal tubule co-localization is not simply driven by the co-incidence of these organelles on ER tubules?

(2) PI(4)P is required for tubule formation. Although the process is blocked by PIK93, is this because post Golgi vesicles are depleted, given the roles for this lipid in Golgi traffic? Furthermore, is the tubule associated PI(4)P really generated by PI4KIIIbeta? Type II PI4K are known to associate with endosomes and lysosomes, and there are commercially available inhibitors such as PI-273. Could that be the source of PI(4)P on tubules? The SAC targeting experiment is convincing, but does not reconcile whether this has anything to do with Arf1 vesicles or PI4KIIIbeta generated PI(4)P.

(3)Even though data in figure 5 clearly supports a requirement for SEC14L2 in tubules fission, its role in the process in not clearly defined. Figure 6 demonstrates a requirement for Vps34-derived PI(3)P in tubules fission, though the only functional data is figure 6A/B, where SEC14L2 knockdown produced a modest depletion of PI(3)P, which is certainly more subtle than the Vps34 inhibitor effect, and (ii), the ability of Arf1-MTM1 to recapitulate the effect of VPS34 inhibition or lysosomal PI(3)P depletion, though as the authors themselves state in the discussion, this could occur in trans and does not support a role for the Arf1 vesicles in tubule fission directly.

To summarize my major concerns, this paper has four main findings:
(a) Golgi-dervied vesicles are enriched at sites of lysosomal tubule fission - but as per my point 1 above, clarity is needed as to whether these are truly enriched given their co-incident localization on the ER
(b) PI(4)P is required for the tubule fission process - though it is not clearly established whether Golgi-dervied PI4KIIIbeta is responsible for this (point 2)
(c) PI(3)P signaling intrinsic to the lysosomal membrane is also required for tubule fission
(d) SEC14L2 is also required for this process, but apart from its localization to Golgi-derived vesicles (fig. 5b), its relation to (b) and (c) are not established, apart from the reference to prior precedence in the literature.

There are also a number of minor technical comments which should be addressed:

(4) Fig 1E: the mean and error not as informative as an indication of the distribution, displayed as perhaps a histogram or scatter blot.

(5) Fig 4 title: "PI(4)P has a pro-tubulation role as lysosomes"; perhaps "at lysosomes" was intended?

(6) in figure 1h, the blue and green are hard to distinguish, especially in the legend. Perhaps more contrasting hues could be used.

(7) Fig 5n: purple and black are also hard to distinguish, and in this figure panel particularly, the reviewer believes the legends have been accidentally swapped.

(8) p. 17 (methods) Refers to a Leica SP8 Lightning microscope. The reviewer believes this should be a "Lightning microscope".

Dear Drs Stenmark and Simon,

Thank you for reviewing our manuscript. We thank all the reviewers for their helpful comments. Their comments have helped us improve our manuscript. This revised manuscript has included new experiments to address the reviewers' concerns and additional ones to enhance the scope of the manuscript. All the significant changes to the manuscript are highlighted in RED font.

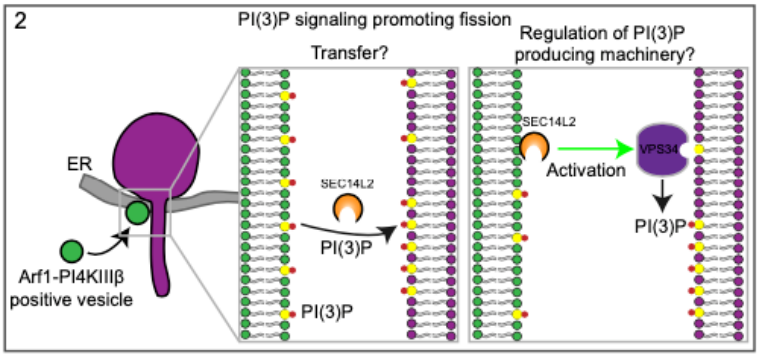
Experimentally, we have added additional experiments in the following areas:

1) We further examined the nature of the vesicles localized to the lysosomal tubule fission sites. We show that these vesicles are not endosomal vesicles. Although these vesicles show all the characteristics of vesicles previously called Golgi-derived vesicles by two recent articles (Nagashima et al. Science 2020; Gong et al. Nat Cell Biol 2021), we elected to call these vesicles Arf1-PI4KIIIβ vesicles as there is no direct evidence that these have emerged from the Golgi Apparatus.

2) One of the major concerns was the need for mechanistic insight into the role of SEC14L2 in lysosomal tubule fission. We now provide new experiments that strengthen the link between SEC14L2, PI(3)P and Arf1-PI4KIIIβ positive vesicles at lysosomal tubule fission sites. Specifically, we show that:

- Lysosomal tubules fission marked by Arf1-PI4KIIIβ positive vesicles is associated with a burst of PI(3)P on tubulating lysosomal just before fission (**Fig 6b,c**).
- Depleting PI(3)P on lysosomes, on Arf1-PI4KIIIbeta positive vesicles or in their immediate proximity prevents tubule fission (**Fig 6g-i; Fig 8j-m**).
- SEC14L2 co-localizes with a subset of Arf1-PI4KIIIβ vesicles with more improved imaging (**Fig 7a**).
- SEC14L2 depletion decreases tubule fission events (**Fig 7j,k**).
- SEC14L2's ability to bind PI(3)P is critical for tubule fission as binding mutant cannot rescue fission (**Fig 8b,c**).
- However, depleting SEC14L2 does not affect the recruitment of Arf1-PI4KIIIβ positive vesicles at Lamp1 tubule necks (where the vast majority of tubule fission events occur) suggesting that SEC14L2 is unlikely to be a tethering factor between the vesicles and the site of lysosome tubule fission (**Fig 8e**).
- Depletion of SEC14L2 decreases levels of PI(3)P at lysosomes, and these levels can be increased by expression of wild-type zSec14l3 (Zebrafish homologue of SEC14L2) but not by that of the PI(3)P binding deficient mutant M5 (**Fig 8g-i**).
- Depletion of PI(3)P at or in immediate proximity to SEC14L2 positive vesicles increase the number of lysosomal tubules, suggesting it impairs their fission (**Fig 8o,p**).

Based on these findings, we propose a new model where SEC14L2 on Arf1-PI4KIIIβ vesicles increases PI(3)P on lysosomal tubules to promote their fission. Mechanistically, we propose that SEC14L2 either transports PI(3)P from the vesicles to lysosomes; or activates VPS34 on lysosomes. These two mechanisms are not mutually exclusive.



Besides these two significant changes, we have added additional experiments to address other concerns of the reviewers, such as replacing FRB-FKBP dimerization experiments with the GAI-GID1 system to avoid the use of rapamycin and using another protein than Rab7 to recruit constructs to lysosomes.

Finally, we modified the text and legends to clarify our interpretation of our data and to avoid any impression of over-interpreting data.

Please find below our detailed point-by-point response to the reviewers' comments. Our responses are in blue font.

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

In this manuscript, Boutry et al suggest that phosphatidylinositol-4-phosphate (PtdIns4P) on Golgi-derived vesicles participates in the scission of tubules that emerge from lysosomes and phagolysosomes during lysosome reformation. Once lysosomes have exhausted their hydrolytic contents following fusion with late endosomes, autophagosomes, or phagosomes, the release of membrane tubules plays an important role in maintaining the pool of functional lysosomes by recycling membrane components to reform competent lysosomes. Based on fluorescence microscopy experiments using overexpressed LAMP1 fluorescent fusion proteins to mark lysosomes and Arf1-GFP to mark the Golgi in several cell systems, Boutry et al implicate Golgi-derived vesicles in the scission of these tubules from lysosomes. Tubule formation is induced in several following induction of engorged endolysosomes, autolysosomes, or phagocytosed particles, and then visualized by live cell imaging. In such experiments, Arf1-GFP is frequently observed at sites of fission of LAMP1-containing tubules emerging from larger LAMP1-containing structures, defined here as lysosomes. Combining quantitative analysis of live cell imaging with a series of clever pharmacological treatments, siRNA knockdowns, labeling with fluorescent fusion protein probes for PtdIns4P and PtdIns3P, and inducible treatments with targeted PtdIns phosphatases and kinases, the authors propose a model in which PtdIns4P derived from PI4KIIIbeta (PI4KIIIb) on non-lysosomal sources is necessary for fission, perhaps to generate Golgi-derived vesicles, and that a Golgi vesicle-localized pool of PtdIns3P that is transferred to lysosomes by SEC14L2 also participates in fission.

The manuscript contributes new data that, in principle, extend the field in several ways. First, they provide additional details regarding the molecular mechanisms underlying the reformation of lysosomes, a critical physiological process in all cells that may be impaired in some heritable disorders or disrupted by infectious agents. Second, the data unequivocally support a role for non-lysosomal membranes in facilitating the fission event, which has been heretofore poorly characterized. Third, the manuscript corrects the literature in documenting that PtdIns4P and the PtdIns-4-kinase, PI4KIIIbeta (PI4KIIIb), that generates it play a positive role, rather than a negative one as previously proposed, in promoting fission of at least some of the tubules that emerge from endolysosomal organelles. It also implicates a phosphoinositide transfer protein, SEC14L2, in this process (although these data are not as strong). With some caveats discussed further below, these points are supported by an extensive body of high quality, quantitative data. Thus, a suitably revised manuscript would be of interest to readers of the JCB.

However, there are several major concerns with the way that the data are presented and interpreted. While the paper clearly documents contacts with non-lysosomal organelles in facilitating the fission of LAMP1-containing tubules from larger LAMP1-containing structures, the data in the paper do not prove that these non-lysosomal organelles are Golgi-derived, and quite frankly, it seems very unlikely that they are (see more below). Moreover, while in some cases (e.g. for phagolysosomes) the source of the LAMP1-containing tubules is clear, the extensive use of only overexpressed LAMP1 fluorescent fusion proteins to define lysosomes and the absence of other labels to validate the source organelle raises concerns that many of the data (e.g. in Figure 6) reflect tubule formation from non-lysosomal organelles such as early and late endosomes rather than from spent lysosomes.

These concerns are compounded by a pervasive problem throughout the manuscript in which results are reported as the interpretation of the data rather than the actual content of the data themselves.

This appears to reflect the assumption that the methods and reagents used are more specific than they are. For example, in all of the experiments, structures labeled by an overexpressed GFP- or mCherry-tagged LAMP1 are interpreted to be lysosomes; however, it is well known that overexpressed LAMP1 labels structures throughout the endocytic system, and even endogenous LAMP1 localizes in part to late endosomes and to transport carriers en route to late endosomes and lysosomes. Similarly, the Results section never even mentions that the FRB fusion partner used to inducibly localize FKBP fusion proteins to "lysosomes" is RAB7, which targets to maturing late endosomes and not lysosomes in the MEFs and HeLa cells used in the manuscript (not to mention the confusion introduced into these experiments of using rapamycin as the dimerizing agent in a system where rapamycin-induced autophagy influences the results). Even more troubling is the use of Arf1-GFP as a "marker" of Golgi-derived vesicles. While it is true that the predominant accumulation of Arf1-GFP in cells is at the Golgi and few readers would question the identity of the massive pericentriolar pool of Arf1, in the periphery there is a substantial pool of endosomal Arf1-GFP that functions in the recruitment of AP-1, AP-3, COPI, and perhaps AP-4 to endosomes; thus, the peripheral punctate structures labeled by Arf1-GFP are not necessarily Golgi-derived and, frankly more likely, may be endosomal. The authors add one modestly quantified supplemental figure in which additional markers (overexpressed fluorescent fusion proteins to TGN46 and PI4KIIIb) are also used to mark LAMP1-mCherry fission sites, but these components also cycle through the endocytic pathway. This lack of specificity in terminology and overinterpretation/ over-presentation of the data detracts largely from an otherwise very interesting and data-filled study that generates (but does not absolutely prove, as the authors suggest) an interesting model in the control of lysosomal tubulation. In order to be a valuable contribution, the hyperbole must be toned down, the data and methods used to obtain them reported accurately and completely, and the nature of the Arf1-labeled structures better defined.

In addition to these overarching concerns, there are a number of additional specific concerns over the interpretation of specific data, validation of the structures shown as lysosomes, incompletely described methods of quantification, experiments that lack controls, and additional lack of clarity in the presentation that are detailed below. Additionally, the data implicating phosphoinositide exchange by SEC14L2 are preliminary and need to be bolstered by additional data.

We thank reviewer #1 for their thorough evaluation of our manuscript. We have taken the reviewers' critiques to significantly improve the overall quality of our study. We addressed the various concerns raised by the reviewer, notably regarding the use of Arf1 and Lamp1 as markers for the vesicles in question and lysosomes and our interpretation of the experiments involving them. Further, we also removed the experiments using the FKBP-FRB system and used instead the GAI-GID1 dimerization system, as well as used another anchor protein (not Rab7) to recruit constructs to lysosomes. Finally, new experiments (**Fig 8**) are now added to strengthen our understanding of the role of SEC14L2 in fission of lysosomal tubules. Please find below a detailed response to the concern raised by this reviewer.

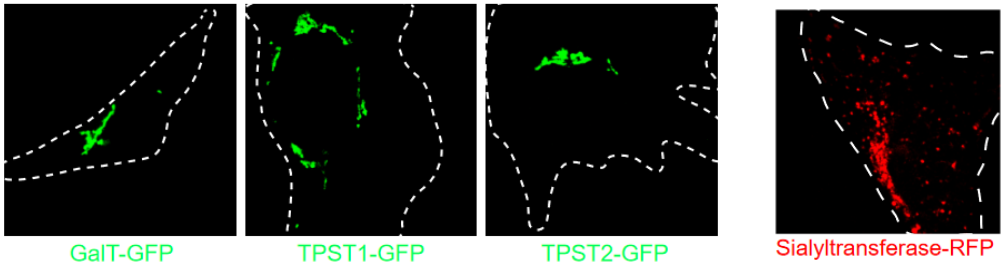
Detailed concerns:

1. The conclusion from Figure 1 is highly premature.

a. As described above, the use of Arf1-GFP as a TGN/ Golgi marker is highly faulted, and the punctate labeling that abuts LAMP1-mCherry labeling may in fact represent Arf1 present on endosomal structures rather than Golgi-derived structures (in fact, at this point in the manuscript - where the

data with TGN46 or PI4KIIIb have not been shown - it could simply represent Arf1-GFP recruitment to late endosomes). Reference to supplementary data shown later and many additional controls will be necessary to conclude whether this reflects Golgi-derived material or endosomes. In addition to the data currently shown in Suppl. Fig. 2 for TGN46-mEmerald and GFP-PI4KIIIb labeling, TGN content that is less prone to recycling (such as galactosyl transferase, sialyl transferase, or sulfotransferase) should be tested, and negative controls should be included, such as RAB5, RAB11, and/or internalized Transferrin for early endosomes; these might label the early endosomal compartments from which Arf1 recruits AP-1 and AP-3 and through which TGN46 (and likely PI4KIIIb) cycle, and this reviewer suspects that they will identify the Arf1-labeled structures as early endosomes.

We thank the reviewer for pointing out the caveat of using Arf1-GFP as a marker for Golgi vesicles. As the reviewer stated, Arf1 can localize to endosomes. To address the nature of the vesicles at the site of fission, we added additional data that further characterize these vesicles (see below). We also modified the text to acknowledge the issues with our markers. We now call these vesicles Arf1-PI4KIIIβ positive vesicles throughout the manuscript as these proteins are both found on the vesicles recruited to lysosomal tubules fission sites. Further, inactivating Arf1 or inhibition of PI4KIIIβ impairs lysosomal tubule fission. We also tested for several Golgi resident proteins as suggested by the reviewer: GalactosylTransferase (GalT-GFP), two turbosyltransferases (TPST1/2-GFP) and Sialyltransferase. We did not find GalT-GFP and TPST1/2 in vesicle-like structures. As for Sialyltransferase-RFP, it appeared on large spherical structures that resemble lysosomes, suggesting mislocalization of the construct. Identical results were found in both MEFs (shown below) and in HeLa cells (not shown). However, we elected not to show these negative results in our manuscript for space consideration.



