An endosome-associated actin network involved in directed apical plasma membrane growth

Luis Rios-Barrera and Maria Leptin

Corresponding Author(s): Maria Leptin, European Molecular Biology Organization and Luis Rios-Barrera, National Autonomous University of Mexico

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Revision 0

Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

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Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

The authors investigate the role of late endosomes in

the context of actin organization during cell morphogenesis. They use as experimental model the polarized terminal cells in the Drosophila tracheal system that forms a sub-cellular projection containing a tube. The authors show that disruption of the sub-cellular localization or maturation of late endosomes leads to increased proportion of terminal cells with mis-guided tubes. Their analysis indicated that endosomal F-actin recruitment is crucial for the directionality of the tube growth. The authors propose a model where, late endosomes control a coordinated crosstalk between endosomal and cortical actin pools to drive subcellular tube-guidance.

Major comments:

1. The conclusion about how WASH functions in the tube-guidance, is not clearly shown and it should be better explained and documented. It is known that loss of function of the WASH leads to dysregulation of endosomal tubulation inducing enlarged endosomes, which in turn affects the endosome-to-plasma-membrane recycling of various cargos (including luminal cargos like Serp) (Gomez, et al., Mol. Biol. Cell, 2012; Dong et al, Nat. Comm. 2013). The authors should clarify if there is a defect in the integrity of endosomes located close to the cell tip in the btl>WASHIR knock down. In the cartoon panel C' (Figure 7), the endosomes in the cell tip are shown intact (Including their relative position to the tip) but no experimental data support this conclusion.

-The functional analysis of WASH was based on RNAi knock down. The authors express a single RNAi construct against WASH. The expression of this RNAi line gave a low penetrance phenotype. A well-known caveat of RNAi is off-targeting. Hence, phenotypic analysis needs to include a verification by a second independent RNAi construct or a rescue of the RNAi phenotype with an overexpressed cDNA of WASH. Ideally, the null wash mutant (Nagel et al. 2017) can be used to confirm the phenotype.

2. The authors claim a role of the late endosomes in subcellular tube growth and guidance. But show no data on lumen formation to prove tube presence in the tracheal terminal cells of V100R755A, btl>WASHIR, shrb mutants or in GrabFP-Bint treated terminal cells. The interpretation and quantification of the phenotypic classes "miss-tube-guided" and "ventrally-tube-guided" are based on membrane markers and not on luminal markers. The presented data with the provided resolution does not prove if the mCD4-mIFP or PH-GFP markers define apical membrane protrusions/extensions or tubular structures. Therefore, the classifications of the tube-guidance phenotype and the quantification of "distance from tube to tip" may be suggestive. The authors need to provide additional confocal data of co.stainings of the endosomal compartments with luminal antigens (i.e. GASP or Serp or Verm).

3. page 8 line 248, the authors interpret that reducing the dose of Shrb by half strongly enhances the wash-RNAi phenotype and suggest that WASH and Shrb act in the same pathway. Shrub is a subunit of the ESCRT-III complex involved in inward membrane budding of endosomes and WASH functions in outward endosomal membrane budding.

The Shrb and WASH form discrete molecular complexes in endosomes. The authors should consider that Shrb and WASH may well act in parallel to control directional tube growth.

4. The authors use nanobody-based GFP trap construct to investigate the effect of Rab7YFP localization. This is an excellent way to provide novel information for protein miss-localization in vivo. Using this method the authors concluded that ... "the correct distribution of late endosomes is required for proper tube guidance" (page 5, lines 157-158). The authors obviously consider that GrabFP-B-Int construct affected the distribution of late endosomes. However, this is unclear and additional control experiments are needed to support the author's claims. For instance, did expression of GrabFP-B-Int, target the Rab7-YFP protein or the Rab7-associated endosomes? With the presented data, it is not clear if the Rab7-YFP positive vesicles are endosomes? or aggregates formed by the trapped Rab7-YFP protein? Co-stainings using GFP in Rab7-YFP terminal cells with another endosomal markers i.e. Avl, or hrs, should be provided. It is also not clear if endocytosis of apical/basal membrane or luminal cargos was affected in GrabFP-B-Int treated terminal cells. The loss of endocytic components has been associated with defects in subcellular tube shape and morphology (Schottenfeld-Roames et al, Cur Biol. 2014). The authors should clarify these issues.

5. In the legends of Figure 7 (C'), the authors stated that.... "lack of actin regulators at the basal cortex prevents the connection of the actin meshwork at the tip to the basal plasma membrane".... by depicting the singed mutant phenotype. singed mutant analysis is not shown in the manuscript.

6. The authors consider the late endosomes nucleate actin ahead of the tube (i.e. page3, line 87-88, page 9, line 285). This is not very convincing from the presented data. The authors should provide some quantitative data showing that lack of WASH (and endosomal F-actin network) effects the apical and basal F-actin pools in the tip of the cell.

Minor concerns:

1. The authors concluded (page 9, line 285) that "endosomes serve as actin nucleating centres that propagate forces within the cell by physically linking different subcellular compartments". The authors may want to consider that endosomes can serve as platforms to assemble important actin polymerization regulators and/or signals in the tip of the terminal cell to instruct tube directionality.

2. The authors should depict in the panels the Ventral/Dorsal axis.

3. Numerous omissions need to be corrected. Labeling is missing in the panels J-M' (Figure 1). The statistical significance and the p values levels are not indicated in Figure 2 (G). The panel figure 5 (D) is miss-labelled. The panels C-C' in figure 7 are not very informative. They do not reflect the general model of the study. How the prevention of actin nucleation at late endosomes, or apical or basal cortex affects tube directionality is not graphically shown.

4. In the section "Crosstalk between cytoskeletal compartments" (Lines 359-400, discussion) the argument about the involvement of microtubules in tube-guidance is a likely scenario. But I found this argument over-extended. WASH interacts with tubulin Derivery et al. Dev Cell (2009) and WASH activity balances the endosomal and cortical F-actin networks during epithelial tube maturation in multicellular tracheal tubes (Tsarouhas et al., Nat. Comm 2019). These results should be considered in the discussion section.

3. Significance:

Significance (Required)

The important role of actin cytoskeleton in the initiation of endocytosis is well established. Actin structures in the plasma membrane are dynamically organized to assist the remodeling of the cell surface and to facilitate the inward movement of vesicles. Similarly actin networks in endosomes are critical for endosomal fusion and fission. In this work, the authors identified an opposing but interesting scenario. They propose a role for the late endocytic pathway in organizing actin networks for proper cell morphogenesis and point out an intracellular crosstalk and coordination between distinct cytoskeletal pools within a cell.

Although the mechanism about how the separate F-actin pools communicate is not shown, the paper is interesting and shows an original contribution in the area of cell morphogenesis. In addition it represents a conceptual advance as it proposes a mechanism through which actin cytoskeleton is coordinated to regulate tube morphogenesis. The proposed mechanism may be relevant for tracheal terminal cells, but could represent a general mechanism in the field of cell biology. The methodology is appropriate and the text flow is well organized. However, as explained, there are few inconsistencies in the manuscript. I believe the above additions would strengthen the conclusion of the paper.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

Rios-Barrera and Leptin investigate the formation and guidance of the subcellular tube that forms in the terminal cell of the dorsal branches of the Drosophila tracheal system. In previous work the authors documented the presence of late endosomes at the tip of the growing terminal cell, ahead of the forming subcellular tube, which are involved in membrane recycling {Mathew, 2020 #1407}. In this present work they analyze the organization of the actin cytoskeleton in the tip terminal cell, in relation with the late endosomes, and assess the guidance of the subcellular tube. They find that the presence and localization of late endosomes play a role in tube guidance. They also find that late endosomes recruit actin around them, mediated by the activity of the actin polymerization regulator Wash, which is recruited to maturing late endosomes. When Wash activity is decreased, actin around late endosomes is decreased and tube guidance is compromised. Based on this observation, laser ablation experiments of actin ahead of the tube and actin staining at the tip of the terminal cell, the authors propose an exciting model: late endosomes recruit actin, which connects the actin pool of the basal membrane and the actin pool of the apical (subcellular tube) membrane thereby directing tube growth and guidance.

The manuscript is well-written and well-presented, the images and movies are of high quality and the experimental data, which is technically challenging, is very good and sufficiently replicated.

Major comments:

1. A critical point in the model that the authors put forward (which is also contained in the title and abstract) is that actin organized at late endosomes anchors apical and basal actin cortices. However, there is no clear and conclusive evidence for this. Clear evidence in this direction should be provided to propose it as a mechanism (as it is in the text, particularly in the first sentences of the discussion) and imply it in the title.

The authors show endogenous actin around late endosomes and actin fibers at the tip of the terminal branch. However, at the level of resolution presented (Fig 3A,B), it is not possible to determine whether the different actin populations are actually "anchored". I suggest to present stronger data supporting this important conclusion.

In the same direction, it would be critical to show that this anchoring of actin fibers is disturbed when actin enrichment at the late endosome is perturbed (see also point 5).

