Journal of Cell Science

Coupling of dynamic microtubules to F-actin by Fmn2 regulates chemotaxis of neuronal growth cones

Tanushree Kundu, Priyanka Dutta, Dhriti Nagar, Sankar Maiti and Aurnab Ghose
DOI: 10.1242/jcs.252916

Editor: Michael Way

Review timeline
Original submission: 11 August 2020
Editorial decision: 20 October 2020
First revision received: 25 May 2021
Accepted: 26 May 2021

Original submission

First decision letter

MS ID#: JOCES/2020/252916

MS TITLE: Coupling of dynamic microtubules to F-actin by Fmn2 regulates chemotaxis of neuronal growth cones

AUTHORS: Tanushree Kundu, Priyanka Dutta, Dhriti Nagar, Sankar Maiti, and Aurnab Ghose
ARTICLE TYPE: Research Article

I am sorry its taken longer then I would like to get back to you but we have been chasing one reviewer for weeks. The good news is we have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, reviewer 1 is very positive and believes the study should be published as is. In contrast, the other two reviewers raise some concerns regarding the conditions of your in vitro actin binding experiments, the use of blebbistatin and analysis of MT tip dynamics. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. In particular, I think you need to try more physiological salt concentrations for your actin pelleting assays and use a UV stable blebbistatin (e.g. para-nitroblebbistatin) if you are not already doing so (no information was provided in materials and methods for blebbistatin). If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript and make a quick decision.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This is a solid advance on the mechanism by which microtubules and actin filaments interact to orchestrate growth cone guidance.

Comments for the author

Excellent manuscript and superb figures. I have no suggestions for improvement and recommend immediate acceptance of this manuscript.

Reviewer 2

Advance summary and potential significance to field

Previous work from the Ghose lab demonstrated a need for the F-actin-binding formin Fmn2 in axon outgrowth and pathfinding, in part through its ability to stabilize adhesions. In this clearly written manuscript, the authors conclude that Fmn2 also binds to microtubules and indeed it helps guide them along actin bundles into extending filopodia on neuronal growth cones, a process that has been linked to growth cone turning in response to guidance cues. Through a number of different assays involving in vivo and in vitro microscopy and sedimentation analysis, the authors show that microtubule guidance along F-actin requires the presence of the C-terminal 24 amino acid FSI domain that is on the second formin homology domain (FH2). Previous studies identified this FH2FSI as the microtubule interacting domain for Fmn2 in meiotic spindles (Kwon S., et al, 2011, Mol Human Reprod. 17, 317-27) although this work was not cited here (but should be). Thus, this advance in structure/function of Fmn2 is only a modest step forward. However, this manuscript is the first demonstration of the importance of Fmn2 to the F-actin-microtubule interaction in growth cone filopodia, a key step in growth cone pathfinding. The in vivo studies in this manuscript were nicely done.

Comments for the author

Through silencing of Fmn2 the authors provide data in Figures 1-3 that demonstrate significant effects of Fmn2 on the organization and dynamics of the microtubules (MT) and actin filaments (A) in filopodia of advancing chick spinal commissural neuronal growth cones. The results suggest that there could be a physical interaction between the three players. They also show this interaction is likely to be significant for growth cone dynamics across phyla by demonstrating that knock down of Fmn2 in zebrafish Rohon-Beard neurons caused similar defects in microtubule extensions into filopodia as observed in chick neurons. The interaction appears to be responsible for the stalling and retrograde movement of microtubules due to retrograde flow of the actin bundles which is also reduced by inhibition of myosin 2.