Representative images of MEF cells overexpressing GalT-GFP, TPST1-GFP, TPST2-GFP or Sialyltransferase-RFP. Localization of GalT-GFP and TPST1/2-GFP appears to be strongly restricted to the Golgi. Sialyltransferase-RFP appears to localize to large vesicles that resemble lysosomes. The outline of cells is indicated by dotted lines.

Additionally, we performed new experiments to determine whether the Arf1 positive vesicles observed at Lamp1 and lysosomal tubule fission sites could be of endosomal nature. We, therefore, assessed the colocalization between Arf1 positive vesicles and several early endosomal markers (Rab5, EEA1 and Rab11) and of PI4KIIIβ. These results show that Arf1-GFP positive vesicles, in our hands, show a limited colocalization with early endosomes while extensively colocalizing with PI4KIIIβ (**Fig 2h,i**).

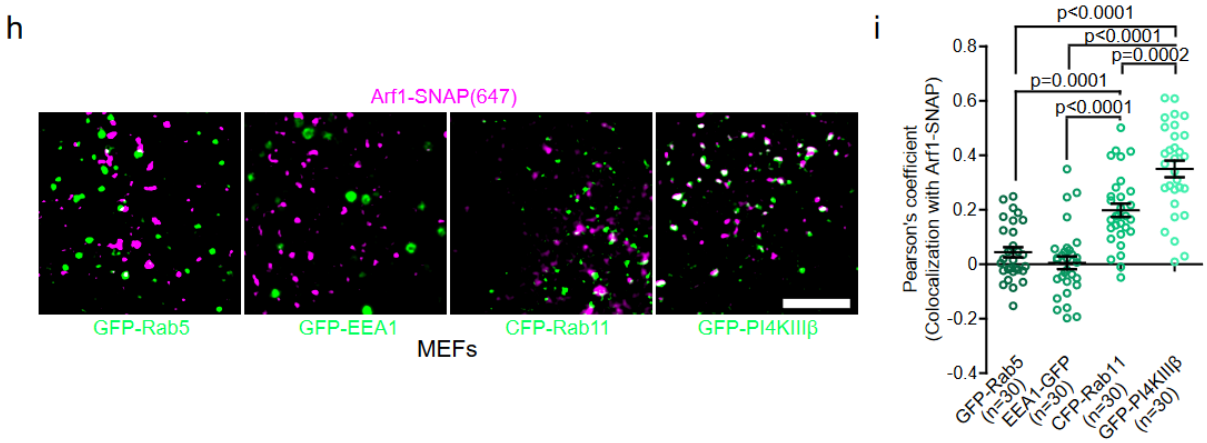
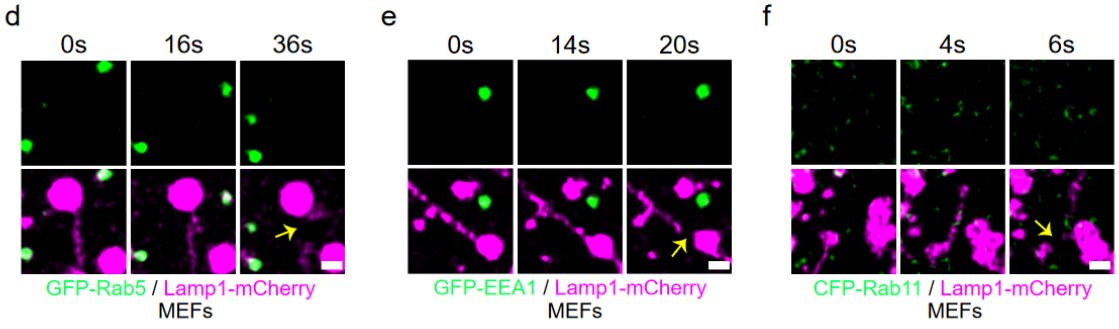


Fig 2h Representative images of a MEFs expressing Arf1-SNAP with GFP-Rab5, GFP-EEA1, CFP-Rab11 or GFP-PI4KIIIβ. Scale bar: 5µm. **i**. Pearson's coefficient measurement of cells is described in (h) as indicated. The graph shows the mean ± SEM from three independent experiments. One-way ANOVA with Dunnett's Multiple Comparison Test.

More importantly, we looked at the recruitment of these endosomal markers at the fission of Lamp1 positive tubules after prolonged HBSS treatment (to trigger the formation and fission of tubules from Autolysosomes) and did not observe any significant recruitment of these markers at the fission sites (**Fig 2d-g**). For instance, about 55% of fission events were positive for Arf1 vesicles while only 20% were for Rab5, which is similar to the 90° rotated control (in which the Lamp1 signal was rotated by 90°).



g

Lamp1 positive tubule fission

Marker	n marker positive / n events	% of events positive for marker	
GFP-Rab5 positive	19/85	22.3%	n = 29 cell
GFP-Rab5 positive 90° rotated control	16/76	21.1%	n = 29 cell
GFP-EEA1 positive	13/54	24.1%	n = 23 cell
GFP-Rab11 positive	16/55	29.1%	n = 25 cell

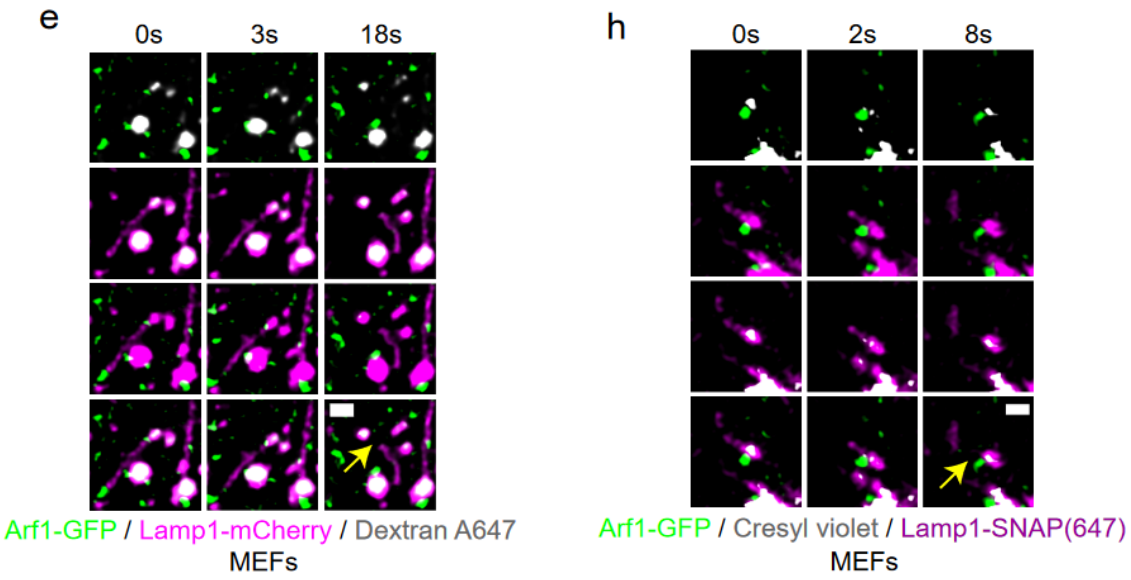
Fig 2d-f. Time-lapse images of MEFs expressing Lamp1-mCherry and (**d**) GFP-Rab5, (**e**) GFP-EEA1 or (**f**) CFP-Rab11 incubated for 8h in HBSS media. Vesicles containing these proteins are absent at a Lamp1 positive tubule fission event. Yellow arrows indicate fission. Scale bars: 1µm. **g**. Quantification of such events (**d**) n=85 events from 29 MEFs, (**e**) n=76 events from 23 MEFs and **(f)** n=55 events from 25 MEFs. Also shown is the quantification of images in (d) where lamp1 was rotated by 90°.

Taken together, analysis of these vesicles strongly suggests that the Arf1 positive vesicles recruited at Lamp1 tubules fission sites are not endosomal vesicles. However, we acknowledge that our data do not prove that these vesicles originate from the Golgi Apparatus. For this reason, we opted to call them: Arf1-PI4KIIIβ positive vesicles. We believe the full characterization of the nature of these

vesicles would require its own study. For the scope of this manuscript, where we explored their role in lysosomal organelle fission, we believe that our characterization is sufficient for the purpose of this manuscript.

b. In addition, given that the cells are transiently transfected and thus likely overexpress the transgenes, it does not seem possible to define the LAMP1-mCherry-labeled structures as late endosomes/ lysosomes. Do the LAMP1-mCherry-labeled structures also label for internalized and chased dextran, DQ-BSA, or Magic Red, or at least Lysotracker?

To confirm that the LAMP1 structures undergoing fission are lysosomes, we now show that these Lamp1 positive structures are positive for overnight chased fluorescent 10kDa dextran (**Sup Fig 1e**) and for Cresyl violet (**Fig 1h**), a dye that was shown to label acidic lysosomes (Ostrowski et al., 2016).



Sup Fig 1e. Representative time-lapse imaging showing an Arf1-GFP vesicle marking fission site of a tubule from a lysosome in a MEF cell. Lysosomes were identified as organelles positive for Lamp1 and overnight chased fluorescent 10kDa Dextran. Yellow arrow indicates fission. Scale bar = 1µm.

Fig 1h. Representative time-lapse imaging showing an Arf1-GFP vesicle marking the fission site of a tubule from a lysosome (Lamp1-SNAP/ cresyl violet positive). Yellow arrow indicates fission. Scale bar: 1µm.

Moreover, to validate that increased tubules from Lamp1 positive organelles are lysosomal tubules, we now provide images and quantification of Lamp1 tubules emerging from Lamp1 and Cresyl violet positive organelles (=Acidic Lamp1 positive organelles). (e.g: **Fig 4c,d**: PI4KIIIβ inhibition and Arf1 inactivation with BFA and **Fig 7e,f**: SEC14L2 depletion).

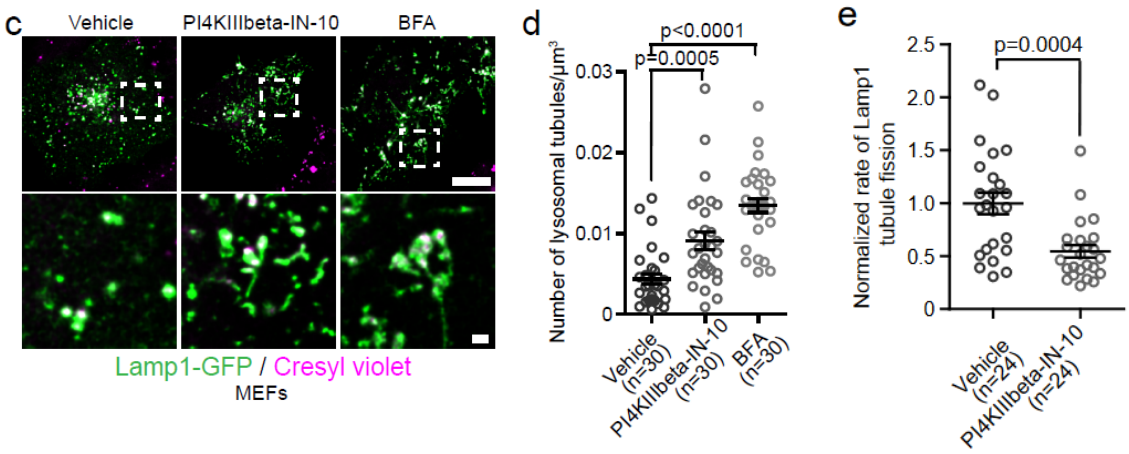


Fig4c-d. MEFs treated with the PI4KIIIβ inhibitor PI4KIIIbeta-IN-10 (25nM for 3 hrs) or Arf1 activation inhibitor Brefeldin A (BFA; 10µg/mL) stained with the acidic organelle marker cresyl violet. Scale bars: 10µm and 1µm (inset). d. Quantification of the number of lysosomal tubules in cells described in (c). One-way ANOVA with Dunnett's Multiple Comparison Test.

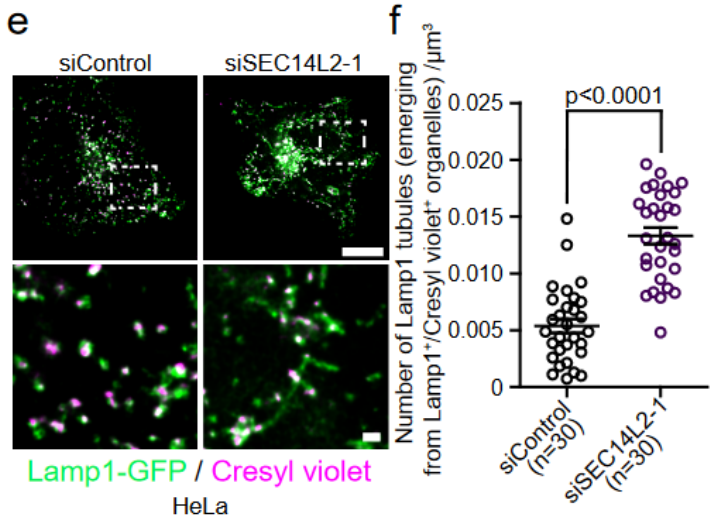


Fig 7e-f. Representative images of lysosomes (Cresyl violet positive Lamp1 organelles) in HeLa cells treated with the indicated siRNAs (e) and quantification of the number of lysosomal tubules (f). Scale bar: 10µm and 1µm (inset). Two-sided unpaired t-test.

2. Fission of LAMP1-mCherry containing structures is not obvious in Suppl. Movie 2 - it is more apparent in Suppl. Movie 3. Movie legends were not provided to the reviewers.

An error was made in the preparation of the supplemental movies. This was corrected, and the movie legends were added to the supplementary information files. We thank the reviewer for identifying this error.

3. The conclusions drawn from Figure 2 suffer from the same caveats as for Figure 1 (point #1).
a. How are the LAMP1-mCherry-labeled structures defined as "autolysosomes"? Simply labeling with LAMP1-mCherry after starvation is not sufficient. Are these any different from the "endolysosomes" described in Figure 1? Do they contain autophagic materials such as degrading mitochondrial or peroxisomal proteins or lipids? Or an acid-resistant LC3b-fusion protein (such as LC3b-mCherry; not LC3b-EGFP)?

To address this concern, we repeated the experiment using both Lamp1 (membrane marker) and LC3 (autophagosomal marker) to more precisely identify tubules emerging from autolysosomes (Lamp1 positive organelles containing LC3 in their lumen). We monitored the percentage of tubule fission emerging from autolysosomes event marked by Arf1-GFP vesicles in cells after prolonged starvation (HBSS). These data are now presented in **Fig 3b**.

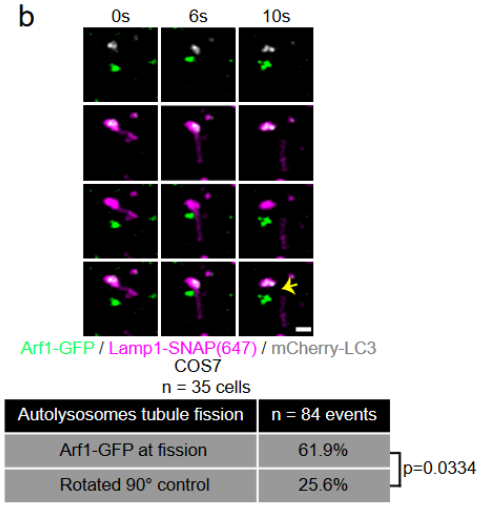


Fig 3b. Time-lapse images of a COS7 cell showing an autolysosomal (Lamp1+/LC3+) tubule fission event marked by an Arf1 positive vesicle and quantification of such events, n=84 events from 35 COS7 cells. Yellow arrow indicates fission. Scale bar: 1µm.