Actually, the authors show that when Vha or Wash activity are downregulated actin accumulation around the CD4 vesicles decrease. However, this experiment has a few inconveniences. First, it is difficult to determine levels of a construct that is overexpressed (UAS-utr::GFP). Could the authors use phalloidin or an actin antibody to confirm the result? Second, I find the result difficult to interpret. In the images provided I see a general decrease of actin (UtrGFP) at the tip, not only around the CD4 vesicles (Fig 6D,F) . Are these mutant conditions also affecting the rest of actin pools? If this is the case, can the authors attribute the defects exclusively to the abnormal recruitment of actin to the late endosomes?

Most importantly, the authors should analyze the pattern of actin distribution (labelling endogenous actin) and determine a possible loss of "anchoring" of fibers when late endosome maturation is perturbed.

2. Another critical point in the model put forward by the authors is that late endosomes drive tube guidance. To test this point the authors use an elegant system to mislocalize Rab7 late endosomes.

However, the effects are not strong (1G), and only a proportion of branches show misguided tubes. Do the cases with a ventrally-guided tube in the experiment Rab7:YFP+/+ (Fig. 1G) have a CD4 endosome (with Rab7YFP) at the tip? This would help to explain the weak effect.

3. What is the cause that preventing proper endosome maturation and acidification leads to misguided tubes (rather than missing ones)?

The authors indicate that downregulating Vha activity leads to defects in acidification, but late endosome-MVB normally form. It is intriguing to see extra CD4 vesicles (like in 1C or 6C).

Wouldn't we expect to see "normal" tip accumulation of CD4 vesicles only, and not extra ones? How relevant are these extra CD4 vesicles?

Wouldn't we expect to see "non functional" CD4 vesicles, unable to recruit actin and lead intracellular tube formation (i.e. no tube) rather than missguidances? (1D shows higher proportion of misguided tubes than no tubes)

Similarly, is Wash-RNAi producing extra CD4 vesicles (as observed in movie 5, fig 6E)?

4. Actin recruitment to late endosomes was already documented, where it plays a role in cargo trafficking.

The authors propose that Wash is recruited to late endosomes upon acidification where it would prime actin nucleation around the endosome. The authors indicate a decrease in Wash accumulation upon expression of Vha dominant negative. However, this decrease is not quantified. In addition, it is difficult to determine levels of a construct when this is overexpressed (UAS-Wash::GFP). It would be desirable to use antibodies against the endogenous protein (Wash in this case) to claim differences in accumulation in mutant conditions.

The results presented do not rule out a requirement of Wash in terminal branching which is not associated with the enrichment in the late endosomes. The genetic interaction observed with Shrub is also compatible with both proteins acting on terminal branching but in different/parallel mechanisms.

5. Laser ablation experiments

The laser ablation experiments are difficult to interpret.

First, it is unclear to me what the results exactly indicate. What does the recoil observed suggest? Does it fit with the expected tension exerted by a link of the actin cytoskeleton relayed by late endosomes?. From the text and figure I don't understand how is the recoil calculated: retraction of the subcellular tube backwards? "enlargement" of the bleached area?

Second, it is unclear to me what laser ablation actually ablates. Does it only affect actin? Or are also CD4-late endosomes and other tip structures affected?

Third, is the recovery observed after ablation correlated with new actin recruitment around old or new late endosomes?

Forth, I find the experiments in cells with secondary subcellular tubes very confusing and the explanations very speculative

Finally, and most importantly. I think that performing laser ablation experiments in mutant conditions that affect actin recruitment (VhaDN and Wash RNAi,....) would be very informative. One would expect to find a decrease in recoil. If this was the case, it would validate, on the one hand, that in control conditions there is a tension that depends (at least in part) on actin organization, and on the other hand it would show that when actin recruitment is affected tension decreases, supporting the "anchoring" model. I understand that laser ablation experiments are not easy to perform, but I think this would be a useful experiment.

To my understanding, as it stands, the laser ablation experiments "....support the notion that adequate cytoskeletal organization at the tip is required for tube guidance and stability" as the authors acknowledge, but they do not convincingly support their "anchoring" model

Other comments:

- From the images presented, it is often difficult to figure out where the subcellular tube forms, the presence of vesicles, the cell morphologies,... and to determine the correlation between the CD4 vesicles and tube guidance.

For instance, in Fig 1H and 1J, is there a "lateral" CD4 vesicle? Why it does not generate a missguided tube?

Fig 1I, are there 2 subcellular tubes? Can the authors mark them? I cannot really visualize them with the CD4 marker, they seem stalled or short or missing.

Fig 1L: what do the authors mean by "corrected" tube sprouts?

It is difficult to identify the cell in Fig 2D-F

- Movie S3: I find it difficult to spot the association of CD4 and utrGFP that the authors point. Can the authors label in the movie the vesicles and the association?

- The results with the Rab7 downregulation and upregulation are not very clear.

Does the downregulation of Rab 7 (Rab7 DN construct) have any effect on tube guidance?

Does it decrease or eliminate actin association with CD4 vesicles in the embryo? The authors show that in the larvae expression of Rab7 DN leads to loss of actin enrichment in Rab7 vesicles. Does this have an effect on terminal branching?

The Rab7 active construct produce effects at larval stages but not in the embryo. Is terminal cell branching in the larvae also dependent on late endosomes? Can the authors show "excess" of late endosomes in the larvae that lead to extra terminal branches? Even that the authors indicate that they cannot detect Rab7Q67L, can they find any effect at embryonic stages (e.g. presence and position of CD4 vesicles, other unrelated effects,...)?

- In some examples in the movies there seem to be a correlation between CD4 vesicles presence/positioning and basal lamellipodia/filopodia or actin enrichment, and also in □-btl experiments. Have the authors explored this? They may want to comment on this in the discussion section.

3. Significance:

Significance (Required)

This work is relevant for the morphogenesis field and deals with the important issue of how the cytoskeleton regulates shape and cellular events. The work represents a deep analysis of a specific issue in the

specialized field of tracheal development, but the results may be relevant for other types of cells forming subcellular tubes. Describing a function of trafficking vesicles (late endosome in this case) in cell morphogenesis (in addition to cargo trafficking) in an in vivo system is also relevant to advance in the cell biology field.

Referees cross-commenting

I agree with the comments of reviewer #3. I find relevant the points raised in "major comments number 2 and 4".

Rios and Leptin, response to referees

GENERAL:

We thank the reviewers for their constructive critique and are pleased they see the results as interesting and of general relevance.

We also acknowledge their concerns on the issue of whether all claims are supported by sufficiently strong data.

Our careful reading and analysis of the points that are raised suggest there are different reasons for the different cases that are brought up:

1. Misunderstandings, due to lack of clarity on our side.
Example: When talking about 'reduced actin', our wording focussed on the *endosome-associated* actin (partly out of consideration for the fact the actual measurements we show come from the area around the endosomes, so we did not want to make any stronger claims), even though we should have made it clear that other areas of the cell tip are also affected. This has been addressed by clearer explanations.

2. Ill-advised wording we chose that is or can be seen as overinterpretation.
Example: 'anchoring' of actin at endosomes. We had not intended to infer anything about specific anchoring sites or mechanisms. We should have used a more neutral term, such as 'associate with' or accumulate around' for the description. This and other cases have also been resolved by rewriting and better wording.

3. Anecdotal evidence or insufficient data.
Example: Images of phalloidin stainings depicting how actin is organized around late endosomes in control embryos. These and other cases have been addressed by adding further examples and additional quantification.

4. Finally, one suggestion was made for obtaining additional experimental data, which would involve laser ablation. While the experiment would provide an interesting extension of our findings, we will sadly not be in a position to carry it out in the foreseeable future, as explained below. We hope the referees will agree that our now extended discussion addresses the point in question sufficiently to support the conclusions from the experiments we do present.

These and all other points are addressed individually below. We highlighted the corresponding text changes in the manuscript file for the reviewers to identify them more easily.

DETAILED RESPONSES (referee comments in grey, our responses in black):

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary

Rios-Barrera and Leptin investigate the formation and guidance of the subcellular tube that forms in the terminal cell of the dorsal branches of the Drosophila tracheal system. In previous work the authors documented the presence of late endosomes at the tip of the growing terminal cell, ahead of the forming subcellular tube, which are involved in membrane recycling {Mathew, 2020 #1407}. In this present work they analyze the organization of the

actin cytoskeleton in the tip terminal cell, in relation with the late endosomes, and assess the guidance of the subcellular tube. They find that the presence and localization of late endosomes play a role in tube guidance. They also find that late endosomes recruit actin around them, mediated by the activity of the actin polymerization regulator Wash, which is recruited to maturing late endosomes. When Wash activity is decreased, actin around late endosomes is decreased and tube guidance is compromised. Based on this observation, laser ablation experiments of actin ahead of the tube and actin staining at the tip of the terminal cell, the authors propose an exciting model: late endosomes recruit actin, which connects the actin pool of the basal membrane and the actin pool of the apical (subcellular tube) membrane thereby directing tube growth and guidance.
The manuscript is well-written and well-presented, the images and movies are of high quality and the experimental data, which is technically challenging, is very good and sufficiently replicated.

