Enabled primarily controls filopodial morphology, not actin organization, in the TSM1 growth cone in Drosophila

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RE: Manuscript #E23-01-0003
TITLE: Enabled primarily controls filopodial morphology, not actin organization, in the TSM1 growth cone in Drosophila

Dear Dr. Giniger:

Thank you for your recent submission to MBoC. We have just received comments from two expert reviewers on your recent manuscript submission, "Enabled primarily controls filopodial morphology, not actin organization, in the TSM1 growth cone in Drosophila." As you will see below, both reviewers appreciated the work, but had multiple suggestions to improve the manuscript. Broadly, they both request major revision of the writing and improved clarity of the manuscript and methods. For example, both reviewers request a better explanation of wavelet analysis as well as improved description of the context and larger question addressed by the study. Reviewer 1 in addition to comments to author, indicated to the editor that they were uncertain of the key messages of the manuscript, indicating improved readability is needed. In addition both reviewers had suggestions for additional and/or improved analysis. While reviewers requested few experiments, there was a question over the localization (or lack of demonstrated localization) of Ena and the interpretation of why loss of Ena did not alter filopodia length. Reviewer one suggests this could be addressed using GFP-tools to investigate growth and retrograde flow. We encourage you to address reviewer comments in a revised manuscript and provide a point-by-point list of how these were addressed.

Sincerely,

Stephanie Gupton
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Giniger,

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

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

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

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

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

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

Sincerely,

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

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors explore the potential role of Ena as downstream mediator of Abl signalling during axon growth of the TSM1 neuron in the developing Drosophila wing at pupal stage. From abstract, end of discussion, beginning of discussion and reading through the results part, I am not certain what the key message of the authors is. As the authors state: "Whereas we had found previously that altering Abl kinase activity primarily modulates the distribution of actin, with only modest effects on growth cone morphology, we now found that the effects of manipulating Ena were opposite, primarily modifying morphology, with only modest effects on actin distribution" - for loss of Abl, the data show axon stall suggesting systemic relevance, but what is the effect of loss of Ena on axon growth? These data seem not to have been provided. If I interpret the data for loss of Ena correctly, mainly filopodia numbers are reduced by ~50%, but the actin core of GCs is pretty unaffected. Given that the main role of filopodia likely is sensing the environment, the sensitivity of GCs may be a bit reduced, but the actual growth capacity not. Hence, does the majority of axons reach their targets, with perhaps a mild increase in aberrant navigation? If this is the case, then authors should build a clear rationale as to whether they think Ena is a mediator, and make clear how they interpret the loss of Ena phenotype.

Another weak point is the localisation of Ena which is not being properly demonstrated at GCs, neither for loss- nor gain of Ena. In the case of loss of Ena, it is surprising that filopodia are not shorter given Ena's involvement in actin polymerisation. There are two possible explanations: First, Ena is not at the tip of filopodia and instead formins take its place; second, the filopodia show very slow backflow dynamics and filopodia have a very long persistence; both aspects can be assessed with available GFP-tagged tools. The reduction in numbers would suggest that less actin filaments are present (are actin amounts comparable in wt and loss of Ena GCs?) or that Ena plays a very important role in filament tip bundling as a prerequisite for filopodia formation. I strongly feel that some of these aspects should be looked at, to provide some more mechanistic detail.

Finally, the manuscript could be improved in its readability (see detailed comments). It would also be of benefit to provide sub-headings that make clear statements, so that arguments are easier to follow.

Overall, I feel that the manuscript is not yet ready for publication, but that the authors should be given the opportunity to re-submit. Happy to give more detailed feedback and engage in discussion: Andreas.Prokop@manchester.ac.uk.

DETAILED COMMENTS

Abstract: in my view, the relevance, rationale and conclusions are not well presented in the abstract. What is the bigger picture problem, where lies the importance and what past work led to the aims of this work? What is the outcome and what does it mean, how does it improve our understanding?

Introduction: I wonder whether the intro should not start a bit more general:
- formation of neural circuits requires axon growth
- axons extend through guided extension implemented by amoeboid GCs at their tips
- GCs are guided through an interplay of spatiotemporal patterns of guidance cues in their environment and the set of receptive systems they present (as a function of specification during early neurogenesis)
- signals translate into changes in cytoskeletal organisation and dynamics, which then implement morphogenetic behaviours of GCs.

Intro, 1st line: "to build a" "during the formation of a"?

Intro, first para: It would be good to illustrate Abl's function as a signal integrator by naming a few upstream and downstream factors.

p.3 (intro): I would disagree that flat lamellar GCs only occur on rigid, highly adherent substrates. All it requires is a less focussed substrate, such as a basement membrane. Accordingly, many spread-out GCs can be found in vivo; examples: https://doi.org/10.1073%2Fpnas.88.6.2293 -- https://doi.org/10.1523/jneurosci.05-09-02345.1985 -- https://doi.org/10.1523/jneurosci.03-01-00020.1983 -- https://doi.org/10.1242/dev.00713 -- https://doi.org/10.1523/JNEUROSCI.2377-04.2005

p.3, last para: Role and structure of filopodial and central actin networks should be explained.

p.3: "of advancing the actin distribution down the axon" -- "towards the tip of the axon" to avoid misunderstanding

bottom of p.3: terms like "condensation" or "contraction" might be imprecise and suggest myosin function. Is this what the authors want to say, or do they want to say "disassembly/reduction"?

p.4: ena bundles actin filament tips, not filaments

p.4: the statement about Ena/formin-dependent filopodial distinctions may not be the case in axons: https://doi.org/10.1371/journal.pone.0018340

p.4: presence of actin in axons: add https://doi.org/10.1371/journal.pone.0018340

Overall, the intro is rather long and authors might want to try compressing it. Definitely, the last para of the intro is far too detailed.

Most details from 1st results section exported into methods?

p.6, last para: what would be an obvious sign of substrate adhesion? either explain or leave out.

p.6, last para: "in the axon shaft" or "at the centre of the GC"?

p.7, first sentence: "In detail..." can you say this simpler? Do you mean that there are more filopodia at the proximal than distal end of the GC?

p.7, top para: Graphs in Fig.2A-D should be a bit better explained in the main text; please, provide correlation in A and provide significances of correlations in all graphs, since there is sufficient space.

p.7, end of 1st para: can you speak of "driving force" here? or is it a mere indicator that the actin core is advancing, i.e. filopodia (which require F-actin networks and and the number of which is likely a function of filament number) lag behind because their anterior translocation is a secondary effect mechanistically downstream of core advance.

p.7, 2nd para: why is the filopodial peak not provided in Fig.2E? It should also be noted that the highest actin density is close to the leading actin edge.

p.7, 2nd para: what is the "distribution midpoint"? Is it not that all three lines show a net forward movement?

Fig.2B and G seem to refer to the same parameters. Why are they separated from each other?

p.8, start of 2nd para: The use of GMR and photoceceptors is confusing. What is it that the authors want to demonstrate here? Also, causing aggregation in Golgi is likely a stress-phenotype? This needs to be interpreted and explained to the reader. Start a new paragraph when explaining the FP4-mito results ("Reducing ena activity...".

p.8/9: "expression ... was readily apparent in wings" -- what do you see? Please, provide a neutral description before coming to conclusions. Since you seem to observe aggregates in the cell body, does mentioning this experiment not fit much better with the GMR experiments above? At lease I now understand what you want to say.

Fig.3G: explain what we see: the cell body or GC? Essential would be show the GC. Why is the tomato channel not shown? Why are there no mitos in the control?

p.9, 1st para: describe the axonal phenotypes in simple terms in the main text. As a second control, also the FP4-mito expression alone has to be shown. Alternatively, it might be better to show the Abl interaction after describing the Ena loss phenotypes, thus confirming that these phenotypes are in agreement with genetic networks described before.

p.9, 2nd para: data in middle part are very difficult to read. Rearrange.

end of p.9: the amplitude of back/forward movements is much larger in the FP4-mito neurons. Is this statistically assessed? Please, comment in the text. In general, the fact that the means do not show differences is not further commented on. What do the authors think this means, or what needs to be done to make sense of it? Is the change in undulation amplitude important here?

p.10, 2nd para: I cannot find that you explained to us what the morphological impact of loss of Ena on axon morphology is. Please, either add this info or make it more prominent. --- when reading on, it becomes clear that some explanations come later. Please, make this clearer in the writing. Also, a colon behind "(Fig.5)" would help.

p.10, 2nd para: "Ena also limited the total length of filopodial" -- "promoted"? Please, provide the data for wild-type controls: ideally, export details into the Figure 5 graphs and name here percent increase/decrease relative to wt.

p.10, middle: The data in Fig.5B and how described in the text are confusing. Leave total filopodia length out (Fig.5B) and rather

focus on average length. Or will you use the total filopodia length data? What is unusual about the data is that filopodia should actually be shorter, unless Ena is not in filopodia. Can you use Ena-GFP to show whether it is in filopodia? Could it be that these neurons have formins at their tips? Does Profilin (which interacts with both Ena and Formins) cause filopodial shortening? This would be a helpful control.

end of page 10: please, describe the axon phenotypes well. It would make sense if a moderate reduction in filopodia numbers would not affect the axon growth, especially since the more essential actin core is unaffected.

First part on page 11 can be said in two sentences. Otherwise, a new para should start at "Therefore,..."

p.11, middle: What is wavelet analysis. Could the fundamental principle briefly be explained, please? How is it different from analyses used so far in this manuscript? Why has wavelet not been used all along?

Fig.5, G-I: Please, could the authors insert a stippled line that represents the mean of all measured curves?

p.12: The descriptions of the Jensen-Shannon divergence make sense only if the parameters are identified that make it less reliable upon loss of Ena. Is it due to the stronger indulation? If axon growth is not distrubed the long term prediction should be the same.

p.12: correlating "filopodial number" and "total filopodial length" is perhaps not the most suitable, since the latter incorporates the first. It would be better to use average filopodial length.

p.12: explain 'filopodial branching complexity': is this how many bifurcating filopodia there are?

p.13, 2nd para: is is unnecessarily complicated and should be simplified. "Suppression of Ena activity by expression of FP4-mito shifted the distribution completely to the
simpler morph" is one of the few clear sentences in this passage.