To demonstrate the lysosomal nature of sucrosomes imaged for tubule fission, the sucrosomes fission experiment was repeated with an overnight chased fluorescent 10kDa Dextran (endocytic cargo). Lamp1 structures with Dextran were quantified for endolysosomal tubule fission events marked by an Arf1 positive vesicle (**Fig 3d**).

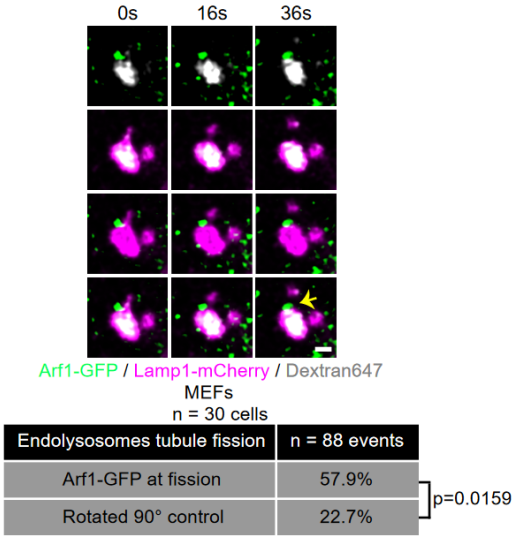


Fig 3d. Time-lapse images of MEFs showing an endolysosomal (Lamp1+/Dextran+) tubule fission event marked by an Arf1 positive vesicle and quantification of such events, n=88 events from 30 MEFs. Lamp1-mCherry signal rotated by 90° was used as a negative control. Yellow arrow indicates fission. Scale bar: 1µm.

b. In panel b the term "expected percentage of Arf1-GFP at fission if due to chance" in the quantification is an interpretation of the data, not a description - it should be labeled for what it is,

i.e. the percentage of LAMP1-mCherry labeled structures that also label for Arf1-GFP. Is this number derived from Figure 1 or from the starved cells used in this experiment?

To address the reviewer's concern about our negative control, we used another method to approximate the random distribution of Arf1-GFP structures to the site of fission. To determine the possibility of Arf1-GFP structures appearing at the site of fission randomly, we quantified the percentage of Lamp1 tubules fission marked by an Arf1-GFP positive vesicle in images where the Lamp1-mCherry channel was rotated by 90°. This method was used in several other studies, such as Abrisch et al. 2020 (https://doi.org/10.1083/jcb.201911122). This allowed us to evaluate the percentage of Lamp1 tubule fission that would be expected to be marked by Arf1 positive vesicles simply due to chance (**Fig 1g**).

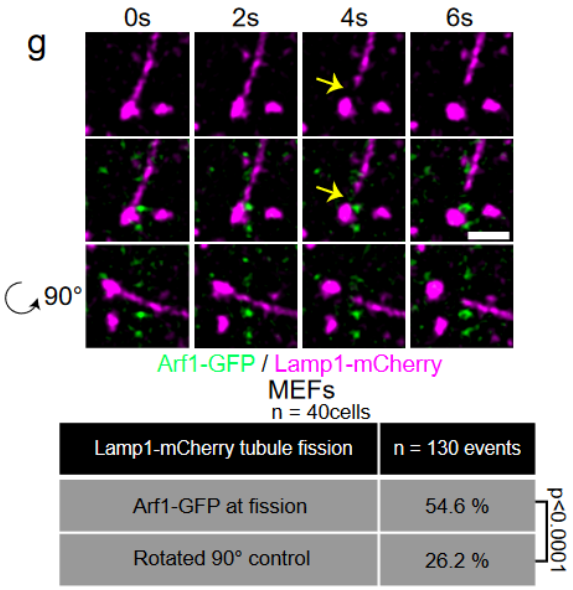


Fig 1g. Representative time-lapse imaging showing an Arf1-GFP positive vesicle marking the fission site of tubule from a Lamp1-mCherry positive organelle in a MEF cell starved for 8h with HBSS (amino acid-free media). Yellow arrows indicate the fission event. The bottom panels are the same images, but the Lamp1-mCherry channel was rotated by 90o. Scale bar: 2µm. The percentage of tubule fission events marked by Arf1-GFP vesicles was quantified. n=130 events from 40 MEFs. Quantification of the same data but where Lamp1-mCherry channel was rotated by 90°. P-value from a Fisher's exact test, two-sided unpaired t-test is shown.

This negative control quantification was also added to several other quantifications throughout the manuscript (**Fig 2d-g, Fig 3b, d and e, Sup Fig 2b).**

c. The data on sucrosomes in Figure 2d and e are nice, but what is the percentage of resolving sucrosomes that co-label for Arf1-GFP? Is it similar to the "autolysosomes" and plain LAMP1-mCherry labeled structures in Fig. 1 and Fig. 2a-c? Same question for the phagolysosomes in Fig. 2g-h.

We found that most resolving sucrosomes and virtually all phagolysosomes had at least one Arf1-GFP vesicles in juxtaposition to them. However, since that these structures are very large (~2-3 µm for sucrosomes and >5µm for phagolysosomes), occupying a high percentage of the cytoplasmic space, we believe these quantifications are not very informative and we felt that such quantification was not useful. For this reason, we only showed the quantification of Lamp1 positive and lysosomal tubule fission sites marked by an Arf1 positive vesicle. In addition, we now provide this quantification

using a 90° rotated Lamp1 signal as a negative control for sucrosomes (**Fig 3d**) and phagolysosomes (**Fig 3e**).

4. Fig. 3 contains a panel showing the absence of GFP-Rab5 from a single fission event, but (a) this needs to be done in the other systems and not just in YM201636 washout cells and (b) it needs to be quantified; 45-50% of the structures in cells expressing Arf1-GFP would look the same as this. In addition, some discussion should be devoted to the potential impact of YM201636 washout on PtdIns3P levels on lysosomes and how that might impact tubule fission.

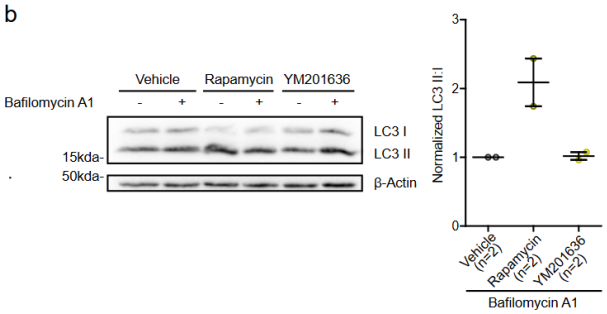
In our revised manuscript, the quantification of Rab5 presence at Lamp1 tubules fission promoted by starvation (8h HBSS treatment) is presented in **Fig 2d, g**. This data is discussed in our response to point 1a. Regarding YM201636, the point of this experiment was to verify that the Lamp1 positive structures are not early endosomes. The purpose of the experiment was not to determine whether vesicles recruited to the fission site are Rab5 positive or negative. We clarified the text (**Line 214-215**).

Regarding the potential impact of YM201636 washout on PI(3)P levels at lysosomes and how that might impact tubules fission: PIKFyve inhibition is expected to lead to an increase of PI(3)P at lysosomes as it is not converted in to PI(3,5)P2 by PIKFyve. However, fission of tubules does not occur since the formation of tubules is abolished by the increased membrane tension resulting from the consequence of PIKFyve inhibition on several lysosomal channels (e.g TPC1/2) (see Freeman et al, 2020. 10.1126/science.aaw9544). After washout, PI(3,5)P2 will be produced enabling reduction of membrane tension and tubulation of lysosomes and their fission. This potentially indicates, that PI(3)P levels could lower after this washout. However, as these levels were very high due to PIKFyve inhibition, that potential transfer of PI(3)P from vesicles or activation of PI(3)P producing machinery could still occur we don't think it would impact the formation o any PI(3)P signal involved in tubule fission. We opted not to include this point in our discussion for space consideration.

5. The data in Sup. Fig. 3b and c that YM201636 treatment did not induce autophagy and that the LAMP1-mCherry structures in these cells are not LC3-containing autolyosomes are weak. In the blot in panel b, there is a clear increase in autophagic flux after YM treatment (and surprisingly not after rapamycin treatment - did the rapamycin actually work in this experiment?). LC3-I levels seem to be high in the YM-treated cells, and so it is possible that LC3 expression was also increased. If this is not representative, then a more representative blot should be shown. In panel c, GFP is a poor fusion partner for LC3 since it is sensitive to pH, and thus might be quenched in active autolysosomes. The authors should consider repeating the experiment using either LAMP1-EGFP and mCherry-LC3 or labeling for GFP-LC3 in the presence of bafilomycin A1. The appropriate experiment here should be done and quantified.

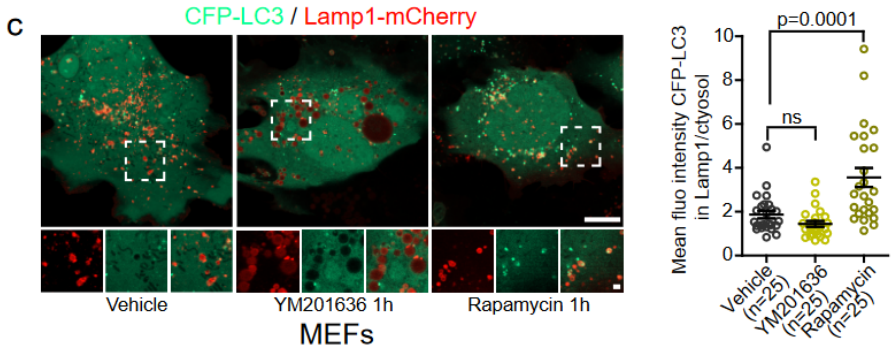
In this experiment, we treated the cells with YM201636 +/- Bafilomycin (1h) and compared it to Rapamycin +/- Bafilomycin for 1 hour. As expected, we do see an increase in LC3B-II/LC3B-I rapamycin compared to vehicle and YM201636. We also observed an increase in the LC3B-II/LC3B-I ratio for Bafilomycin-treated Vehicle and YM201636, as expected. However, we did not observe an increase in the LC3B-II/LC3B-I ratio between Rapamycin alone vs Rapamycin + Bafilomycin. This was because of the short time of treatment (1 hour), which was sufficient to observe upregulating of autophagosomes but not sufficient to result in a significant decrease of the LC3-II above the upregulated amount of LC3. We now provide a better representative blot for this experiment that

clearly shows an increase in the autophagic flux in rapamycin-treated cells and not in the YM201636-treated cells (**Sup Fig 3b**).



Sup Fig 3b. Representative western blot images of MEFs treated 1 hour with YM201636 (1µM) or vehicle control, rapamycin (10µg/mL) was used as a positive control. Bafilomycin A1 treatment (500nM, 1 hour) was used to inhibit degradation of autophagosomes allowing to evaluate whether YM201636 treatment induced formation of autophagosomes. Levels of LC3II were normalized to LC3I to quantify autophagosomes. β-Actin was used as a positive control. The graphs show the mean ± SEM, cells from two independent experiments.

Additionally, we repeated the LC3 experiment with CFP-LC3 as CFP pkA (4.7) is amenable to pH-sensitive experiments, and CFP-LC3 was already previously used to look at LC3 accumulation in lysosomes (Yu et al., 2010) (**Sup Fig 3c**).



Sup Fig 3c. Representative images of MEFs cells expression CFP-LC3 and Lamp1-mCherry and treated with YM201636 (1µM for 1h) or Rapamycin (10µg/mL) as a positive control. Scale bars: 10µm and 2µm (inset). Quantification of the mean fluorescence intensity of CFP-LC3 colocalizing with Lamp1 normalized to that of the cytosol. The graphs show the mean ± SEM, cells from three independent experiments. One-way ANOVA with Dunnett's Multiple Comparison Test. ns = 0.4570.

6. The experiment shown in Fig. 3g, h is described in the text as documenting autolysosome tubule fission, but there is no description in the figure legend of autophagy induction in this experiment; were these cells starved for 8 h prior to analysis? In Figure 3k-l, the legend indicates no time scale for image acquisition after SRBC phagocytosis. The length of treatments with various drugs is not indicated in the figure legend. In addition, it would be very helpful to indicate what is being analyzed (e.g. sucrosome, phagosome, autolysosome) on the figure itself without having to refer to the legend. Note, while the data are consistent with the conclusion drawn, they do not provide proof that Golgi-derived vesicles, as opposed to some process dependent on the intact functional Golgi or BFA-sensitive recruitment of Arf1 to endosomes, are the agents that support lysosomal tubule scission.

We apologize for omitting some critical details to the legend of this figure. We addressed this point by modifying the text and adding more details regarding the time of treatments to the figures and legends, as suggested by this reviewer.

See lines 240-244; Lines 247-205 as well as the legend of **Fig 4e,f and i**.

We did not add the mention of sucrosomes or phagolysosomes directly to the figure as we felt it would overload them and decrease the readability, and this information is clearly written in the text and legends.

Regarding the latter point of the reviewer that our data do not preclude "…the possibility process dependent on the intact functional Golgi or BFA-sensitive recruitment of Arf1 to endosomes …". Although we agree with the reviewer that the data presented in Fig 3g,h (now **Fig 4 c, d and f**) do not provide absolute proof that it is the Arf1-PI4KIIIβ positive vesicles that are mediating fission and that we cannot rule of the possibility that other Golgi functions may play a role in lysosomal tubule fission, our data shows that Arf1-PI4KIIIβ positive vesicles formation or function is required for efficient lysosomal tubule fission. To further demonstrate this point, we have added new data showing that SEC14L2 localizes to a subset of Arf1-PI4KIIIβ positive vesicles and is required for efficient fission, while it is not involved in the formation of these vesicles. Below, we provide the various evidence presented in our manuscript that supports our conclusion that these Arf1-PI4KIIIβ positive vesicles are required for fission.

1) We show that Arf1 (**Fig 1g; Fig 3b, d and e**), TGN46 (**Fig 2b**) and PI4KIIIβ (**Fig 2a**) positive vesicles are recruited to a variety of lysosomal tubule fission sites, and we provide evidence supporting that these vesicles are not endosomes.

2) We show that treatment with BFA or PI4KIIIβ inhibitors inhibits fission (**Fig 4c-f**). Both BFA and PI4KIIIβ inhibitors are expected to inhibit the function and/or formation of Arf1-PI4KIIIβ positive vesicles. We find that these drugs cause an increase in Lamp1 tubule number and decrease Lamp1 tubule fission rate, demonstrating impaired fission. Note that the overall morphology of the Golgi does not appear to be strongly modified by the PI4KIIIβ inhibitors and that PI4KIIIβ was not reported (to the best of our knowledge) to control Arf1 localization to endosomes.

3) Existing literature implicating Arf1 positive vesicles identified as Golgi-derived vesicles as being recruited to membrane fission events (mitochondria, Nagashima et al., 2020 and early endosome (Gong et al., 2021) whose action at fission events were shown to be inhibited by BFA treatment or Arf1/PI4KIIIβ depletion.

