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A pair of E3 ubiquitin ligases compete to regulate filopodial dynamics and axon guidance

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March 20, 2019

Re: JCB manuscript #201902088

Prof. Stephanie L Gupton
University of North Carolina at Chapel Hill
111 Mason Farm Road 4332 MBRB, CB 7090
Chapel Hill, North Carolina 27599

Dear Prof. Gupton,

Thank you for submitting your manuscript entitled "A pair of E3 ubiquitin ligases compete to regulate filopodial dynamics and axon guidance". Thank you very much for your patience with the peer review and editorial process. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

We're pleased to share that the reviewers were supportive of the work and found the results interesting and of high quality. They provided comments that we find constructive and important to ensure the data are solid, clear, and convincing. They requested quite a number of clarifications not requiring novel experiments that we find relevant (including about the methods and approaches used) and we think that these helpful suggestions from experts in the field are valid and need to be addressed. In addition, in revising the work, we suggest you focus experimental efforts on clarifying whether TRIM67 mutant growth cones are enlarged compared to WT (Ref #1, point #1) and addressing the reviewers' questions about the hierarchy between TRIM9 and TRIM67 in the pathway. We also think that the reviewers' points about the ubiquitination data (in particular Rev#3's points #6 #7 #9) should be addressed to strengthen these studies. Please also address Referee #3's question #10 (whether control images have been reused in different figure panels) as well as the referees' requests for more convincing and better-controlled data throughout. Providing evidence of endogenous interactions via coIPs (Rev#1 point #3) would provide additional support for the conclusions, but if this is technically challenging, we would find the current biochemical data acceptable. Lastly, while Rev#3 suggests an interesting experiment in point #5, we do not find these mutant analyses indispensable to support your current conclusions and the potential mislocalization resulting from the lack of some domains is a general limitation of the approach that weakens the conclusions, so we would not require these data for publication.

Please let us know if you have any questions about the revisions or anticipate any issues addressing the reviewers' comments. We would be happy to discuss the revisions further if helpful.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does

not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Valerie Castellani, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

In this paper, Boyer et al. al demonstrate the involvement of the E3 ubiquitin ligase TRIM67 in netrin-1-mediated filopodial dynamics, axon branching, and guidance in embryonic cortical neurons. Interestingly, they show an antagonistic role of TRIM67 over TRIM9 regulation of VASP ubiquitination and its role in filopodial dynamics in response to netrin-1. They show the interaction between VASP and TRIM67 and a competitive inhibition of TRIM9-VASP interaction by TRIM67.

Overall, this is a very interesting study, combining in vivo and in vitro analysis, to demonstrate the coordinated regulation of VASP ubiquitination as a critical requirement for netrin-1-mediated growth cone dynamics and axon guidance. In general, the data are convincing, well performed with appropriate controls. The quantification of the data and statistics are properly done. However, there are some issues to be clarified, see comments below:

Comments:

1) Figure 3 shows that TRIM67 is required for the axon and growth cone response to netrin-1. However, in the absence of netrin-1, there is a significant increase in growth cone area, number of filopodia per growth cone, and filopodia length, which would argue that these effects could be netrin-1-independent. In fig S1D and E, the authors are testing the response of neurons to other factors, FGF2 and Slit2N. TRIM67 -/-neurons are responding to the factors, but is there a significant difference between untreated wt neurons and TRIM67 -/- neurons, as in fig3? This is less convincing and should be clarified.
2) Fig3D: Trim67-/- + netrin-1: does it induce growth cone collapse? It seems that there is less branching and shorter neurite, the images do not match the quantification in 3E and 3F.
3) Figure 5: the authors show coIP with overexpression of TRIM67 and VASP. Do endogenous proteins interact in neurons? This should be shown.
4) I have problems with Fig 5E and F: the most convincing data is indeed the increase of VASPub in TRIM67-/- neurons, which is reduced with netrin-1. However, the reduced VASPub in WT neurons treated with netrin-1 or in TRIM9-/- neurons is not convincing in the blot and does not reflect the graph in F. perhaps replacing the blot would help.
5) Fig. 5G: the expression of the myc-tagged proteins should be shown as well.
6) Fig. 5J: IF VASPub is increased in TRIM67 -/- (Fig.5E,F), why the fluorescence recovery halftime is not significantly slower?
7) Fig. 6E: it would be nice to show Ponceau S staining of the GST proteins.
8) Fig. 7A,B: something is wrong with the blot in A. if the blot is labeled properly, why do we see bands of VASP and p-VASP in the ubiquitin IB? again, the blot does not reflect the quantification data and is not convincing. I don't see a reduction in VASPub in wt cells, treated with netrin-1, and there is a higher signal in KO cells.
9) Finally, I don't think Fig7 is showing that TRIM67 is acting upstream of TRIM9. To prove this, it would be nice to see if expression of TRIM9 would induce more VASPub in TRIM67 -/- HEK293 cells.