Reviewer #2 (Remarks to the Author):

In this paper, the authors study the role of Ena in growth cone morphology and actin distribution in the TSM1 axon of the Drosophila wing by spinning disk confocal imaging and detailed analysis of various morphology and actin intensity parameters. Besides single parameter analysis, they also performed pairwise correlations and PCA. Ena levels were controlled by either targeting Ena to mitochondria (reducing Ena levels from the plasma membrane) or overexpressing Ena. The main finding of the study is that Ena mainly regulates morphological features of the growth cone, such as filopodia number and filopodia length, and to a much lesser actin-dependent features such the size of the actin peak. This is in contrast to the effects of Abl, which was previously reported by the same authors to have opposite effects. Abl is a regulator of Ena. The authors suggest that Abl affects several actin regulatory proteins and that negative regulation of Ena by Abl may have some buffering effects with respect to filopodia formation. Furthermore, the authors speculate that Ena might have a bigger effect on membrane-actin linkage than actin organization, at least in these TSM1 axons.

This study is experimentally well conducted and provides a detailed analysis of growth cone morphology and actin peak in growth cones with different amounts of Ena. The paper overall is well written, at times with redundant statements. Strong points of the study are the pairwise correlations and PCA, which further emphasize the effects of Ena on morphology than actin distribution. What is evident is that there does not seem to be simple Abl-Ena-F-actin-filopodia protrusion pathway in these growth cones. However, the imaging data as provided does not allow to make any conclusions about detailed changes in F-actin organization and dynamics beyond the size and position of the actin peak. Overall it is not obvious that this study makes a significant advance in our knowledge of the mechanism of protrusive axonal growth over the previous two studies from this group using the same system (Clarke et al., 2020a; Clarke et al., 2020b) beyond highlighting the difference in effects by Ena and Abl.

Major concerns:

1. Introduction: The authors distinguish two major modes of growth cone advance: (i) traction force/adhesion-mediated growth and (ii) protrusive growth involving actin assembly and turnover. It is suggested not to discuss these modes in a way that they appear to be mutually exclusive. The traction force model also involves actin turnover and a shift of the F-actin rich domain. The main difference between these two modes is whether myosin and adhesions contribute to advance or not, and this might be context-dependent. The previous work by these authors has shown that the growth cone protrusion correlates with the position of the actin peak, which would be expected in either mode of growth cone advance.

2. The authors report on fluctuations of peak actin distribution with a net forward trend, e.g. on the bottom of p.7 "Together, these observations suggest that TSM1 growth cone advance is driven by forward-biased fluctuations of the actin distribution."

From the correlation between actin peak and filopodia density peak (basically the center of the growth cone), we can only conclude that F-actin appears to be involved in growth cone advance but not more about the mechanism which aspect of actin dynamics is the driving force (assembly vs myosin-driven traction force) since corresponding quantification has not been performed.

3. Fig. 5F: Wavelet analysis: it would be good to show example images of different actin clusters that were used for wavelet analysis. It would help the reader to show more images of individual actin peaks and clusters at different time points in addition to the quantification.

4. Fig. 6: The authors should explain better what the correlation values mean. What number reflects a strong correlation, what number a weak correlation.

5. Authors need to explain how the sample size was chosen.

Minor concerns:

1. Introduction: "Abl is also an upstream regulator of many aspects of cytoskeletal organization, including polymerization, branching, bundling, severing, and contractility of actin networks." Since it is relevant to this paper, I suggest naming some of the proteins that affect the cytoskeleton and are regulated by Abl.

2. Results: p.6 bottom, "We saw no evidence of large lamellipodial structures or obvious signs of substratum adhesion." What would be an obvious sign of adhesion in such recordings? Filopodia could protrude and adhere at the same time. How can the authors be certain that no adhesions are involved?

3. Figure 2A: please plot the two axes at the same scale for easier comparison of the two variables.

4. Figure 3A-B: it would be helpful to draw the outline of the cell(s).

5. Beginning of discussion p.14: "and to compare it with the effects of the Ena regulator, Abl tyrosine kinase." In this paper, the authors did not look at Abl, whereas they studied Abl with the same approach in a previous study. Thus, the way it is phrased, this first statement in the discussion is not fully appropriate.

6. In the methods section, more details about how the PCA analysis was carried out should be provided.

RE: Manuscript #E23-01-0003
TITLE: Enabled primarily controls filopodial morphology, not actin organization, in the TSM1 growth cone in Drosophila

Author response to referee comments

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors explore the potential role of Ena as downstream mediator of Abl signalling during axon growth of the TSM1 neuron in the developing Drosophila wing at pupal stage. From abstract, end of discussion, beginning of discussion and reading through the results part, I am not certain what the key message of the authors is. As the authors state: "Whereas we had found previously that altering Abl kinase activity primarily modulates the distribution of actin, with only modest effects on growth cone morphology, we now found that the effects of manipulating Ena were opposite, primarily modifying morphology, with only modest effects on actin distribution" - for loss of Abl, the data show axon stall suggesting systemic relevance, but what is the effect of loss of Ena on axon growth? These data seem not to have been provided. If I interpret the data for loss of Ena correctly, mainly filopodia numbers are reduced by ~50%, but the actin core of GCs is pretty unaffected. Given that the main role of filopodia likely is sensing the environment, the sensitivity of GCs may be a bit reduced, but the actual growth capacity not. Hence, does the majority of axons reach their targets, with perhaps a mild increase in aberrant navigation? If this is the case, then authors should build a clear rationale as to whether they think Ena is a mediator, and make clear how they interpret the loss of Ena phenotype.

We now present quantification of the terminal phenotype of TSM1 in Ena gain- and loss-of function. In brief, ~30% of TSM1 axons fail to develop properly (stall, misroute) in the LOF and 20% have defects in the GOF. (Fig 3).

If I understand the question properly, the referee asks us to discuss why we see axon stalling and misrouting in the altered Ena conditions even though the detailed phenotypes in the growth cone, particularly the actin phenotypes, are generally relatively subtle. We now have two paragraphs in the Discussion that present possible reasons for this. In brief, we suggest that the aberrant terminal axonal phenotypes upon altering Ena level arise (a) from absence of an orderly evolution of the actin distribution over time, and (b) from failure to buffer GC morphology against the morphological effects of changing Abl activity as the

kinase responds to changing inputs from external cues. This interpretation is discussed in detail in the text (pgs 21-22).

Another weak point is the localisation of Ena which is not being properly demonstrated at GCs, neither for loss- nor gain of Ena. In the case of loss of Ena, it is surprising that filopodia are not shorter given Ena's involvement in actin polymerisation. There are two possible explanations: First, Ena is not at the tip of filopodia and instead formins take its place; second, the filopodia show very slow backflow dynamics and filopodia have a very long persistence; both aspects can be assessed with available GFP-tagged tools. The reduction in numbers would suggest that less actin filaments are present (are actin amounts comparable in wt and loss of Ena GCs?) or that Ena plays a very important role in filament tip bundling as a prerequisite for filopodia formation. I strongly feel that some of these aspects should be looked at, to provide some more mechanistic detail.

We now show localization of Ena in the TSM1 growth cone in the UAS-ena condition (Fig 3G). We cannot examine Ena localization in wild type TSM1 GCs because the small amount of Ena immunoreactivity in this growth cone is completely masked by the high level of Ena in the adjacent epithelia (stated on pgs 10/11). Consequently, we also can't show reduction of membrane-associated Ena in the growth cone upon expression of FP4-mito since we cannot see the signal above background even in the WT, preventing comparison.

Regarding the reasons why average filopodial length is not a simple function of Ena level, a few points can be made.

- First, the referee is surprised and concerned because we find Ena affecting filopodial number in a consistent way but not average filopodial length. We point out in the text, however (pg 19), that exactly this pattern was also reported from *in vivo* live imaging of motoneuron growth cones in *C. elegans* (Norris (2009) *Neur Dev*). Perhaps it is the expectation based on prior studies of *in vitro* growth cones that is the problem, rather than the data reported here.
- Second, while we do sometimes detect accumulation of Ena at filopodial tips upon Ena overexpression in TSM1, this is not what we see most frequently (as shown in the Ena immunolocalization figure that has been added – Fig 3G, discussed on pg 11). Ena is not excluded from filopodia, but it is typically detected throughout the structure, without obvious evidence for selective concentration at the tip. This by itself may answer the referee's question, as he points out. We have no data as to whether the effect of Ena on filopodial length may be connected to presence or absence

of formins, and there is no way for us to find out short of starting a whole new research program devoted to investigation of those proteins.

- We cannot say anything about the relative amount of actin in filopodia in the different genotypes. Given the variability in the expression of a UAS-transgene from cell to cell and animal to animal, never mind in different genetic backgrounds, together with technical variation of microscope function from day to day, we cannot compare absolute levels of LifeAct signal intensity between cells or between genotypes.
- We agree that the likeliest explanation for the effect of Ena on filopodial number is related to a role for Ena in filopodial initiation. This is commented on in the text (pg 22).
- The referee points out that there are transgenes available that could be used to "look at" details of actin flow in filopodia. This may be true, but simply collecting those transgenes and validating them (to ensure they don't disrupt TSM morphology, dynamics, and function) would take at least 6-12 months, and probably a minimum of another 1-2 years to establish conditions for the relevant experiments and do a careful, rigorous, quantitative study of actin flow. This could not possibly be done in the 90 days available for revision. There is no point whatever in "looking at" these processes unless one does it thoroughly and rigorously.
- The referee suggests that such measurements of actin flow and filopodial dynamics are important to provide "mechanistic detail". ***I completely disagree****. Everything in this paper, and in our previous papers, argues that the details of filopodial dynamics have little effect on the growth of this axon. The data here, and the data we have published previously, show that axon growth, at least for this cell, does **NOT** correlate with any of the filopodial parameters we can measure, or any combination of them, not (1) total filopodial length, (2) average filopodial length, (3) filopodial number, (4) filopodial branching complexity, or (5) the length of the filopodial-rich domain. There is therefore nothing in the data that provides any reason to think that a detailed analysis of actin flow in these filopodia will shed meaningful mechanistic insight into the growth of this axon. Indeed, in the process of our previous study of the effects of Abl on TSM1 we did initially examine kinetics of filopodial emergence and persistence and we did not even bother to include it in the published manuscript as it was not informative.