4) We show new data demonstrating the role of SEC14L2 in the fission of lysosomal tubules. We show that SEC14L2 localizes to a subset of Arf1-PI4KIIIβ positive vesicles (**Fig 7a**) and that its depletion impairs the fission of lysosomal tubules (**Fig 7e,f**). In these experiments, we also show that knockdown of SEC14L2 expression did not affect the number of Arf1 puncta (**Fig 7j,k**), suggesting that SEC14L2 does not affect Arf1 recruitment at Golgi vesicles or endosomes and does not appear to affect Golgi morphology, overall.

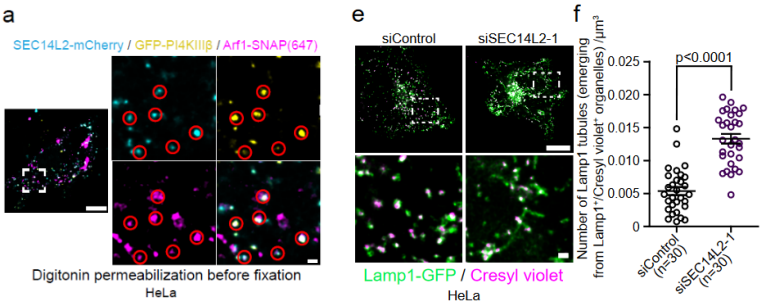
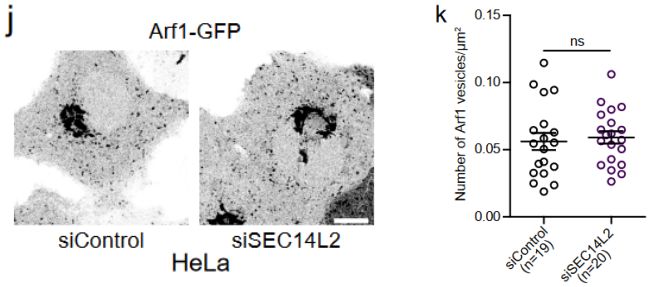


Fig 7a. Representative live image of a HeLa cell expressing SEC14L2-mCherry, GFP-PI4KIIIβ and Arf1-SNAP. Excess cytosolic signal was removed by short-term permeabilization with digitonin before fixation. Red circles show SEC14L2 colocalization with Arf1-PI4KIIIβ positive vesicles. Scale bar: 10µm and 1µm (inset). **e-f**. Representative images of lysosomes (Cresyl violet positive Lamp1 organelles) in HeLa cells treated with the indicated siRNAs (**e**) and quantification of the number of lysosomal tubules (**f**). Scale bar: 10µm and 1µm (inset). Two-sided unpaired t-test.



Sup Fig7j-k. (**j**) Representative Airyscan images of HeLa cells expressing Arf1-GFP and treated with indicated siRNAs. Scale bar: 10µm. (**k**) Quantification of the number of Arf1-GFP positive vesicles per cell. Two-sided unpaired t-test. ns = 0.6999.

Collectively, the data presented in our manuscripts support that Arf1-PI4KIIIβ vesicles are the agent that supports the fission of lysosomal tubules.

7. In Fig. 4g, h, how do tubule numbers in the cells expressing active or mutant Sac1 fusion protein compare in cells that have not been transfected with any LysoGFP-Sac1 construct? Is it the same as in the catalytically inactive transductants? Also, it is intriguing that the LysoGFP-Sac1 construct is more effective in reducing tubule formation (Fig. 4g, h; Suppl. Fig. 6e-h) than the GFP-ORPSAC1 construct (Suppl. Fig. 6c, d). Might this reflect the limited distribution of ORP1L to ER-lysosome contacts, and if so, how would the lower impact of GRP-ORPSAC1 be interpreted in the context of the ER contacts at the fission site shown in Suppl. Fig. 2C?

We find that the Lamp1 tubule numbers of the mutant Sac1 are similar to that of Lamp1-GFP expressing cells in DMEM and HBSS (8 hours) media, while the Sac1 is significantly less than lamp1-GFP (**see Fig 5e vs 5h**).

Regarding the difference in the effectiveness of LysoGFP-Sac1 and GFP-ORPSAC1, we believe, as suggested by this reviewer, that this is due to differences in the localization of the constructs. LysoGFP-Sac1 localizes to lysosomes thanks to the n-terminal sequence of p18/LAMTOR, whereas ORPSAC1 is recruited to Rab7 positive late endosomes and lysosomes. In addition, ORPSAC1 expression is expected to localize more specifically to ER-late endosomes/lysosomes contact. As for the interpretation in the context of ER presence at Lamp1 tubules fission sites, it is important to note

that while we show that the ER is contacting Lamp1-positive tubule necks during fission, we do not know if tubule formation occurs at the site of contact. Recently Levin-Konigsberg et al. (PMID: 31570833) provided evidence that the appearance of tubules from phagolysosomes occurs at the site where the ER is excluded. Although the question of ER-lysosome contact is very intriguing, it is outside the scope of this paper. The question that needs to be addressed differs significantly from the current study, and it would be better served in another manuscript.

We believe these data indicate that lysosomal PI(4)P is required for the formation of lysosomal tubules.

8. The data and interpretation of Figure 5 raise some concerns.

a. In Fig. 5a,b and Suppl. Fig. 7a, b, the conclusion that SEC14L2 localizes to Golgi-derived vesicles is premature. Again, the authors use Arf1 instead of a more specific Golgi/ TGN marker to define Golgi, invalidating the conclusion. This is also a problem in concluding that "formation of Golgi vesicles is unaffected" in panels c and d; what is measured is the number of Arf1-containing puncta, only some of which would be expected to be Golgi-derived. Moreover, in panels a and b there is no quantification of the overlap. These conclusions should be softened or bolstered by additional data using Golgi/TGN-specific contents. The same problem applies to the interpretation of data in Fig. 7e and f and Fig. 6f that VPS34-IN1 does not block Golgi vesicle formation (I suspect this is true but the data do not show this) but increases "Golgi vesicle" contacts with lysosomes. Again, the graph should state what the data are, not their overinterpretation.

We now provide more evidence that SEC14L2 is localized to Arf1-PI4KIIIβ positive vesicles and provide quantifications of Arf1 and of PI4KIIIβ positive vesicles to demonstrate that depletion of SEC14L2 or VPS34-IN1 treatment does not prevent the formation of these Arf1-PI4KIIIβ positive vesicles. As we explained in our response to point 1, we acknowledge the caveats of Arf1 as a marker and that Arf1 alone is not sufficient to identify these vesicles as Golgi-derived and are now calling them Arf1-PI4KIIIβ positive vesicles. We now show images indicating that overexpressed SEC14L2 localizes to a subset of Arf1-PI4KIIIβ positive vesicles in HeLa cells (**Fig 7a**). This finding is consistent with a previous study showing that SEC14L2 localizes to Arf1-positive structures that were identified as Golgi-derived vesicles (Gong et al., 2021).

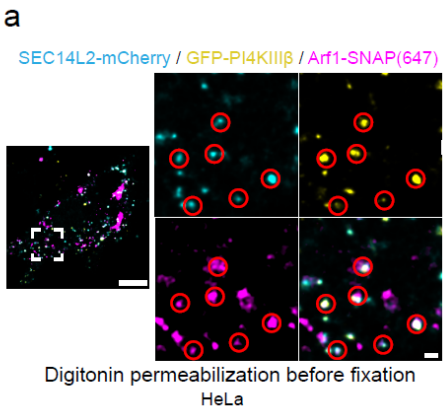
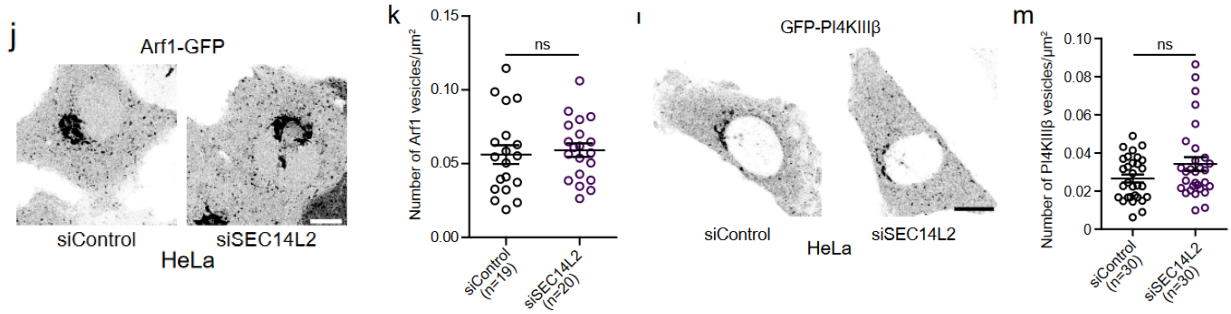


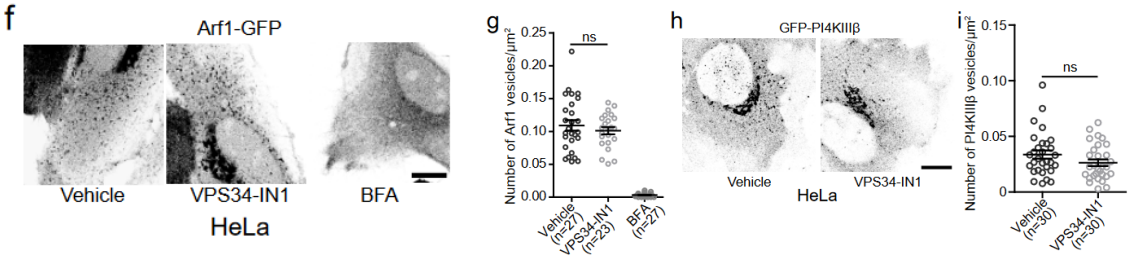
Fig 7a. Representative live image of a HeLa cell expressing SEC14L2-mCherry, GFP-PI4KIIIβ and Arf1-SNAP. Excess cytosolic signal was removed by short-term permeabilization with digitonin before fixation. Red circles show SEC14L2 colocalization with Arf1-PI4KIIIβ positive vesicles. Scale bar: 10µm and 1µm (inset).

We also examined whether SEC14L2 depletion using siRNA affected the number of such vesicles by quantifying the number of Arf1 puncta and PI4KIIIβ puncta structures in cells using fluorescently tagged constructs in SEC14L2 depleted cells. We found that SEC14L2 depletion does not affect the number of such vesicles, suggesting that this protein does not control the formation of the Arf1-PI4KIIIβ positive vesicles (**Sup Fig 7j-m**).



Sup Fig 7j-m. (**j**) Representative Airyscan images of HeLa cells expressing Arf1-GFP and treated with indicated siRNAs. Scale bar: 10µm. (**k**) Quantification of the number of Arf1-GFP positive vesicles per cell. Two-sided unpaired t-test. ns = 0.6999. l-m. (**l**) Representative images of HeLa cells expressing GFP-PI4KIIIβ and treated with the indicated siRNAs. (**m**) Quantification of the number of GFP-PI4KIIIβ positive vesicles in these cells. Two-sided unpaired t-test.

The same experiments were performed to test whether VPS34-IN1 treatment impaired the formation of Arf1-PI4KIIIβ positive vesicles. We find that this treatment had no effect on the number of Arf1 puncta and of PI4KIIIβ compared to cells treated with vehicle control, using fluorescently tagged constructs (**Sup Fig 7f-i**).



Sup Fig 7 f-g. (**f**) Representative images of HeLa cells expressing Arf1-GFP and treated with VPS34-IN1 (1µM) or BFA (10µg/10mL) for 1 hour. Scale bar: 10µm. (**g**) Quantification of the number of Arf1-GFP positive vesicles per cell. One-way ANOVA with Dunnett's multiple comparison test. ns = 0.5088. **h-i**. (**h**) Representative images of HeLa cells expressing GFP-PI4KIIIβ and treated with the indicated drug for 1H. Ethanol was used as a Vehicle control. Scale bar: 10µm. (**i**) Quantification of the number of GFP-PI4KIIIβ positive vesicles in these cells. Two-sided unpaired t-test. ns = 0.1194.

Finally, we also revised the manuscript to avoid the impression that we are over interpretating our data.

b. The impact of knocking down SEC14L2 expression in Figure 5 is clear and the quantification of the rescue experiment in panel f is nice, but these data alone (or in combination with the data in Figure 6) do not prove that SEC14L2 lipid transfer activity is required for LAMP1 tubule formation. Lipid binding residues in SEC14L2 have been predicted and tested for function (Gong et al., Nat. Cell Biol. 23: 782), and others could be predicted based on homology to the well-studied yeast Sec14. To affirm that SEC14L2 lipid transfer activity is required, it should be tested whether lipid binding mutants are able to rescue the SEC14L2 knockdown as in Figure 5f.

We believe that the reviewer misunderstood our conclusion to these sets of experiments as we are not claiming that SEC14L2 is involved in tubulation but instead in fission of tubules. We have modified the text to clarify our interpretation and conclusion to these data. We found that depleting SEC14L2 increased the number of tubules on the lysosomal organelles (**Fig7 d-f**) and impaired the fission of Lamp1 tubules (**Fig 7j**) suggesting a role of SEC14L2 in the fission of lysosomal tubules but not in their formation.

Regarding the experiment to test the lipid transfer activity of the protein, we thank the reviewer for suggesting this experiment. We examined whether SEC14L2 lipid binding/transfer function was required for the fission of the tubules, as suggested by the reviewer. Here, we performed complementation experiments using the previously described PI(3)P binding deficient mutant zSec14l3 M5 (Gong et al., Nat. Cell Biol. 23: 782) and two domain-deleted constructs. These results showed that only wild-type zSec14l3 was able to rescue the loss of fission activity in cells depleted of SEC14L2. Expressing PI(3)P binding mutants of zSec14l3 (zSec14l3-M5) did not decrease the number of tubules or increase the rate of Lamp1 positive tubule fission (**Fig 8a-c**).

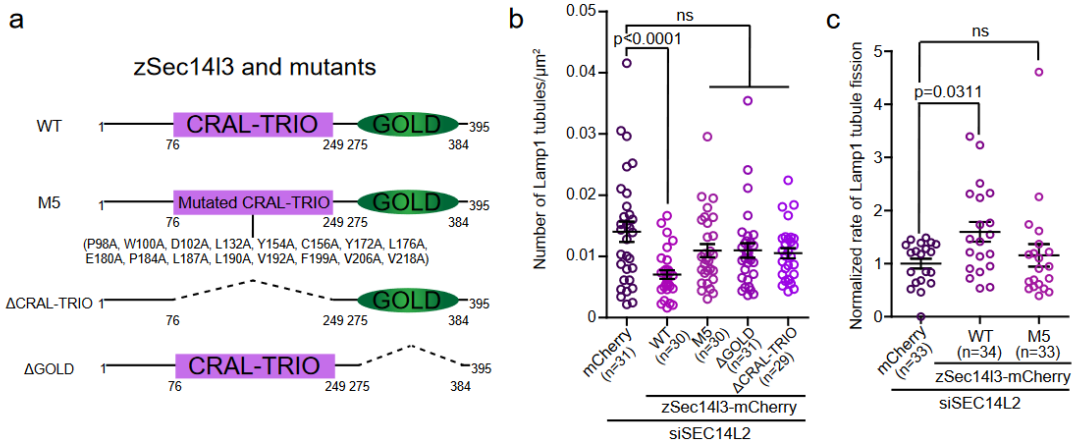


Fig 8a-c: **a**. Cartoon illustration of the various zSec14l3 constructs used in the study. **b**. Quantification of the number of Lamp1 positive tubules in cells treated with indicated siRNA and expressing the indicated constructs. One-way ANOVA with Dunnett's Multiple Comparison Test. p-value = 0.1776 (M5), 0.1808 (ΔCRAL-TRIO) and 0.1069 (ΔGOLD). **c**. Normalized rate of Lamp1 positive tubule fission in cells treated with indicated siRNA and expressing the indicated constructs. One-way ANOVA with Dunnett's Multiple Comparison Test. ns = 0.7442.