Minor comments:

-Mislabeling in a few figures: S2 and 6.
Figure 1C is not mentioned in the text.
Fig5B: should be mentioned that this is done in TRIM67-/- cells.

Reviewer #2 (Comments to the Authors (Required)):

These investigators have shown previously that response to the axon guidance cue Netrin in cultured mouse cortical neurons is gated by non-degradative ubiquitylation of the actin polymerase VASP by the E3 ubiquitin ligase TRIM9. In the current ms, they extend these studies to provide evidence that a TRIM9 paralog, TRIM67, blocks VASP ubiquitylation by TRIM9, thereby providing the background conditions against which the action of Netrin can occur. The data are thorough, convincing and rigorously analyzed. The result is interesting in that it dissects an unexpected biochemical relay underlying the action of a key regulator of morphogenesis. I have no significant issues with the paper.

1. There is really only one detail that left me puzzled, which is why a TRIM67 derivative lacking ligase activity failed to inhibit TRIM9 ubiquitylation of VASP. As far as I can see, there is nothing in

the authors' binding-competition model that predicts (or accounts for) activity-dependence for TRIM67, particularly as the authors demonstrate that the LD mutation, or even wholesale deletion of the RING domain, do not block binding of TRIM67 to VASP. This observation does not by any means invalidate the proposed model, but it also does not fit in it very obviously. It would be worthwhile for the authors to comment on this.

Reviewer #3 (Comments to the Authors (Required)):

This study demonstrates the role of TRIM67 in netrin-1-dependent development of the neuronal growth cone. Authors extend their own published works reporting functions of TRIM9 and TRIM67. TRIM67 is crucial for the netrin-dependent expansion of the growth cones, filopodial formation and elongation. They further study the molecular machinery of netrin-1- and TRIM67-dependent growth cone development. Unexpectedly, TRIM9-dependent ubiquitination of VASP is antagonized by TRIM67 in its ligase activity independent manner. I appreciate their live imaging and immunohisto/cytochemistry results, and EM data in Figures 1, 2, 3, and 4. Characterization of TRIM67f/f and TRIM67-/- neurons is thorough and deep in this regard. However, biochemistry and mouse genetics are not designed well enough and the results do not always support their conclusions. Overall, although this manuscript has a good potential to be an excellent work, it is too preliminary to be published in a highly appreciated journal such as JCB in the current form.

Major comments;
1) Details of quantification and statistics should be described in individual figure legends or as tables. Numbers of cells, animals, and/or culture experiments as well as statistics (e.g. t-test, one-way or two-way ANOVA, post-hoc tests) need to be documented for each individual figure. Some quantifications are shown as box-dot-plots others are as bar diagrams. They should always use the same way to present their data if they don't have particular reasons. In some bar diagrams, authors don't provide error bars in the control (e.g. the first bars in Fig 5F, 5H, 6D, and 7B). This should be corrected too (See Fig. 1B, Blanco-Suarez et al., Neuron, 100,1116, 2018 for example).

2) Do they use littermate controls for experiments using mutant mice? For instance, TRIM67-/-, TRIM9-/- double KO should be compared with TRIM67+/+, TRIM9+/+ from the same litter in the most ideal case. However, I realize that it is unreasonable to request authors to repeat experiments with the littermate control, especially using TRIM67-/-, TRIM9-/- double KO. The chance to have these two genotypes (i.e. WT and double KO) in the same litter is extremely low. If they used the non-littermate control, authors should state this point in the legend or in Methods.

3) In Fig 3C, TRIM67 KO neurons show increases in growth cone size and number of filopodia/growth cone in mock treated cells, while FigS1E showed no such phenotype. They need to explain the discrepancy. I am afraid that changes in growth cone/filipodia morphology in FGF-treated neurons might be due to relatively small values in mock-treated cells in Fig.S1E.