Finally, the manuscript could be improved in its readability (see detailed comments). It would also be of benefit to provide sub-headings that make clear statements, so that arguments are easier to follow.

We have tried to simplify the wording as suggested, as we enumerate in the detailed response below. However, I need to point out that there are also places (again, specified below) where this was not possible. All of the processes, and all of the functional relationships, that we discuss are statistical, not deterministic. That means that for any given measurement we describe there will be cases that look superficially like counterexamples, or as if they contradict our description. We have found in the past that giving too simple a description of the average condition for any one of these observations immediately confuses readers when they see a case in the data that does not seem to conform to that description. In fact, there is no contradiction; it is simply a reflection of the statistical nature of the effects. Therefore, there are many places where we have no choice but to be very exact in our wording, even if it means that a seemingly simple idea requires a detailed, and apparently lengthy, presentation.

Overall, I feel that the manuscript is not yet ready for publication, but that the authors should be given the opportunity to re-submit. Happy to give more detailed feedback and engage in discussion: Andreas.Prokop@manchester.ac.uk.

DETAILED COMMENTS

Abstract: in my view, the relevance, rationale and conclusions are not well presented in the abstract. What is the bigger picture problem, where lies the importance and what past work led to the aims of this work? What is the outcome and what does it mean, how does it improve our understanding?

We have signposted the Abstract (pg 2) so it is clearer about what each sentence is providing. In 200 words there is a limit to how much we can say about each of these points, but it seems to me that the Abstract does, in fact, explicitly address every one of the questions that are raised.

Introduction: I wonder whether the intro should not start a bit more general:
- formation of neural circuits requires axon growth
- axons extend through guided extension implemented by amoeboid GCs at their tips
- GCs are guided through an interplay of spatiotemporal patterns of guidance cues in their environment and the set of receptive systems they

present (as a function of specification during early neurogenesis)
- signals translate into changes in cytoskeletal organisation and dynamics, which then implement morphogenetic behaviours of GCs.

We have added a bit of explanation along the lines requested by the referee (pgs 2-6). However, the flow of the Intro has to fit the story we are telling, and the flow of the Results that will follow, which are perhaps somewhat different from the organization typically used by the referee. The Intro gets to GCs, cytoskeleton, and signaling as they are needed, though perhaps not in the order and timing the referee would have chosen for one of his own papers.

Intro, 1st line: "to build a" "during the formation of a"?

Intro, first para: It would be good to illustrate Abl's function as a signal integrator by naming a few upstream and downstream factors.

This has been added (pg 2).

p.3 (intro): I would disagree that flat lamellar GCs only occur on rigid, highly adherent substrates. All it requires is a less focussed substrate, such as a basement membrane. Accordingly, many spread-out GCs can be found in vivo; examples:
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https://doi.org/10.1242/dev.00713 -- https://doi.org/10.1523/JNEUROSCI.2377-04.2005

We have reworded this sentence to be less dogmatic (pg 3).

We must point out, however, that we do not agree with the premise of the referee's comment. All but one of the papers he cites in support of his view rely on use of PFA-fixed samples for their high-resolution pictures of lamellar GCs. However, we have found in TSM1 that standard PFA fixation induces a lamellar appearance in non-lamellar growth cones as a systematic artifact. Live imaging, or faster, more efficient methods of fixation, do not show this. If we restrict our literature search to in vivo, live imaging, then flat, lamellar, adhesive growth cones are indeed rare, and when we do see them, more often than not they are either in places where the growth cone is stationary (typically because it is in the process of reorienting in place), or the images are pictures of mutant conditions.

Nonetheless, lamellar growth cones can sometimes be found by live imaging in vivo so we have reworded the relevant sentence.

p.3, last para: Role and structure of filopodial and central actin networks should be explained.

We briefly introduce the filopodial vs central actin networks (pg 3). We do not go into much detail about the filopodial actin network because it is not measured or analyzed anywhere in our study. Therefore, too much detail about this in the Introduction would be distracting and misleading.

p.3: "of advancing the actin distribution down the axon" -- "towards the tip of the axon" to avoid misunderstanding

We have introduced this terminology for clarification in various places (eg., pg 4).

bottom of p.3: terms like "condensation" or "contraction" might be imprecise and suggest myosin function. Is this what the authors want to say, or do they want to say "disassembly/reduction"?

We definitely do not want to call it disassembly! That is NOT what we are talking about. We are discussing the actomyosin network becoming more compact – the same amount of actin being condensed into a smaller volume. As it happens, myosin activity is one of the contributors to compaction, but the referee make a sensible point that a naïve reader may overestimate its role if we use the word "contraction", so we will avoid that word, and instead stick to the term "compaction" throughout the manuscript (eg., pg 4).

p.4: ena bundles actin filament tips, not filaments

Actually, according to Blanchoin, et al (Phys Rev 2014), Ena also bundles actin filaments per se and not just tips, by virtue of its actin binding domain and multimerization activity. Those authors suggest that this contributes, for example, to the stiffness of actin bundles. This reference is now cited for that comment (pg 5).

p.4: the statement about Ena/formin-dependent filopodial distinctions may not be the case in axons: https://doi.org/10.1371/journal.pone.0018340

This may be true, and we do not insist that the interaction is relevant in the axon. However, we also have no direct experimental evidence that *excludes* this possibility in the specific case of TSM1. Indeed, this effect could theoretically contribute to the unexpected relationship between Ena activity and average filopodial length in this study. Therefore it seems prudent to mention the

possibility that this well-established property could potentially come into play (pg 5).

p.4: presence of actin in axons: add https://doi.org/10.1371/journal.pone.0018340

Referenced in the text at various points.

Overall, the intro is rather long and authors might want to try compressing it. Definitely, the last para of the intro is far too detailed.

Earlier versions of the manuscript had a shorter Intro but outside readers asked for additional background (just as the Referee has asked for substantial additional details).

Specifically regarding the final paragraph, the only way to shorten it would be to remove either central conclusions of the paper or any mention of the data that support them. We feel that stating the key argument of the paper in both the last paragraph of the Intro and the first paragraph of the Discussion is essential to ensure that the message gets through to the reader.

Most details from 1st results section exported into methods?

Again, earlier drafts segregated the experimental info into the Methods but readers who were not so familiar with our earlier papers felt strongly that we should include a more detailed description of the experimental method in the main text.

p.6, last para: what would be an obvious sign of substrate adhesion? either explain or leave out.

An explanation is now given (pg 7). There are at least 6 distinct features that contribute to this assertion, 5 of which can be seen by simple visual inspection of the movies. The key ones are now summarized briefly in the text.

- The axon extends in a ~20micron thick open space between the dorsal and ventral epithelia and is not associated consistently with either one in our movies
- The filopodia splay out as much in the z-axis as in x or y; they do not lie flat against one epithelium
- The axon shaft translocates laterally relative to the epithelia between any two successive time points, showing that it is not anchored along its length
- Individual filopodia translocate laterally relative to the epithelia between any two successive time points, showing that they are not anchored along their length

- The lengths of filopodia change substantially between time points as their tips move back and forth relative to the epithelia. This suggests that the tips are not strongly anchored to the substratum
- Co-imaging of actin and zyxin does not reveal any consistent relationship between local concentrations of actin and adhesive complexes. These data are not included in the manuscript since it is difficult to prove a negative, but had it been observed it would have been an indication of potential substrate adhesion. It was not observed.

p.6, last para: "in the axon shaft" or "at the centre of the GC"?

As stated explicitly in the text (pg 7), actin density “along the axon shaft” is measured along the entire length of the axon by integrating LifeAct intensity across the entire diameter of the axon shaft at each successive bin of axon length. To say "the center of the growth cone” would be both ambiguous and inaccurate. We do not limit our measurements to a line along the radial center of the axon, nor do we limit it to just a portion of the axon length in the vicinity of the actin peak or domain of high filopodial density (depending on how one defines the GC).

p.7, first sentence: "In detail..." can you say this simpler? Do you mean that there are more filopodia at the proximal than distal end of the GC?

No, it is NOT necessarily the case that there are more filopodia at the proximal vs the distal end of the growth cone.
We have now tried to simplify the wording here (pg 8), but this is one of the places where too much simplification leads to confusion for the reader. First, while *on average* the actin peak leads the filopodial peak, these are both dynamic, statistical entities. Because of random fluctuation, individual cases can be found that appear to contradict the statement (quantified in Fig 2B). That does not, in fact, contradict the fundamental interpretation, since the growth cone essentially averages over time and space to make a growth or guidance choice, but it does mean we have to be careful in stating the observation to ensure that our description is accurate.

Confusion also arises because the mass of actin covers a substantial range along the length of the axon, as does the domain of abundant filopodia (each ~12-15 microns). As a result, the two “peaks” actually overlap significantly. Our metric allows us to define a unique position for each so we can quantify the spatial relationship between these two broad peaks, but again, we have found that if we are not precise about our wording the reader quickly gets lost.

p.7, top para: Graphs in Fig.2A-D should be a bit better explained in the main text; please, provide correlation in A and provide significances of correlations in all graphs, since there is sufficient space.

The Spearman correlation and p-value of Panel 2A are now given in the text, as requested (pg 8). We must point out, however, that this particular correlation is NOT actually relevant to the phenomenon under discussion in this paragraph, so these values have not been added to the figure. As stated in the legend, and marked on the figure, the dashed line shown on the figure is the line x=y; the line where the data would lie if the actin and filopodial peaks were coincident. It is NOT a least-squares line through the data. The point of the figure, again as discussed in the text and pointed out specifically in the legend (pg 34), is that the datapoints are strongly biased to lie below the line x=y, demonstrating that the filopodial peak tends preferentially to lag behind the actin peak. We have added additional wording to explain this more explicitly.

We have also reworded the explanation of Fig 2C and D to try to simplify the descriptions of these (pg 8), and we have added p-values to all graphs of correlation plots.

p.7, end of 1st para: can you speak of "driving force" here? or is it a mere indicator that the actin core is advancing, i.e. filopodia (which require F-actin networks and and the number of which is likely a function of filament number) lag behind because their anterior translocation is a secondary effect mechanistically downstream of core advance.

We no longer use the term “driving force”.

p.7, 2nd para: why is the filopodial peak not provided in Fig.2E? It should also be noted that the highest actin density is close to the leading actin edge.