These data indicate that the lipid binding/transfer activity, and particularly the PI(3)P binding activity, of SEC14L2 is required for SEC14L2 function in the fission of Lamp1 positive tubules and strengthen the link between SEC14L2 and PI(3)P in the fission of lysosomal tubules.

c. In Fig. 5i and j, remind us in the text of how the rate of lysosomal fission was measured. In Fig. 5k and l, how are "enlarged LAMP1 positive structures" defined? Were the structures measured? How? Arrows in the figures would help here, but some objective definition of what was measured needs to be provided.

We added the clarification regarding the quantification of the rate of fission in the text when we used this quantification for the first time for **Fig4e, f** (lines 240-244) and described it in the methods (lines 700-702).

For the enlarged Lamp1 structures, these structures were measured and considered as enlarged if they were of more than 1µm². However, as this data is redundant with the enlarged autolysosome

quantification presented in **Fig 7m-n**), we removed the data initially presented in fig 5k in our revised manuscript for the sake of simplicity.

d. Finally, in Fig. 5m, n, the observation of GFP signal from LC3-GFP associated with LAMP1-positive structures in SEC14L2-depleted cells suggests either that these compartments are not acidic - and perhaps not fused to lysosomes or de-acidified. How do the authors explain their presence in SEC14L2-depleted cells despite the conclusion that autophagy is not affected by tubule impairment? Does SEC14L2 play another role in this process besides tubule severing?

We thank this reviewer for pointing out a potential caveat with our GFP-LC3 experiment. We opted to repeat the experiments with LC3 tagged to CFP as it has a lower pKa of 4.7. We now provide new results using CFP-LC3. These new data indicate that SEC14L2 depletion results in an increased number of enlarged autolysosomes (Lamp1+/LC3+ organelles) (**Fig 7m-n**), suggesting that autophagic lysosome reformation, a late step of the autophagic process requiring formation and fission of tubules from autolysosomes (yu et al., 2010) was impaired. This is consistent with an important role for SEC14L2 in the fission of autolysosomal tubules.

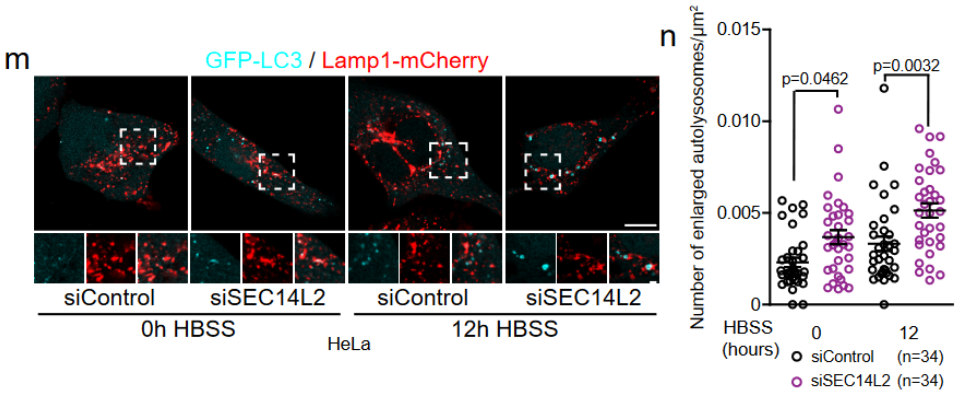


Fig 7m-n. (**m**) Representative images of HeLa cells expressing CFP-LC3 and Lamp1-mCherry treated with the indicated siRNAs and incubated in HBSS for the indicated time. Scale bars: 10µm and 1µm. **n**. Quantification of the number of enlarged autolysosomes (>1µm²) of cells in (**m**).

9. The approach to quantify labeling of "lysosomes" by PX-GFP in Fig. 6a, b is confusing. Most PtdIns(3)P in cells accumulates on early endosomal vacuoles, not on lysosomes (or the Golgi - see below). Is this again a problem of using overexpressed LAMP1-mCherry as way to mark "lysosomes", or was the lysosomal signal a small proportion of the punctate structures observed? It is confusing that the quantification was of signal overlapping lysosomes relative to cytosolic PX-GFP as opposed to early endosomal GFP, where most would be expected to accumulate. This concern extends into Fig. 6c-e, as the appearance of the enlarged LAMP1-mCherry-containing structures in the VPS34-IN1-treated cells in 6c look more like the enlarged early endosomal vacuoles that are well known to accumulate in cells with impaired PtdIns-3-kinase activity. Are these lysosomes or early endosomes? They should be labeled by cargo (e.g. chased dextran) and not just by LAMP1-mCherry.

The reviewer is correct in that PI(3)P is enriched in early endosomes, and genetically encoded fluorescent PI(3)P biosensors such as PX will localize mainly to this enriched pool. The signal overlapping with Lamp1 will thus represent only a small proportion of the structure observed. The presence of PI(3)P at lysosomes is well established (Posor et al, 2022). For instance, it is well known that PIKFyve produces PI(3,5)P2 on lysosomes by using PI(3)P as a substrate. Moreover, several studies detected PI(3)P in lysosomes (e.g., Munson et al., 2016). Therefore, we feel using PX as a

PI(3)P biosensor is a valid way of evaluating PI(3)P levels at lysosomes. However, to provide more evidence of the nature of these PI(3)P positive structures, we examined PX-GFP with cresyl violet, a dye that marks acidic lysosomes (Ostrowski et al., 2016). These data are now presented in **Fig 8g,h.**

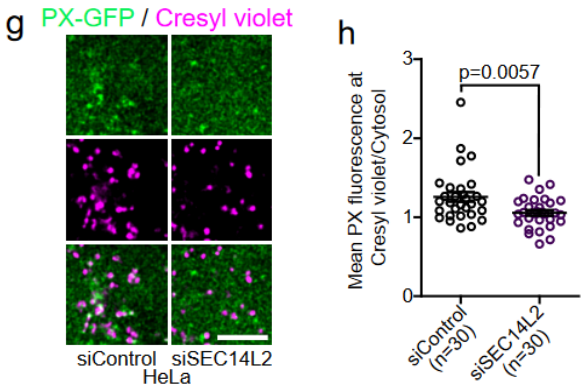


Fig 8g-h. (g) Representative images of a 10 µm x 10 µm section of a HeLa cell treated with the indicated siRNA expressing the PI(3)P biosensor PX-GFP and where acidic lysosomes were marked using cresyl violet Scale bar: 5µm. **h**. Quantification of the PX levels colocalizing with cresyl violet normalized to the cytosolic level of the probe of cells in (**g**). Two-sided unpaired t-test.

Regarding the VPS34-IN1 experiment, we feel it is not required to further label Lamp1 positive structures as this inhibitor has been well characterized to act on lysosomes, and our data are in line with published data. For instance, similar to what was reported by Munson et al. 2016, we show that VPS34 inhibition causes an increase in the number of lamp1 positive tubules. Moreover, we show that acute depletion of PI(3)P at lysosomes using the GAI-GID1 system to acutely recruit MTM1 to lysosomes increases the number of lysosomal tubules and decrease the rate of tubule fission from lysosomes (**Fig 6g-I**, see below). Further, similar to other reports, we also show that VPS34 inhibition leads to the accumulation of very enlarged lysosomes, a phenotype very likely due to loss of PI(3,5)P2 production by PIKFyve.

In this manuscript, we offer new data that shows that depleting PI(3)P at structures marked by the lyso (n-terminal lysosomal anchoring sequence of p18/LAMTOR) increases the number of tubules from lysosomes (defined here as positive for the lyso tag and cresyl violet) (**Fig 6e-h).** These results indicate that the loss of PI(3)P at lysosomes leads to the persistence of lysosomal tubules in cells, consistent with a defective tubule fission process.

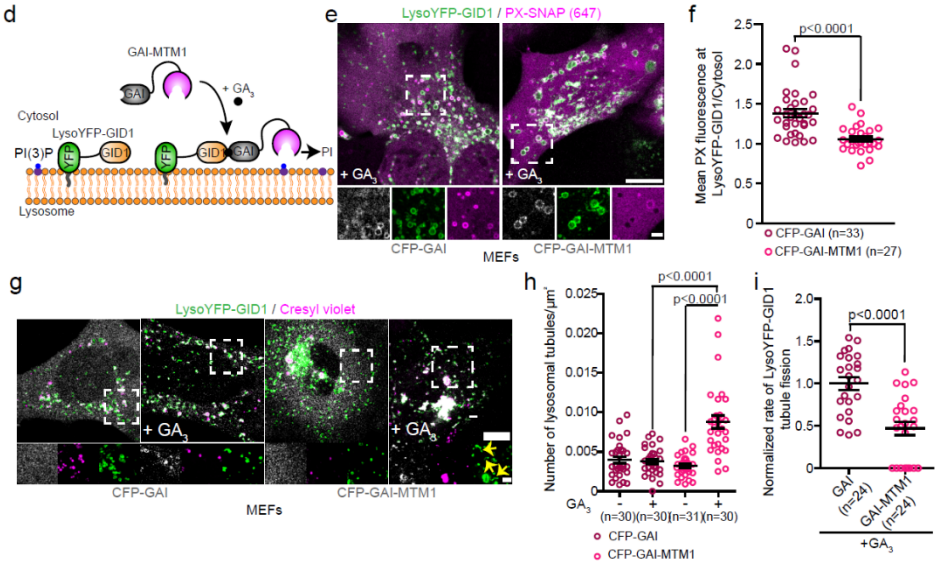


Fig 6d. **d**. Cartoon illustration of the GAI-GID1 dimerization system used to acutely recruit the PI(3)P phosphate MTM1 to lysosomes using the LysoYFP-GID1 construct as an anchor. **e-f**. (**e**) Representative Airyscan images of MEFs expressing LysoYFP-GID1, PX-SNAP and CFP-GAI or CFP-GAI-MTM1 and treated with GA3-AM (10μM for 1H). Scale bar: 10μm and 2μm (insets) (**f**) Quantification of the mean fluorescence intensity of PX at LysoYFP-GID1 positive organelles normalized to cytosolic levels. Two-sided unpaired t-test. **g**. Representative Airyscan images of MEFs expressing LysoYFP-GID1 and CFP-GAI or CFP-GAI-MTM1 before and after treatment with GA3-AM (10μM for 1H). Acidic (Cresyl violet) LysoYFP-GID1 positive organelles were considered lysosomes. Scale bars: 10μm and 2μm (inset). Yellow arrows indicate tubules. **h**. Quantification of the number of lysosomal tubules in cells in (**g**). Two-way ANOVA with Tukey's multiple comparison tests. **i**. Normalized rate of tubule fission from LysoYFP-GID1 positive organelles in MEFs (starved for 8h in HBSS) after recruitment of indicated construct by GA3-AM treatment (10μM) for 1h. Two-sided unpaired t-test.

10. I find a great deal about the data presentation, conclusions, and documentation for Fig. 6 to be very frustrating.

We apologize for the frustration we caused the reviewer and thank the reviewer for taking the time to provide this insightful critique of our manuscript. We believe that the reviewer's comments were instrumental in preparing a manuscript that we feel that provides great insight into the field of lysosome biology

a. The quantification data in Fig. 6 are presented with axes labeling the data interpretation rather than what was actually measured. This must be remedied. For example, the y-axis in panels b and d should read something like "PX-GFP overlapping with LAMP1-mCherry relative to cytosolic PX-GFP" and the y-axis in panel f should read "% of LAMP1-mCherry structures contacting Arf1-GFP structures".

We modified the labelling of our figures and figures legends in all of our figures to refer more precisely to what was measured and not our interpretation of the data. For this particular figure, the Y-axis now reads: "Mean PX at Lamp1/cytosol".

b. Panel g (and the text of the Results - incredibly frustrating that the information is not there) should indicate that the cells are expressing RAB7-FRB - note this should target the FKBP fusions to late endosomes, not lysosomes, and thus the conclusions drawn need to be reconsidered.

In order to simplify the interpretation of these experiments, we performed this experiment using the GAI-GID1 dimerization system (Miyamoto et al., 2012). This system allows dimerization of GAI with GID1 upon the addition of cell-permeant Giberellin (GA_3-AM), a drug with no known target in mammalian cells. We used this system to recruit GAI-MTM1 (MTM1 =PI(3)P phosphatase) to LysoYFP-GID1 positive structures in order to deplete PI(3)P at lysosomes. We obtained similar results to the previously used FRB-FKBP system. This set of new data is presented in **Fig 6d-i** and already presented above in our response to concern 9).

c. It is not clear why in panel g the localization of the mRFP or mCherry fusion is shown, whereas the quantification in h documents PX-GFP association with lysosomes, which is not shown at all. How do the authors explain the accumulation of the FKBP-MTM1 fusion in aggregates that do not localize to the LAMP1-containing structures? Does this not alter the interpretation of the result and suggest that the fusion protein is not actually removing PtdIns3P from lysosomes per se?

We apologize for this mistake, panel **g** was mislabelled, and the green signal referred to PX-GFP and not Lamp1-GFP. This figure was removed as the experiment was performed using the GAI-GID1 dimerization system and is presented in **Fig 6e, f**. (see response to point 9 above).

d. In the experiments of 6j-l, it is not stated in the figure legend or the text how long the cells were treated with rapamycin - was this a brief treatment to induce FRB-FKBP interaction or was this long-term treatment to activate autophagy as in Fig. 2b, c? Because of this complication, this experiment would be much more clear with a non-functional rapalog dimerization agent.

We removed the data using the FKBP-FRB system and are now showing data with the GAI-GID1 dimerization system (see above our response to point 9). PX at LysoYFP-GID1 and the number of lysosomal tubules were measured after 1 hour of treatment with GA_3-AM to induce dimerization. The time of treatment and concentration of GA_3-AM are indicated in the figure legend.

e. The effect of the MTM1 recruitment to phagolysosomes has an extraordinarily modest effect on tubule number, and one wonders whether the statistical significance was assessed correctly. Was the ANOVA test done using each measured cell as a replicate, or was it done properly in which the mean from each time the experiment was done was used as a replicate (see Lord et al., J. Cell Biol. 219: e202001064)?

As for lysosomes, we performed this experiment using the GAI-GID1 system. Note that for this experiment, we used iRFP-GID1-Rab7 as an anchor to recruit GAI-MTM1 to phagolysosomes as this construct (iRFP-GID1-Rab7) was previously successfully used to recruit constructs to phagolysosomes (Levin-Konigsberg et al., 2019). This experiment shows an increased number of Lamp1-positive tubules per phagolysosome (**Fig 6j**). We changed our style of presentation to simplify this panel. The statistical analysis was performed as indicated by this reviewer.