The domain interactions responsible for the microtubule binding of Fmn2 were then studied both in vivo and in vitro by expressing or using just the FH2FSI region of the protein either with or without the FSI region (24 amino acid C-terminal domain). The in vivo studies certainly support the authors' conclusions about the necessity of the 24 amino acid FSI C-terminal domain for the microtubule interactions of Fmn2 in their guidance along actin bundles in filopodia. However, the in vitro studies trying to quantify the strength of the microtubule and F-actin interactions for the FH2FSI and FH2ΔFSI through supernatant and pellet gel quantification leave much to be desired. First of all the buffers used for quantifying the interactions are at different pH (6.9 for MTs and 8.0 for F-

actin). Second, neither of these buffers is anywhere near physiological ionic strength or osmolarity (should be about 300 mOsM). Since the interactions between the FH2FSI domain and the cytoskeletal elements are mostly electrostatic, co-sedimentation does not tell us whether these are specific interactions of just the co-sedimentation of a charged domain with a polymer that has an oppositely charged region. The FSI domain has a very positive charge with only one acidic residue but with 6 lysine, one arginine, one histidine plus 4 hydrophilic residues out of 24 total. Binding studies should be done over a range of salt concentrations to show specificity in binding that does not disappear at a physiologically relevant ionic strength- and binding both polymers need to be studied at the same physiological pH (7.2 to 7.4- not 6.9 and 8.0). Furthermore, it is necessary to show that the expression of short domain pieces of Fmn2 retain their normal 3D structure when expressed and used in these types of studies. If this has been done elsewhere (e.g. these domains have been studied previously), the reference to this structural information should be provided. Otherwise, the entire protein, or at the very least, the FH1/FH2FSI domains, should be expressed for comparison. If the behavior of the combined FH1/FH2FSI domain protein (or full length if available) behaves similarly to the FH2 fragments, the use of the latter is then justified for in vitro binding studies under physiological conditions. This point is important because almost any basic protein will co-sediment with F-actin whether or not the association is of any physiological relevance. Since the in vivo activities of the FH2FSI and FH2ΔFSI support the authors' conclusions, perhaps the in vitro binding study should be moved to a supplementary figure and the caveats in its interpretation in support of the in vivo work explained when mentioned in the text?
One important consequence of the Fmn2 interaction with actin bundles and microtubules is addressed in Figure 7 – turning movements of growth cones as a chemotactic response. It would be nice to confirm that this response is exhibited between two different natural substrates rather than just between the artificial poly-D-lysine and the more physiological laminin/fibronectin surface. For instance, what would happen between laminin/fibronectin and collagen, which also supports growth, or between laminin/fibronectin and a natural repulsive cue such as aggrecan if the FSI region is present or absent? Of course the latter repulsive cue may be mediated by much more than just filopodia recruitment of microtubules.

Minor corrections:
Line 112: …rich structures, such as…. (NOT like)

Line 196: …filopodia… (not a filopodia)

Line 234: replace "indicating" with suggesting Line 268: …stabilized MTs from implicating…

Line 283: …no protein controls…

Line 286: … centrifuged at very low speeds (please give rpm or g value- "very low" is not helpful)

Line 298… (a fraction of the …) give the percent of label since it might have an effect on the outcome Line 486: … crosstalk in growth cones and…

Line 671: … 100,000 (no space)

Reviewer 3

Advance summary and potential significance to field

This paper identifies the formin protein Fmn2 as an integrator of microtubule and actin filament dynamics in neurons. They provide evidence of the importance of Fmn2 function in axon growth and guidance capability.

Comments for the author

In this study, the authors examined the role of the formin Fmn2 in microtubule-actin interactions and its influence on the directional motility of chick commissural neurons in culture. Using knock down assays in these cultured neurons, the authors revealed that Fmn2 is important for interaction of exploratory microtubules with filopodial F-actin in grow cones, stabilization of these microtubules, and for growth cone turning in a chemotaxis assay. Using in vitro biochemistry assays

they provide evidence that the FSI domain of Fmn2 is critical for this F- actin and microtubule interaction. They also provide evidence that Fmn2 can stabilize exploratory microtubules in RB neurons of zebrafish, suggesting this role is conserved in growth cones in vivo. Overall this is a well-executed study with solid imaging and result quantification. They provide strong biochemical evidence that Fmn2 can crosslink actin filaments and that Fmn2 is a critical mediator of microtubules-actin interactions in growth cone filopodia. While the data in this study largely supports the authors conclusions, there are some areas of concern with respect to interpretation of the results that need to be addressed as outlined below.

