Initiation and disassembly of filopodia tip complexes containing VASP and lamellipodin

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RE: Manuscript #E20-04-0270
TITLE: "Initiation and disassembly of filopodia tip complexes containing VASP and lamellipodin"

Dear Dyche,

Two experts in the field reviewed your manuscript. You will see that both reviewers found your manuscript very interesting. They have some minor comments/questions that should be easy to address. I am looking forward to receive a revised version of your very interesting study.

All the best,

Laurent Blanchoin
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Mullins,

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.

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Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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Reviewer #1 (Remarks to the Author):

This is a very nice paper that demonstrates a lamellipodin-dependent mechanism of VASP clustering at the leading edge of lamellipodia, which in turn leads to the formation filopodial actin bundles. The key point of the mechanism is multivalent interaction between these two proteins and actin filament barbed ends. Along the way, the authors also clarify an ambiguous situation in the literature regarding the localization and role of IRSp53 in filopodia. The data are beautifully illustrated and thoroughly analyzed. I have only minor comments that need to be addressed and a couple of optional ones.

1) p.5. The knockin cell lines used in the study are referred to as being "monoclonal". However, no cell line cloning procedures are described in Methods. It appears that the cell lines were not cloned, but selected by FACS. For the purposes of this study, it is fully acceptable, but the terminology should be corrected.

2) p. 8. "...filopodial protrusions containing mRuby2-IRSp53, most of which localized along filopodia shafts but rarely extended out to the tips". It would be nice to have a high magnification image to illustrate this point.

3) p. 8. "...only a few of these ectopic, IRSp53-rich filopodia contained detectable VASP-eYFP at their tips." Does this statement refer to what appears to be static retraction fibers at the cell rear? It seems that most filopodia at the leading edge in the shown image have VASP.

4) Fig. 3A legend. "Blue" dotted lines are actually red.

5) (Optional) Can authors briefly comment (e.g. in Discussion) on "skating" of lamellipodin alone? Based on the behavior of Lpd before VASP joining, as well as on experiments with CytoD and with Lyn11-C-term-Lpd, it appears that VASP enhances, or even is necessary for cluster fusion.

6) p. 10. "...a large bolus of VASP and Lpd abruptly detaches from the original VASP/Lpd cluster". Detachment of lamellipodin is not obvious. Showing separate channels may help. Also, the description in Results of this phenomenon conflicts with the relevant statement in Discussion (p. 17: "During splitting, a bolus of VASP detaches from a cluster and moves into the cytoplasm while an equimolar amount of Lpd redistributes evenly back into the plasma membrane." Please, clarify what really happens to Lpd during tip complex splitting.

7) Arrowhead in Figure 4C, 32 sec blocks the nascent focal adhesion. It should be moved slightly away.

8) p. 11. "We quantified the relative change in lamellipodin clustering..." The figure 5D legend says that both lamellipodin and VASP were quantified, although it seems that only one protein is shown on the graph. Please, clarify.

9) p. 14 "Similar to previous studies, we find that overexpressing the membrane-associated protein, IRSp53 strongly induces formation of filopodia." These previous studies are not cited.

10) (Optional) p. 17. "We propose that the size-dependent instability of VASP clusters reflects the difficulty of coordinating VASP's interaction with the two binding partners that hold the cluster together: FPPPP-containing proteins and free barbed ends of actin filaments. " This is a reasonable hypothesis, but I am curious whether there is a thermodynamic optimum for the size of clusters, which are driven by multivalent interactions.

Reviewer #2 (Remarks to the Author):

Cheng and Mullins used CRISPR to tag endogenous VASP and lammelipodin within B16 cells. Their data shows that puncta of lammelipodin precede the formation of puncta of VASP, suggesting that lammelipodin is part of precursor complex upstream of VASP. Once they are co-localized, VASP and lamelipodin maintain relatively constant stoichiometry. Further descriptions made possible by these endogenously tagged cell lines include some confirmations of previous literature using proteins that were exogenously expressed. The data presented here also shows that there is a form of dynamic instability in which their seems to be a size limit of a cluster containing VASP and lammelipodin beyond which the cluster splits. These descriptions alone would make this study interesting to the readers of MBoC. However, Cheng and Mullins do not stop at description but also present an elegant structure/function analysis revealing that lamellipodin needs membrane-binding capability and EVH1-binding sequences.

The data in this paper is clear and well documented. I only have two minor questions:

1) What is "SNAPjf646-actin bundle" probe in Figure 1C? Video 2 calls it just actin. Is this beta actin?

2) The edge analysis presented is intriguing. The 'image analysis' section of the methods states that there was a custom Matlab code used. Will this code be provided with the manuscript? A well annotated MatLab script would be a valuable tool for

the field.