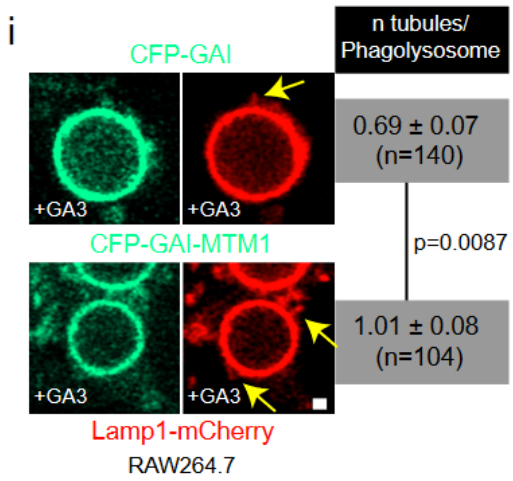


Fig 6j. Representative Airyscan images of phagolysosomes from RAW 264.7 cells phagocyting SRBCs expressing iRFP-GID1-Rab7 (not imaged), Lamp1-mCherry and CFP-GAI or CFP-GAI-MTM1 after treatment with GA3-AM (10µM for 1H) (Yellow arrows show tubules. Scale bar: 2µm) and quantification of the number of tubules per phagolysosome. Two-sided unpaired t-test.

f. The use of Arf1 as a fusion partner to target MTM1 to the TGN is inappropriate, as discussed at length above. The data in no way suggest that PtdIns3P on the Golgi plays any role in the lysosomal tubulation process, and again in fact suggests that the Arf1 labeling observed in the periphery is

largely on early endosomes rather than Golgi-derived vesicles. To test this more directly, why not anchor MTM1 to the TGN with an FRB fusion to galactyosyl transferase, sulfotransferase, or sialyl transferase?

We thank the reviewer for suggesting this interesting experiment. However, as described above (see our response to concern 1)a)) none of the markers suggested here appear to be incorporated in vesicles and only show a localization at the Golgi. Thus, anchoring MTM1 to these markers would not allow depleting PI(3)P at Golgi-derived vesicles but only at the Golgi. Therefore, although anchoring MTM1 to Arf1 is not without its own issues, as raised by this reviewer, Arf1 localizes to the vesicles implicated in the fission of lysosomal tubules and is thus represents a better option. In addition, we also performed a similar experiment in which we fused MTM1 to SEC14L2 in order to deplete PI(3)P whereSEC14L2 localizes and on its immediate proximity. SEC14L2-MTM1 fusion protein still shows colocalization with Arf1 positive vesicles (**Fig 8n**), and its expression in cells leads to an increase in the number of tubules emerging from lysosomes (Lamp1+/cresyl violet+) (**Fig 8o,p**), suggesting it impairs the fission of lysosomal tubules.

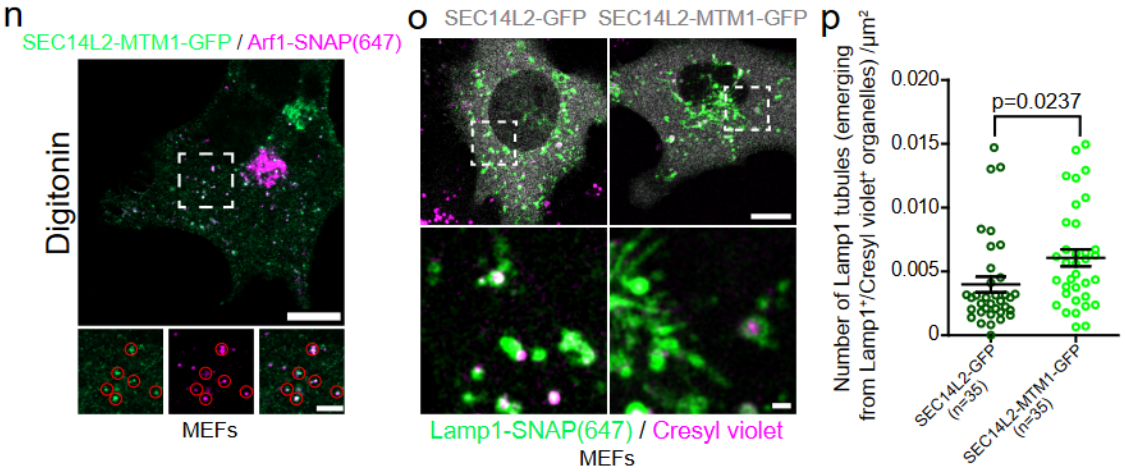


Fig8 n. Representative Airyscan image of a MEF cell expressing SEC14L2-MTM1-GFP and Arf1-SNAP. Red circles show SEC14L2-MTM1 colocalizing with Arf1 positive vesicles. Scale bar: 10µm and 1µm (inset). **o-p**. Representative Airyscan images of MEFs expressing Lamp1-SNAP and SEC14L2-GFP or SEC14L2-MTM1-GFP and treated with Cresyl violet to mark acidic lysosomes (scale bar: 10µm and 1µm (inset)) (**o**) and the quantification of the number of lysosomal tubules (**p**).

While this construct suffers from some of the caveats of the Arf1-MTM1 one, such as the possible activity in trans, we believe this experiment indicates that depletion of PI(3)P where SEC14L2 localizes or at the immediate proximity of its location impairs the fission of lysosomal tubules and strengthen the link between SEC14L2 and PI(3)P in the fission of lysosomal tubules. We modified the text to make clear that depleting PI(3)P where Arf1 and SEC14L2 localize and in their immediate proximity was the goal of these constructs.

11. The discussion lacks any reference to any of the caveats raised in this review regarding data interpretation, and starts with assuming that their model is correct. This must be amended.

We thank this reviewer for this important point. We modified the discussion to address this issue.

12. The summary cartoon in Figure 7 refers to PI(4)P being involved in tubule formation, which was

not at all discussed in this manuscript, but omits the requirement shown in the manuscript that PI(4)P is required for tubule scission (by their interpretation, via conversion to PtdIns3P). The cartoon should be amended to focus on the main point of the paper.

We have revised the manuscript in our description of our PI(4)P experiments (**Fig 5**). We have tested and discuss the role of PI(4)P in tubule formation in the manuscript (see **Fig 5** ,where we show that depletion of PI(4)P at lysosomes strongly decreases the number of tubules). Importantly, it is not our interpretation that a conversion mechanisms (PI(4)P to PI(3)P) is required for tubule scission. The text was clarified to avoid any misunderstanding.

We decided to keep this role of PI(4)P production in the formation of tubules in the cartoon of our proposed model as it is one important part of our manuscript. Indeed, this part shows that contrary to what was proposed in a previous study (Sridhar et al., 2013), PI(4)P does not have an anti-tubulation role at lysosomes but, on the contrary, appears to be required for their formation. This is consistent with several recent studies implicating PI(4)P in the formation of tubules from endosomes (Atul Jani et al., 2022 and Zhu et al., 2022) or phagolysosomes (Levin-Konigsberg et al., 2019)

Minor concerns.

13. General - as different cell types are used in different experiments, it would be helpful to have the cell type used indicated on the figures themselves.

We indicated the cell types used in all the figures of the manuscript.

14. The rationale for documenting the long-term association of Arf1-GFP-labeled structures with LAMP1-mCherry-containing structures in Figure 1 is not clear, given that the rest of the study focuses on the rapid association of Arf1 with tubules in the process of severing.

We believe it provides evidence that Arf1 positive structures are bona fide vesicles that are independent of Lamp1 positive vesicles and therefore do not represent recruitment of Arf1 to Lamp1 positive membranes. We also think it suggests that Arf1 positive vesicles and Lamp1 positive vesicles are making contact which is important to this study and is of interest to the readers. This rationale was clarified in the text.

15. Regarding the quantification of the rate of lysosomal tubule fission, the authors should clarify how they define the fission (e.g. fission occurs at the neck or the middle of the tubule that is associate with a lysosome, or at a tubular structure).

The vast majority (>95%) of fission events that we observed were at the neck of the tubules, even though fission at the middle of tubules was sometimes observed (see **Fig 1F**). However, we defined fission as any event showing clear scission of the tubule (neck or middle) and the main organelle body. We added this precision to the methods (lines 690-694).

16. In Suppl. Fig. 4e, it would be helpful to indicate the outline of the cells.

We added the outline of the cells to this figure as suggested.

17. The quantification in Supp. Fig. 5c is unclear and needs to be clarified. Were 345 lysosomes counted in each sample (vehicle- and BFA-treated), and among those, the indicated number of tubulation and vesiculation events were counted? Over what time frame? This might be more clearly presented as a percentage of total lysosomes.

Indeed, 345 Lamp1 positive organelles (15 Lamp1 positive organelles per cell, n=23 cell) in each condition were counted, and among those, the indicated number of fissions by tubulation and vesiculation was observed. We clarified this in the figure and its legend and added the time frame. However, we feel it is not preferable to present this as a percentage of total lysosomes as it was sometimes possible to observe more than 1 fission event for a single Lamp1 positive organelle.

18. In the text describing Fig. 3g, h (lines 170-172), the authors should briefly indicate how the rate of tubule fission was quantified. The figure legend here is not very clear - were these manually counted over a given area? What area?

We added this information in the text and legend. In addition, the way fission was counted was more thoroughly described in the method section (lines 700-702).

19. In Figure 4e, it is not clear what is actually being quantified and how the experiment was done. Were both LAMP1-GFP and LysoGFP-PI4KB cotransduced with LAMP1-mCherry, and the number of LAMP1-mCherry tubules quantified? Or something else? How did the number of

Indeed, both were co-transfected with Lamp1-mCherry. We clarified the text (lines 298-302) and the legend of the figure.

20. In Fig. 4h and Suppl. Fig. 6j and l, the blue and green colors are difficult to distinguish.

We changed the colours to increase the contrast between the two, as suggested.

21. In Suppl. Fig. 6j stats should be shown between all 0, 4, and 8 h groupings.

We feel that adding all the stats for this panel would overload it and decrease its readability. For this reason, we only show the most important stats comparing the effect of the two conditions and indicating that anchoring Sac1 to lysosomes do not appear to affect the fusion of lysosomes with autophagosomes and thus formation of autolysosomes.

22. Quantification of fission rate is needed for Figure 6c, I, k, and o.

The effect of VPS34-IN1 treatment on lysosome fission was already reported (see. Munson et al., 2015). We quantified the fission rate for tubules extending from LysoYFP-GID1 positive organelles after PI(3)P depletion induced by GAI-MTM1 recruitment. This new data, which is presented in **Fig 6i** and described above in our response to point 9, further suggests that PI(3)P depletion at lysosomes impairs fission of their tubule.

23. In general, arrows and arrowheads should be used liberally to point to structures of interest in the fluorescence micrographs, particularly to illustrate examples of tubules or of marker overlap. One particular site of importance for the latter is Suppl. Fig. 7h, in which an overlap image of the inset is not provided.

We removed this panel from our manuscript to simplify it.

24. Line 281 - reference 38 does not mention PX-GFP as a PtdIns3P probe.

This reference does mention PX-GFP as a PI(3)P probe. See table 1 (PX p40phox). We modified the text to make it clear that this is the construct we used in the study. (See line 338-339).

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

The manuscript by Boutry et al describes how vesicles derived from the Golgi generated by the action of PI4KB/PI4P regulate PI3P signalling to control formation and fission of tubules from lysosomes. The authors carried out extensive high resolution cell microscopy following the formation of endolysosomes, autolysosomes, and phagolysosomes.

While I am not an expert in the microscopy approaches utilised, the methods are clearly described and the conclusions with one exception appear to be well justified.

Major concerns.
1. My major concern is the PI4KB inhibitor utilised. As the authors note, PI3P plays a very important role in the processes under study. The authors utilised a non-specific PI4KB inhibitor for a variety of their tests (see Fig 3C, 3G, etc). As PIK93 equally inhibits vps34 (the main source of PI3P), PI4KB, as well as some class I PI3Ks, this complicates the analysis of these experiments. While there are knockout experiments of PI4KB, and experiments showing the critical role of PI4KB localisation, to fully prove their underlying hypothesis would require testing a more PI4KB selective inhibitor (many commercially available, see https://www.medchemexpress.com/Targets/PI4K.html as a reference point).

I would suggest the authors repeat the PI4K inhibitor experiment in Fig 3C+G with one of the more selective PI4Kb inhibitors to validate that this also leads to increased number of lysosomal tubules and decreased rate of tubule fission.

We thank the reviewer for their assessment of the manuscript and for pointing out this caveat of using PIK93 as an inhibitor. As suggested, we repeated these experiments using another, more specific, inhibitor called PI4KIIIb-IN-10 and obtained the same results (now in **Fig 4c,d** and **e**).

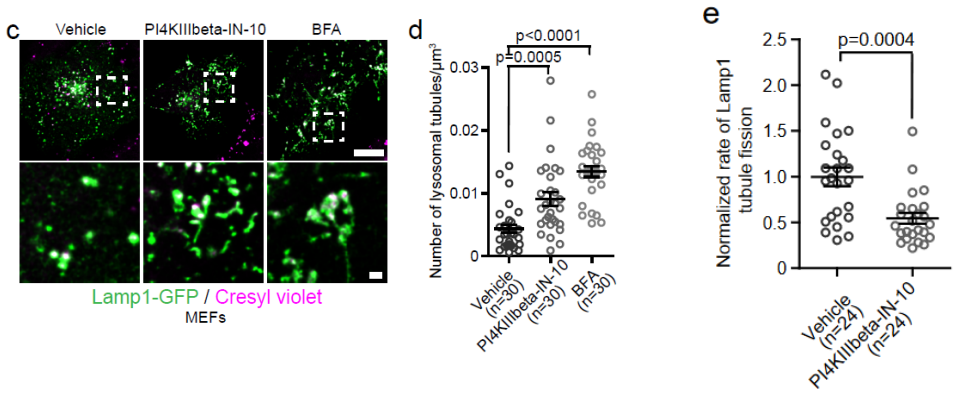


Fig 4c-d. c. MEFs treated with the PI4KIIIβ inhibitor PI4KIIIbeta-IN-10 (25nM for 3 hrs) or Arf1 activation inhibitor Brefeldin A (BFA; 10µg/mL) stained with the acidic organelle marker cresyl violet. Scale bars: 10µm and 1µm (inset). **d**. Quantification of the number of lysosomal tubules in cells described in (**c**). One-way ANOVA with Dunnett's Multiple Comparison Test. **e**-f. Normalized rate of Lamp1 positive tubule fission in MEFs starved for 8H in HBSS treated with PI4KIIIbeta-IN-10 (25nM, 3 hrs) (**e**) before imaging. Two-sided, unpaired t-test.

These results are consistent with the increased number of lysosomal tubules observed when PI4KIIIβ was depleted using shRNA (Sridhar et al., 2013).

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

The manuscript builds on the burgeoning line of evidence that Golgi-derived vesicles assist in organelle fission reactions via membrane contact sites, in conjunction with the ER. Arf1 positive vesicles are shown to make frequent (~1/5 lysosomes) and stable (> 30s) contacts, and to be present at more than half of observed tubule fission events from autolysosomes, phagolysosomes and endolysosomes. Chemical inhibition of Arf1 or its effector, PI4KIIIbeta, increases lysosomal tubules and decreases their fission rate, suggesting a role in the process. Mechanistically, the lipid product of PI4KIIIbeta is shown to be present on the tubules. Depletion of this lipid by enzymatic removal causes loss of the tubules. The effects of Arf1 or PI4KIIIbeta inhibition are phenocopied by knockdown of the PI transfer protein, SEC14L2, which is shown to localize in part to Arf1-positive vesicles. Intriguingly, SEC14L2 knockdown prevents PI3P enrichment at lysosomes (as does inhibition of VPS34), which also increase lysosomal tubules. Depletion of PI3P by a Arf1-targeted PI3P phosphatase also increases tubulation, indicating aberrant fission. The paper concludes that "Golgi-derived vesicles contribute to the fission of lysosomal tubules of a wide range of lysosomal organelles by promoting a SEC14L2 dependent PI(3)P signaling at the site of fission."