1) Caveats of using EB3 to infer MT dynamics: The +end TIP protein EB3 can be used to track MT ends and can be used to assess MT assembly rates; however, this can only be done reliably if the body of the MT body is stationary or a fiducial mark is placed on the MT proximal to the +end so that the instantaneous position of the MT body can be co-assessed with that of the +end position in order to separate MT assembly/disassembly parameters from MT translocation. The extent of this problem becomes apparent if one examines Fig. 3G in this paper, since coupling to retrograde F-actin flow and MT motor actions could both affect MT body translocation rates; which, will in turn, influence EB3 +end positions. The same argument holds for experiments where GFP-tubulin was used as a marker to assess dynamics. Specifically, unless a fiducial mark is made on the MT so that the distance from the +end to a known position on the MT can be assessed for every measurement, dynamic MT parameters cannot be inferred. The data presented is indeed suggestive of the author's conclusions but should be tempered to reflect the limitations alluded to above. Better yet, experiments using internal fiducial MT marks could be added to quantitatively address out how how MT translocation versus assembly/disassembly events are related to one another under the various conditions examined. To this end, one might employ a photoactivatable tubulin construct to place marks on MTs in tandem with a EB3 +end tip label. This addition would significantly strengthen the impact of this study. Also, the plethora of evidence for both kinesin and dynein based MT translocation (sliding) in axons and growth cones should be cited (e.g. Peter Baas's lab's work in this area).

2) There may be a critical problem with the blebbistatin data presented in Fig. 3I. Specifically it is known that exposure to blue light rapidly inactivates blebbistatin and can result in non-specific cell toxicity. Thus, if pCAG-EB3-GFP was used in these experiments the data is not only invalid, but, the conclusions may be inverted given that blue light excitation of GFP would disinhibit blebbistatin's effects on non-muscle myosin II.

3) I think the Fmn2-MO data presented in Fig. 3H and 3J clearly suggests Fmn2 is involved in regulation of MT-actin filament interactions. With that said, direct assessment of retrograde actin flow rates in tandem with EB3 comets would make this figure significantly more compelling. In particular it would let the author's directly compare and contrast the rates of actin filament and MT +end tip movement.

Note, however, that the same caveats would hold here for the use of EB3 as a MT marker. Perhaps a better way to assess MT-actin filament coupling would be to use a photoactivatable probes to co-assess actin filament and MT dynamics.

First revision

Author response to reviewers' comments

Editor's summary:
As you will see, reviewer 1 is very positive and believes the study should be published as is. In contrast, the other two reviewers raise some concerns regarding the conditions of your in vitro actin binding experiments, the use of blebbistatin and analysis of MT tip dynamics. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. ***In particular, I think you need to try more physiological salt concentrations for your actin pelleting assays and use a UV stable blebbistatin (e.g. para-nitroblebbistatin) if you are not***

already doing so (no information was provided in materials and methods for blebbistatin)*. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript and make a quick decision.*
We thank the editor for the summary and focussing us towards the two major concerns raised by the referees.

The blebbistatin experiments were conducted using EB3-mCherry, precisely to avoid toxicity issues arising under blue light conditions. We missed mentioning this fact in the main text of the original manuscript (had indicated the construct used in Table S1) and thank the reviewer for pointing this out. It is now indicated in the text.

We have conducted fresh experiments using a range of salt concentrations, this data is now included as supplementary data. Both actin and microtubules continue to bind Fmn2 despite increasing salt concentrations, though the microtubule binding is more sensitive. This is in line with the fact that the Fmn2 – Microtubule interaction has a substantial electrostatic component. Previous work on Cappuccino, the fly orthologue of Fmn2, has demonstrated Fmn2-microtubule interactions to be mediated by the electrostatic interactions of the Fmn2 C-terminal tail (Roth-Johnson et al, 2014). As we have shown here (Figure S4 A), this region is highly conserved in vertebrate Fmn2 and is indeed also the region that is critical for Fmn2-mediated actin-microtubule crosstalk in neuronal growth cones. For more details see response to Reviewer 2.

Reviewer 1 Advance Summary and Potential Significance to Field:
This is a solid advance on the mechanism by which microtubules and actin filaments interact to orchestrate growth cone guidance.