We appreciate all the reviewers' thoughtful comments, and addressing them helped us significantly improve the paper. Below we respond point-by-point to each comment and sketch the changes in the manuscript that they prompted:

Reviewer #1:

1) p.5. The knockin cell lines used in the study are referred to as being "monoclonal". However, no cell line cloning procedures are described in Methods. It appears that the cell lines were not cloned, but selected by FACS. For the purposes of this study, it is fully acceptable, but the terminology should be corrected.

We recognize that the original description of our methods was a bit ambiguous. To clarify, we used FACS to identify cells expressing fluorescent protein. We then seeded single fluorescent cells into 96-well plates and expanded each into a separate clonal cell line (Supplemental Figure 1a). For each clonal line we confirmed correct knock-in by fluorescence microscopy as well as PCR and DNA sequencing. We have rewritten the Methods section in the revised paper to include these details.

2) p. 8. "...filopodial protrusions containing mRuby2-IRSp53, most of which localized along filopodia shafts but rarely extended out to the tips". It would be nice to have a high magnification image to illustrate this point.

This was a very good suggestion. To address it, we added a higher magnification image of filopodia containing mRuby2-IRSp53 to Supplementary Figure 3c of the revised manuscript.

3) p. 8. "...only a few of these ectopic, IRSp53-rich filopodia contained detectable VASP-eYFP at their tips." Does this statement refer to what appears to be static retraction fibers at the cell rear? It seems that most filopodia at the leading edge in the shown image have VASP.

The reviewer's comment about 'static retraction fibers' is interesting and highlights a key problem in the study of filopodia. Namely, that actin-rich, finger-like cell protrusions can be created by multiple mechanisms and/or described by different names (e.g. filopodia, retraction fibers, microspikes, nanotubes, cytonemes, etc.). The distinction between filopodia and retraction fibers is particularly fraught because the long filopodial bundles that project through lamellipodial actin networks often show up as 'retraction fibers' after the lamellipod retreats. Generally we have followed Tanya Svitkina's convention of lumping all of these structures together and calling them filopodia bundles. We have expanded on this point in the corresponding results section.

4) Fig. 3A legend. "Blue" dotted lines are actually red.

Oops! We fixed this problem in the figure legend of the revised paper.

5) (Optional) Can authors briefly comment (e.g. in Discussion) on "skating" of lamellipodin alone? Based on the behavior of Lpd before VASP joining, as well as on experiments with CytoD and with Lyn11-C-term-Lpd, it appears that VASP enhances, or even is necessary for cluster fusion.

Thank you for this astute observation! We observed that lamellipodin-only clusters exhibit significantly less lateral mobility than clusters that contain VASP and they only rarely fuse (Supplemental figure 2b). Lateral 'skating' of VASP-containing clusters occurs when the associated filopodial actin bundle is tilted away from normal to the leading edge (Katoh et al. 1999). The fact that lamellipodin-only clusters do not 'skate' in the same way suggests that (1) small Lpd clusters are not as strongly coupled to the growing actin filaments beneath them (purified Lpd has weak affinity for actin filaments in vitro; K_d~200 uM; (Hansen and Mullins 2015)) and (2) that lamellipodin by itself is not sufficient to locally rearrange lamellipodial actin filaments into parallel bundles.

VASP appears to be required for both coupling to and rearranging the underlying filaments. This explanation also fits with the effects of perturbations such as the addition of cytochalasin D and the overexpression of Lyn_{11}-C-term Lpd, which drive VASP out of the clusters, leaving behind small Lpd-only clusters behind that cannot skate or fuse. In other words, the dynamics of the Lpd-only clusters produced by these perturbations mirror the dynamics of endogenous Lpd-only clusters found in wild type cells. We have included an additional paragraph about these findings in the Discussion section.

6) p. 10. "...a large bolus of VASP and Lpd abruptly detaches from the original VASP/Lpd cluster". Detachment of lamellipodin is not obvious. Showing separate channels may help. Also, the description in Results of this phenomenon conflicts with the relevant statement in Discussion (p. 17: "During splitting, a bolus of VASP detaches from a cluster and moves into the cytoplasm while an equimolar amount of Lpd redistributes evenly back into the plasma membrane." Please, clarify what really happens to Lpd during tip complex splitting.

We have sharpened our language in describing tip complex splitting. We have included a separate Lpd channel in Figure 4C (middle), which illustrates a tip complex splitting event. Our previous statement that, *"a large bolus of VASP and Lpd abruptly detaches…"* was meant to describe the end result of splitting in which equivalent amounts of VASP and Lpd are lost. We recognize that the different dynamics of VASP and lamellipodin during a splitting event render this statement confusing and/or misleading.