4) CC is required for TRIM67's binding to VASP (Fig 5B) and deltaCC cannot rescue the phenotypes (Fig S2 and S3), indicating that TRIM67-binding to VASP is crucial (Fig 7F). However, other CC containing mutant cannot rescue the phenotype either, e.g. LD, deltaRING, deltaSPRY in Fig S2C. This result is confusing. What puzzles me more is the rescue of filopodial density by expressing Nterm (Fig S2D). This region has nothing to do with VASP-binding of TRIM67. I suggest to remove rescue data, except for the ones using FL, LD and deltaCC mutants, from the manuscript. FL and LD as well as deltaCC should stay in the manuscript since they include very important messages (i.e.

catalytic activity is needed for the role of TRIM67).

5) Authors should rescue TRIM67 KO by expressing only CC. If it does not work, they should discuss the reason in DISCUSSION, for instance by showing the disturbed localization of CC in the cells. In some cases, truncated mutant of TRIM proteins seem to be unstable in cells (Fig. 6 in Reymond et al., 2001, EMBOJ).

6) The straightforward explanation of the lack of rescue by LD mutant in Fig.S2 could be the lack of TRIM67-mediated ubiquination and degradation of TRIM9. TRIM9 regulation by TRIM67 could be done in two ways; competing TRINM9/VASP interaction and degradation of TRIM9. Authors' arguments that regulations of VASP and of TRIM9 by TRIM67 are proteasome/degradation-independent are weak in Fig. S4I, S4J, S4K, and S4L. They used homogenates for the quantification. They showed that the regulations are local at the growth cone in immunocytoechemistry. If the majorities of VASP and TRIM9 are in the cell body and if only small fractions of VASP and TRIM9 is regulation at the growth cone, their local changes in the TRIM67 KO at the growth cone could be masked by the unchanged levels in the cell body. To circumvent this, authors should show the level of VASP and TRIM9 in TRIM67 KO by immunocytochemistry at the growth cone. They have good antibodies for this purpose.

7) If they think that this ubiquitination is proteasome independent, western blot using anti-K6, ati-K11, and anti-K63 antibodies is interesting. At least some of them should be tried with proper positive controls (i.e. purified polyubiquitin chains of each type). Antibodies and positive controls are available commercially (e.g. from BostonBiochem). If they are negative, authors should also discuss the possibility of multiubiquitination.

8) I realize that quantification of ubiquitination level is often challenging. To support the estimation of ubiquitin western blots in Fig. 5E, 5G, and 7A, authors should show entire gel images with and without regions of interest (ROIs). The result of quantification of ubiquitin western blot depends on how one chooses ROIs.

9) Ubiquitination experiment in Fig. 5G is very nice since they use TRIM67 KO HEK293 cells. However, GFP is not the best tag for the substrate. This tag has several lysine residues and could be conjugated by K48-linked polyubiquitin chains. Indeed, estimation of GFP variant turnover (decay time) is often used to monitor the proteasome activity in the live cell imaging. Authors should repeat this experiment using a small tag, such as myc, flag or HA. Also the difference of the molecular weights of ubiquitinated and non-ubiquitinated GFP-VASP is smaller than 24 kDa for me. Using a small tag will solve this problem too.

10) I found images used for the first, second, seventh, and eighth lanes in Fig.5E and first four lanes in Fig.7A seem to be identical. If they are indeed same and if data used for the wild type in quantifications in Fig.5F and Fig.7B are same, it should be stated in the legends. If they are same, did authors perform experiments for Fig.5F and 7B at the same time as a single set of experiments? It is not allowed to compare the values from a control and from a KO taken in two independent experiments.

11) In discussion, authors argument that 'netrin responses in a Trim67:Trim9 double knockout neuron resemble that of a Trim9-/- neuron. This suggests that TRIM9 acts downstream of TRIM67.' This argument is weak. If they want to show that TRIM67 functions as an upstream inhibitor of TRIM9, TRIM67 KO phenotype should be restored by additional knocking out or knocking down of TRIM9. They should change this part of DISCUSSION.