Reviewer 1 Comments for the Author:
Excellent manuscript and superb figures. I have no suggestions for improvement and recommend immediate acceptance of this manuscript.
We thank the reviewer for appreciating our manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:
Previous work from the Ghose lab demonstrated a need for the F-actin-binding formin Fmn2 in axon outgrowth and pathfinding, in part through its ability to stabilize adhesions. In this clearly written manuscript, the authors conclude that Fmn2 also binds to microtubules and indeed it helps guide them along actin bundles into extending filopodia on neuronal growth cones, a process that has been linked to growth cone turning in response to guidance cues. Through a number of different assays involving in vivo and in vitro microscopy and sedimentation analysis, the authors show that microtubule guidance along F-actin requires the presence of the C-terminal 24 amino acid FSI domain that is on the second formin homology domain (FH2). Previous studies identified this FH2FSI as the microtubule interacting domain for Fmn2 in meiotic spindles (Kwon S., et al, 2011, Mol Human Reprod. 17, 317-27) although this work was not cited here (but should be). Thus, this advance in structure/function of Fmn2 is only a modest step forward. However, this manuscript is the first demonstration of the importance of Fmn2 to the F-actin-microtubule interaction in growth cone filopodia, a key step in growth cone pathfinding. The in vivo studies in this manuscript were nicely done.

We thank the reviewer for appreciating our manuscript and the role of Fmn2 in growth cone filopodia dynamics.
We have now included the Kwon et al, 2011 in the Discussion section of the manuscript, which suggested that a microtubule interacting function for Fmn2 in oocytes. Beyond this early suggestion of microtubule interaction, our work directly demonstrates a F-Actin – microtubule crosslinking function for Fmn2 mediated by the FSI tail domain in neuronal growth cones.

Reviewer 2 Comments for the Author:
Through silencing of Fmn2 the authors provide data in Figures 1-3 that demonstrate significant effects of Fmn2 on the organization and dynamics of the microtubules (MT) and actin filaments (A) in filopodia of advancing chick spinal commissural neuronal growth cones. The results suggest that there could be a physical interaction between the three players. They also show this interaction is likely to be significant for growth cone dynamics across phyla by demonstrating that knock down of Fmn2 in zebrafish Rohon-Beard neurons caused similar defects in microtubule extensions into

filopodia as observed in chick neurons. The interaction appears to be responsible for the stalling and retrograde movement of microtubules due to retrograde flow of the actin bundles which is also reduced by inhibition of myosin 2.

The domain interactions responsible for the microtubule binding of Fmn2 were then studied both in vivo and in vitro by expressing or using just the FH2FSI region of the protein either with or without the FSI region (24 amino acid C-terminal domain). The in vivo studies certainly support the authors' conclusions about the necessity of the 24 amino acid FSI C-terminal domain for the microtubule interactions of Fmn2 in their guidance along actin bundles in filopodia. However, the in vitro studies trying to quantify the strength of the microtubule and F-actin interactions for the FH2FSI and FH2ΔFSI through supernatant and pellet gel quantification leave much to be desired. First of all the buffers used for quantifying the interactions are at different pH (6.9 for MTs and 8.0 for F-actin). Second, neither of these buffers is anywhere near physiological ionic strength or osmolarity (should be about 300 mOsM). Since the interactions between the FH2FSI domain and the cytoskeletal elements are mostly electrostatic, co-sedimentation does not tell us whether these are specific interactions of just the co-sedimentation of a charged domain with a polymer that has an oppositely charged region. The FSI domain has a very positive charge with only one acidic residue but with 6 lysine, one arginine, one histidine plus 4 hydrophilic residues out of 24 total. Binding studies should be done over a range of salt concentrations to show specificity in binding that does not disappear at a physiologically relevant ionic strength- and binding both polymers need to be studied at the same physiological pH (7.2 to 7.4- not 6.9 and 8.0). Furthermore, it is necessary to show that the expression of short domain pieces of Fmn2 retain their normal 3D structure when expressed and used in these types of studies. If this has been done elsewhere (e.g. these domains have been studied previously), the reference to this structural information should be provided. Otherwise, the entire protein, or at the very least, the FH1/FH2FSI domains, should be expressed for comparison. If the behavior of the combined FH1/FH2FSI domain protein (or full length if available) behaves similarly to the FH2 fragments, the use of the latter is then justified for in vitro binding studies under physiological conditions. This point is important because almost any basic protein will co-sediment with F-actin whether or not the association is of any physiological relevance. Since the in vivo activities of the FH2FSI and FH2ΔFSI support the authors' conclusions, perhaps the in vitro binding study should be moved to a supplementary figure and the caveats in its interpretation in support of the in vivo work explained when mentioned in the text?
We appreciate the very valid concerns raised by the reviewer regarding our *in vitro* experimentation and have conducted a series of new experiments to address some of these.

