LIM and SH3 Protein 1 Localizes to the Leading Edge of Protruding Lamellipodia and Regulates Axon Development

Stephanie Pollitt, Kenneth Myers, Jin Yoo, and James Zheng

Corresponding author(s): James Zheng, Emory University

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-06-0366
TITLE: LIM and SH3 Protein 1 Localizes to the Leading Edge of Protruding Lamellipodia and Regulates Axon Development

Dear Dr. Zheng:

Thank you for submitting this very nice manuscript to MBoC. As you can see, the reviewers were very positive, and made constructive suggestions to improve the experiments and clarity of the manuscript. In your revised manuscript, please respond to the reviewers' comments point-by-point. In particular, please address their concerns regarding LASP1 knockdown verification, drug treatments, and quantification of Drosophila experiments. The additional analyses they suggested (cross-correlation analysis of LASP1 at the leading edge, analysis of growth cone morphology, quantifying changes in GFP-LASP1 after drug treatments) should be feasible and are important. If you can perform their suggested additional experiments (e.g. investigating barbed ends/actin dynamics in the absence of LASP1,) it will greatly enhance our understanding of LASP1 mechanisms in axon growth. Finally, to comply with the author submission checklist, please add an identifier (e.g. stock number or Flybase identifier) for the Drosophila RNAi line used in your experiments.

Sincerely,

Avital Rodal
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Zheng,

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):

This manuscript by Pollitt et al, is a seminal study of the cell biology of the actin-binding protein LASP1 (LIM and SH3 Protein 1) and its role in growth cone motility and neuronal development. Although LASP1 is highly expressed in multiple tissues including the brain, and its dysregulation implicated in several neurological disorders including ASD and schizophrenia, little is known about its role in developing neurons. Here the authors characterized the localization of LASP1 in CAD cells and hippocampal neurons, and phenotypes associated with knockdown of LASP1. They found that LASP1 is enriched in the growth cone periphery and the lamellipodial edge of CAD cells. LASP1 colocalizes with free barbed ends of filamentous actin. Interestingly this localization was sensitive to cytoD, but not other pharmacological inhibitors of actin polymerization. Knockdown of LASP1 was associated with shorter axons, slower axon growth, and reduced axon branching. The authors than switch from murine LASP1 to the drosophila homolog, and find knockdown of lasp in drosophila is associated with subtle axon phenotypes in the ventral nerve cord. Overall the study is sound with beautiful images and image analysis. I have a couple of minor experimental concerns, one experimental suggestions, as well as a few easily addressable suggestions for clarity and writing outlined below.

1). Figure 1A: You state that LASP1 expression is relatively high at E18 and this persist through to adulthood. Relative to what? Rather, your data to me suggest only that LASP1 is present throughout all developmental stages investigated to adulthood is a better interpretation of this data. B-D: these images are beautiful!

2) Figure 2: the kymographs showing lasp1 enriched at the leading edge, particularly during protrusion is really nice. I wonder if you could perform a temporal cross correlation analysis that could illuminate the relationship between LASP1 enrichment and leading edge behavior. Similar to correlation analysis done by K. Lee, et al, Danuser 2015, Cell Systems paper.. While this isn't critical, this type of analysis could determine if LASP1 starts to arrive or depart before a change in protrusion/retraction occurs.

3) Figure 4 beautiful demonstrates with the barbed end assay that lasp1 is concentrated where Barbed ends are. The sensitivity of this to low doses of cytochalasin is nice, demonstrating the requirement of free barbed ends for LASP1 enrichment at the lamellipodia. However, I am confused that the other pharmacological inhibitors have no effect on LASP1. For example, doesn't Arp2/3 generate most of the barbed ends at the leading edge? For this reason I can't see how CK666 treatment doesn't affect LASP1 localization. Are you sure the CK666 worked? Is there an associated barbed end assay showing that barbed ends were reduced? The latrunculin A treatment is less concerning, since this is monomer sequestering. The SMIFH2 drug has recently been shown by the Sellers lab at NHLBI to inhibit myosinII better than formins, so there is a large caveat there.

4) You nicely show that LASP1 localizes with barbed ends and requires barbed ends for its leading edge localization. Is LASP1 responsible for these barbed ends? The only new experiments I would suggest: Does knockdown of LASP1 reduce barbed ends? Doing the barbed end assay after knockdown of LASP1 would greatly enhance the potential mechanism that you are getting here. Alternatively, would actin FRAP analysis reveal any changes in the dynamics of actin +/- LASP1?

5) Figure 6a: the blot shown is NOT quantifiable, with multiple bubbles disrupting signal. Presumably this is the best blot, which causes concern for the validity of the quantification of LASP1 knockdown efficiency. Please improve quality of this and redo quantification.