Adding the position of the filopodial peak to the graph would cause unnecessary confusion for the reader. As discussed above, although the two peaks have a consistent relationship *when averaged over the population*, at any given instant they can either coincide, or indeed the value used to define the position of the filopodial peak can advance ahead of the one that defines the position of the actin peak. This can be a transient statistical fluctuation, or it can be an adventitious consequence of the way things are calculated. We could, of course, cherry-pick a trajectory where everything fits the average description, but that would not be a typical case – usually things are messier than that. Again, this was all discussed in detail in previously published work (cited in the text, eg. Pg 8). The statistical nature of these observations is discussed at numerous points throughout the current manuscript so we see no need to make things more complex with another long explanation.

It is true that the “leading edge” of the actin peak is typically closer to the actin maximum than is the “trailing edge”. This is an intriguing observation, and we have various guesses for why it may be true, but we have no firm data on the point so we have no grounds to discuss it.

p.7, 2nd para: what is the "distribution midpoint"? Is it not that all three lines show a net forward movement?

We now refer to the actin mass as moving forward as a whole (pg 8).
We note, however, that the 3 lines do not move in lockstep. In any given timestep, the front, the back, or both can move opposite to the direction of the intensity peak. The midpoint of the distribution (the middle of the window that contains the maximum integrated actin intensity) is a useful fiduciary mark for tracking the motion of the mass of actin, but it is separate from the stochastic fluctuations of the front and back of the mass.

Fig.2B and G seem to refer to the same parameters. Why are they separated from each other?

Figure 2B simply tabulates the value of a specific measurement across the dataset (offset). Figure 2G uses that offset value to investigate correlation to a different measurement (length of the actin peak). The significance of assessing that particular correlation is not obvious until various other properties have been introduced, so the comparison does not appear until some lines further down in the text. In between, we present the correlation of the offset to various other features of the data. The values in 2B show up when they are needed; the correlation in 2G is introduced when it makes sense in the argument. The figure panels are then labeled and cited in the order they are used, as the journal requires.

p.8, start of 2nd para: The use of GMR and photoceceptors is confusing. What is it that the authors want to demonstrate here? Also, causing aggregation in Golgi is likely a stress-phenotype? This needs to be interpreted and explained to the reader. Start a new paragraph when explaining the FP4-mito results ("Reducing ena activity...".

We have now added wording explaining the motivation behind the use of this and the other measurements introduced in this figure (pg 9-10). We first needed to verify that the UAS-ena transgene to be used in this study was competent to produce a phenotype in Drosophila neurons. We therefore needed a characterized neuronal phenotype of Ena overexpression that we could use as an in vivo functional assay. There actually aren’t very many of those. However, we have demonstrated previously that overexpression of Ena reliably changes Golgi organization in fly photoreceptors, so that is the test case we used here solely to validate the reagents.

As it happens, the Golgi aggregation phenotype has nothing to do with stress responses. Rather, Ena alters the relative rates of fission and fusion of Golgi leaflets by changing actin

organization. This is discussed in detail in a previous paper of ours (referenced in the text; pg 9) but the underlying mechanism is irrelevant to the use of this assay for the current paper, so it would be distracting and confusing to go into detail in the text.

As an aside, this is NOT a GMR experiment. The point of this experiment was to test whether expression of the UAS-untagged Ena transgene, under control of the GAL4 driver used in the current study, neur-GAL4, was sufficient to reproduce the relocalization phenotype observed previously with GMR and tagged Ena in photoreceptors. The data show that it is. GMR is not used anywhere in the current study.

The ISNb analysis, like the photoreceptor experiment, is used simply to validate a published reagent using a well-characterized, in vivo, neuronal bioassay. We see no need to give these validations excessive prominence by splitting them into multiple paragraphs.

p.8/9: "expression ... was readily apparent in wings" -- what do you see? Please, provide a neutral description before coming to conclusions. Since you seem to observe aggregates in the cell body, does mentioning this experiment not fit much better with the GMR experiments above? At lease I now understand what you want to say.

"Readily apparent" just means one can see it easily. However, we have changed the wording to sound more operational (pg 10). This image does not go better with the photoreceptor experiments because we have no evidence as to whether the puncta observed in these wing neurons are cis-Golgi (though that seems plausible), and it doesn't matter for the purpose of the experiment. Moreover, the point of the experiment is to show successful overexpression of this transgene, in this cell, at the stage of interest, whereas the photoreceptor experiment investigates the capability of this transgene to mimic a particular Ena OE phenotype by exploiting a different neuron type at a different time in development.

Fig.3G: explain what we see: the cell body or GC? Essential would be show the GC. Why is the tomato channel not shown? Why are there no mitos in the control?

We now specify in the figure legend that the previous Fig 3G (now Fig 3H & I) shows the cell body, not the growth cone. As discussed above, the modest signal from Ena immunoreactivity in the wild type TSM1 growth cone is completely overwhelmed by the Ena signal in the adjacent epithelia. This is particularly problematic in fixed samples where the growth cone becomes tightly adhered to both the dorsal and ventral epithelia by the fixation process. Since we can't see Ena in the WT growth cone, we also can't use growth cone staining to show reduction of signal at the membrane upon expression of FP4-mito-eGPF. We therefore have to examine the cell body, not the growth cone, to be able to compare directly the level and localization of Ena in the same location in WT, UAS-Ena, and FP4-mito.

As an aside, it is only very rarely that we can detect signal from the mitochondrial marker in the distal axon or growth cone of TSM1 at this stage. We therefore suspect that much of

the axonal phenotype of FP4-mito reflects not just trapping of Ena at mitochondria, but trapping specifically at mitochondria in the cell body, with consequent exclusion from the axon and growth cone. However, we cannot prove that so we prefer not to speculate in the text.

The mitochondria are not visible in the control panel because, as stated in the text (pg 10), we use the FP4-mito-eGFP itself to identify the mitochondria. Obviously, this reagent is not present in the control. However, it is obvious by inspection of the image that there are no puncta of Ena of any sort in the control condition that are analogous to those seen upon expression of FP4-mito, so there is no punctate signal in the control whose localization site needs to be identified.

The tomato channel is not shown to avoid confusion. *neur-GAL4* expresses in all the cells of the sense organ, not just the neuron, so the tomato channel shows signals outside the cell of interest, and lengthy explanations would be required to explain the image. It is also not an important point for the purposes of this experiment since we do, in fact, see recruitment of the endogenous Ena to mitochondrial puncta in all cells expressing the FP4 reagent, not just the neuron.

p.9, 1st para: describe the axonal phenotypes in simple terms in the main text. As a second control, also the FP4-mito expression alone has to be shown. Alternatively, it might be better to show the Abl interaction after describing the Ena loss phenotypes, thus confirming that these phenotypes are in agreement with genetic networks described before.

We now specify that the most frequent Abl RNAi phenotype in TSM1 is axon stalling (pg 11), and we include the expressivity of the FP4-mito TSM1 phenotype in the same figure that quantifies phenotypic suppression of Abl LOF by ena LOF (in the subsequent panel quantifying the phenotypes of ena GOF and LOF; Fig 3Q).

We feel it is important to validate the individual reagents before we use them to generate and describe *ena* phenotypes. Suppression of the Abl LOF phenotype is the defining characteristic of ena LOF. We therefore prefer to provide these data in the paragraph on validation of the reagent.

p.9, 2nd para: data in middle part are very difficult to read. Rearrange.

We have rearranged this sentence (pg 11) and moved the description of the sample sizes to the Methods (pg 24).

end of p.9: the amplitude of back/forward movements is much larger in the FP4-mito neurons. Is this statistically assessed? Please, comment in the text. In general, the fact that the means do not show differences is not further commented on. What do the authors think this means, or what needs to be done

to make sense of it? Is the change in undulation amplitude important here?

While the variance in the amplitude of actin excursions in FP4 mito looks larger than that in WT, the difference is not statistically significant. When calculating significance, one must take into account the internal correlation of values for each cell – each cell is more like itself than it is like the other cells in the dataset (discussed in the Methods in the section about Statistical Methods, pg 26). Since the apparent differences are not significant there is nothing to discuss.

The mean excursion of the actin peak, on the other hand, is by definition simply the average growth rate of the axon. (The justification for this definition is one of the major results of the Clarke papers and is discussed there in great detail.) The lack of difference between the growth rates among the three ena genotypes is pointed out on pg 11, and as the referee points out, is consistent with the overall observation of minimal effects of ena on actin distribution.

p.10, 2nd para: I cannot find that you explained to us what the morphological impact of loss of Ena on axon morphology is. Please, either add this info or make it more prominent. --- when reading on, it becomes clear that some explanations come later. Please, make this clearer in the writing. Also, a colon behind "(Fig.5)" would help.

We now have a brief statement summarizing the effect of Ena on filopodial pattern before we present and quantify the details (pg 12). We have also now added pictures and quantification of the terminal axonal phenotype of TSM1 (Fig 3 N-Q; text pg 11).

p.10, 2nd para: "Ena also limited the total length of filopodial" -- "promoted"? Please, provide the data for wild-type controls: ideally, export details into the Figure 5 graphs and name here percent increase/decrease relative to wt.

To say ena level was “limiting” implied that increasing ena increases total length and decreasing ena decreased total length. But we have reworded the paragraph to change “limited” to “increased”, as requested (pg 12). The data for the WT controls are given in the text, as is the relative percent of the effect due to loss vs gain of Ena (pg 12).

p.10, middle: The data in Fig.5B and how described in the text are confusing. Leave total filopodia length out (Fig.5B) and rather focus on average length. Or will you use the total filopodia length data? What is unusual about the data is that filopodia should actually be shorter, unless Ena is not in filopodia. Can you use Ena-GFP to show whether it is in filopodia? Could it be that these neurons have formins at their tips? Does Profilin (which interacts with both Ena and Formins) cause filopodial shortening? This would be a helpful control.

First, concerning the use of total vs average filopodial length, total length shows a simple, global correlation with Ena activity. In contrast, the effect of Ena on average filopodial length is confusing, non-obvious, and seemingly at odds with expectation from previous studies of growth cones in vitro (as the referee points out). That would seem to make total filopodial length the natural choice for trying to make sense of the effect of Ena in this experimental system, not average filopodial length. Second, we will need to use total filopodial length later, in the PCA. Total filopodial length is a simple, unbiased measurement. Average length depends on filopodial number and branching organization, both of which are also terms in the PCA. Total length is therefore a better choice for the later statistics so it makes sense to use that parameter throughout.