As suggested by the reviewer and editor, we conducted the F-actin and microtubule binding experiments across different salt conditions, including 0, 50, 100 and 150 mM (300 mOsM). These are now presented in the revised manuscript (Figure S5). Fmn2 binds both F-actin and microtubules across these salt concentrations though as expected the Kd changes. For F-actin, the Kd ranges from 5 μM (0 mM salt) to 11.2 μM (150 mM salt). For microtubules, the Kd of Fmn2 binding ranges from 1.8 μM (0 mM salt) to 14.2 μM (150 mM salt). The increase in Kd, especially for microtubule binding, is consistent with our assertion that the interaction is substantially electrostatic in nature. In fact, similar findings have been reported for the fly orthologue of Fmn2. In both flies (Roth-Johnson et al., 2014) and chick (our data), the highly conserved, positively charged C-terminal FSI region is the primary mediator of microtubule binding. In accordance with these observations, our cell biological data strongly support the role of the FSI region in mediating Fmn2-microtubule interactions. While the Kd is weaker at physiological 150 mM salt concentration, it is important to note that Fmn2 is highly enriched in the growth cone filopodia (Sahasrabudhe et al., 2016). This latter observation suggests substantially high local concentrations in this region of the growth cone, where the cellular functions of Fmn2- microtubule interactions are most prominent.

Interaction of microtubule regulatory proteins with microtubules via electrostatic interactions is not uncommon. The well characterised microtubule binding activity of EB1 also has a significant electrostatic component (Hayashi and Ikura, 2003; Zhu et al., 2016) with the interaction being abrogated at a moderate salt concentration of 100 mM salt (Zhu et al., 2016).
The choice of pH (6.9 for microtubules and 8.0 for F-actin) was based on the commonly used protocols for such assays (Dutta et al., 2017, Elie et al., 2015, Szikora et al, 2017). In the single molecule experiments involving both actin and microtubule we had used pH 7.5, again in accordance with literature (Elie et al., 2015). The new binding experiments described above, have

been conducted at pH 7.5 for both F-actin and microtubules (Figure S5). There is no significant difference in the Kd's (at similar salt concentrations) between assays conducted at pH 6.9 (microtubule) or pH 8 (F- actin) and those performed at pH 7.5.

The reviewer also raises the pertinent question whether the short protein fragments of Fmn2 used in our *in vitro* studies are stable. Analogous fragments (FH2FSI and FH2_ΔFSI) of mouse Fmn2 have been previously used for *in vitro* studies (Montaville et al., 2016). However, in the absence of structural data for these fragments from chick Fmn2, we have evaluated the actin nucleating activity of the chick FH2FSI and the FH2_ΔFSI fragments. Fluorimetric analysis revealed that the FH2FSI domain is capable of nucleating F-actin filaments from monomeric G-actin (Figure S6). The FH2_ΔFSI fragment also shows F-actin nucleation function, albeit at higher protein concentrations (Figure S4). The latter observation is consistent with mouse Fmn2 studies where the C-terminal FSI domain is known to facilitate the actin nucleation activity of the FH2 domain (Montaville et al., 2016). We have been unable to purify full length Fmn2 protein to compare directly with the activity of the purified fragments.

However, we have taken a well characterised fragment of human DAAM1 formin (Dutta et al., 2017) as positive control in these experiments.
We would like to highlight that the cell biological data, where the full length Fmn2 but not Fmn2 with only the FSI region deleted rescues the effects of Fmn2 knockdown, are consistent with our conclusions from the biochemical interaction experiments.