Major comments:
1. A critical point in the model that the authors put forward (which is also contained in the title and abstract) is that actin organized at late endosomes anchors apical and basal actin cortices. However, there is no clear and conclusive evidence for this. Clear evidence in this direction should be provided to propose it as a mechanism (as it is in the text, particularly in the first sentences of the discussion) and imply it in the title.
The authors show endogenous actin around late endosomes and actin fibers at the tip of the terminal branch. However, at the level of resolution presented (Fig 3A,B), it is not possible to determine whether the different actin populations are actually "anchored". I suggest to present stronger data supporting this important conclusion.
In the same direction, it would be critical to show that this anchoring of actin fibers is disturbed when actin enrichment at the late endosome is perturbed (see also point 5). Actually, the authors show that when Vha or Wash activity are downregulated actin accumulation around the CD4 vesicles decrease. However, this experiment has a few inconveniences. First, it is difficult to determine levels of a construct that is overexpressed (UAS-utr::GFP). Could the authors use phalloidin or an actin antibody to confirm the result? Second, I find the result difficult to interpret. In the images provided I see a general decrease of actin (UtrGFP) at the tip, not only around the CD4 vesicles (Fig 6D,F) . Are these mutant conditions also affecting the rest of actin pools? If this is the case, can the authors attribute the defects exclusively to the abnormal recruitment of actin to the late endosomes?
Most importantly, the authors should analyze the pattern of actin distribution (labelling endogenous actin) and determine a possible loss of "anchoring" of fibers when late endosome maturation is perturbed.

We understand the referee addresses three issues here, to which we will respond in turn below:

(a) As already mentioned above, the referee interprets the term ‘anchoring’ in a more specific meaning than we had intended it to have. We have now rephrased these statements.
(b) A technical critique of the use of an overexpressed construct to visualize actin, which in turn has two sub-points: potential physiological effects on actin, and potentially inaccurate localisation. Both are valid points, but in our view do not undermine our conclusions. We have raised and discussed these concerns in our revised text.
(c) The specificity of the effects of reducing Vha and Wash function on actin associated with endosomes versus throughout the growth cone of the cell – a very good point, about which we should have been much clearer and we hope that our current phrasing is better.

(a)

Considering the use of the term ‘anchoring’, and the referee’s concern over whether we provide the appropriate evidence gave us with a good starting point to re-think what we actually show and how it can be interpreted.
Put in neutral terms, what [we felt] we had shown was an accumulation or enrichment of actin around endosomes that was dependent on proper functioning of vATPase and Wash. We agree that the term ‘anchoring’ cannot be justified by the description of actin localisation alone. The term implies a physical (and perhaps strong or long term) interaction between the endosome and the surrounding actin.
We see a strong enrichment of actin around endosomes, including in experiments in which we use phalloidin to visualize actin (Fig. 3B). The resolution of our images is approximately 200nm so they are able to reveal the very close association. The question is what the mechanistic basis for this closeness is. It is unlikely to be random, as shown by a quantification we have now included (Figure S3C). It is difficult to imagine how it could persist without at least transient physical interaction between the two components. The association is indeed highly dynamic and is constantly being re-established. This must mean that something ‘attracts’ actin to endosomes, most likely a molecule that is itself associated with endosomes. The presence or accessibility of such a molecule depends on the proper maturation of endosomes, as shown by the results of $Vha100^{R755A}$ expression, and the ability of actin to associate depends on Wash. Together these findings suggest to us the existence of a (dynamic) molecular link between the endosomes and the actin network.
In order not to give the impression that we are claiming a permanent ‘anchor’, we now use more general terms such as ‘associates’ or ‘accumulates’, but also include the clarification on our thinking in the text. Furthermore, to illustrate a representative range of cases, we have added more examples of late endosomes and the actin meshwork surrounding them (Figure S3A, B). These images should give a broader reflection of the actin populations and their dynamism during tube growth.

(b)
A major reason why we use live imaging with actin reporters is that the distribution of actin around late endosomes and the tip compartment in general is very dynamic, so capturing cells at the right time point can be challenging from fixed samples. This problem is exacerbated by a technical limitation: For the actin cytoskeleton to be well preserved during fixation, embryos have to be manually dechorionated which limits the throughput of the experiment. We therefore found that analysing cells over time is more informative than analysing cells fixed at a given time point.
As the reviewer points out, using an overexpressed reporter can have drawbacks. With regard to the problem of not representing the endogenous distribution faithfully, this can be the case when making statements about the absolute distribution. However, what we are looking at here is not absolute quantities of actin but relative changes in the area of interest with respect to other, unaffected regions of the cells, and then comparing these between mutant conditions and the control. We do this by normalizing the signal to the levels seen in the subcellular tube, using it as an internal control that allows us to adjust for variation in expression levels.
There is on case where such a normalization could be problematic, and that is when comparing actin levels in cells expressing *bitesize* RNAi, because Bitesize is itself involved in organizing the actin cytoskeleton in the tube membrane (JayaNandanan et al., 2014). However, in this experiment, the analysis still shows that actin levels at late endosomes do not correlate with the tube misguidance phenotype.
With regard to potential physiological effects of an over-expressed construct, some of the commonly used actin reporters have subtle effects on actin physiology, whereas Utr-ABD has negligible or no effects on the actin cytoskeleton, and it also reproduces actin dynamics faithfully (Spracklen et al., 2014). It is therefore generally considered the most reliable tool for live imaging of actin in Drosophila.
We have adapted the text and commented on these issues and hoped we have achieved more clarity (page 6, lines 189-191).

(c)

We agree that upon Vha100^{R755A} expression or when Wash is downregulated, actin levels are overall reduced in the growth cone of the cells, while this is not the case in other regions, for example at the base of the cell. Although we had not explicitly stated this (but now have), this is a further indication that the different actin populations in the growing tip interact with each other.

For the downregulation of Wash, this could potentially have been due to a direct effect of Wash on the apical and basal actin, but then we would have expected a similar result in other parts of the cell, including the cell body and the proximal part of the branch, but we do not see that. Even more importantly, the expression of Vha100^{R755A} has the same effect and this cannot be easily explained by a direction action on actin. Together, these findings therefore indicate that depletion of actin around endosomes has a knock-on effect on the basal and apical actin cortex in the vicinity. We have included this reasoning in the paper now (Page 8, lines 271-277).

2. Another critical point in the model put forward by the authors is that late endosomes drive tube guidance. To test this point the authors use an elegant system to mislocalize Rab7 late endosomes.

However, the effects are not strong (1G), and only a proportion of branches show misguided tubes. Do the cases with a ventrally-guided tube in the experiment Rab7:YFP+/+ (Fig. 1G) have a CD4 endosome (with Rab7YFP) at the tip? This would help to explain the weak effect.

This is an excellent point, and it is indeed what we observe: all cells with ventrally guided tubes have a late endosome that is positive for the YRab7-nanobody-membrane complex at the tip of the cell (n=42), whereas only 2/3 of misguided tubes do (n = 12), and those always have the additional endosome at the tip of the misguide tube. As the reviewer suggests, this provides an obvious explanation for why these cells do not have a tube misguidance phenotype. We have added a representative image of this condition (Rab7::YFP+/+, ventrally-guided tube) in Figure 2 to illustrate the phenotype.

3. What is the cause that preventing proper endosome maturation and acidification leads to misguided tubes (rather than missing ones)?

A complete loss of late endosome activity would indeed result in the absence of the subcellular tube. However, we and others have shown that partial loss of function (as caused by RNAi) can have more subtle effects. For instance, fully blocking endocytosis using the *shibire*^{ts} line completely prevents proper tube extension (Mathew et al., 2020), but expression of a *shibire* RNAi still allows tube formation to proceed, albeit in a defective manner (Schottenfeld-Roames et al., 2014). Similarly, the misguidance phenotypes resulting from Vha100^{R755A} expression likely reflect weaker loss of late endosome function. These perturbations would allow initial tube growth to proceed, but later on they would uncover this later function of the endocytic pathway in regulating tube guidance.

We believe that what we see as this weaker defect is an uncoupling of direction from growth *per se*. The cells still receive their growth-inducing signals from the FGF-receptor, and this leads to directed *cell* growth in the direction of the chemotactic signal. The normal trafficking of membrane material from the apical to the basal domain is also not disrupted. Thus, membrane keeps being added to both domains and both the tube and the basal domain continue to growth. However, the growing tube has been disconnected from its guiding structure at the tip of the cell (our speculation: because failed endosome maturation no longer allows proper actin coordination) and therefore follows a random path. We had not been sufficiently clear about this but have now hopefully remedied this in the text (Page 10, lines 338-344).