The referee says the filopodia "should actually be shorter" (I presume he means in the LOF condition). I can't say what the answer "should" be, only what it is. I must also point out that our data are in agreement with results from *in vivo* live imaging of motoneuron growth cones in *C. elegans* (as mentioned earlier, and commented on and referenced in the text). If there is a discrepancy with in vitro growth cone data, perhaps it is the in vitro data that are in need of explanation, not the data from multiple, independent in vivo systems. However, as requested by the referee, in the Discussion we now comment on the apparent difference between the effect of Ena on average filopodial length in this study vs that seen in published work in other systems, and speculate that it may be related to the failure to see reliable accumulation of Ena at filopodial tips in this non-adherent system, as opposed to what has been seen in the adhesion-dominated contexts where most of the prior Ena work has been done (pg18).

We now include a picture of anti-Ena immunostaining of UAS-ena, since in the overexpression condition, unlike WT, we can easily distinguish the growth cone signal from that of the underlying epithelium (Fig 3G). Ena is present in filopodia, though we generally do not see evidence for it accumulating at their tips. Perhaps this helps the referee come to an interpretation of the result. I must point out, however, that this is an overexpression condition, and one that we already know changes the filopodial parameters. What it implies about the WT condition is entirely unclear to me.

We have no data as to whether formins are present, or which ones, or where they may be localized, or how their properties may change in response to changes in Ena, and we have no desire to speculate without data. Investigation of these questions would require an entirely new, multi-year project. The same is true for profilin. In the case of profilin, moreover, the predictions are far from obvious. The effects of profilin are extremely complex, they can be opposite at different ends of the protein's concentration range, and they are entirely dependent on the balance of other factors in any given biological context (Arp 2/3, ena, cofilin, and others; see, for example, Dilley, et al. (2009) *Biophys J* **vol 96**, p. 3529). To call investigation of the effects of altering profilin a "control" is entirely inaccurate.

end of page 10: please, describe the axon phenotypes well. It would make sense if a moderate reduction in filopodia numbers would not affect the axon growth,

especially since the more essential actin core is unaffected.

A description of axon phenotypes in altered ena conditions is now provided, as discussed above (pgs 11,12; Fig3; images Fig 4 C, D; quantification Fig 5 A-D). The relationship of the actin and filopodial phenotypes to one another, to growth cone morphology, and to overall axon growth is the essential subject of the entire paper. It is considered in detail in the Discussion

First part on page 11 can be said in two sentences. Otherwise, a new para should start at "Therefore,..."

We disagree with this suggestion. The peak length data are important and they need to be presented accurately and thoroughly. The wavelet analysis then extends and clarifies the result of the simpler peak length measurement. We therefore prefer to keep these together in a single paragraph (pg 13).

p.11, middle: What is wavelet analysis. Could the fundamental principle briefly be explained, please? How is it different from analyses used so far in this manuscript? Why has wavelet not been used all along?

Since both referees expressed substantial confusion about the meaning and interpretation of wavelet analysis, we have now given an explanation in the text (pg 13) that is more intuitive, and less mathematical, (though that necessarily means it is also less precise, unfortunately). Perhaps the very simplest way to describe it is that the wavelet analysis gives a measure of how clumpy or homogeneous the actin distribution is, how broad the clumps tend to be, and how far they are spaced from one another along the length of the axon (though this is a pretty gross oversimplification of what the analysis tells us). Note that the wavelets quantify the complete distribution of values for each of these facets, at multiple spatial scales, they do NOT just report a single, average value. Please see the text for more details. Also note that the wavelet calculation takes into account the distribution of actin along the entire length of the axon in a single calculation, and it quantifies the axon properties globally. It is NOT done by selecting individual features for analysis. (Note also that in extracting the spatial scales of intensity correlations the wavelet calculation is sensitive to both the widths of features and the spacing of features; it does NOT discriminate whether a correlation length arises from a peak or a trough in the absolute intensity value.)

As stated in the text, the wavelet analysis differs from the measurement of actin peak length in that rather than measuring just the outline of a single feature (the actin peak), it quantifies the distribution of actin intensity within that peak, and indeed everywhere in the axon, at all possible scales of spatial separation (sub micron, 1-2 micron, 2-5, 5-10, etc). It is far richer in information content than is the simple measurement of peak length. Note also that Supplemental Fig 2 includes a table specifying the spatial scale that is quantified by each wavelet order.

Given the confusion the wavelets caused both referees it should be obvious why we did not start with this in the text or use it in place of reporting measurement of the length of the actin peak. The concepts behind the wavelet analysis are far less familiar to many cell biologists than those for a simple measurement of a defined feature, so there was a value in giving the simple idea first. However, even for an audience of physicists accustomed to this analytical method we would have presented the data both ways (and the simpler way first). The peak length measures a specific property of the growth cone, and one that we have previously shown is meaningfully connected to the mechanism of growth cone advance. The wavelets tell us something else about both the fine structure and gross structure of the actin distribution across the whole axon. Both are useful, both are informative, and indeed their messages, while distinct, reinforce one another.

Fig.5, G-I: Please, could the authors insert a stippled line that represents the mean of all measured curves?

The mean of all curves is not a meaningful parameter in this case. This analysis quantifies how predictable the evolution of the actin distribution is over time. As can be seen from inspection of the curves (Fig 3 G-I; Suppl Fig 3), the effect of Ena is statistical – in the altered conditions, a fraction of cells develop like WT and a fraction do not. The *distribution* of different curve shapes is what matters. A mean curve would average-away exactly what is the key observation – the presence and frequency of outlier cells with stochastic disruption of actin evolution. It would also be contaminated by a variety of technical issues that are not relevant to the interpretation.

p.12: The descriptions of the Jensen-Shannon divergence make sense only if the parameters are identified that make it less reliable upon loss of Ena. Is it due to the stronger indulation? If axon growth is not distrubed the long term prediction should be the same.

We have also rewritten and simplified the description of divergence to make this more intuitive (pgs 14-15).

- The divergence is a simple, unbiased way to measure the similarity between two curves. If the curves are identical, there is no divergence – it is 0. If there is (hypothetically) no overlap between the curves, their divergence is complete – it is 1.
- The divergence between two curves (in this case, between the profile of actin intensity along the axon at two different times) is calculated as a simple point-to-point comparison of the (normalized) actin intensity at corresponding positions in the two profiles.
- The divergence does NOT include any “parameters”. It is not like a PCA. It is just a number that quantifies similarity of curve shape
- As an aside, there is no strong connection between the variance of actin peak step size and the value of the divergence in this dataset.

We now comment in the Discussion about whether the presence of cells with unpredictable changes in actin distribution from frame to frame may be related to the probability of axon stalling and misrouting in the altered ena conditions (which we think is very likely; pg 21).

p.12: correlating "filopodial number" and "total filopodial length" is perhaps not the most suitable, since the latter incorporates the first. It would be better to use average filopodial length.

The referee is mistaken. Filopodial number and total filopodial length are both simple, naïve, raw measurements that can be made without any artificial definitions. Average filopodial length is a derived measurement that depends on how one defines the branching pattern of a filopodial tree. Moreover, it is not true that total length incorporates filopodial number. Two trees can be exactly the same total length but have one consist of a single long branch and the other be a large number of very short branches, or two trees can have the same number of branches but differ in total length by any arbitrary amount depending on the lengths of individual branches. The fact that filopodial number and total length turn out to correlate in this dataset gives meaningful insight into the nature of axon morphology. It is not a trivial consequence of the definitions of the terms.

We explain above why we prefer not to use average filopodial length. The very fact that prior work done in vitro has given the referee has such strong opinions about how this measurement "should" behave, but those expectations are contradicted by the data presented here and also in the *C. elegans in vivo* data, suggests that this measure is far less transparent than it has been assumed to be.

p.12: explain 'filopodial branching complexity': is this how many bifurcating filopodia there are?

We define this in the text and the methods (pg 13, pg 25). Branching complexity is defined here just as it is defined for all analyses of dendritic branching and axonal branching. Primary branches come directly off the axon shaft; secondaries branch off of primaries, tertiaries branch off of secondaries, and so forth (called "branch order"). The measure we report is the average value of branch order.

p.13, 2nd para: is is unnecessarily complicated and should be simplified. "Suppression of Ena activity by expression of FP4-mito shifted the distribution completely to the
simpler morph" is one of the few clear sentences in this passage.

We have tried to simplify the wording in this paragraph (pg 16). However, this is another place where if we were to say too baldly that 'WT growth cones come in two types; FP4 only have the simple type, and U-Ena fall into a single type that is intermediate between the two WT forms', the referee would have objected strenuously – and in this case correctly - that we were substituting interpretation for data and we should present the facts in a neutral way. These are highly derived statistical measures; there is a significant gray area in the description and distinction of morphs; individual cells can switch between morphs during an imaging session (see Clarke(2020a)). So we have again done our best to simplify the description, but we must retain the subtleties of the data to be accurate.

Reviewer #2 (Remarks to the Author):

In this paper, the authors study the role of Ena in growth cone morphology and actin distribution in the TSM1 axon of the Drosophila wing by spinning disk confocal imaging and detailed analysis of various morphology and actin intensity parameters. Besides single parameter analysis, they also performed pairwise correlations and PCA. Ena levels were controlled by either targeting Ena to mitochondria (reducing Ena levels from the plasma membrane) or overexpressing Ena. The main finding of the study is that Ena mainly regulates morphological features of the growth cone, such as filopodia number and filopodia length, and to a much lesser actin-dependent features such the size of the actin peak. This is in contrast to the effects of Abl, which was previously reported by the same authors to have opposite effects. Abl is a regulator of Ena. The authors suggest that Abl affects several actin regulatory proteins and that negative regulation of Ena by Abl may have some buffering effects with respect to filopodia formation. Furthermore, the authors speculate that Ena might have a bigger effect on membrane-actin linkage than actin organization, at least in these TSM1 axons.