12) Details of FRAP estimation are not documented in Methods section. It seems to be incorrect, judging based on the top image in Fig 5I. If a part of fluorescence remains after photobleaching (t=0' in Fig.5I), they need to subtract the residual fluorescence intensity from intensities at all imaging time-points. Do they take the background signals for the estimation in account? This should be also subtracted. They should re-estimate using the existing dataset based on a detailed method paper (McNally, Methods in Cell Biology, 85, 329, for instance). If they want to estimate the turn over of the protein (i.e. protein degradation), half-time recovery should be estimated. If they aim to study the local exchange rate of VASP, which is dependent on protein-protein interactions, they should try to fit the FRAP curve to single- and multi-exponential curves. If fitting works the best with double exponential curve for instance, they could discuss that the exchange of VASP is possibly dependent on two protein-protein interactions.

Minor comments;

1) There are some inconsistency and mistakes in the manuscript. In page 14, 'mcherry' is used while 'mCherry' is used in page 19. In the second paragraph of page 6, 'Fig. 6D, E' should be 'Fig. 6C, D'. 'Fig. 6F' should be 'Fig. 6E'.

2) Some abbreviations are used without explanation. For instance, in the third paragraph in page 6, '2XKO' appears without explanation. This abbreviation is not used afterwards. In the manuscript in JCB, abbreviations need to be explained in their first appearance and need to be used several times. '2XKO' appears only once.

3) Fig S4K, why does each genotype have two lanes? Are they samples from different mice with same genotypes? Did authors load samples in a single gel? This should be stated in the legend.

4) They need to describe more precise molecular weight in their western blot results. Tick marks on the right side of the molecular weight number are necessary.

5) They should explain what PR-619 is in the main text. Readers do not always have a strong background of ubiquitination biochemistry.

6) In page 5, line 10 from the bottom, "To assay effects of increased VASP ubiquitination on FRAP t1/2 we treated neurons with PR-619; in Trim67+/+ neurons PR-619 increased GFP-VASP FRAP t1/2, consistent with an increase in ubiquitination (Fig.5J)." They never showed an increase in ubiquitination of VASP upon PR-619 treatment in WT cells. The text needs to be changed.

7) In general, methods are not well documented. It would be difficult to repeat experiments only with this description. Authors should document the detailed methods as other manuscripts published in JCB recently.

8) In the method section, animal background is explained as C57BL/6J. However, in the recently published paper from authors' group (Boyer et al., eNeuro, 5, e0186, 2018), authors explain that TRIM67 KO animals have C57BL/7 background. If so, they should have crossed TRIM67 KO with C57BL/6J wild type several times. How many times they backcrossed needs to be explained. The phenotype of midline crossing is highly depends on the genetic background of mice.

9) Authors may want to acknowledge Dr. Simon Rothenfusser for his providing them a material.

10) In the section of Immunoblotting and precipication assay in page 19, they need to explain why they included MG132. As far as VASP ubiquitination is nothing to do with proteasomal degradation, MG132 increases the background. It does not make sense to include MG132. The rationale should be explained.

11) In the section of Immunoblotting and precipication assay in page 19, 270 ul should be 270 µl.

12) In Figure 3B legend, "B) Growth cones from primary neuronal cultures stained for filamentous actin (phalloidin), β-III-tubulin, and TRIM67". However, they never showed staining of TRIM67 in the figure.

13) Figure 7A has four panels; the first and third ones shows a similar pattern and the second and fourth have a similar pattern. I assume that the first and third panels are from the anti-VASP and the second and fourth ones are from the anti-ubiquitin antibody. If I am right, authors should correct the labeling of the figure.

1st Revision - Authors' Response to Reviewers: September 25, 2019

We thank the reviewers for their helpful comments and suggestions and for appreciating the significance and rigor of our studies. We have addressed your concerns for clarity and additional experiments, etc point-by-point below, with our comments in blue text in this response, and in red in the manuscript.

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

In this paper, Boyer et al. al demonstrate the involvement of the E3 ubiquitin ligase TRIM67 in netrin-1-mediated filopodial dynamics, axon branching, and guidance in embryonic cortical neurons. Interestingly, they show an antagonistic role of TRIM67 over TRIM9 regulation of VASP ubiquitination and its role in filopodial dynamics in response to netrin-1. They show the interaction between VASP and TRIM67 and a competitive inhibition of TRIM9-VASP interaction by TRIM67.