The authors indicate that downregulating Vha activity leads to defects in acidification, but late endosome-MVB normally form. It is intriguing to see extra CD4 vesicles (like in 1C or 6C).
Wouldn't we expect to see "normal" tip accumulation of CD4 vesicles only, and not extra ones? How relevant are these extra CD4 vesicles?
Wouldn't we expect to see "non functional" CD4 vesicles, unable to recruit actin and lead intracellular tube formation (i.e. no tube) rather than missguidances? (1D shows higher proportion of misguided tubes than no tubes)
Similarly, is Wash-RNAi producing extra CD4 vesicles (as observed in movie 5, fig 6E)?
We do not postulate that the late endosomes are morphologically normal – there are vesicles carrying the CD4 marker (which is only a membrane marker, not specific for endosomes), but the literature indicates that the endosomes do not undergo their normal maturations, and we would have no reason to claim otherwise. So we agree that the ones we see in the Vha100^{R755A} cells are not fully functional, and this is indeed confirmed by their inability to recruit actin.
With regard to the number of large CD4 vesicles at the tip, terminal cells can normally have from 1 to 3 in the growth cone, and the fact that the experimental cells we showed were at the upper range whereas the control at the lower end was pure chance. We have now quantified the number of vesicles in the abnormal conditions and see that there is no increase (Figure S5F).

4. Actin recruitment to late endosomes was already documented, where it plays a role in cargo trafficking.
The authors propose that Wash is recruited to late endosomes upon acidification where it would prime actin nucleation around the endosome. The authors indicate a decrease in Wash accumulation upon expression of Vha dominant negative. However, this decrease is not quantified. In addition, it is difficult to determine levels of a construct when this is overexpressed (UAS-Wash::GFP). It would be desirable to use antibodies against the endogenous protein (Wash in this case) to claim differences in accumulation in mutant conditions.
We have quantified the amount of Wash::GFP in CD4 vesicles. As mentioned, the vesicles are very dynamic, and so is their recruitment of Wash::GFP, and doing the analysis in the live cells is therefore more meaningful than extracting information from fixed samples. We appreciate that as discussed above for actin, results using overexpressed constructs have to be interpreted with care, but here again, we mitigate against this by assessing relative changes rather than absolute amounts and mitigate against misinterpretation by normalizing the signal to the one seen in the cytoplasm (Figure 5M).

The results presented do not rule out a requirement of Wash in terminal branching which is not associated with the enrichment in the late endosomes. The genetic interaction observed with Shrub is also compatible with both proteins acting on terminal branching but in different/parallel mechanisms.
While the fact that expression of Vha100^{R755A} has the same effect cannot be explained in this manner, we agree with the reviewer and have rephrased this section in the paper (Page 8, lines 253-259)

5. Laser ablation experiments
The laser ablation experiments are difficult to interpret.
First, it is unclear to me what the results exactly indicate. What does the recoil observed suggest? Does it fit with the expected tension exerted by a link of the actin cytoskeleton relayed by late endosomes?.
The observed recoil suggests that there was tension across the ablated area. The laser ablation experiments were one way to evaluate whether the actin cytoskeleton within the tip of the cell was continuous between the subcellular tube and the leading edge of the cell. Tension along this axis would support such a model. We assumed that if the actin

cytoskeleton at the tip is continuous with both membrane compartments it was likely to be under tension, and our laser ablation experiments showed that is indeed the case. We have rewritten this section to make it clearer (Page 7, lines 207-210).

From the text and figure I don't understand how is the recoil calculated: retraction of the subcellular tube backwards? "enlargement" of the bleached area?

Briefly, we had used three measuring points: the backward displacement of (i) the subcellular tube and (ii) of the plasma membrane adjacent to the ablated area, which both retract towards the cell body, and we also measured (iii) the forward displacement of plasma membrane on the other side of the ablated area. We then calculated the average of these for each experiment.

However, we have now redone the evaluations of these experiments using PIV, an established method that is commonly used to calculate initial recoil after ablation and have explained this in the text (Page 16, lines 535-546).

Second, it is unclear to me what laser ablation actually ablates. Does it only affect actin? Or are also CD4-late endosomes and other tip structures affected?

The laser ablations with the conditions we use have in the past been shown to temporarily disrupt the actin cytoskeleton without otherwise damaging the cell (Rauzzi et al., 2015). The ablations were done in cells that express the actin reporter Utr::GFP together with the membrane marker CD4::mIFP but we have no reason to believe that CD4 containing structures were damaged. For example, upon ablation, the CD4 vesicles in the ablated area are bleached, but in the recovery phase, we observe actin puncta in the positions where CD4 vesicles were originally located, suggesting that the vesicles themselves persist. Our interpretation of these observations is that the bleached CD4 vesicles do not recover their fluorescence (CD4::mIFP is an integral membrane protein and cannot simply be re-inserted within short periods), but they are still capable of recruiting actin. We have added a representative image of this to better describe the experiment (Fig. S4).

Third, is the recovery observed after ablation correlated with new actin recruitment around old or new late endosomes?

Actin rapidly reappears in the bleached area and the region that recoiled, where it is first seen in the basal cortex and filopodia. The tube re-extends towards the ablated area, and actin reassembles around the tube within seconds. During further recovery, actin reappears in puncta ahead of the tube and we assume that this is partly *de novo* assembly around the existing vesicles (Fig. S4A, B). At the same time, we also see new CD4 vesicles reaching the tip, so it is likely that both populations (old and new vesicles) mediate the recovery phase. We have added images of additional examples that illustrate these points (Fig. S4).

Forth, I find the experiments in cells with secondary subcellular tubes very confusing and the explanations very speculative

The data on cuts in cells with tube duplications are indeed difficult to interpret, and because the emergence of secondary branches is unpredictable, it is not easy to obtain large numbers of observations. Figure S4 is another example of the response of these cells to the laser cut, and we have made clear that our interpretations are merely speculative.

Finally, and most importantly. I think that performing laser ablation experiments in mutant conditions that affect actin recruitment (VhaDN and Wash RNAi,....) would be very informative. One would expect to find a decrease in recoil. If this was the case, it would validate, on the one hand, that in control conditions there is a tension that depends (at least in part) on actin organization, and on the other hand it would show that when actin recruitment is affected tension decreases, supporting the "anchoring" model. I understand that laser ablation experiments are not easy to perform, but I think this would be a useful experiment.

To my understanding, as it stands, the laser ablation experiments "....support the notion that adequate cytoskeletal organization at the tip is required for tube guidance and stability" as the authors acknowledge, but they do not convincingly support their "anchoring" model
Laser cuts on cells that express Vha100^{R755A} or wash-RNAi would be a nice addition that would take the work to the next level. But sadly, these are among the experiments that right now are impossible to carry out because of all the logistical and other problems resulting from the Covid pandemic, as explained in the cover letter.

Other comments:

- From the images presented, it is often difficult to figure out where the subcellular tube forms, the presence of vesicles, the cell morphologies,... and to determine the correlation between the CD4 vesicles and tube guidance.
This is the result of a frustrating technical limitation. In experiments in the past we have used markers for the outline of the cell, as we do here, too. Thus, where CD4 is expressed under the *btl-gal4* driver it marks the entire outline of the cell against a completely negative background. Even for other markers, if expressed under *btl-gal4*, the outline of the cell is visible against the dark background. However, for endogenously marked proteins that are expressed ubiquitously, this is no longer true, and as we add more markers to follow different structures, we run out of fluorescent colours for everything we would like to highlight (and genetically, out of chromosomes to accommodate the necessary transgenic or endogenously modified constructs). We have added tracings of the outlines of the cells to make the images clearer (Figs. 1, 2, and S1).

For instance, in Fig 1H and 1J, is there a "lateral" CD4 vesicle? Why it does not generate a missguided tube?
Yes, there are also CD4 vesicles closer to the proximal part of the cell. They are enriched at but not restricted to the tip of the cell. As we have shown previously (Mathew et al., 2020), they emerge along the subcellular tube, and most are transported towards the tip (also seen in Fig. 1A, for example). Why the remaining ones do not affect the guidance of the tube is unclear, but it is almost certain that the growth of the tip of the cells towards the chemotactic FGF signal plays a role: the basal membrane is constantly moving away from the tip of the tube at this location, but not at the sides further down the branch.

Fig 1I, are there 2 subcellular tubes? Can the authors mark them? I cannot really visualize them with the CD4 marker, they seem stalled or short or missing.
In Fig 1I, the tube is curled up inside of the cell, a phenotype often seen in larval terminal cells with excessive FGF signaling (for instance see Ukken et al., 2014). We added diagrams that explain the morphology of the tubes in this figure.

Fig 1L: what do the authors mean by "corrected" tube sprouts?
This is not well phrased, and we have also improved the figure to make the point clearer. Panels 1K-M (now 1H-J) show snapshots from a movie of a cell that originally had only a misguided tube (at the top left) and is here in the process of forming its ‘correct’ tube growing in the ventral direction. In 1L (now 1I) this second tube is showing first signs of emerging, in 1M (now 1J) it is clearly visible. We have changed the wording in the figure and added an explanation in the legend. We also added a second example of this process in Figure S1.

It is difficult to identify the cell in Fig 2D-F
We added a dotted line in one of the channels showing the general morphology of the cell.

- Movie S3: I find it difficult to spot the association of CD4 and utrGFP that the authors point. Can the authors label in the movie the vesicles and the association?