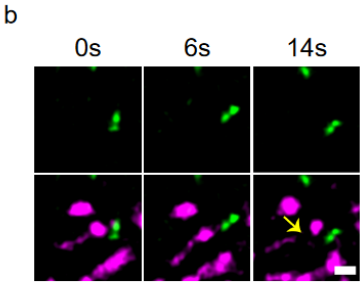
Overall, the manuscript is clearly written, the experiments are thorough and analyzed with appropriate, statistically rigorous approaches, and the data are overall clear and convincing. The linking of Golgi-dervied vesicles to lysosomal tubules formation and membrane recycling is novel and adds to to the field. That said, the mechanistic model is not clearly defined or compelling. Specific areas needing clarification to support this model include:

We thank the reviewer for assessing our manuscript, raising these concerns and suggesting various experiments to improve our study. We now provide a revised version of the manuscript with additional data that strengthen the link between SEC14L2, PI(3)P and Arf1-PI4KIIIβ positive vesicle and support the proposed model.

(1) Enrichment of Golgi-derived vesicles at fission sites. Controls are performed to show that this occurs more frequently than expected by chance for random distributions. However, given that (as shown in fig S2), the ER is present at most fission sites, and that many organelles, including lysosomes, maintain tight contact with the ER, can the authors rule out that Arf1-vesicle and lysosomal tubule co-localization is not simply driven by the co-incidence of these organelles on ER tubules?

To address this concern, we took two approaches. First, instead of using a calculated random distribution, we rotated the Lamp1-mCherry channel by 90° and quantified the number of Arf1-vesicles at the site of fission. This resulted in 26% of fission sites with Arf1-vesicles in the rotated images compared to 55% in the normal images (**Fig 1g**). Secondly, we also examined the number of peroxisomes at these sites of fission. Peroxisomes are virtually all in contact with the ER (Hua et al., 2017), therefore, they can be used to test whether co-incidence contact with the ER can drive the co-incidence of two organelles on the ER. As shown in this quantification (**Sup Fig 2b**), peroxisomes are observed at only 20% of fission events, which was similar to 90° as a negative control (20%) but lower than Arf1 positive vesicles supporting that Arf1 vesicles are not found at fission sites simply because they are contacting the ER. This indicates that contact with the ER is not enough to be recruited to the site of Lamp1 tubule fission sites and supports that the Arf1 positive vesicles are not recruited

there by chance. However, we believe the ER may be actively involved in the recruitment of the Arf1 vesicles to the site of fission, and we discussed that in the discussion section.



Lamp1 tubule division	n = 49 events
ub-GFP-SKL positive	20.4 %
90° rotated control	20.5 %

Sup. Fig 2b. Representative time-lapse images showing the absence of ub-GFP-SKL (Peroxisome) at a Lamp1 positive tubule fission site in MEFs starved (HBSS) for 8 hours and quantification of percentage of fission events marked by ub-GFP-SKL. Negative control analysis was performed with the Lamp1-mCherry signal rotated by 90°. n = 49 events from 22 cells. Yellow arrow indicates fission. Scale bar = 1µm.

(2) PI(4)P is required for tubule formation. Although the process is blocked by PIK93, is this because post Golgi vesicles are depleted, given the roles for this lipid in Golgi traffic? Furthermore, is the tubule associated PI(4)P really generated by PI4KIIIbeta? Type II PI4K are known to associate with endosomes and lysosomes, and there are commercially available inhibitors such as PI-273. Could that be the source of PI(4)P on tubules? The SAC targeting experiment is convincing, but does not reconcile whether this has anything to do with Arf1 vesicles or PI4KIIIbeta generated PI(4)P.

We do not propose in this manuscript that PI4KIIIβ is producing the PI(4)P required for tubulation of lamp1 positive organelles. With respect to PI4KIIIβ, our data support that this enzyme is likely not responsible for the production of PI(4)P required for Lamp1 tubule formation as we found that its inhibition leads to an increased number of tubules. Our data support this increase is due to its role in the formation and/or function of Arf1-PI4KIIIβ positive vesicles at the tubule fission site.

Although we agree with the reviewer that identifying the specific PI4K producing PI(4)P required for the formation of lysosomal tubules would be of great interest, such studies are outside the scope of this manuscript which is to determine the role of Arf1-PI4KIIIβ vesicles in the fission of lysosomal organelle tubules. We believe that identifying the specific PI4k controlling tubulation would require its own study.

To prevent any misunderstanding about our views on PI4KIIIβ, we clarified the text and discussion about this point, notably by discussing the potential source of PI(4)P involved in tubulation (Lines 565-569).

(3)Even though data in figure 5 clearly supports a requirement for SEC14L2 in tubules fission, its role in the process in not clearly defined. Figure 6 demonstrates a requirement for Vps34-derived PI(3)P in tubules fission, though the only functional data is figure 6A/B, where SEC14L2 knockdown produced a modest depletion of PI(3)P, which is certainly more subtle than the Vps34 inhibitor

effect, and (ii), the ability of Arf1-MTM1 to recapitulate the effect of VPS34 inhibition or lysosomal PI(3)P depletion, though as the authors themselves state in the discussion, this could occur in trans and does not support a role for the Arf1 vesicles in tubule fission directly.

In this manuscript, we have four additional experiments to further describe the role of SEC14L2 in lysosome tubule fission. First, we show that both the lipid binding domain and the PI(3)P binding ability of the protein are required for fission by performing experiments using mutants version of zSec14l3 (the SEC14L2 homologue in zebrafish): the zSec14l3-M5 that is unable to bind PI(3)P (as shown in Gong et al., 2021) and the zSec14l3 ΔCRAL-TRIO that lacks the lipid binding domain. Overexpression of these constructs did not decrease the number of Lamp1 positive tubules in cells depleted of SEC14L2 using siRNA, while overexpression of wild-type zSec14l3 was able to rescue the phenotype. Moreover, the rate of Lamp1 tubule fission in cells depleted of SEC14L2 using siRNA was only increased by overexpression of wild-type zSec14l3, while expression of the PI(3)P binding deficient mutant M5 had no effect. Together, these experiments demonstrate that SEC14L2 needs both its PI(3)P binding ability and lipid binding domain to mediate the fission of Lamp1-positive tubules. These data are shown in **Fig. 8a-c**.

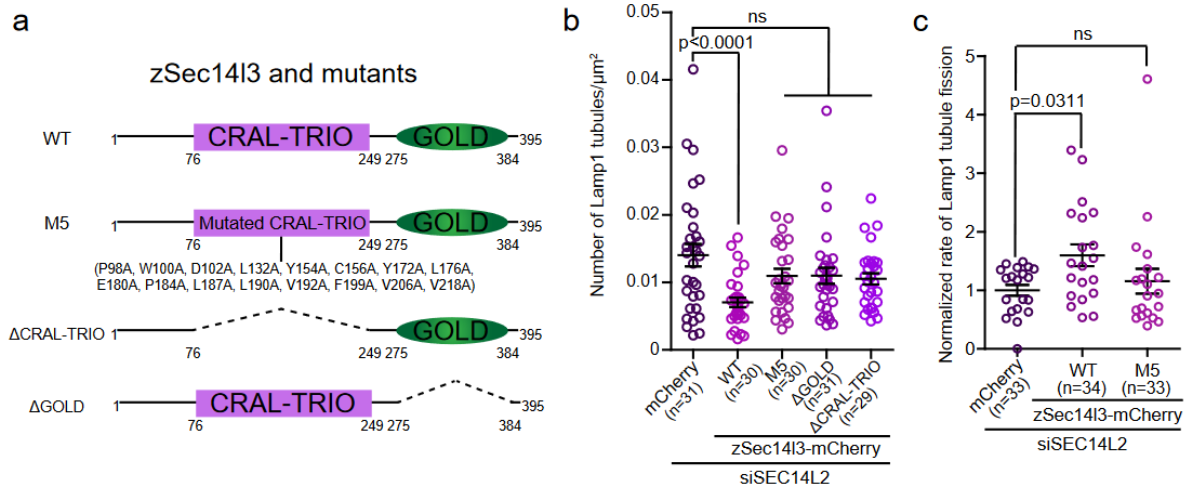


Fig 8a-c. a. Cartoon illustration of the various zSec14l3 constructs used in the study. **b**. Quantification of the number of Lamp1 positive tubules in cells treated with indicated siRNA and expressing the indicated constructs. One-way ANOVA with Dunnett's Multiple Comparison Test. p-value = 0.1776 (M5), 0.1808 (ΔCRAL-TRIO) and 0.1069 (ΔGOLD). **c**. Normalized rate of Lamp1 positive tubule fission in cells treated with indicated siRNA and expressing the indicated constructs. One-way ANOVA with Dunnett's Multiple Comparison Test. ns = 0.7442.

Second, we looked at PI(3)P levels at lysosomes in cells depleted of SEC14L2 using cresyl violet, which marks acidic lysosomes (Ostrowski et al., 2016) and observed that SEC14L2 depletion leads to a decreased level of PI(3)P at lysosomes (**Fig 8h**). This experiment replaces a previous experiment using Lamp1, as this marker is not restricted to lysosomes. Moreover, overexpression of wild-type zSec14l3 but not of the PI(3)P binding deficient mutant M5 increased the level of lysosomal (identified using overnight chased fluorescent 10kDa Dextran) PI(3)P in cells depleted of SEC14L2 (**Fig 8i**). This shows that SEC14L2 plays an important role in the regulation of lysosomal PI(3)P in a mechanism dependent on its PI(3)P binding ability.

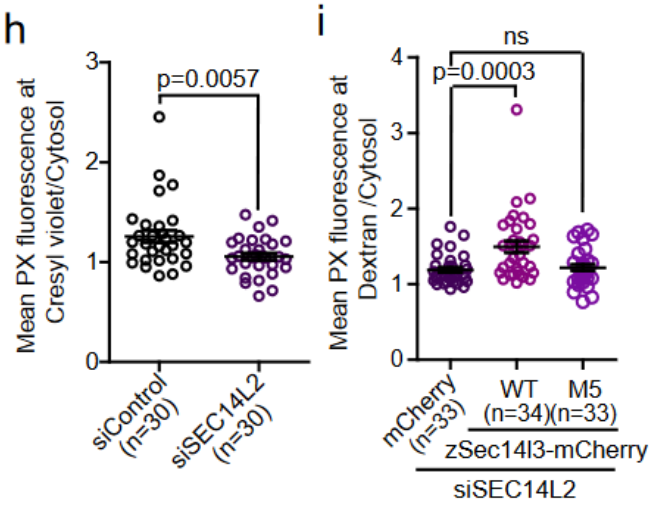


Fig 8h. Quantification of the PX levels colocalizing with cresyl violet normalized to the cytosolic level of the probe of cells in (**Fig 8g**). Two-sided unpaired t-test. **i**. Quantification of the PX levels colocalizing with overnight chased 10kDa fluorescent Dextran normalized to the cytosolic level of the probe in HeLa cells treated with indicated siRNA and expressing the indicated constructs. One-way ANOVA with Dunnett's Multiple Comparison Test. ns = 0.8898.

Third, we fused MTM1 to SEC14L2 and evaluated the effect of its expression on the number of Lamp1-positive tubules. We find that, similar to Arf1-MTM1, expression of this construct leads to an increased number of Lamp1 tubules (**Fig 8o,p**). While this construct suffers from the same caveat as the Arf1-MTM1 one in terms of possible activity in *trans*, we believe it strengthens the link between SEC14L2 and PI(3)P regulation as it demonstrates that depleting PI(3)P where SEC14L2 localizes, or on its immediate proximity, affects the fission of Lamp1 positive tubules.

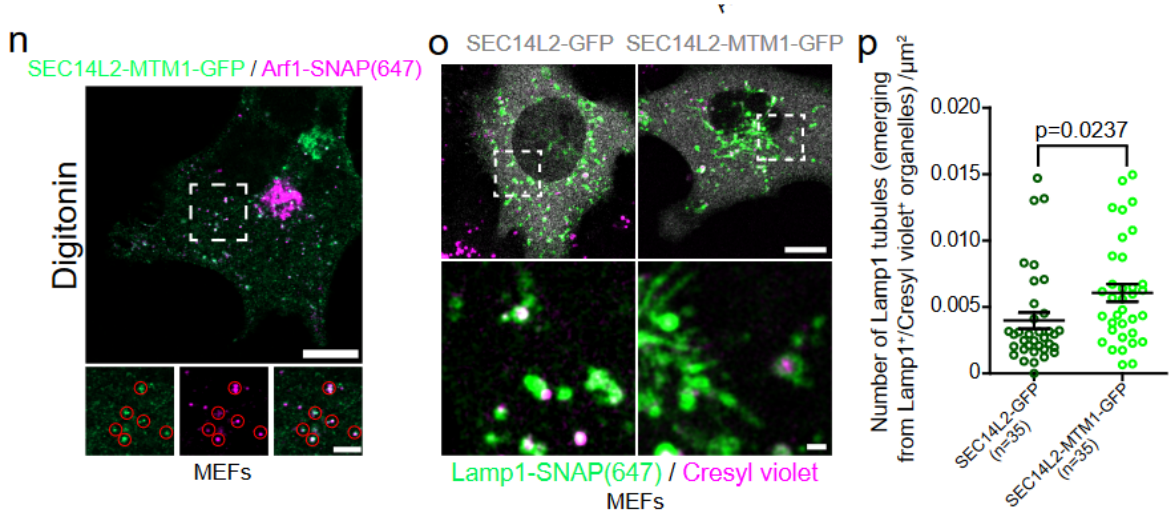
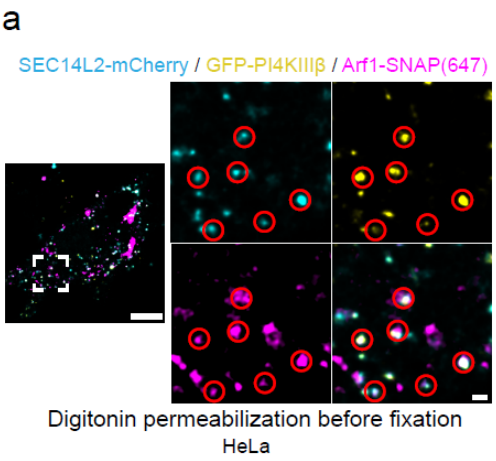


Fig 8n. **n**. Representative Airyscan image of a MEF cell expressing SEC14L2-MTM1-GFP and Arf1-SNAP. Red circles show SEC14L2-MTM1 colocalizing with Arf1 positive vesicles. Scale bar: 10µm and 1µm (inset). **o-p**. Representative Airyscan images of MEFs expressing Lamp1-SNAP and SEC14L2-GFP or SEC14L2-MTM1-GFP and treated with Cresyl violet to mark acidic lysosomes (scale bar: 10µm and 1µm (inset)) (**o**) and the quantification of the number of lysosomal tubules (**p**). Two-sided unpaired t-test.

Finally, we also provide further evidence that SEC14L2 is directly involved in the fission of lysosomal tubules mediated by the Arf1-PI4KIIIβ positive vesicles as we show that overexpressed SEC14L2 localizes to a subset Arf1-PI4KIIIβ positive vesicles (**Fig 7a**) and that its depletion decreases not only the rate of lysosomal fission but also the percentage of Lamp1 tubules fission events marked by Arf1 positive vesicles (**Fig 7l**).



I

n = 27 cell

	% of Lamp1 tubules fission marked by Arf1-GFP
siControl	62.1% (54/87)
siSEC14L2	35.6% (21/59)

Fig 7a. Representative live image of a HeLa cell expressing SEC14L2-mCherry, GFP-PI4KIIIβ and Arf1-SNAP. Excess cytosolic signal was removed by short-term permeabilization with digitonin before fixation. Red circles show SEC14L2 colocalization with Arf1-PI4KIIIβ positive vesicles. Scale bar: 10μm and 1μm (inset). **I**. Quantification of the number of Lamp1 positive tubule fission events marked by an Arf1 positive vesicle in HeLa cells treated with indicated siRNAs and starved (HBSS) for 8 hours.