Overall, this is a very interesting study, combining in vivo and in vitro analysis, to demonstrate the coordinated regulation of VASP ubiquitination as a critical requirement for netrin-1-mediated growth cone dynamics and axon guidance. In general, the data are convincing, well performed with appropriate controls. The quantification of the data and statistics are properly done. However, there are some issues to be clarified, see comments below:

Comments:

1) Figure 3 shows that TRIM67 is required for the axon and growth cone response to netrin-1. However, in the absence of netrin-1, there is a significant increase in growth cone area, number of filopodia per growth cone, and filopodia length, which would argue that these effects could be netrin-1-independent. In fig S1D and E, the authors are testing the response of neurons to other factors, FGF2 and Slit2N. TRIM67 -/-neurons are responding to the factors, but is there a significant difference between untreated wt neurons and TRIM67 -/- neurons, as in fig3? This is less convincing and should be clarified.

We do in fact see an increase in growth cone size, number of filopodia and filopodial length in *Trim67-/-* neurons that are independent of netrin, but that is not further increased by netrin. The increase in filopodia number is likely due to the increase in growth cone size, as filopodia density is unchanged. Intriguingly we saw a similar increase in both growth cone size and filopodial length in *Trim9*$^{-/-}$ neurons (Menon, Boyer, et al., 2015), however pursuing the mechanisms behind these effects is beyond the scope of this study. The experiments with FGF and slit address the concern that basal increases in area and filopodia could mask response to guidance cues, as *Trim67*$^{-/-}$ neurons can still respond to these cues, although they don't respond to netrin. We determined the number of growth cones needed for the FGF2/Slit2N experiments based on previously published experiments in the literature (Szebenyi 2001), in which sample sizes were 50-52 growth cones per group. As the FGF2 and Slit2N effects on growth cones are both quite robust, the estimated number of samples required is considerably less than the sample size predicted to be necessary to confidently detect a statistically significant difference between untreated *Trim67*$^{+/+}$ and *Trim67*$^{-/-}$ growth cone areas. This is due to the small effect size of TRIM67 knockout on growth cone area coupled with the inherently large deviation of these measurements. The sample sizes were larger in the experiment testing netrin-1 effect on *Trim67-/-* growth cones, and as such we were able to detect this less apparent increase in growth cone area. This information has been added to the methods to clarify this potential confusing factor. Since the difference between *Trim67*$^{+/+}$ and *Trim67*$^{-/-}$ growth cones was already established in figure 3, we did not increase the biological replicates in Figure s1 to repeat these findings.

2) Fig3D: Trim67-/- + netrin-1: does it induce growth cone collapse? It seems that there is less branching and shorter neurite, the images do not match the quantification in 3E and 3F.

The branching assay employs a longer timeframe for netrin-1 stimulation (24hrs) as compared to the shorter growth cone assay (40mins). We did not measure growth cone parameters at 24hrs after treatment, as effects of bath-applied netrin-1 on growth cone morphology have been shown to be acute in the literature (Lebrand et al., Neuron, 2004). We clarified the timepoints of each experiment in the figure legend, and replaced the example images with neurons which more accurately reflect the population averages. The text now reads as follows:

"**C)** Individual data points and box and whisker plots showing the data spread in the interquartile range (box) and min and max (whiskers) of growth cone responses after acute netrin-1 treatment (40min), including increase in growth cone area, filopodial density, filopodia number, and filopodia length."

3) Figure 5: the authors show coIP with overexpression of TRIM67 and VASP. Do endogenous proteins interact in neurons? This should be shown.

We have added a coimmunoprecipitation blot showing interaction between endogenous TRIM67 and VASP from embryonic cortical neurons (**now Figure 5C**), with the following text now in the figure legend:

"**C)** Immunoprecipitation of endogenous TRIM67 from cultured embryonic cortical neurons, showing co-precipitation of VASP."

and in the results section:

"We confirmed the TRIM67:VASP interaction by immunoprecipitating endogenous TRIM67 from cultured embryonic cortical neurons, which co-precipitated endogenous VASP (**Fig.5C**)."

4) I have problems with Fig 5E and F: the most convincing data is indeed the increase of VASPub in TRIM67-/- neurons, which is reduced with netrin-1. However, the reduced VASPub in WT neurons treated with netrin-1 or in TRIM9-/- neurons is not convincing in the blot and does not reflect the graph in F. perhaps replacing the blot would help.