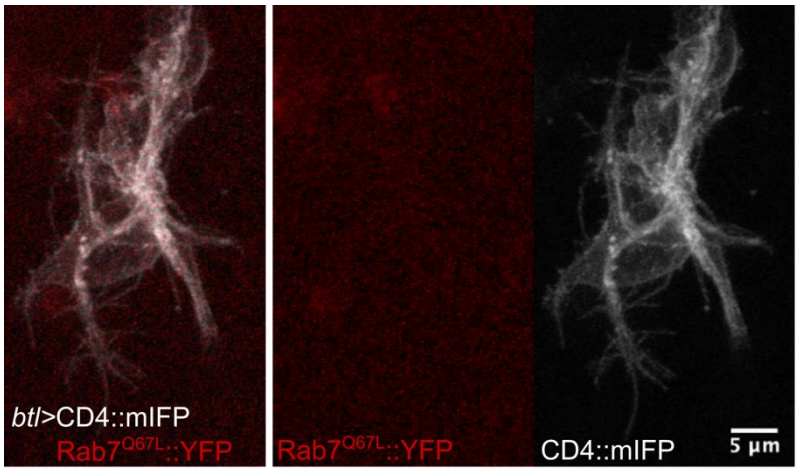
We added pauses in the movie and arrows to the frames where actin is seen surrounding late endosomes.

- The results with the Rab7 downregulation and upregulation are not very clear. Does the downregulation of Rab 7 (Rab7 DN construct) have any effect on tube guidance? Does it decrease or eliminate actin association with CD4 vesicles in the embryo? The authors show that in the larvae expression of Rab7 DN leads to loss of actin enrichment in Rab7 vesicles. Does this have an effect on terminal branching?

Rab7DN is not visible in the embryo so we did not pursue further experiments in those stages and we previously showed that loss of Rab7 does not affect branching in larvae (Best and Leptin 2019). However, as the reviewer rightfully pointed out, expression of Rab7DN prevents actin nucleation at late endosomes in larval stages, so having the phenotypic consequence of this experiment would be informative and we are grateful for the observation. We had done the experiment, and we found no difference in the number of branches compared to controls. This suggests either that at larval stages actin recruitment at late endosomes is no longer required for branching or that there are redundant mechanisms that can balance the lack of actin nucleation. We favour the second model, because it has been shown that microtubules also play a role in tube branching and in coordinating the actin cytoskeleton (Araujo lab, 2021), so it is possible that actin nucleation can be bypassed. This is also consistent the fact that the phenotypes we describe are not all fully penetrant, again pointing to redundant mechanisms ensuring consistent directed growth.
We added the data regarding Rab7DN to the manuscript (Figure S2).

The Rab7 active construct produce effects at larval stages but not in the embryo. Is terminal cell branching in the larvae also dependent on late endosomes? Can the authors show "excess" of late endosomes in the larvae that lead to extra terminal branches? Even that the authors indicate that they cannot detect Rab7Q67L, can they find any effect at embryonic stages (e.g. presence and position of CD4 vesicles, other unrelated effects,...)?

Expression of Rab7CA in the embryo generates similar defects as nanobody-mediated mislocalisation of Rab7. We include below an example for the reviewer, but we did not feel comfortable including these data in the paper because some technical complications made them impossible to document and interpret with the certainty that we would wish. Most importantly, the YFP fusion protein is not detectable at embryonic stages, even with the most sensitive microscopes and detectors available to us. This means that we cannot correlate the observed phenotypes with the presence or absence of Rab7CA, which in our view makes them too weak for publication. At face value, these results suggest that Rab7CA begins to trigger branching during embryonic development, which eventually leads to the excess number of branches we see in the larva, but alas, we think this is too speculative to include in the paper.



Example of a cell expressing Rab7CA (not detectable, middle panel) with abnormal branching pattern and tube morphology. This seen in 4 out of 9 terminal cells.

- In some examples in the movies there seem to be a correlation between CD4 vesicles presence/positioning and basal lamellipodia/filopodia or actin enrichment, and also in -btl experiments. Have the authors explored this? They may want to comment on this in the discussion section.

That is a very pertinent point, and we should indeed have commented on it. If we assume the reviewer is looking at examples such as the one in Fig. 1I (currently S1C), then the explanation is the following. The terminal cells in the embryo often form transient side-branches, presumably in response to a low level FGF signals from the environment. In those cases, the basal actin cytoskeleton rearranges in the branching area to form the filopodia that lead the outgrowth of the branch, and what the reviewer observed is that this transient branch also forms the late endosome structure that we see in the main or proper growth cone. Thus, the guiding FGF-signal leads to a reorganisation of the entire actin cytoskeleton in the growth cone, and the formation of the actin-covered endosome is part of that process. We have included this in the discussion (Page 12, line 390-396).

Reviewer #2 (Significance (Required)):

This work is relevant for the morphogenesis field and deals with the important issue of how the cytoskeleton regulates shape and cellular events. The work represents a deep analysis of a specific issue in the specialized field of tracheal development, but the results may be relevant for other types of cells forming subcellular tubes. Describing a function of trafficking vesicles (late endosome in this case) in cell morphogenesis (in addition to cargo trafficking) in an in vivo system is also relevant to advance in the cell biology field.

Referees cross-commenting

I agree with the comments of reviewer #1. I find relevant the points raised in "major comments number 2 and 4".

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary:

The authors investigate the role of late endosomes in
the context of actin organization during cell morphogenesis. They use as experimental model the polarized terminal cells in the Drosophila tracheal system that forms a sub-cellular projection containing a tube. The authors show that disruption of the sub-cellular localization or maturation of late endosomes leads to increased proportion of terminal cells with mis-guided tubes. Their analysis indicated that endosomal F-actin recruitment is crucial for the directionality of the tube growth. The authors propose a model where, late endosomes control a coordinated crosstalk between endosomal and cortical actin pools to drive subcellular tube-guidance.

Major comments:

1. The conclusion about how WASH functions in the tube-guidance, is not clearly shown and it should be better explained and documented. It is known that loss of function of the WASH leads to dysregulation of endosomal tubulation inducing enlarged endosomes, which in turn affects the endosome-to-plasma-membrane recycling of various cargos (including luminal cargos like Serp) (Gomez, et al., Mol. Biol. Cell, 2012; Dong et al, Nat. Comm. 2013). The

authors should clarify if there is a defect in the integrity of endosomes located close to the cell tip in the btl>WASHIR knock down. In the cartoon panel C' (Figure 7), the endosomes in the cell tip are shown intact (Including their relative position to the tip) but no experimental data support this conclusion.

Given the fact that Wash contributes to proper late endosome morphology we do not necessarily expect the endosomes to look normal. We had not shown this in the diagram because our own data do not directly address this point, but the literature is of course clear enough about this, so we have modified our diagrams so that they better reflect the expected phenotypes and included a reference to the relevant literature.

We and others have shown the important role of late endosomes in plasma membrane and luminal cargo delivery, and as elaborated in the response to referee 2's point 3, complete loss of endosomal function blocks these processes. Here, at reduced but not abolished function plasma membrane delivery is clearly still functional.

-The functional analysis of WASH was based on RNAi knock down. The authors express a single RNAi construct against WASH. The expression of this RNAi line gave a low penetrance phenotype. A well-known caveat of RNAi is off-targeting. Hence, phenotypic analysis needs to include a verification by a second independent RNAi construct or a rescue of the RNAi phenotype with an overexpressed cDNA of WASH. Ideally, the null wash mutant (Nagel et al. 2017) can be used to confirm the phenotype.

Analysing *wash* mutants would provide a welcome additional confirmation of the knockdown results, and it is true in general that poorly characterised RNAi lines can have off target effects. However, this is a well validated line: Nagel et al. (2017) showed that the same RNAi line that we used fully recapitulates the phenotype seen in *wash* mutants: In both cases, actin fails to localize to late endosomes, and this is what we also found in terminal cells. Whereas we believe therefore that the experiment is not essential to support our conclusions, we agree it is desirable and have ordered these flies. However, progress is being hampered by import restrictions at the first author's lab: the necessary paperwork for flies to be imported for his work is still under revision by officials. The experiment thus cannot be done at the moment.

2. The authors claim a role of the late endosomes in subcellular tube growth and guidance. But show no data on lumen formation to prove tube presence in the tracheal terminal cells of V100R755A, btl>WASHIR, shrb mutants or in GrabFP-Bint treated terminal cells. The interpretation and quantification of the phenotypic classes "miss-tube-guided" and "ventrally-tube-guided" are based on membrane markers and not on luminal markers. The presented data with the provided resolution does not prove if the mCD4-mIFP or PH-GFP markers define apical membrane protrusions/extensions or tubular structures. Therefore, the classifications of the tube-guidance phenotype and the quantification of "distance from tube to tip" may be suggestive. The authors need to provide additional confocal data of co.stainings of the endosomal compartments with luminal antigens (i.e. GASP or Serp or Verm).

We are very unsure as to what this would add and in what context it would be necessary. Membrane and actin markers have been widely used to follow the formation of the subcellular tube by all groups working in this field. There is ample documentation in the literature that the subcellular tube, as defined by luminal content (Serp, Verm, Gasp, CBP-GFP, ANF-GFP) is surrounded by apical plasma membrane which carries apical transmembrane proteins (Crb, Uif) and their associated apical cytoplasmic complexes (Par3, Par6, aPKC, pMoesin), and apical phospholipids which can be visualized by specific PIP-binding markers, e. g. The PLC-d PH-domain that binds to PIP_2 (see, e.g., Kato et al., 2004; Oshima et al., 2006; Okenve-Ramos & Llimargas, 2014 (here they use both luminal and actin reporters); Ochoa-Espinosa et al., 2017; and from our lab: JayaNandanan et al., 2014; Mathew et al., 2020. Therefore, all labs in this field have used these markers interchangeably to visualize the subcellular tube and we are not aware of a single case where luminal shape and apical membrane shape were not exactly congruent.