Collectively, we believe this set of new data strengthens the links between SEC14L2, Arf1-PI4KIIIβ positive vesicles and PI(3)P regulation at lysosomes and supports our proposed model.

To summarize my major concerns, this paper has four main findings:
(a) Golgi-dervied vesicles are enriched at sites of lysosomal tubule fission - but as per my point 1 above, clarity is needed as to whether these are truly enriched given their co-incident localization on the ER

We discussed and provided new data to answer this concern above in our response to concern 1)

(b) PI(4)P is required for the tubule fission process - though it is not clearly established whether Golgi-dervied PI4KIIIbeta is responsible for this (point 2)

We propose that PI(4)P is important for the formation of tubules (**see Fig 5**). However, we do not propose a role for PI(4)P but for PI(3)P in the fission process. Whether PI(4)P plays a direct role in the fission process was not tested in this study. We clarified the manuscript in various parts and also discussed the potential source of PI(4)P production for the formation of lysosome tubules (Lines 565-569).

(c) PI(3)P signaling intrinsic to the lysosomal membrane is also required for tubule fission
(d) SEC14L2 is also required for this process, but apart from its localization to Golgi-derived vesicles (fig. 5b), its relation to (b) and (c) are not established, apart from the reference to prior precedence in the literature.
We discussed and provided new data to answer this concern above in our response to concern 3)

There are also a number of minor technical comments which should be addressed:

(4) Fig 1E: the mean and error not as informative as an indication of the distribution, displayed as perhaps a histogram or scatter blot.

The figure was modified as suggested.

(5) Fig 4 title: "PI(4)P has a pro-tubulation role as lysosomes"; perhaps "at lysosomes" was intended?

We thank the reviewer for pointing out this error. We corrected the title of the figure.

(6) in figure 1h, the blue and green are hard to distinguish, especially in the legend. Perhaps more contrasting hues could be used.

We modified the colours to increase the contrast as suggested.

(7) Fig 5n: purple and black are also hard to distinguish, and in this figure panel particularly, the reviewer believes the legends have been accidentally swapped.

We slightly modified the colors on this particular figure to increase the contrast with black.

(8) p. 17 (methods) Refers to a Leica SP8 Lightning microscope. The reviewer believes this should be a "Lightning microscope".

Yes. The reviewer is correct, and we have made the change.

May 8, 2023

RE: JCB Manuscript #202205128R-A

Prof. Peter K Kim
Hospital for Sick Children
686 Bay St
Rm 19.9708
Toronto, Ontario M5G 1X8
Canada

Dear Prof. Kim,

Thank you for submitting your revised manuscript entitled "Arf1-PI4KIIIβ positive vesicles regulate phosphatidylinositol-3-phosphate signalling to facilitate the fission of lysosomal tubules." We would be happy to publish your paper in JCB pending final text revisions necessary to address the remaining reviewer comments and to meet our formatting guidelines (see details below). As you will also see below, Reviewer #1 suggests an additional experiment to confirm that PtdIns3P are required for tubule scission. This experiment is not required for resubmission but we would certainly welcome such data, if you wish to add it, as it would further enhance your very interesting study.

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 title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figure formatting: Articles may have up to 10 main text figures. Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add scale bars to Figure S5c and the magnification images in Figures 6k & 7c.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

3) 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. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

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 (both in the figure legend itself and 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."

4) The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership. Your current title exceeds the limit and we therefore suggest shortening it to:
"Arf1-PI4KIIIβ positive vesicles regulate PtdIns3P signaling to facilitate lysosomal tubule fission"

5) 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 (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Please also indicate the type of membrane used for immunoblotting/western blots.

6) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible.

7) 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.).

8) 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.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. You currently exceed this limit but, in this case, we will be able to give you the extra space but please try not to add to the current total. 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. Please include one brief sentence per item.

10) Video legends: Should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

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. It should begin with "First author name(s) et al..." to match our preferred style.

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) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

14) 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.

15) JCB 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 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

16) Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will

be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

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
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

The manuscript by Boutry et al has been extensively revised to address reviewer concerns. I am extraordinarily impressed with the improvement to the manuscript. I found the revised manuscript much easier to read and to follow the results presented in the figures, the conclusions were nearly completely modified to an appropriate level, and the new data make a significant addition to the story that was offered in the original manuscript. The discussion is now much more scholarly and complete with an appropriate balance of critical evaluation and speculation. The paper now conclusively documents the role of Arf1/PI4KIIIbeta-containing membranes in facilitating severing of lysosome- and phagosome-derived membrane tubules, and also documents a role for phosphatidylinositol-3-phosphate (PtdIns3P), enriched on lysosomes in a SEC14L2-dependent manner, in facilitating this process. The paper represents an important advance that also clears up a misconception from the literature, and is thus suitable for readers of The JCB.

The new data added are largely complete and convincing, but there is one conclusion drawn that is not fully supported, as indicated in comment #1 below. This can be addressed without adding new data simply by toning down the conclusion. In addition, the data in one panel of Figure 7 are not particularly convincing; I suspect they can be replaced with other available data. Lastly, there are a few minor concerns with the text that can be easily addressed.

1. While the new data in Figure 6 and Suppl. Fig 7 documenting the increase in PtdIns3P on lysosomes in Arf1-containing lysosomes and phagosomes and its requirement for severing tubules, there are no experiments indicating that Arf1 or PI4KIIIbeta are actually required for this increase; the data provide only a correlation of the PtdIns3P increase with Arf1 recruitment. Does treatment with BFA or targeted recruitment of Sac1 impair the increase in PtdIns3P? Without such experimental support, the authors can only speculate that PtdIns3P is required for the scission event.

2. While the quantification in Figure 7n is convincing, it is difficult to see the enlarged lysosomes in the example images shown in Figure 7m.

Minor concerns:

3. Line 56: Spastic is misspelled.

4. Text lines 296-304 vs. Suppl. Fig. 6a, b: The naming of PI4KIIIbeta used throughout the manuscript is altered in the labeling of the Figure in Fig. 6a, b, where it is called PI4KB. It would be helpful to readers to be consistent in using the PI4KIIIbeta nomenclature here (or, if preferred, PI4KB throughout).

5. Text lines 310-316 and Figs. 5f-h, Suppl. Figs.6c-h: The authors claim that fusing active SAC1 to lysosomal targeting constructs decreases tubule formation, but they do not compare the numbers obtained with these constructs to control cells - only to cells expressing catalytically inactive construct (a nice control, but its expression might induce its own effect). A comparison to the numbers from controls (even if based on other experiments in the paper) would be important to draw the conclusion that digestion of PtdIns4P on lysosomes impairs tubulation.

6. Text line 361: Please indicate that acute recruitment is mediated by treatment with the cell permeant GA3-AM reagent that joins the GID1 and GAI elements of the fusion proteins. Similarly on line 369, please indicate that PI3K inhibition was mediated by treatment with the chemical inhibitor VPS34-IN1.

7. Figure 7a is confusing in that there are no labels to indicate what is shown in each panel. It looks like the upper left is SEC14L2 alone, upper right is PI4KIIIbeta alone, lower left is Arf1 alone, and lower right is a merge of all 3. This should be indicated on the figure itself (if I am correct, or some other way if I am not).

8. While I understand that it is defined in the figure legend, it would be super helpful to readers if on line 415, the definition of enlarged - just "(>1 micron)", only 4 characters - would be added.

9. In the discussion, is it possible that PtdIns4P generated by type II PI4Ks on lysosomes might be exchanged by SEC14L2 for PtdIns3P? This could provide roles for both PtdIns4P and PtdIns3P in tubule fission. Note, a positive role for PtdIns4P in lysosomal or phagosomal tubule formation was also documented by López-Haber et al., 2020, PNAS 117: 28251 (PMID: 33109721).

10. The manuscript should be carefully vetted throughout for grammar, syntax, and spelling mistakes.

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

The authors have thoroughly addressed all of my concerns.

I have one, additional minor point. Of the two models for SEC14L2 function, one is PI3P transfer and the other is a yet undefined VPS34 activation. Is a real sub-possibility for this not supply of PI substrate to the VPS34 from the Arf1 vesicles? This is probably worth a mention in the discussion, since this is an original function of the Sec14 domain.

2nd Revision - Authors' Response to Reviewers: May 17, 2023

We thank the reviewers for taking the time to review our manuscript again. As we mentioned in our last response, we found the reviewers' constructive comments extremely helpful and have helped us greatly improve our manuscript. Below are our responses to the latest comments.

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

The manuscript by Boutry et al has been extensively revised to address reviewer concerns. I am extraordinarily impressed with the improvement to the manuscript. I found the revised manuscript much easier to read and to follow the results presented in the figures, the conclusions were nearly completely modified to an appropriate level, and the new data make a significant addition to the story that was offered in the original manuscript. The discussion is now much more scholarly and complete with an appropriate balance of critical evaluation and speculation. The paper now conclusively documents the role of Arf1/PI4KIIIbeta-containing membranes in facilitating severing of lysosome- and phagosome-derived membrane tubules, and also documents a role for phosphatidylinositol-3-phosphate (PtdIns3P), enriched on lysosomes in a SEC14L2-dependent manner, in facilitating this process. The paper represents an important advance that also clears up a misconception from the literature, and is thus suitable for readers of The JCB.

The new data added are largely complete and convincing, but there is one conclusion drawn that is not fully supported, as indicated in comment #1 below. This can be addressed without adding new data simply by toning down the conclusion. In addition, the data in one panel of Figure 7 are not particularly convincing; I suspect they can be replaced with other available data. Lastly, there are a few minor concerns with the text that can be easily addressed.

1. While the new data in Figure 6 and Suppl. Fig 7 documenting the increase in PtdIns3P on lysosomes in Arf1-containing lysosomes and phagosomes and its requirement for severing tubules, there are no experiments indicating that Arf1 or PI4KIIIbeta are actually required for this increase; the data provide only a correlation of the PtdIns3P increase with Arf1 recruitment. Does treatment with BFA or targeted recruitment of Sac1 impair the increase in PtdIns3P? Without such experimental support, the authors can only speculate that PtdIns3P is required for the scission event.

We agree with the reviewer that our data only show a correlation between the Arf1-PI4KIIIβ vesicle recruitment at the site of lysosomal tubule fission and the PI(3)P increase on the lysosome. We do not demonstrate that Arf1 or PI4KIIIβ or the vesicles that contain them directly cause an increase in PI(3)P that is required for the fission event. However, our data demonstrate the need for Arf1-PI4KIIIβ vesicles and of PI(3)P on lysosomes for the fission of lysosomal tubules. Since we do not directly show the connection between the two events, as the reviewer correctly points out, we modified two sentences to make this point clear.

Line 552-553: "Collectively, these data suggest that Arf1-PI4KIIIβ positive vesicles mediate a PI(3)P signalling to drive the fission of lysosomal tubules."

Line 605-508: "Our study supports that Arf1-PI4KIIIβ positive vesicles play a significant role in promoting SEC14L2-dependent PI(3)P signaling at the site of fission, leading to the fission of lysosomal tubules in a wide range of lysosomal organelles"

Furthermore, we modified the title of Fig. 6 to "Arf1 positive vesicle-mediated Lamp1 tubule fission events are associated with a PI(3)P increase and depletion of lysosomal PI(3)P impairs tubule fission.". Finally, we modified the subheading of the corresponding section of the results to split it in

two separate subheadings titles: Line 351-352: “Arf1-PI4KIIIβ positive vesicles at Lamp1 tubule fission sites are associated with a PI(3)P signal” and line 380 “Depletion of lysosomal PI(3)P impairs the fission of lysosomal tubules”

2. While the quantification in Figure 7n is convincing, it is difficult to see the enlarged lysosomes in the example images shown in Figure 7m.

We thank the reviewer for pointing out this, we increased the size of the insets of the images of Fig. 7 M.

Minor concerns:

3. Line 56: Spastic is misspelled.

The text was corrected.

4. Text lines 296-304 vs. Suppl. Fig. 6a, b: The naming of PI4KIIIbeta used throughout the manuscript is altered in the labeling of the Figure in Fig. 6a, b, where it is called PI4KB. It would be helpful to readers to be consistent in using the PI4KIIIbeta nomenclature here (or, if preferred, PI4KB throughout).

We modified the text, legends and figures. PI4KB was changed to PI4KIIIβ for the LysoGFP-PI4KIIIβ construct.

5. Text lines 310-316 and Figs. 5f-h, Suppl. Figs.6c-h: The authors claim that fusing active SAC1 to lysosomal targeting constructs decreases tubule formation, but they do not compare the numbers obtained with these constructs to control cells - only to cells expressing catalytically inactive construct (a nice control, but its expression might induce its own effect). A comparison to the numbers from controls (even if based on other experiments in the paper) would be important to draw the conclusion that digestion of PtdIns4P on lysosomes impairs tubulation.
We modified the text to indicate that the comparison was made to cells overexpressing the catalytically dead construct.

Line 332-333: “Overexpression of ORPSAC1 in MEFs led to fewer Lamp1 tubules after starvation compared to overexpression of the catalytic dead mutant C392S”

The tubule numbers obtained for the catalytic dead are remarkably similar to that obtained from Lamp1 overexpression, as seen in Fig. 5 E. We did not add the comparison of the number of lamp1 tubules from other controls that were used in other experiments, as the catalytically dead mutant is the best possible control for these experiments.

6. Text line 361: Please indicate that acute recruitment is mediated by treatment with the cell permeant GA3-AM reagent that joins the GID1 and GAI elements of the fusion proteins. Similarly on line 369, please indicate that PI3K inhibition was mediated by treatment with the chemical inhibitor VPS34-IN1.
We added both pieces of information to the text.

7. Figure 7a is confusing in that there are no labels to indicate what is shown in each panel. It looks like the upper left is SEC14L2 alone, upper right is PI4KIIIbeta alone, lower left is Arf1 alone, and lower right is a merge of all 3. This should be indicated on the figure itself (if I am correct, or some other way if I am not).

We added these labels to Fig. 7 A.

8. While I understand that it is defined in the figure legend, it would be super helpful to readers if on line 415, the definition of enlarged - just "(>1 micron)", only 4 characters - would be added.
This was added to the text.

9. In the discussion, is it possible that PtdIns4P generated by type II PI4Ks on lysosomes might be exchanged by SEC14L2 for PtdIns3P? This could provide roles for both PtdIns4P and PtdIns3P in tubule fission. Note, a positive role for PtdIns4P in lysosomal or phagosomal tubule formation was also documented by López-Haber et al., 2020, PNAS 117: 28251 (PMID: 33109721).

We thank the reviewer for suggesting this idea. Indeed, we cannot exclude this possibility. However, it is unlikely since we did not see any decrease in PI(4)P levels at lysosomes showing fission of a tubule (Fig. S7 C) suggesting that PI(4)P levels are not decreasing in the seconds before fission, while we detected a clear increase in the levels of PI(3)P. This suggest that PI(4)P and PI(3)P are not exchanged. We elected to not include this possibility in the discussion for space consideration.

10. The manuscript should be carefully vetted throughout for grammar, syntax, and spelling mistakes.
We had the manuscript edited by two other readers.

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

The authors have thoroughly addressed all of my concerns.

I have one, additional minor point. Of the two models for SEC14L2 function, one is PI3P transfer and the other is a yet undefined VPS34 activation. Is a real sub-possibility for this not supply of PI substrate to the VPS34 from the Arf1 vesicles? This is probably worth a mention in the discussion, since this is an original function of the Sec14 domain.

Indeed, this model is consistent with our observations. However, SEC14L2 was shown to bind only weakly to PI compared to PI(3)P (Gong et al., 2021). We discuss this point in the discussion in lines 576-580.