One important consequence of the Fmn2 interaction with actin bundles and microtubules is addressed in Figure 7 – turning movements of growth cones as a chemotactic response. It would be nice to confirm that this response is exhibited between two different natural substrates rather than just between the artificial poly-D-lysine and the more physiological laminin/fibronectin surface. For instance, what would happen between laminin/fibronectin and collagen, which also supports growth, or between laminin/fibronectin and a natural repulsive cue such as aggrecan if the FSI region is present or absent? Of course the latter repulsive cue may be mediated by much more than just filopodia recruitment of microtubules.
The reason for choosing the artificial poly-l-lysine was based on our observation that Fmn2 modulates adhesive contacts in an integrin-dependent manner (Sahasradhudhe et al., 2016; Ghate et al., 2020). Thus, we wanted a condition with maximum dynamic range by contrasting an integrin substrate with non-integrin interaction. However, we agree that experiments with other extracellular matrix molecules (including ones with active guidance function) could extend our results. We have been unable to conduct experiments with other substrate combinations due to disruption of supply chains and protracted closure of the laboratory.

We would like to point out that Fmn2 does contributes to axonal pathfinding of chick spinal neurons *in vivo* (Sahasrabudhe et al, 2016). In the latter paper we had highlighted the role of filopodial stability and it now appears from our current work that filopodial microtubule capture contributes significantly to this phenomenon.

Minor corrections:
Line 112: …rich structures, such as…. (NOT like) Line 196: …filopodia… (not a filopodia)

Line 234: replace "indicating" with suggesting Line 268: …stabilized MTs from implicating… Line 283: …no protein controls…

Line 286: … centrifuged at very low speeds (please give rpm or g value- "very low" is not helpful)

Line 298… (a fraction of the …) give the percent of label since it might have an effect on the outcome Line 486: … crosstalk in growth cones and…

Line 671: … 100,000 (no space)

We thank the reviewer for pointing out these errors. These have been corrected in the revised manuscript.

Reviewer 3 Advance Summary and Potential Significance to Field:

This paper identifies the formin protein Fmn2 as an integrator of microtubule and actin filament dynamics in neurons. They provide evidence of the importance of Fmn2 function in axon growth and guidance capability.

Reviewer 3 Comments for the Author:
In this study, the authors examined the role of the formin Fmn2 in microtubule-actin interactions and its influence on the directional motility of chick commissural neurons in culture. Using knock down assays in these cultured neurons, the authors revealed that Fmn2 is important for interaction of exploratory microtubules with filopodial F-actin in grow cones, stabilization of these microtubules, and for growth cone turning in a chemotaxis assay. Using in vitro biochemistry assays they provide evidence that the FSI domain of Fmn2 is critical for this F- actin and microtubule interaction. They also provide evidence that Fmn2 can stabilize exploratory microtubules in RB neurons of zebrafish, suggesting this role is conserved in growth cones in vivo. Overall this is a well-executed study with solid imaging and result quantification. They provide strong biochemical evidence that Fmn2 can crosslink actin filaments and that Fmn2 is a critical mediator of microtubules-actin interactions in growth cone filopodia. While the data in this study largely supports the authors conclusions, there are some areas of concern with respect to interpretation of the results that need to be addressed as outlined below.

1) Caveats of using EB3 to infer MT dynamics: The +end TIP protein EB3 can be used to track MT ends and can be used to assess MT assembly rates; however, this can only be done reliably if the body of the MT body is stationary or a fiducial mark is placed on the MT proximal to the +end so that the instantaneous position of the MT body can be co-assessed with that of the +end position in order to separate MT assembly/disassembly parameters from MT translocation. The extent of this problem becomes apparent if one examines Fig. 3G in this paper, since coupling to retrograde F-actin flow and MT motor actions could both affect MT body translocation rates; which, will in turn, influence EB3+end positions. The same argument holds for experiments where GFP-tubulin was used as a marker to assess dynamics. Specifically, unless a fiducial mark is made on the MT so that the distance from the +end to a known position on the MT can be assessed for every measurement, dynamic MT parameters cannot be inferred. The data presented is indeed suggestive of the author's conclusions but should be tempered to reflect the limitations alluded to above. Better yet, experiments using internal fiducial MT marks could be added to quantitatively address out how how MT translocation versus assembly/disassembly events are related to one another under the various conditions examined. To this end, one might employ a photoactivatable tubulin construct to place marks on MTs in tandem with a EB3 +end tip label. This addition would significantly strengthen the impact of this study. Also, the plethora of evidence for both kinesin and dynein based MT translocation (sliding) in axons and growth cones should be cited (e.g. Peter Baas's lab's work in this area).