We have in fact used luminal markers in some experiments here, but we believe there is no reason to assume that luminal markers would have a different distribution compared to membrane, apical or actin reporters in any the experiments described here. Finally, the focus of the paper is on the behaviour of the early out-growing membrane rather than the mature tube, and on how membrane is remodelled in this process by modifications in the actin cytoskeleton. Including confirmation of the presence of luminal material would not add to the paper.

3. page 8 line 248, the authors interpret that reducing the dose of Shrb by half strongly enhances the wash-RNAi phenotype and suggest that WASH and Shrb act in the same pathway. Shrub is a subunit of the ESCRT-III complex involved in inward membrane budding of endosomes and WASH functions in outward endosomal membrane budding.
The Shrb and WASH form discrete molecular complexes in endosomes. The authors should consider that Shrb and WASH may well act in parallel to control directional tube growth.
This is a good point and we have now rephrased our conclusions from this experiment (Page 8, lines 256-259).

4. The authors use nanobody-based GFP trap construct to investigate the effect of Rab7YFP localization. This is an excellent way to provide novel information for protein miss-localization in vivo. Using this method the authors concluded that ... "the correct distribution of late endosomes is required for proper tube guidance" (page 5, lines 157-158). The authors obviously consider that GrabFP-B-Int construct affected the distribution of late endosomes. However, this is unclear and additional control experiments are needed to support the author's claims. For instance, did expression of GrabFP-B-Int, target the Rab7-YFP protein or the Rab7-associated endosomes? With the presented data, it is not clear if the Rab7-YFP positive vesicles are endosomes? or aggregates formed by the trapped Rab7-YFP protein? Co-stainings using GFP in Rab7-YFP terminal cells with another endosomal markers i.e. Avl, or hrs, should be provided. It is also not clear if endocytosis of apical/basal membrane or luminal cargos was affected in GrabFP-B-Int treated terminal cells. The loss of endocytic components has been associated with defects in subcellular tube shape and morphology (Schottenfeld-Roames et al, Cur Biol. 2014). The authors should clarify these issues.
The nanobody would of course bind both to free Rab7::YFP (if there is any available) and to endosome-associated Rab7::YFP. However, in addition to Rab7::YFP we also assayed the distribution of CD4::mIFP, a membrane-associated protein that is seen at very low levels in all membranes (Mathew et al., 2020), but highly enriched in cytoplasmic vesicles, which we showed by co-expressed markers to correspond to endosomes (Mathew et al., 2020). If the nanobody sequestered free Rab7::YFP, we would expect little overlap between Rab7::YFP and CD4::mIFP puncta. Instead, we see that the large Rab7::YFP/nanobody puncta have membrane associated with them (63% of vesicles are triple positive, vs 8% of Rab7::YFP-GrabFP vesicles) indicating that they are not merely Rab7 aggregates. We have included a quantification of the degree of overlap between these components.

Regarding the question of whether endocytosis is affected, we believe this is unlikely, or if it is at all, only to a minimal extent, since growth of the outer membrane, which crucially depends on endocytosis, continues in these cells. We have added a comment to this effect in the text. The cells look very different from cells in which endocytosis has been inhibited.

5. In the legends of Figure 7 (C'), the authors stated that.... "lack of actin regulators at the basal cortex prevents the connection of the actin meshwork at the tip to the basal plasma membrane".... by depicting the singed mutant phenotype. singed mutant analysis is not shown in the manuscript.
Singed/Fascin has previously been shown to be required for actin organization in fillopodia (Okenve-Ramos & Llimargas, 2014). We have now included new data that show that cells expressing *singed* RNAi also have reduced amounts of actin at late endosomes, and that reduced actin correlates strongly with tube misguidance. This shows that an actin bundling

protein that has previously been shown to be needed for actin bundles in filopodia again affects actin around endosomes, providing another illustration that these compartments interact with each other.
Our quantifications on actin around late endosomes show that interfering with endosome maturation, actin nucleation via Wash and basal/filopodial actin all lead to loss of actin around endosomes, and the misguidance phenotype correlates with actin loss (Figure 6J). By contrast, disruption of the apical actin cortex does not affect endosomal actin but does lead to misguidance. This establishes a hierarchy of actin organisation in the tip of the cell: basal actin affects endosomal actin, loss endosomal actin affects both apical and basal actin, but apical actin does not feed back on endosomal. All three pools are nevertheless required for tube guidance.

6. The authors consider the late endosomes nucleate actin ahead of the tube (i.e. page3, line 87-88, page 9, line 285). This is not very convincing from the presented data. The authors should provide some quantitative data showing that lack of WASH (and endosomal F-actin network) effects the apical and basal F-actin pools in the tip of the cell.
If we understand the reviewer correctly, there are two comments included in this point: (i) whether actin is nucleated at late endosomes, and (ii), whether reducing endosomal F-actin affects apical-basal actin pools in the tip of the cell.
(i) As stated above in the response to reviewer #2, we have added quantitative data illustrating actin recruitment at late endosomes with phalloidin stainings. Actin association with endosomes is also confirmed by the Rab7 stainings in larval terminal cells in Fig. 3G-H that show actin puncta associated with endosomes.
(ii) Again, as mentioned in the response to reviewer #2, we do think that all actin pools in the growth cone are affected. We are glad that the reviewers encouraged us to make this more explicit and have now discussed more clearly how endosomal F-actin could affect apical and basal F-actin pools.

Minor concerns:

1. The authors concluded (page 9, line 285) that "endosomes serve as actin nucleating centres that propagate forces within the cell by physically linking different subcellular compartments".
We agree with the reviewer, this is a good way of phrasing it, and we have rewritten this conclusion accordingly.

2. The authors should depict in the panels the Ventral/Dorsal axis.
All images are positioned in the same orientation, but we have ensured that the D/V axis orientation is stated in the manuscript.

3. Numerous omissions need to be corrected. Labeling is missing in the panels J-M' (Figure 1). The statistical significance and the p values levels are not indicated in Figure 2 (G). The panel figure 5 (D) is miss-labelled. The panels C-C' in f igure 7 are not very informative. They do not reflect the general model of the study. How the prevention of actin nucleation at late endosomes, or apical or basal cortex affects tube directionality is not graphically shown.
We thank the reviewer for noticing these omissions, we have now fixed them. Having added more discussion about the general organization of actin at the tip of the cell, we think the relevance of panels 7C is justified.

4. In the section "Crosstalk between cytoskeletal compartments" (Lines 359- 400, discussion) the argument about the involvement of microtubules in tube-guidance is a likely scenario. But I found this argument over-extended. WASH interacts with tubulin Derivery et al. Dev Cell (2009) and WASH activity balances the endosomal and cortical F-actin networks

during epithelial tube maturation in multicellular tracheal tubes (Tsarouhas et al., Nat. Comm 2019). These results should be considered in the discussion section.

We have incorporated these references to the discussion, they have for sure enriched it (Page 12, lines 391-397; page 13, lines 423-431).

Reviewer #1 (Significance (Required)):

The important role of actin cytoskeleton in the initiation of endocytosis is well established. Actin structures in the plasma membrane are dynamically organized to assist the remodeling of the cell surface and to facilitate the inward movement of vesicles. Similarly actin networks in endosomes are critical for endosomal fusion and fission. In this work, the authors identified an opposing but interesting scenario. They propose a role for the late endocytic pathway in organizing actin networks for proper cell morphogenesis and point out an intracellular crosstalk and coordination between distinct cytoskeletal pools within a cell.

Although the mechanism about how the separate F-actin pools communicate is not shown, the paper is interesting and shows an original contribution in the area of cell morphogenesis. In addition it represents a conceptual advance as it proposes a mechanism through which actin cytoskeleton is coordinated to regulate tube morphogenesis. The proposed mechanism may be relevant for tracheal terminal cells, but could represent a general mechanism in the field of cell biology. The methodology is appropriate and the text flow is well organized. However, as explained, there are few inconsistencies in the manuscript. I believe the above additions would strengthen the conclusion of the paper.

August 20, 2021

RE: JCB Manuscript #202106124T

Prof. Maria Leptin
European Molecular Biology Organization
Meyerhofstraße 1
Heidelberg 69117
Germany

Dear Prof. Leptin:

Thank you for submitting your revised manuscript entitled "An endosome-associated actin network involved in directed apical plasma membrane growth". As you will see, only one of the two Reviewers was available to review the revision. They think the work is substantially improved. However, they still have a small number of issues that need to addressed. We have examined these. Both this Reviewer and the other Reviewer in the Review Commons round felt that the conclusions you draw about the distribution of actin need to be strengthened and they offer what seems to be a straightforward suggestion to accomplish this. They also request some clarification of the effect of btsz loss, suggest you remove the data on Rab7 Q67L, and request some minor modifications to Figure S4A. If you can satisfactorily address these remaining concerns, we will be ready to move forward.