In the earliest phase of a tip complex splitting event, before two clearly separated VASP clusters emerge, we note that *both* VASP *and* Lpd take on an elongated shape, suggesting that they remain together as the complex begins to deform (Figure 4c, yellow oval outline at 20 seconds). As splitting progresses, however, Lpd is lost from the shedding bolus of VASP and redistributes along the membrane. We reasoned that this differential localization is due to differential binding interactions with the plasma membrane. VASP, which does not directly bind the plasma membrane, is lost into the cell body where it becomes either diffuse in the cytoplasm or incorporates into nearby focal adhesions. In contrast, lamellipodin, which binds directly to

phospholipids via its RAPH domain, remains associated with the membrane. We clarify this point in the revised manuscript.

7) Arrowhead in Figure 4C, 32 sec blocks the nascent focal adhesion. It should be moved slightly away.

This is a good point. We have shifted the arrowhead in new Figure 4C so that it no longer obscures the focal adhesion at the 32 sec time point.

8) p. 11. "We quantified the relative change in lamellipodin clustering..." The figure 5D legend says that both lamellipodin and VASP were quantified, although it seems that only one protein is shown on the graph. Please, clarify.

This is a good catch. We have changed the Figure 5D legend to note that only lamellipodin intensity is quantified in the plot. In an earlier analysis of the cytochalasin D experiment (not included in the manuscript) we quantified changes in both lamellipodin and VASP clustering. Both proteins showed clear disruption in clustering upon cytochalasin D treatment. We chose not to display the VASP data because, in addition to disrupting clusters, the drug causes VASP detach from the membrane. We therefore felt that it would be more correct to quantify and plot only the lamellipodin data in which its total protein fluorescence remained the same before and after drug treatment.

9) p. 14 "Similar to previous studies, we find that overexpressing the membrane-associated protein, IRSp53 strongly induces formation of filopodia." These previous studies are not cited.

This is another good catch. We have added the appropriate citations in the text of the revised manuscript.

10) (Optional) p. 17. "We propose that the size-dependent instability of VASP clusters reflects the difficulty of coordinating VASP's interaction with the two binding partners that hold the cluster together: FPPPP-containing proteins and free barbed ends of actin filaments. " This is a reasonable hypothesis, but I am curious whether there is a thermodynamic optimum for the size of clusters, which are driven by multivalent interactions.

This is an excellent question that has intrigued us throughout the entire project. The system cannot be in equilibrium, since its dynamic behaviors are driven by continuous polymerization of associated actin filaments, but we wondered whether some steady-state approximations and some assumptions about ‘effective temperature’ might give us mechanistic insight into the distribution of VASP/Lpd cluster sizes. For example, a recent paper by Chattaraj et al (2019) argued that binding affinity, protein flexibility/geometry, and valency of interactions all affect cluster size at steady state. We do not have direct access to some of these parameters (e.g. flexibility) and we do not have a quantitative constraint on the effects of continuous actin assembly, so we are not confident that a thermodynamic model would add much mechanistic insight at present. This is, however, the direction we plan to take the project in the future.

Reviewer #2:

1) What is "SNAPjf646-actin bundle" probe in Figure 1C? Video 2 calls it just actin. Is this beta actin?

Yes, $SNAP_{JF646}$-actin refers to beta actin fused to SNAP-tag at the N-terminus that was subsequently labeled with a SNAP ligand called Janelia Fluor 646 (JF646) which we used as a live cell imaging probe. We have changed the nomenclature of this construct to be “SNAP-beta-actin” in the text for clarity.

2) The edge analysis presented is intriguing. The 'image analysis' section of the methods states that there was a custom Matlab code used. Will this code be provided with the manuscript? A well annotated MatLab script would be a valuable tool for the field.

We are big believers in sharing computer code. We have posted the annotated Matlab code on the Mullins lab website: mullinslab.ucsf.edu > protocols as well as on the Mullins lab GitHub repository: https://github.com/mullinslabUCSF/edge-kymograph.

References

Chattaraj, A., Youngstrom, M. and Loew, L.M. 2019. The interplay of structural and cellular biophysics controls clustering of multivalent molecules. *Biophysical Journal* 116(3), pp. 560–572.

Hansen, S.D. and Mullins, R.D. 2015. Lamellipodin promotes actin assembly by clustering Ena/VASP proteins and tethering them to actin filaments. *eLife* 4.

Katoh, K., Hammar, K., Smith, P.J. and Oldenbourg, R. 1999. Birefringence imaging directly reveals architectural dynamics of filamentous actin in living growth cones. *Molecular Biology of the Cell* 10(1), pp. 197–210.

RE: Manuscript #E20-04-0270R
TITLE: "Initiation and disassembly of filopodia tip complexes containing VASP and lamellipodin"

Dear Dyche,

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

Sincerely,
Laurent Blanchoin
Monitoring Editor
Molecular Biology of the Cell

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