6) Figure 7: are there associated movies that can be included to see difference in growth cone motility and branching? This would be powerful. What do you mean by the termination of new branches? Their disappearance? Their growing stopping? This is unclear.

7) The transition to drosophila is abrupt. Please provide a little more information about lasp homolog in drosophila. What are the similarities both sequence and domain architecture. Are there other nebulin family member homologs in drosophila? Is there information known about lasp expression in drosophila?

8) Regarding the axon defasiculation in drosophila: how was this quantified, presumably from the tau:GFP images, but what was the definition and how this was quantified is unclear. The images of the CNS axons look pretty similar between control and knockdown, so I worry about this quantification.

Reviewer #2 (Remarks to the Author):

Overall, this is a well-documented study of the function of LASP1 in early neuronal development. Using a combination of primary hippocampal neuronal culture, CAD cell lines and drosophila they define the function of LASP1 in growth cone function, axon outgrowth and guidance. This group recently published a paper on the function of LASP proteins in dendrite and dendritic spine function and this work now shows how LASP1 functions in an earlier stage of development. For the most part the study is well conceived and the data that are presented are of high quality. It is well written and easy to follow. However, there are a few

concerns that should be easily addressable. They are outlined below.

Although there is quantification of growth cone speed and persistence in Fig. 7B after LASP1 knockdown, there is no data presented showing growth cone morphology after knockdown. If LASP1 is important for actin dynamics, presumably there would be some sort of shape change in the growth cone. Are there growth cones smaller or larger? Are the growth cones more lamellar or filopodial?

Fig. 1A has uneven loading of the tubulin making it difficult to determine how LASP1 is expressed in hippocampus. Furthermore, E18 has one high concentration and two lower concentrations. It would be important to know if LASP1 is really highly expressed at E18 or not.

Fig. 1B - This doesn't appear to be a representative neuron. The cell body consists of a large lamellar/filopodial protrusion without any minor processes. An example of a neuron with both clear axonal as well as dendritic (minor process) growth cones would be useful. Also, a single label LASP1 image would be useful. With high actin and tubulin labeling it is hard to determine where LASP1 is localized in these neurons.

Fig. 1C. Better examples of growth cones with clear f-actin concentration in the growth cone would be useful. This lab generally publishes very nice growth cone images with well-labeled linear actin structures. These don't seem to be of the quality that they usually publish.

Fig. 5 - Some sort of quantitation of the change in GFP-LASP1 at the leading edge, after drug treatments, would be useful.

Fig. 2 legend refers to blue arrowheads in the kymographs in C. In the figure the arrowheads are orange.

We sincerely thank the reviewers for their time and effort in evaluating our study on LASP1 in cell motility and axon development. We are particularly grateful for the extremely positive feedback on our work and greatly appreciate the insightful and constructive comments. We have revised the manuscript to address the reviewers' comments, including the LASP1 knockdown verification, drug treatments, and quantification of *Drosophila* experiments. Due to the current restriction on bench research however, we were unable to perform some of the additional experiments that the reviewers suggested. We hope that the reviewers and the editorial office of MBoC will find the revision satisfactory and the work is suitable for publication. Details of the revision and responses to reviewers' comments are provided below. Changes are also indicated in the revised manuscript (light grey highlighted).

Reviewer #1 (Remarks to the Author):

Figure 1A: You state that LASP1 expression is relatively high at E18 and this persist through to adulthood. Relative to what? Rather, your data to me suggest only that LASP1 is present throughout all developmental stages investigated to adulthood is a better interpretation of this data. B-D: these images are beautiful!

> The reviewer makes a good point. We have revised the manuscript to state that LASP1 is expressed at all of the developmental stages that we investigated. In addition, we have included a new blot that has more even levels of the tubulin loading control in the revised Figure 1. We thank the reviewer for his/her comment on the quality of our images!

2) Figure 2: the kymographs showing lasp1 enriched at the leading edge, particularly during protrusion is really nice. I wonder if you could perform a temporal cross correlation analysis that could illuminate the relationship between LASP1 enrichment and leading edge behavior. Similar to correlation analysis done by K. Lee, et al, Danuser 2015, Cell Systems paper.. While this isn't critical, this type of analysis could determine if LASP1 starts to arrive or depart before a change in protrusion/retraction occurs.

> We thank the reviewer for this suggestion and indeed this temporal cross-correlation analysis would provide the further support for the apparent relationship between LASP1 edge enrichment and protrusion/retraction behaviors. We tried to perform this analysis, but were unsuccessful due to the insufficient temporal resolution of our time lapse sequences. Thus, to successfully perform this temporal cross-correlation analysis, we would have to repeat the live cell imaging with increased frame rates. Given that the reviewer felt this analysis wasn't critical, we hope they will agree that the kymographs are sufficient to demonstrate the tight association between LASP1 edge enrichment and membrane protrusion.