We thank the reviewer for pointing out this shortcoming in the example data, and we have taken multiple steps to address this concern, discussed at the end of this paragraph. We now include several examples of VASP-Ub assays with endogenous proteins in neurons, as well as GFP-VASP and Myc-VASP in HEK293 cells. As recognized in the field, and we now note in the text, endogenous ubiquitination of proteins is notoriously difficult to detect, particularly when the substrate is not modified by multiple ubiquitins (poly-ubiquitinated). Further, acquiring sufficient material from timed pregnant litters of multiple genotypes is limiting. The quantification we show in Figure 5 and 8 (previously 7) are from multiple experiments (multiple litters each) and show the overall consistency of our data, but perfectly representative blots are difficult to come by. For this reason, and in response to this concern and those of reviewer three, we now include additional example ubiquitination assay blots in figures 5 and 8 (previously figure 7), and supplemental figures 5 and 6, and show larger fields of view, pointing out comigrating bands of VASP and Ubiquitin (Red arrowheads), unmodified VASP (black arrowheads) and spurious background bands in the ubiquitin channel (*). We clarify our language regarding the results and observations in the results and drawn clearer comparisons between difficult to see endogenous ubiquitination and more easily detectable ubiquitnation in HEK293 cells. In addition, we provide more transparency in the text regarding the limitations of this assay. Further, we dial back our interpretation regarding molecular weight shifts and numbers of ubiquitin, due to well documented apparent molecular weight shift of VASP after a simple phosphorylation (~4 kDa)[1, 2]

[1]**Reinhard, M., Halbrügge, M., Scheer, U., Wiegand, C., Jockusch, B. M. and Walter, U.** (1992). The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. *EMBO J.* **11**, 2063–2070.
[2]**Butt, E., Abels, K., Kriegers, M., Palmn, D., Hoppen, V., Hoppen, J. and Walter, U.** (1994). CAMP-and cGMP-dependent Protein Kinase Phosphorylation Sites of the Focal Adhesion Vasodilator-stimulated Phosphoprotein (VASP) in Vitro and in Intact Human Platelets*. *J. Biol. Chem.* **269**, 14509–14517.

5) Fig. 5G: the expression of the myc-tagged proteins should be shown as well.

The myc channel of this blot has been added to the figure.

6) Fig. 5J: IF VASPub is increased in TRIM67 -/- (Fig.5E,F), why the fluorescence recovery halftime is not significantly slower?

Our extant set of data for untreated *Trim67*$^{-/-}$ VASP FRAP had a large standard deviation compared to the mean, which was masking the significance of the comparison with wild-type. After performing a post-hoc power analysis, we determined that we should perform further experiments. With the new data included, we found that there was a significant increase in FRAP halftime in the knockout cells. This is now in Figure 6.

7) Fig. 6E: it would be nice to show Ponceau S staining of the GST proteins.

We added panel 7E showing the Coomassie staining of our purified GST and GST-EVH1 proteins.

8) Fig. 7A,B: something is wrong with the blot in A. if the blot is labeled properly, why do we see bands of VASP and p-VASP in the ubiquitin IB? again, the blot does not reflect the quantification data and is not convincing. I don't see a reduction in VASPub in wt cells, treated with netrin-1, and there is a higher signal in KO cells.

We thank the reviewer for pointing out the error in our labeling. Blots in Fig7A were incorrectly labeled for both genotype and probe. In light of this concern, and those made above in your point #4, we have replaced the blot in Fig7A (now figure 8) with two newer experiments (Now Fig. 8A and 8A'), which more clearly shows the effect of netrin on VASP ubiquitination levels, and we have confirmed that all panels are appropriately labeled.

9) Finally, I don't think Fig7 is showing that TRIM67 is acting upstream of TRIM9. To prove this, it would be nice to see if expression of TRIM9 would induce more VASPub in TRIM67 -/- HEK293 cells.

In response to comments from reviewer 1 and 3 we have performed this suggested experiment and removed our all language suggesting that TRIM67 acts upstream of TRIM9, and alternatively suggest that TRIM67-mediated regulation of VASP involves antagonizing the function of TRIM9.