This study is experimentally well conducted and provides a detailed analysis of growth cone morphology and actin peak in growth cones with different amounts of Ena. The paper overall is well written, at times with redundant statements. Strong points of the study are the pairwise correlations and PCA, which further emphasize the effects of Ena on morphology than actin distribution. What is evident is that there does not seem to be simple Abl-Ena-F-actin-filopodia protrusion pathway in these growth cones. However, the imaging data as provided does not allow to make any conclusions about detailed changes in F-actin organization and dynamics beyond the size and position of the actin peak. Overall it is not obvious that this study makes a significant advance in our knowledge of the mechanism of protrusive axonal growth over the previous two studies from this group using the same system (Clarke et al., 2020a; Clarke et al., 2020b) beyond highlighting the difference in effects by Ena and Abl.

We agree that one of the major outcomes of this study is simply to highlight the differences between Abl and Ena, but we feel this in itself does provide a significant advance to the field. It has been pretty generally agreed for many years that Abl plays a central role in the function of many growth cones, and for many years there has been agreement that the essence of growth cone mechanism can be found somewhere in the cytoskeletal events downstream. So what are those events? That is the key question in GC mechanism. Since the early 90's it has been believed that much of Abl function is channeled through Ena/VASP. It is therefore clearly important, and unexpected, to discover that most of the Abl-dependent regulation of actin distribution (at least in this axon) does NOT go through Ena; it must be looked for elsewhere (probably in a Trio-Rac-Arp2/3 pathway, among others). The implications of the current study, moreover, go far beyond a negative finding of Ena having less of a role in actin organization than one had thought. We also make the positive finding, first, that what matters about Ena level is that it is critical both for the reliable evolution of the actin distribution over time and for determining the efficiency with which local actin density is translated into a local density of filopodial formation, and second, that the complementarity of Ena and Arp2/3 allows the growth cone to modulate Abl activity over a broad range, as needed for processive axon growth, while still maintaining growth cone organization within the narrow envelope that is permissive for orderly axonal development. To my knowledge, it has never been suggested that these are the two key functions of Ena for growth cone signaling.

Major concerns:

1. Introduction: The authors distinguish two major modes of growth cone advance: (i) traction force/adhesion-mediated growth and (ii) protrusive growth involving actin assembly and turnover. It is suggested not to discuss these modes in a way that they appear to be mutually exclusive. The traction force model also involves actin turnover and a shift of the F-actin rich domain. The main difference between these two modes is whether myosin and adhesions contribute to advance or not, and this might be context-dependent. The previous work by these authors has shown that the growth cone protrusion correlates with the position of the actin peak, which would be expected in either mode of growth cone advance.

We are now more measured in our description of these models, and distinguish between traction-based and protrusion-based models of adherent axon growth in vitro, as well as distinguishing both from non-adherent protrusive growth in vivo (pgs 3, 4).

2. The authors report on fluctuations of peak actin distribution with a net forward trend, e.g. on the bottom of p.7 "Together, these observations suggest that TSM1 growth cone advance is driven by forward-biased fluctuations of the actin distribution." From the correlation between actin peak and filopodia density peak (basically the center of the growth cone), we can only conclude that F-actin appears to be involved in growth cone advance but not more about the mechanism which aspect of actin dynamics is the driving force (assembly vs myosin-driven traction force) since corresponding quantification has not been performed.

We have modified the wording to emphasize correlation and association rather than causality (pgs 7. 8).

However, as a matter of clarification, this question is related to Minor concern #2 below, and to one of the comments of Referee 1 (above) asking what is the evidence that adhesive traction forces are not involved in forward motion for TSM1. As commented on in the response to Referee 1 and now summarized in the text (pgs 7,8), there are at least 6 lines of evidence that argue against the presence of significant adhesive interactions of TSM1 with the substratum (and therefore against a role for traction forces requiring such adhesions). These include (among others) apparently free lateral motion of both the axon shaft and individual filopodia relative to the epithelium between time points (even when the frame rate was one z-stack per 60 seconds, or one stack per 15 seconds, rather than one per 3' as in the current dataset) and apparently free motion of filopodial tips relative to the substratum in the same time intervals. All of these seem inconsistent with the anchorage required for generation of traction force. Moreover, we failed to detect any consistent correlation between local actin distribution and localized focal adhesion proteins in fast frame-rate imaging, again arguing against the traction models. Therefore, we never tried to develop technology for measuring traction forces in this system because we never observed any interaction that we could set out to measure.

3. Fig. 5F: Wavelet analysis: it would be good to show example images of different actin clusters that were used for wavelet analysis. It would help the reader to show more images of individual actin peaks and clusters at different time points in addition to the quantification.

As now stated specifically in the text (pg 13), and discussed in the response to Referee 1, above, wavelet analysis is a calculation performed on the entire length of the actin distribution for any given timepoint. It is NOT performed on individual features or segments of an axon.

Actin peaks and clusters are transient, dynamic, statistical entities. Images of single features like these are not informative since the range of features observed is exactly the same in all genotypes; what varies is their relative frequency. That can only be detected by global quantification. Desired images could, of course, be cherry-picked, but that would not be meaningful.
We have also rewritten the explanation of the wavelet method to make it more intuitive and less mathematical since our previous explanation was evidently unclear to both of the referees (pg 13).

4. Fig. 6: The authors should explain better what the correlation values mean. What number reflects a strong correlation, what number a weak correlation.

As stated in the legend to Fig 6A, all the correlation values that were statistically significant, and only the correlation values that were statistically significant, are shown in Fig 6 (Benjamini-Hochberg FDR < 5%). Additionally, Suppl Fig 4B shows the P-values for all of the comparisons in the table. Here we consider any correlation that is statistically significant as being strong enough to deserve our attention.

5. Authors need to explain how the sample size was chosen.

As stated in the Reproducibility section in the Methods (pg 28), sample size was selected based on “leave-out” analyses of our previous datasets.

Minor concerns:

1. Introduction: "Abl is also an upstream regulator of many aspects of cytoskeletal organization, including polymerization, branching, bundling, severing, and contractility of actin networks." Since it is relevant to this paper, I suggest naming some of the proteins that affect the cytoskeleton and are regulated by Abl.

This has now been added (pg 2,3).

2. Results: p.6 bottom, "We saw no evidence of large lamellipodial structures or obvious signs of substratum adhesion." What would be an obvious sign of adhesion in such recordings? Filopodia could protrude and adhere at the same time. How can the authors be certain that no adhesions are involved?

See Major comment #2 above. An abbreviated version of that response is now included in the text (pg 7)

3. Figure 2A: please plot the two axes at the same scale for easier comparison of the two variables.

This has been done.

4. Figure 3A-B: it would be helpful to draw the outline of the cell(s).

We attempted to add cell outlines but they completely obscured the small, membrane-proximal Ena puncta, particularly in the wild type condition. We therefore chose not to include this. The membranes are in any event clearly visible in the micrographs, in green in the wild type image (panel A) and in red in the U-ena image (panel B).

5. Beginning of discussion p.14: "and to compare it with the effects of the Ena regulator, Abl tyrosine kinase." In this paper, the authors did not look at Abl, whereas they studied Abl with the same approach in a previous study. Thus, the way it is phrased, this first statement in the discussion is not fully appropriate.

We have reworded the text to specify that the Abl data come from a previous manuscript (pg 17).

6. In the methods section, more details about how the PCA analysis was carried out should be provided.

We have expanded the description of the PCA in the Methods (pg 26).

RE: Manuscript #E23-01-0003R
TITLE: "Enabled primarily controls filopodial morphology, not actin organization, in the TSM1 growth cone in Drosophila"

Dear Dr. Giniger:

We have just received comments from reviewers regarding your revised manuscript. As you can see, while they appreciate revisions that were made in this version, there remains some concern in the presentation of the manuscript. I appreciate the difficulty in condensing complicated information into a small space, but I also agree with reviewers that readability could be improved. We hope you will consider a revision of the introduction to streamline and simplify the major points of the manuscript, and a simplified explanation of the wavelet analysis as requested by reviewer 1, which at this point remains cryptic and thus may be under-appreciated by your audience. Please return a revised manuscript with text changes marked in red, as requested by reviewer two.

Sincerely,
Stephanie Gupton
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Giniger,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

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

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

Authors are allowed 15 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
Link Not Available

MBoC offers the option to publish your work with immediate open access. Open access can increase the discoverability and usability of your research. If you would like to publish your paper with immediate open access but did not select this option during initial submission, please contact us at mbc@ascb.org..

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

Thank you for submitting your manuscript to MBoC. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker

Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The authors have clearly responded to some of my points with regard to Ena tip localisation (which seems absent consistent with the unaffected filopodial length upon Ena manipulation) and providing data on axon growth upon Ena manipulation. However, the text is still very hard work to read. It would benefit from shortening (easily to half the length) and a more focussed discussion, ideally with headlines that raise clear statements. For example, the second half of the results part deals with very abstract readouts to describe Ena phenotypes which show mild or now changes. Why does the reader have to go through such length of text for a simple message? As indicated in my detailed comments, some conclusions seem to be missing.

DETAILED COMMENTS

Title and abstract: Enabled controls filopodial NUMBER - morphology seems little changed as shown in Fig.5

Abstract: there is no mentioning of Ena's impact on axon growth, neither of the one experiment that shows that Ena can suppress loss of Abl phenotypes, i.e. substantiating of the downstream statement

The introduction is far too long, lacks focus and contains misleading elements:
1) Description of the growth cone in the introduction is misleading; some suggested formulations:
- core of microtubules from where individual microtubules splay into a meshwork of .... in the growth cone periphery
- From this meshwork, actin bundles emerge to form filopodia that emanate into the growth cones surrounding.
2) the two modes of axon growth are inappropriate likely even wrong; current thoughts about axons growth need to be dealt with more carefully and perhaps and need to be explained with greater care. For example, the role of actin could be the focus, not the two suggested ways of growth. What we definitely know is that actin can generate forces that can generate morphogenetic changes but also mediate mechanosensing, that actin can drive protrusion of lamellipodia and filopodia, that actin can influence microtubule behaviours through structural guidance, through providing an anchor for cross-linkers or through mediating signalling, that actin is essential for the exo-/endocytosis of receptors. The key question is how these activities translate into axon growth, which may be implemented through mechanical pulling followed by microtubules in one school of thought, or implemented by microtubules downstream of actin in another school.

3) As pointed out previously, narrow growth cones are a common phenomenon also in culture. The way in which phenomena are classified in the introduction is just not helpful.