3) Figure 4 beautiful demonstrates with the barbed end assay that lasp1 is concentrated where Barbed ends are. The sensitivity of this to low doses of cytochalasin is nice, demonstrating the requirement of free barbed ends for LASP1 enrichment at the lamellipodia. However, I am confused that the other pharmacological inhibitors have no effect on LASP1. For example, doesn't Arp2/3 generate most of the barbed ends at the leading edge? For this reason I can't see how CK666 treatment doesn't affect LASP1 localization. Are you sure the CK666 worked? Is there an associated barbed end assay showing that barbed ends were reduced? The latrunculin A treatment is less concerning, since this is monomer

sequestering. The SMIFH2 drug has recently been shown by the Sellers lab at NHLBI to inhibit myosinII better than formins, so there is a large caveat there.

The Arp2/3 complex does nucleate branched actin filaments, however Arp2/3 does not generate all of the barbed ends at the leading edge. Formin- and Mena/VASP-dependent polymerization is still occurring in these cells. Thus, cells treated with 100 µM of CK-666 (which was previously shown to be specific at this concentration (Wu et al. 2012 Cell, Vitriol et al. 2015 Cell Reports)) are expected to change the composition of their lamellipodial actin network. Indeed, we observed an increase in the number of long parallel actin bundles in the lamellipodia after treatment with CK-666, similar to previous reports (Vitriol et al. 2015 Cell Reports, Skruber et al. 2020 Current Biology). This confirms that CK-666 works in our hands (Figure 5). Finally, the acute nature of CK-666 application will unlikely result in a complete elimination of actin barbed ends generated by Arp2/3 before CK-666 treatment.

The caveat that SMIFH2 affects MyosinII contractility has been added to the manuscript.

4) You nicely show that LASP1 localizes with barbed ends and requires barbed ends for its leading edge localization. Is LASP1 responsible for these barbed ends? The only new experiments I would suggest: Does knockdown of LASP1 reduce barbed ends? Doing the barbed end assay after knockdown of LASP1 would greatly enhance the potential mechanism that you are getting here. Alternatively, would actin FRAP analysis reveal any changes in the dynamics of actin +/- LASP1?

We acknowledge that the experiments suggested by the reviewers here are interesting and would certainly be a valuable contribution to the manuscript. We hope that we will be able to perform this line of experiments in the near future when the research restriction has been lifted. We have revised the discussion to address this possibility that LASP1 might influence the number of barbed ends.

5) Figure 6a: the blot shown is NOT quantifiable, with multiple bubbles disrupting signal. Presumably this is the best blot, which causes concern for the validity of the quantification of LASP1 knockdown efficiency. Please improve quality of this and redo quantification.

The reviewer raises a valid concern about the effects of the bubbles on our quantification. Therefore, we have re-run the blots (there are now no bubbles in any of the blots used for analysis) and reanalyzed them to quantify the extent of LASP1 knockdown.

6) Figure 7: are there associated movies that can be included to see difference in growth cone motility and branching? This would be powerful. What do you mean by the termination of new branches? Their disappearance? Their growing stopping? This is unclear.

We have now included a supplemental video clip that shows the dynamic nature of axon/dendrite growth and retraction of control and LASP1-knockdown neurons. With regard to the branching phenotype, we have changed the phrasing in the manuscript and methods section to clarify that we are referring to the complete retraction of new axon branches.

7) The transition to drosophila is abrupt. Please provide a little more information about lasp homolog in drosophila. What are the similarities both sequence and domain architecture. Are there other nebulin family member homologs in drosophila? Is there information known about lasp expression in drosophila?

We have revised this section of the manuscript to include more information about Lasp in *Drosophila*. We have also cited previous papers showing Lasp expression and sequence/domain architecture.

8) Regarding the axon defasiculation in drosophila: how was this quantified, presumably from the tau:GFP images, but what was the definition and how this was quantified is unclear. The images of the CNS axons look pretty similar between control and knockdown, so I worry about this quantification.

We have revised the results section of the manuscript to clarify how the quantification was performed, and have expanded the methods section to address this. Before analysis, each z-stack was blinded, then analyzed for axons and commissures that do not follow the typical trajectory as described previously (O'Donnell and Bashaw, 2013). Each segment of the ventral nerve cord was analyzed for three developmental abnormalities: 1) commissures that do not complete the journey to their contralateral target, 2) individual axons that leave the main commissural tract, and 3) commissures with axons that have spread apart, such that gaps are visible within the commissure. The number of segments with each phenotype were expressed as a percentage of the total number of segments in each individual embryo, and then the samples were unblinded.