To avoid unnecessary delays in the potential acceptance and publication of your paper, please read the following information carefully.

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6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

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a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses

c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Sincerely,

Mark Peifer
Monitoring Editor

Andrea L. Marat
Senior Scientific Editor

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

The authors have extensively addressed most of the concerns raised in the first revision of this manuscript. They have clarified several points, added more data and quantifications, addressed specific questions, and tone down some of their initial conclusions that I considered overinterpretations. To my understanding they have changed and clarified the message of the manuscript adjusting better their results with their conclusions. I do think that in general they have done a good job and improved the manuscript. Besides adding some new results and quantifications, they have greatly improved the figures, providing the manual tracing of the cell contours and outlines which help to observe the effects they describe.

Most of the major and minor points I had indicated previously have been satisfactorily addressed. The only points that I still feel remain weakly demonstrated are the following ones:

1) One of the main criticisms I raised was the association of actin in CD4 vesicles with actin in apical and basal membrane. The authors indicate (line 184) "While actin was always seen enriched around these vesicles, its distribution varied from small puncta in the vicinity of the endosomes to thick networks that extended to the apical membrane surrounding the lumen". I still find it difficult to clearly observe this. The authors have made an effort to document this point using also live imaging, but I still find it hard to visualize it. The association of actin with late endosomes is very clear, but the association with the apical (or basal) membrane is still unclaer to me, at the level of resolution provided by live imaging.

In line 283 the authors state "Based on this, and on the phalloidin stainings in control embryos and the laser ablations, we propose that late endosomes at the tip serve as actin-organizing centres that contribute to the formation of a continuous actin meshwork from the tube to the basal plasma membrane.". Their results indicate effects on the levels of actin in the region, but are not demonstrating differences in continuity of the actin network

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This is a very interesting hypothesis. It would require to explain why disruption of actin recruitment at CD4 also affects basal actin, and to show that btsz affects apical actin.

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Ríos and Leptin, response to the reviewer.

We have followed a variety of approaches to obtain more data to respond to the concerns of the referee, but the outcomes are slightly frustrating (though not a problem for our conclusions). We outline below what we attempted, which obstacles we faced, and what the remaining options are. We also present again the evidence we have that we believe supports our conclusions, and will be happy to enter into a conversation with the referee and the editor to discuss what would be the most sensible way forward. Finally, we include additional data and we have modified the text in response to the referee's comments (major changes are highlighted in the manuscript).

The authors have extensively addressed most of the concerns raised in the first revision of this manuscript. They have clarified several points, added more data and quantifications, addressed specific questions, and tone down some of their initial conclusions that I considered overinterpretations. To my understanding they have changed and clarified the message of the manuscript adjusting better their results with their conclusions. I do think that in general they have done a good job and improved the manuscript. Besides adding some new results and quantifications, they have greatly improved the figures, providing the manual tracing of the cell contours and outlines which help to observe the effects they describe.

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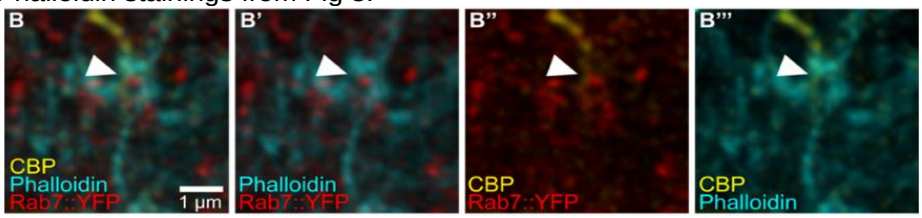
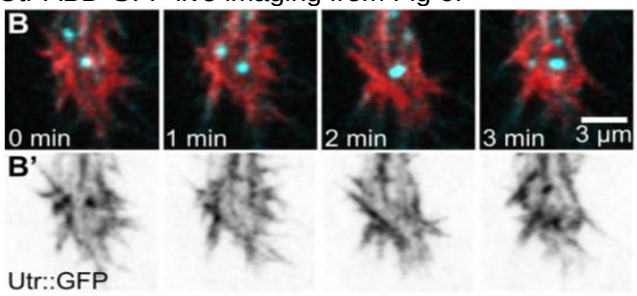
The results on which these interpretations were based are the following. The referee has clearly read the paper carefully, so we are just re-listing this as a basis for discussion to see if they might not be sufficient (or if we have perhaps used wording that exaggerated our claims/interpretations and that should be tuned down):

- We showed in control cells that actin forms thick meshworks around endosomes, and these often associate closely to the subcellular tube (Fig. 3, Fig S3). In these experiments we do not have a marker for the cell outline to show the proximity to the basal plasma membrane, but this is complemented with our Utrophin-ABD-GFP experiments in live embryos, particularly as shown in Figure 6. Thus, the three markers that would ideally have been shown together are shown pairwise in separate experiments.

Phalloidin stainings from Fig 3:

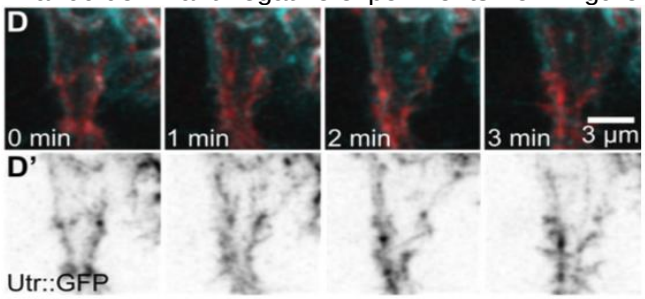


Utr-ABD-GFP live imaging from Fig 6:

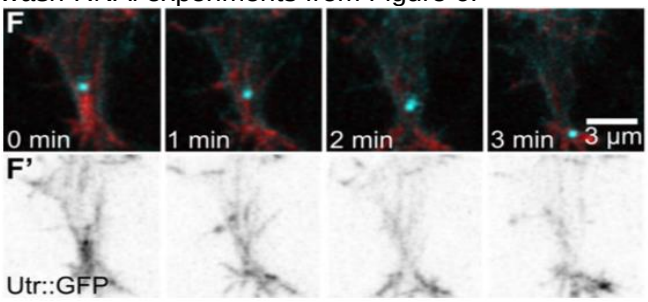


- Our experiments using RNAi against *wash* and the dominant negative form of a vATPase subunit (Vha100) show a significant reduction of actin in late endosomes; the images also show that actin does not reach either the subcellular tube or the basal plasma membrane.

Vha100 dominant negative experiments from Figure 6:

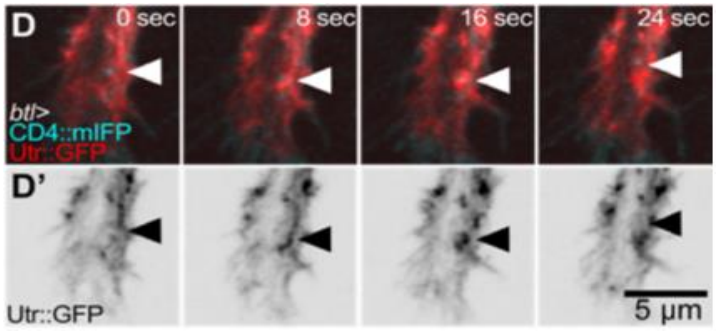


wash RNAi experiments from Figure 6:

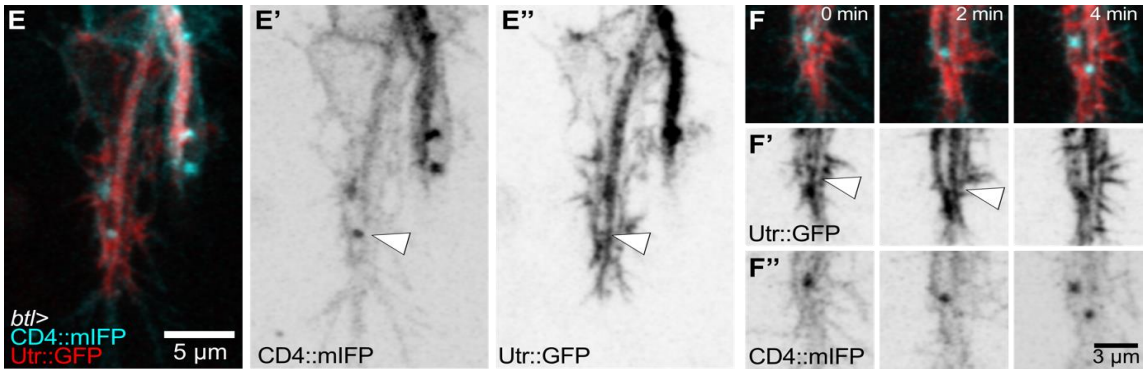


We see how our images may complicate the interpretations of our results. In Figure 3, we show a high temporal resolution micrograph of how actin associates to endosomes in a very dynamic way, but we agree that this short movie does not show the association of actin all the way from the apical to the basal plasma membrane. We will now also add images that better reflect these associations using Utrophin-ABD-GFP:

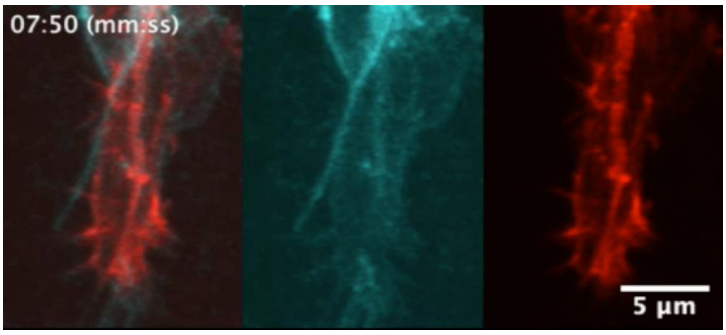
High temporal resolution micrographs of Utr-ABD-GFP and the membrane marker CD4-mIFP presented in Figure 3 (And Video 3):



New examples are now included in Figure 3, accompanying the fast imaging. These images are acquired at a slower rate but have better spatial resolution. Arrowheads point to actin structures spanning from the tube to the basal plasma membrane at the leading growth tip of the cell:



A similar association can be seen at several time points in Movie 3, for instance:



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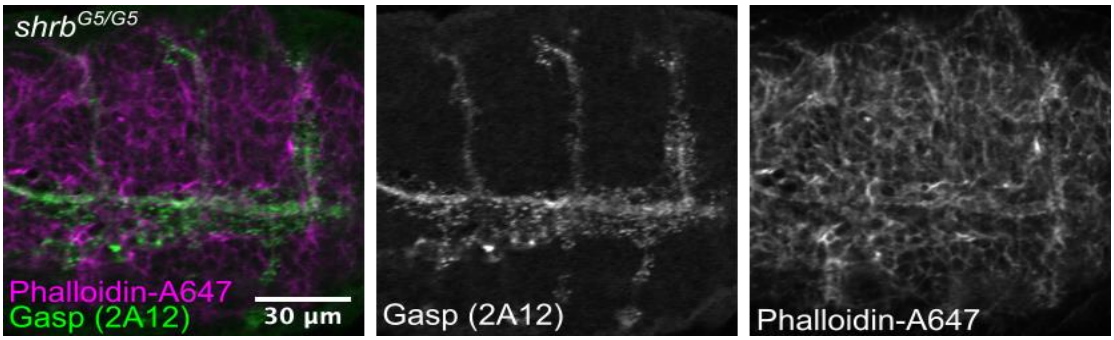
To follow the referee's request for a higher resolution illustration of the discontinuity of the actin meshwork in mutant conditions we used embryos mutant for *shrb*, which show misguidance phenotypes (Fig. 1B). We stained for Gasp (antibody 2A12), an enzyme that is secreted into the lumen of the tracheal system and is commonly used to mark the lumen, together with phalloidin to label the actin cytoskeleton. We observed that Gasp formed aggregates in the cell cytoplasm throughout the tracheal system (see figure below) and it thus did not fulfil the intended function of outlining the tube, a phenotype that complicates analyses of actin distribution at the tip of terminal cells. In hindsight, we realize this is an expected result; Dong & Hayashi (2014b) have shown that *shrb* mutants show cytoplasmic accumulations of Serp, another secreted luminal marker, a finding we confirmed using a Shrb-GFP dominant negative construct and Serp (Mathew et al., 2020).



Alternative experiments to address this point would be to use a chitin-binding probe, as we did in Figures 3A-B and S3. However, this reagent was generated at EMBL in Heidelberg, and the current new experiments are now being carried out by the first author in Mexico where he does not have access to the reagent. The paperwork to import reagents to Mexico is extremely slow, and even with the adequate permits, release from customs can take several weeks. Even if Daniel were able to overcome the bureaucratic hurdles, it is likely that

the probe would not survive the transport as it is usually stored at -20°C and very sensitive to temperature fluctuations.

A second possibility is to carry out these experiments in other mutants like *wash*$^{\Delta 185}$ (Verboon et al., 2018) or to use other transgenes like a fluorescent chitin probe (UAS-CBP-GFP). Unfortunately, fly import permits are also frustratingly slow: We submitted paperwork to import fly lines from Germany to Mexico and this took 10 months (February until November). Getting lines from Bloomington is not yet a possibility due to similar delays.

The third option is to carry out the experiments in Germany, which might be feasible now that travel restrictions are loosened (who knows for how long?), but the flies would still have to be ordered and crossed, a process that will take time.

We hope the reviewer and the editor find our arguments and new modifications sufficient to justify our model. We have also rephrased our interpretations to reflect that our experiments show a close association of endosomal actin to the vicinity of the apical and basal plasma membranes.

2) The authors observe that a dominant negative form of Rab7 showed no obvious effect on larval branching, and suggested that "terminal cells may not rely on late endosomes to build subcellular tubes at the larval stage, or that larval branching uses other compensatory mechanisms."

I am not sure that, if this is the case, the results with Rab7 Q67L can be directly attributed to an increase of late endosomes that actually they do not show.

I understand what the authors suggest in the response letter, but as they acknowledge, they cannot attribute with certainty the phenotype observed (Fig S2) to the mechanism that they are describing is operating in the embryo.
This experiment is not essential and could be omitted

We agree with the reviewer and we have removed these experiments from the paper.

3) In the absence of btsz the authors do not detect clear effects on actin around CD4 vesicles. Do they detect effects on apical actin, as expected?
Because when they affect the basal actin network using singed downregulation they also affect actin associated with CD4 vesicles, their results suggest that affecting the basal actin network, but not the apical one, has an effect on actin recruitment to endosomes.

The authors point in the discussion that it is "reasonable to assume that FGF signalling could control microtubule orientation by locally organizing F-actin at the leading edge, which in turn serves as a cue to organize microtubules via Shot"

This is a very interesting hypothesis. It would require to explain why disruption of actin recruitment at CD4 also affects basal actin, and to show that btsz affects apical actin.

Our group has previously shown (JayaNandanan et al., 2014) that knocking down *btsz* expression in the embryo with the same RNAi line that we use here phenocopies *btsz* loss of function alleles, with shorter subcellular tubes compared to control cells. In larval terminal cells Utr::GFP recruitment to the apical plasma membrane is indeed reduced, however this is difficult to quantify in embryonic cells. For this, one would need to normalize the Utr::GFP signal in the subcellular tube to that of Utr::GFP in the basal plasma membrane, but the signal and its distribution in this domain is much more dynamic than in the tube, so a true 'baseline' cannot be reliably determined. However, since this is a tested RNAi that

recapitulates the embryonic phenotype, we are confident that it is effectively knocking down *btsz* expression.

As the reviewer points out, this then suggests that affecting the basal actin pool (through *sn* knockdown), but not the apical one, affects the organization of actin at CD4 vesicles. We see two alternative explanations that we now discuss in the paper: 1) Fascin/Singed may be directly involved in the organization of the actin meshwork around the endosomes at the tip, or 2) The effect may be indirect, for instance, through effects on FGF signaling. Loss of *singed* is known to affect the expression of SRF, a target gene of FGF signaling (Okenve-Ramos et al., 2014). It would be possible that an unknown effector of FGF signaling is involved in the organization of the endosomal actin pool.

4) Laser ablation experiments
In the response to reviewer's letter lines 286-299 I cannot interpret what the authors indicate. They claim that CD4 vesicles remain intact upon laser ablation and that they can see actin puncta in originally bleached CD4 vesicles. However, if the vesicles and actin association are so dynamic as the authors point, it is difficult to attribute an actin puncta to a bleached CD4 vesicle. In Fig S4A it is difficult to understand whether the actin puncta (black arrow) is or is not associated with an old bleached CD4 vesicle or a non-bleached CD4 vesicle. The authors should put both arrows (black and white) in the individual (actin and CD4) panels

We apologize for the confusion. Our intention with this figure was to point that after laser ablation, the recovery could be mediated by ‘old’, bleached vesicles at the tip as well as new vesicles arriving afterwards. The figure shows that in the ablated area, there are Utr::GFP puncta reappearing within seconds after the laser cut, which presumably would correspond to preexisting, bleached endosomes. We also see new endosomes reaching the tip of the cell, therefore these may also contribute to the recovery after laser ablation. We have now used arrows of different colors in Figure S4 to differentiate between these two types of puncta: reappearing Utr::GFP dots and new endosomes reaching the tip.

December 14, 2021

RE: JCB Manuscript #202106124R

Prof. Maria Leptin
European Molecular Biology Organization
Meyerhofstraße 1
Heidelberg 69117
Germany

Dear Prof. Leptin:

Thank you for submitting your revised manuscript entitled "An endosome-associated actin network involved in directed apical plasma membrane growth". Your response to the reviews seems reasonable--you have clearly accomplished much of what the Reviewer initially requested and we are satisfied with your response to their continuing concerns. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). Please also take one more look at the text and Figure presentation to ensure the caveats and clarifications included in your response to the Reviewer are now incorporated into the manuscript.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium

e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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