We agree fully with the reviewer's comments. Indeed, employing a fiducial mark of the body of the microtubule and evaluating EB3 dynamics at the tip would be ideal while estimating growth parameters. Unfortunately, we have been unable to implement this reliably in growth cone filopodia for multiple technical reasons. Microtubule polymerisation rates are significantly faster than the reported bulk translocation rates of the microtubule network (Miller and Sheetz, 2006; Szikora et al, 2017; this manuscript). Thus, our growth rate measurements of solitary exploratory microtubules within filopodia, though slightly imprecise, should adequately reflect the changes between control and Fmn2 knockdown growth cones. Similarly, the F-actin retrograde flow influences the evaluation of microtubule growth dynamics, especially if the microtubules are coupled to F-actin. In fact, it is precisely because of the caveats highlighted by the reviewer, we have evaluated other parameters like dwell time and characteristics of the kymographs to assess the microtubule-F-actin coupling by Fmn2 in filopodia. As suggested by the reviewer, we have modified the Discussion section to reflect the limitations of the measurements. We have now modified the Discussion to also include references to motor protein-based microtubule sliding in order to place our work in context.
Our primary conclusion in this work is the microtubule – F-actin cross bridging function of Fmn2 and the significance of this function in growth cone filopodia. We think that the combined evaluation of multiple parameters associated with microtubule dynamics in growth cones and complementary biochemical evidence strongly support our conclusion.

2) There may be a critical problem with the blebbistatin data presented in Fig. 3I. Specifically, it

is known that exposure to blue light rapidly inactivates blebbistatin and can result in non-specific cell toxicity. Thus, if pCAG-EB3-GFP was used in these experiments the data is not only invalid, but, the conclusions may be inverted given that blue light excitation of GFP would disinhibit blebbistatin's effects on non-muscle myosin II.

The experiments reported in the manuscript using blebbistatin were carried out using pCAG-EB3-mCherry, precisely to avoid the issues of inactivation and toxicity indicated by the reviewer. While we had indicated the use of this constructs in Table S1, we failed to report it in the main text of the manuscript. This is now clearly indicated. We thank the reviewer for pointing out this omission.

3) I think the Fmn2-MO data presented in Fig. 3H and 3J clearly suggests Fmn2 is involved in regulation of MT-actin filament interactions. With that said, direct assessment of retrograde actin flow rates in tandem with EB3 comets would make this figure significantly more compelling. In particular it would let the author's directly compare and contrast the rates of actin filament and MT +end tip movement. Note, however, that the same caveats would hold here for the use of EB3 as a MT marker. Perhaps a better way to assess MT-actin filament coupling would be to use a photoactivatable probes to co-assess actin filament and MT dynamics. Again, we agree with the reviewer. Indeed, simultaneous imaging of F-actin flow and microtubule polymerisation, corrected for bulk translocation, would have been ideal to generate precise quantitative data. However, we have been unable to implement this multi-parametric analysis in our laboratory not in the least because of the pandemic- related restrictions and closures which are still continuing.

We have recently demonstrated that the rate of F-actin retrograde flow increases in Fmn2 depleted growth cones due to compromised adhesive contacts between the growth cone and the extracellular matrix (Ghate et al., 2020). As Fmn2 also cross- links microtubules to filopodial F-actin, Fmn2 depletion decouples the two polymers and reduces the F-actin retrograde flow driven backward movement (retrograde events) and stalling of filopodial microtubules. If F-actin-microtubule coupling were unchanged upon Fmn2 depletion, one would have expected and increase in such events. Thus, while we have been unable to image F-actin retrograde flow and microtubule polymerisation simultaneously in growth cones, the above evidence, albeit indirectly, strongly supports the crosslinking function of Fmn2 in neurons.