Reviewer #2 (Remarks to the Author):

Overall, this is a well-documented study of the function of LASP1 in early neuronal development. Using a combination of primary hippocampal neuronal culture, CAD cell lines and drosophila they define the function of LASP1 in growth cone function, axon outgrowth and guidance. This group recently published a paper on the function of LASP proteins in dendrite and dendritic spine function and this work now shows how LASP1 functions in an earlier stage of development. For the most part the study is well conceived and the data that are presented are of high quality. It is well written and easy to follow. However, there are a few concerns that should be easily addressable. They are outlined below.

Although there is quantification of growth cone speed and persistence in Fig. 7B after LASP1 knockdown, there is no data presented showing growth cone morphology after knockdown. If LASP1 is important for actin dynamics, presumably there would be some sort of shape change in the growth cone. Are there growth cones smaller or larger? Are the growth cones more lamellar or filopodial?

The questions posed by the reviewers about growth cone morphology and actin composition are highly interesting, and would add to our findings and model of LASP1 activity. Unfortunately, the time-lapse recordings were performed with low magnification to capture the entire cells, thus lacking the resolution for analyzing growth cone morphology and actin structures/dynamics.

Fig. 1A has uneven loading of the tubulin making it difficult to determine how LASP1 is expressed in

hippocampus. Furthermore, E18 has one high concentration and two lower concentrations. It would be important to know if LASP1 is really highly expressed at E18 or not.

A new blot with relatively even loading tubulin control is provided in the revised Figure 1. We have also revised the manuscript to state that LASP1 is expressed at relatively consistent levels of expression at each stage of hippocampal development that we investigated.

Fig. 1B - This doesn't appear to be a representative neuron. The cell body consists of a large lamellar/filopodial protrusion without any minor processes. An example of a neuron with both clear axonal as well as dendritic (minor process) growth cones would be useful. Also, a single label LASP1 image would be useful. With high actin and tubulin labeling it is hard to determine where LASP1 is localized in these neurons.

We have performed a new round of immunofluorescence labeling and included a new neuron for Figure 1B. Please note that hippocampal neurons in culture for only two days (DIV2) do not have elaborated dendritic processes. Instead, DIV2 hippocampal neurons only have minor processes, which are evident in the new image. We have also provided individual channels to better depict the patterns of MTs, F-actin, and LASP1.

Fig. 1C. Better examples of growth cones with clear f-actin concentration in the growth cone would be useful. This lab generally publishes very nice growth cone images with well-labeled linear actin structures. These don't seem to be of the quality that they usually publish.

We thank the reviewer for his/her positive comment on our track record on growth cone imaging. However, unlike growth cones from frogs (that we used to work on), hippocampal neurons do not extend large growth cones with uniform morphology. The two growth cones shown in Figure 1C represent the diversity of hippocampal growth cones, highlighting the spatial distribution of LASP1 with respect to the F-actin structures. Together with the new cell in Figure 1B, the reviewer will find our representative images of hippocampal growth cones are of sufficient quality to demonstrate the unique LASP1 spatial distribution.

Fig. 5 - Some sort of quantitation of the change in GFP-LASP1 at the leading edge, after drug treatments, would be useful.

We have provided quantification of the change in GFP-LASP1 after each drug treatment.

Fig. 2 legend refers to blue arrowheads in the kymographs in C. In the figure the arrowheads are orange.

We thank the reviewer for pointing out this mistake. We have revised the figure and legends to correct the mistake.

RE: Manuscript #E20-06-0366R
TITLE: "LIM and SH3 Protein 1 Localizes to the Leading Edge of Protruding Lamellipodia and Regulates Axon Development"

Dear Dr. Zheng:

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

Thank you for resubmitting your thoughtfully revised manuscript. As you can see, both reviewers found that the revisions addressed their concerns.

Sincerely,
Avital Rodal
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Zheng:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Your paper is among those chosen by the Editorial Board for Highlights from MBoC. Hightlights from MBoC appears in the ASCB Newsletter and highlights the important articles from the most recent issue of MBoC.

All Highlights papers are also considered for the MBoC Paper of the Year. In order to be eligible for this award, however, the first author of the paper must be a student or postdoc. Please email me to indicate if this paper is eligible for Paper of the Year.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

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

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns from the previous round and I think this is ready for publication in MBoC. Well done!

A small suggestion that edition of labels and elapsed time on the movie would be great!

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my concerns and strengthened the manuscript. Although the quantification of growth cone morphology after LASP1 knockdown, that I suggested in the previous review, would be a good addition to the manuscript, I understand the limitations on research due to COVID restrictions and do not think it is worth holding up publication of the manuscript for those data.