4) the term "non-adherent mode" is unnecessarily overstated and there is simply no proof for this as a general phenomenon in vivo. It is unconceivable that no adhesion takes place to even lose networks of ECM, especially when considering that many signalling receptors require a certain amount of pulling force for their action.

5) p.5: Ena links the TIPS of adjacent filaments

p.7 "the morphological "growth cone", contained a high local concentration of actin intensity in the axon shaft" - the use of axon shaft is misleading here

p.7 The formation of filopodia as a function of actin availability was also suggested by studies of actin nucelators (Goncalves-Pimentel 2011).

p.37 "the protrusive region of the axon, the morphological "growth cone", contained a high local concentration of actin intensity in the axon shaft" - the use of axon shaft is misleading here.

p.10: images of Ena expression are insufficient. In each case, growth cones need to be shown, of wt, Ena overexpression and FP4. FP4 traps Ena in the cell body, but is potentially locally translated Ena in GCs also away from the periphery? Where are the mitochondria in GCs? Is Ena attached to mitochondria still able to polymerise actin, therefore not eliminate but rather relocate actin polymerisation? Similarly, is there more Ena in the periphery of GCs when overexpressed?

p.11: Fig.3G - show a wt GC with anti-Ena taken at the same camera settings as the one overexpressing Ena. It is important to support the statement made, i.e. that endogenous Ena cannot be distinguished from background, and that ena overexpression overcomes this.

p.11: the absence of Ena at filopodial tips might be explained by the presence of formins; it needs to be highlighted more in the

text and used to provide a potential explanation as to why average filopodial length does not change upon Ena manipulation.

p.12 bottom half: the data explanations are unnecessarily complicated. The matter is simple: show data for filopodia numbers (which differ for FP4) and average length (slightly differ); total length is irrelevant since it is a mere product of the two and not very telling if average length is hardly changed anyway. - A reduction in filopodia numbers is consistent with Goncalves-Pimentel 2011 and was suggested to be due to a role of Ena in supporting nucleation events (promoting the polymerisation of new seeds); in that system Ena was at the tip of filopodia and loss of Ena affected their average length.

p.13: The wavelet data are not well explained and I cannot see what they may tell us. Please, could this be accompanied by a simple schematic or microscopic images? What are the mechanistic implications?

p.14: what would have been learned if Jensen-Shannon divergence had shown differences?

Reviewer #2 (Remarks to the Author):

The authors have significantly improved the manuscript based on both reviewers' comments.

Comments:

1. It would be helpful to mark the text changes in red font to see the differences.

2. Major concern 2 was addressed by changing the wording from "Together, these observations suggest that TSM1 growth cone advance is driven by forward-biased fluctuations of the actin distribution." To "Together, these observations suggest that TSM1 growth cone advance arises from forward-biased fluctuations of the actin distribution." I do not agree that this modification changed the emphasis on causality much. They should rather use the word "is correlated with".

3. Initial minor concern 3: scaling of axes of Fig. 2A. An adjustment was done, but it is unclear why the axes scaling is still different between x and y axes. Please use the same distance for corresponding values and start both scales at 0. Font on axis of Fig. 2B should also be increased.

4. Initial minor concern 4: Figure 3A-B: it would be helpful to draw the outline of the cell(s). I do not agree with the author. You can use a fine dashed line tool in ImageJ to outline one cell without obscuring things.

Response to referee comments on revised version of Fang, et al.

Editor comments:

We have just received comments from reviewers regarding your revised manuscript. As you can see, while they appreciate revisions that were made in this version, there remains some concern in the presentation of the manuscript. I appreciate the difficulty in condensing complicated information into a small space, but I also agree with reviewers that readability could be improved. We hope you will consider a revision of the introduction to streamline and simplify the major points of the manuscript, and a simplified explanation of the wavelet analysis as requested by reviewer 1, which at this point remains cryptic and thus may be under-appreciated by your audience. Please return a revised manuscript with text changes marked in red, as requested by reviewer two.

Our general comments:

1. Nearly all the wording concerns of Ref 1 related to the way our Introduction described old ideas about growth cone mechanics. None of this historical background, however, is necessary to present or understand the data we report here. With the permission of the Editor, therefore, we have simply deleted this superfluous background material. This shortens the Introduction, as requested by the Referee and removes all the wording he evidently found objectionable.
2. Regarding the request for an expanded explanation of wavelet analysis, we have elected to provide this in the form of a supplemental file. The explanation of the method was necessarily too lengthy to include in the main text.
3. Ref 2 requested that some Figure panels have additional annotation, and Ref 1 requested inclusion of images documenting that we cannot see the anti-Ena immunoreactivity in wild type and FP4-mito growth cones above the background of anti-Ena signal in the associated epithelia. We have included these images in an additional supplemental figure (the new Suppl Fig 2). Showing the annotated images alongside the unmanipulated images (which seemed best) would have required either that we shrink all the figures excessively or obscure part of the images had we kept them in the main figure. Including images in the main text in which we cannot discern the relevant features seemed to us to be confusing to a reader who would be wondering what they were trying to show.
    a. To clarify, we note that none of the newly-added material introduces new data to the paper.

Reviewer #1 (Remarks to the Author):

The authors have clearly responded to some of my points with regard to Ena tip localisation (which seems absent consistent with the unaffected filopodial length upon Ena manipulation) and providing data on axon growth upon Ena

manipulation. However, the text is still very hard work to read. It would benefit from shortening (easily to half the length) and a more focussed discussion, ideally with headlines that raise clear statements. For example, the second half of the results part deals with very abstract readouts to describe Ena phenotypes which show mild or now changes. Why does the reader have to go through such length of text for a simple message? As indicated in my detailed comments, some conclusions seem to be missing.

As stated above, we have shortened the Introduction by removing the parts that the referee found objectionable, and that were not essential to our presentation.

We have added headings to portions of the Discussion to break up the presentation for the reader.

It is true that the readouts of growth cone function that we use are unfamiliar to many cell biologists and, in some cases, are grounded in mathematical methods that may seem rather abstract to some readers. However, these are the readouts we were driven to use by the nature of the system. We did not resort to them by choice. Cell biologists, in general, are not accustomed to thinking about noisy, stochastic systems. However, as many of us are learning, that is the nature of cytoskeletal cell biology. We can try to pretend it is simple and deterministic, but that doesn't make it so. We therefore have used the analyses that we were driven to by the need to extract the meaningful signals hiding in the stochastic noise, even when it required us to learn new ideas and new analytical methods. These methods are not more abstract than other kinds of mathematical analyses, they are simply less familiar to some readers.

DETAILED COMMENTS

Title and abstract: Enabled controls filopodial NUMBER - morphology seems little changed as shown in Fig.5

In the title, it is the global morphology of the growth cone that we refer to, of which one aspect is filopodial number. We therefore choose not to change the title.

Abstract: there is no mentioning of Ena's impact on axon growth, neither of the one experiment that shows that Ena can suppress loss of Abl phenotypes, i.e. substantiating of the downstream statement

We have modified the Abstract to include the terminal axonal phenotypes of *ena* in TSM1.

Regarding the next several referee comments, as discussed above, we have simply deleted the offending paragraph in its entirety. Therefore, these comments are no longer germane.

The introduction is far too long, lacks focus and contains misleading elements:
1) Description of the growth cone in the introduction is misleading; some suggested formulations:
- core of microtubules from where individual microtubules splay into a meshwork of .... in the growth cone periphery
- From this meshwork, actin bundles emerge to form filopodia that emanate into the growth cones surrounding.

Description deleted; suggested formulation therefore not relevant. (As an aside, we could not have used these formulations for TSM1 in any event since they do not correspond well to our experimental observations of this particular neuron).

2) the two modes of axon growth are inappropriate likely even wrong; current thoughts about axons growth need to be dealt with more carefully and perhaps and need to be explained with greater care. For example, the role of actin could be the focus, not the two suggested ways of growth. What we definitely know is that actin can generate forces that can generate morphogenetic changes but also mediate mechanosensing, that actin can drive protrusion of lamellipodia and filopodia, that actin can influence microtubule behaviours through structural guidance, through providing an anchor for cross-linkers or through mediating signalling, that actin is essential for the exo-/endocytosis of receptors. The key question is how these activities translate into axon growth, which may be implemented through mechanical pulling followed by microtubules in one school of thought, or implemented by microtubules downstream of actin in another school.

Two hypothetical modes of growth are not discussed; no need to explain them further.

3) As pointed out previously, narrow growth cones are a common phenomenon also in culture. The way in which phenomena are classified in the introduction is just not helpful.

Narrow growth cones not discussed; no need to classify them or ask whether they are or are not the same as the structures observed for TSM1.

4) the term "non-adherent mode" is unnecessarily overstated and there is simply no proof for this as a general phenomenon in vivo. It is unconceivable that no adhesion takes place to even lose networks of ECM, especially when considering that many signalling receptors require a certain amount of pulling force for their

action.
Adherent vs non-adherent modes no longer discussed.

5) p.5: Ena links the TIPS of adjacent filaments
We have restricted this sentence to “tips” in the text as the referee insists, since the distinction is not important to us. We suggest, however, that the referee take up this matter with Blanchoin, Boujemaa-Paterski, Sykes, and Plastino, who are expert about Ena, and who evidently feel the referee is mistaken (based on the review by these authors that was recommended to us by the referee, and that we cite in the text).

p.7 "the morphological "growth cone", contained a high local concentration of actin intensity in the axon shaft" - the use of axon shaft is misleading here
Here and below the referee objects to our use of the term “axon shaft” to refer to the part of the axon from which projections emerge and which will eventually become the definitive axon and finally the mature axon. We cannot imagine, however, what term he would rather that we use. I have routinely heard this structure referred to as “axon shaft” by dozens of investigators over the past 35 years. We cannot simply say “a region of the axon with a high local concentration…”, without additional qualification, because that would include the filopodia as well as the structure from which they emerge, and that is inaccurate. As discussed in the comments on the previous revision, terms like “core” or “central part” would also be ambiguous and misleading. We really have no idea what the referee has in mind as an alternative and better name for this structural element. Therefore, we have left it as “axon shaft”.

p.7 The formation of filopodia as a function of actin availability was also suggested by studies of actin nucelators (Goncalves-Pimentel 2011).