References:

1. Dutta P, Das S, Maiti S. Non diaphanous formin delphilin acts as a barbed end capping protein. Exp Cell Res. 2017 Aug 15;357(2):163-169. doi: 10.1016/j.yexcr.2017.05.014. Epub 2017 May 17.

2. Elie A, Prezel E, Guérin C, Denarier E, Ramirez-Rios S, Serre L, Andrieux A, Fourest-Lieuvin A, Blanchoin L, Arnal I. Tau co-organizes dynamic microtubule and actin networks. Sci Rep. 2015 May 5;5:9964. doi: 10.1038/srep09964.

3. Ghate K, Mutalik SP, Sthanam LK, Sen S, Ghose A. Fmn2 Regulates Growth Cone Motility by Mediating a Molecular Clutch to Generate Traction Forces. Neuroscience. 2020 Nov 10;448:160-171. doi: 10.1016/j.neuroscience.2020.09.046. Epub 2020 Sep 29. PMID: 33002558.

4. Hayashi I, Ikura M. Crystal structure of the amino-terminal microtubule-binding domain of end-binding protein 1 (EB1). J Biol Chem. 2003 Sep 19;278(38):36430-4. doi: 10.1074/jbc.M305773200. Epub 2003 Jul 11.

5. Kwon S, Shin H, Lim HJ. Dynamic interaction of formin proteins and cytoskeleton in mouse oocytes during meiotic maturation. Mol Hum Reprod. 2011 May;17(5):317-27. doi: 10.1093/molehr/gaq088. Epub 2010 Oct 22.

6. Miller KE, Sheetz MP. Direct evidence for coherent low velocity axonal transport of mitochondria. J Cell Biol. 2006 May 8;173(3):373-81. doi: 10.1083/jcb.200510097.

7. Montaville P, Kühn S, Compper C, Carlier MF. Role of the C-terminal Extension of Formin 2 in Its Activation by Spire Protein and Processive Assembly of Actin Filaments. J Biol Chem. 2016 Feb 12;291(7):3302-18. doi: 10.1074/jbc.M115.681379. Epub 2015 Dec 14.

8. Roth-Johnson EA, Vizcarra CL, Bois JS, Quinlan ME. Interaction between microtubules and the Drosophila formin Cappuccino and its effect on actin assembly. J Biol Chem. 2014 Feb 14;289(7):4395-404. doi: 10.1074/jbc.M113.499921. Epub 2013 Dec 20.

9. Sahasrabudhe A, Ghate K, Mutalik S, Jacob A, Ghose A. Formin 2 regulates the stabilization of filopodial tip adhesions in growth cones and affects neuronal outgrowth and pathfinding in vivo. Development. 2016 Feb 1;143(3):449-60. doi: 10.1242/dev.130104. Epub 2015 Dec 30.

10. Szikora S, Földi I, Tóth K, Migh E, Vig A, Bugyi B, Maléth J, Hegyi P, Kaltenecker P, Sanchez-Soriano N, Mihály J. The formin DAAM is required for coordination of the actin and

microtubule cytoskeleton in axonal growth cones. J Cell Sci. 2017 Aug 1;130(15):2506-2519. doi: 10.1242/jcs.203455. Epub 2017 Jun 12.
11. Zhu ZC, Gupta KK, Slabbekoorn AR, Paulson BA, Folker ES, Goodson HV. Interactions between EB1 and microtubules: dramatic effect of affinity tags and evidence for cooperative behavior. J Biol Chem. 2009 Nov 20;284(47):32651-61. doi: 10.1074/jbc.M109.013466. Epub 2009 Sep 23.

Second decision letter

MS ID#: JOCES/2020/252916

MS TITLE: Coupling of dynamic microtubules to F-actin by Fmn2 regulates chemotaxis of neuronal growth cones

AUTHORS: Tanushree Kundu, Priyanka Dutta, Dhriti Nagar, Sankar Maiti, and Aurnab Ghose
ARTICLE TYPE: Research Article

I hope you and your lab are keeping well during this difficult COVID-19 situation. I have been through your responses to the reviewers original questions and do not feel there is a need to go back to the reviewers. Moreover, I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.