As requested, we have put in an additional callout to the paper from the Referee’s lab (already cited multiple times elsewhere in the manuscript).

p.37 "the protrusive region of the axon, the morphological "growth cone", contained a high local concentration of actin intensity in the axon shaft" - the use of axon shaft is misleading here.

Please see reply above.

p.10: images of Ena expression are insufficient. In each case, growth cones need to be shown, of wt, Ena overexpression and FP4. FP4 traps Ena in the cell body, but is potentially locally translated Ena in GCs also away from the periphery? Where are the mitochondria in GCs? Is Ena attached to mitochondria still able to polymerise actin, therefore not eliminate but rather relocate actin polymerisation? Similarly, is there more Ena in the periphery of GCs when overexpressed?

As mentioned in the General Comments, above, we now show images of anti-Ena staining of WT and FP4-mito TSM1 growth cones to document that we can't reliably distinguish Ena signal in the growth cone in these genotypes above the background of Ena immunoreactivity in the associated epithelia (Suppl Fig 2 C, D).

Regarding the specific questions asked by the referee:
- We have no idea whether Ena may be locally translated in these growth cones, nor do we have grounds to speculate. We are not aware of any evidence to suggest this.
- We do not know where mitochondria are localized in these growth cones in wild type. We can only ask in the context of FP4-mito expression, and in that case, as discussed in the previous submission and shown now in the additional figure, it is only rarely that the FP4-mito-eGFP reagent provides evidence for small puncta in the growth cone or distal axon.
- We do not know with certainty whether Ena linked to mitochondria can still polymerize actin. We presume that it probably does.

We presume that the unstated intent of the comments above is to question whether expression of FP4-mito does, in fact, reduce the ability of Ena to contribute to axon growth. It seems to us, however, that the most relevant response to this question is provided by the functional data. In the manuscript, we show (1) that expression of FP4-mito faithfully recreates the well-characterized *ena* genetic loss of function phenotype in ISNb axons, (2) the detailed effects of FP4-mito on various parameters of TSM1 growth cones are opposite to Abl loss of function and match Abl gain of function, consistent with the well-established antagonism between Abl and Ena, and (3) expression of FP4-mito suppresses the axonal defects produced by Abl loss of function in TSM1. Together, these data provide strong evidence that expression of FP4-mito in this cell produces an effective axonal loss of function condition for Ena, just has been demonstrated in numerous contexts in a number of organisms. We also point out that neither referee has offered any specific argument that contradicts this interpretation.

- It is apparent that overexpression of Ena does increase the amount of Ena in the periphery of the growth cone relative to wild type, since we can see the growth cone outline against the background of epithelial staining in the *UAS-ena* condition but not in the wild type (Fig 3G vs Suppl Fig 2C).

p.11: Fig.3G - show a wt GC with anti-Ena taken at the same camera settings as

the one overexpressing Ena. It is important to support the statement made, i.e. that endogenous Ena cannot be distinguished from background, and that ena overexpression overcomes this.

See above. Now shown in Suppl Fig 2C.

p.11: the absence of Ena at filopodial tips might be explained by the presence of formins; it needs to be highlighted more in the text and used to provide a potential explanation as to why average filopodial length does not change upon Ena manipulation.

The referee insists that we should highlight the idea that absence of Ena from filopodial tips may be explained by the presence of formins. It is, of course, conceivable that presence of formins could have something to do with the absence of Ena at filopodial tips in these axons. However, there are numerous other possible explanations. Perhaps there is a different, non-formin, actin polymerase at the tips. Perhaps the absence of Ena at tips is due to an absence of focal adhesion proteins at tips to provide Ena tethering sites. Perhaps Ena is trapped by FP4-bearing protein complexes elsewhere in the axon so it can't make it to filopodial tips. Perhaps Ena needs an active mechanism, such as a specific myosin, to transport it to filopodial tips and that hypothetical protein is not present (see, for example, Young, et al (2018)). We have no data whatsoever as to the presence or absence of formins, or their connection or lack of connection to the relevant observations. We are confused as to why the referee feels it is essential for us to highlight a specific speculation that entirely lacks experimental support. Indeed, in the initial review, the Referee was equally insistent that we delete the suggestion that there may be an interplay between Ena and formins in generating the properties of growth cone filopodia as it "may not be the case in axons" (for which he then references one of his own publications). We therefore have chosen not to add lengthy speculations about something these axons do not do, preferring to concentrate on the properties that they DO have.

At a technical level, it is also not correct that "average filopodial length does not change upon Ena manipulation". The increase in filopodial number in UAS-ena, together with the absence of a change in total filopodial length, causes the average filopodial length to decrease significantly in this genotype relative to wild type or FP4-mito (Fig 5C).

p.12 bottom half: the data explanations are unnecessarily complicated. The matter is simple: show data for filopodia numbers (which differ for FP4) and average length (slightly differ); total length is irrelevant since it is a mere product of the two and not very telling if average length is hardly changed anyway. - A reduction in filopodia numbers is consistent with Goncalves-Pimentel 2011 and was suggested to be due to a role of Ena in supporting nucleation events (promoting the polymerisation of new seeds); in that system Ena was at the tip of filopodia and loss of Ena affected their average length.

The referee has strong opinions about how we ought to present our data. However, he does not offer any evidence, or even suggestion, that our presentation is technically inaccurate. We have reasons why we think it best to present the data as we do. As it is our data, we feel we have a right to present it in the way we feel is most clear.

It is also true, as we explained previously, that the referee is simply wrong on the facts concerning the relative value of average vs total filopodial length as metrics to assess the role of Ena. Unless all filopodia are primary branches that come directly off the axon shaft (they are not), average filopodial length cannot be measured directly. It depends on the pattern of branching, and therefore can only be derived by a calculation: total filopodial length divided by filopodial number. Calculating the correlation between average filopodial length and filopodial number, therefore, would be statistically inappropriate as one is calculated from the other. Frankly, our statistician refuses to do it (and rightly so). Total filopodial length is a direct, objective, unbiased measurement. Moreover, it is simply untrue that average length is an inherently superior measure, and total length irrelevant, for reasons discussed in the Response to Referee Comments on the previous version. It may well be that average length had value for analyses the referee performed previously in a different experimental system to ask different scientific questions, but for us it is a suboptimal measurement.

The interpretation that Ena affects filopodial initiation is discussed in the text already (and was also present in both previously submitted versions).

p.13: The wavelet data are not well explained and I cannot see what they may tell us. Please, could this be accompanied by a simple schematic or microscopic images? What are the mechanistic implications?

We have now added an additional Supplement to give a more detailed explanation of wavelets. It is far too lengthy to put into the main text. As stated in the text, the wavelet analysis gives a global measure of how actin density is distributed in the axon. Here, it is used to provide rigorous mathematical support for the interpretation that reducing Ena activity causes actin to spread out in the axon on a multi-micron scale. This supports and extends the conclusions we draw based on direct measurement of the width of the actin peak. The mechanistic implications of the differences in actin distribution revealed by the totality of the analyses in our various experimental settings are quite wide-ranging, and were discussed at length in 3 previous papers, in addition to the discussion here of this phenomenon. Here, however, as it happens the critical result is that the effect of Ena on actin distribution is quite muted, though it can be detected and quantified by using the

wavelet method. In the paper, we discuss at length the significance of the unanticipated finding that Ena has only modest effects on actin organization in TSM1.

p.14: what would have been learned if Jensen-Shannon divergence had shown differences?

We are confused by this question. The JS divergence does show differences, in all comparisons. There is no comparison for which the divergence is 0, and both altered-Ena genotypes are different from wild type in the way that the actin distribution changes with time in individual axons, as measured by JSD. The basic point is that over time, the actin peak evolves in a consistent, systematic way in wild type as the axon grows. In contrast, in the altered-Ena conditions, there is a subset of cells in which evolution of the actin pattern is disordered and unpredictable. We speculate in the Discussion that the outlier trajectories with the most disorderly development are the ones most likely to show overt mutant phenotypes at the end of development.

Reviewer #2 (Remarks to the Author):

The authors have significantly improved the manuscript based on both reviewers' comments.

Comments:

1. It would be helpful to mark the text changes in red font to see the differences.

We have now done this

2. Major concern 2 was addressed by changing the wording from "Together, these observations suggest that TSM1 growth cone advance is driven by forward-biased fluctuations of the actin distribution." To "Together, these observations suggest that TSM1 growth cone advance arises from forward-biased fluctuations of the actin distribution." I do not agree that this modification changed the emphasis on causality much. They should rather use the word "is correlated with".

The evidence for causality was in our previous publications so we did not repeat those experiments here. Nonetheless, we have changed the wording to say "Together with other experiments (Clarke, et al., 2020a; Clarke, et al., 2020b), these observations demonstrate that TSM1 growth cone advance **is correlated with** forward-biased fluctuations of the actin distribution." (Note that correlation is “demonstrated” by the data, not simply “suggested”, as appropriate tests

presented in the manuscript show the correlations are statistically significant.)

3. Initial minor concern 3: scaling of axes of Fig. 2A. An adjustment was done, but it is unclear why the axes scaling is still different between x and y axes. Please use the same distance for corresponding values and start both scales at 0. Font on axis of Fig. 2B should also be increased.

We have extended the axis, as requested, and increased the font size on panel B.

4. Initial minor concern 4: Figure 3A-B: it would be helpful to draw the outline of the cell(s). I do not agree with the author. You can use a fine dashed line tool in ImageJ to outline one cell without obscuring things.

We have now included this. In order to have room to show both the annotated image and the unmanipulated parent image we have put these panels in a supplemental figure (and left the main text figure as the unannotated image).

RE: Manuscript #E23-01-0003RR
TITLE: "Enabled primarily controls filopodial morphology, not actin organization, in the TSM1 growth cone in Drosophila"

Dear Dr. Giniger:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Thank you for addressing remaining reviewer concerns and adding the additional description of wavelet analysis to the supplement.

Sincerely,
Stephanie Gupton
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Giniger:

Congratulations on the acceptance of your manuscript! Thank you for choosing to publish your work in Molecular Biology of the Cell (MBoC).

Within 10 days, an unedited PDF of your manuscript will be published on MBoC in Press, an early release version of the journal. The date your manuscript appears on this site is the official publication date.

Your copyedited and typeset manuscript will be scheduled for publication in the next available issue of MBoC. Our production team will notify you when the page proofs of your paper are ready for your review.

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We look forward to publishing your paper in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
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