Results now read:

"If TRIM67 regulates VASP dynamics and filopodia by antagonizing TRIM9-mediated ubiquitination of VASP, we predicted that in the absence of both TRIM proteins, the phenotype of *Trim9*$^{-/-}$ neurons (Menon et al., 2015) would dominate. Indeed in *Trim9*$^{-/-}$*:Trim67*$^{-/-}$ neurons we observed a decrease in VASP-Ub (**Fig.8A,B**, p = 0.002), similar to *Trim9* $^{-/-}$ neurons. The FRAP $t_{1/2}$ of GFP-VASP expressed in *Trim9*$^{-/-}$*:Trim67*$^{-/-}$ embryonic cortical neurons was lower than in untreated wild-type neurons (p=.018), and displayed an increase following addition of PR-619 (**Fig. 8C**, p=.002), consistent with the hypothesis that VASP has faster dynamics at filopodia tips than VASP-Ub. As with previous FRAP assays we saw no differences in % recovery with any condition (**Fig.S5F**). Analysis of *Trim9*$^{-/-}$*:Trim67* $^{-/-}$ axonal growth cones (**Fig. 8D**) showed that similar to those of *Trim9*$^{-/-}$ neurons, basal filopodial number and filopodial density increased in the absence of both TRIM proteins and did not increase in response to netrin-1 treatment (**Fig. 8E**). These data suggest that TRIM67-mediated regulation of VASP and the growth cone involves antagonizing the function of TRIM9. Consistent with TRIM67 regulating VASP via TRIM9, overexpression of TRIM9 in absence of TRIM67 did not further increase VASP-Ub (**Fig.S5G,H**)."

Minor comments:

-Mislabeling in a few figures: S2 and 6.
Figure 1C is not mentioned in the text.
Fig5B: should be mentioned that this is done in TRIM67-/- cells.

We have made these changes to the text and figures. The text for figure 1 now includes:

"We performed similar sectioning and staining at P4 (**Fig.1C**), when the callosum completed midline crossing (Wahlsten, 1984). The callosum extended a shorter distance caudally in *Trim67*$^{Fl/Fl}$ littermates (**Fig.1D**, p = 0.040), and the posterior portion of the callosum was thinner at this time point (**Fig.1E**, p = 0.008)."

The figure 5 legend now reads:

"**B)** Coimmunoprecipitation assays from *TRIM67*$^{-/-}$ HEK293 cells transfected with the shown TRIM67 and VASP constructs…"
and the results takes states;

"To map the domains of TRIM67 necessary for VASP interaction, we generated a HEK293 cell line in which *TRIM67* was deleted via CRISPR/Cas9 genome editing (*TRIM67*$^{-/-}$ HEK293, **Fig.S4A-E**) and performed co-immunoprecipitation assays using domain-deletion constructs of TRIM67."

Reviewer #2 (Comments to the Authors (Required)):

These investigators have shown previously that response to the axon guidance cue Netrin in cultured mouse cortical neurons is gated by non-degradative ubiquitylation of the actin polymerase VASP by the E3 ubiquitin ligase TRIM9. In the current ms, they extend these studies to provide evidence that a TRIM9 paralog, TRIM67, blocks VASP ubiquitylation by TRIM9, thereby providing the background conditions against which the action of Netrin can occur. The data are thorough, convincing and rigorously analyzed. The result is interesting in that it dissects an unexpected biochemical relay underlying the action of a key regulator of morphogenesis. I have no significant issues with the paper.

1. There is really only one detail that left me puzzled, which is why a TRIM67 derivative lacking ligase activity failed to inhibit TRIM9 ubiquitylation of VASP. As far as I can see, there is nothing in the authors' binding-competition model that predicts (or accounts for) activity-dependence for TRIM67, particularly as the authors demonstrate that the LD mutation, or even wholesale deletion of the RING domain, do not block binding of TRIM67 to VASP. This observation does not by any means invalidate the proposed model, but it also does not fit in it very obviously. It would be worthwhile for the authors to comment on this.

We agree that this is a curious result, and needs to be addressed in the text. The following has been added to the Discussion section:

"The results of our structure-function VASP ubiquitination assay in HEK293 cells suggest that TRIM67 has other functions in addition to competitively inhibiting TRIM9-dependent VASP ubiquitination. The ligase-dead TRIM67 mutant was unable to reduce levels of VASP ubiquitination, suggesting that either the ligase function of TRIM67 is necessary to inhibit TRIM9 or another unknown pathway that regulates VASP-Ub. Our structure-function growth cone assay indicates that TRIM67 ligase function is also necessary for filopodial responses to netrin-1, as neither the ligase-dead nor RING domain deleted mutants were able to rescue the filopodial phenotype of *Trim67*$^{-/-}$ neurons. Determining the function and substrates of TRIM67 ligase activity will require further studies."

Reviewer #3 (Comments to the Authors (Required)):

This study demonstrates the role of TRIM67 in netrin-1-dependent development of the neuronal growth cone. Authors extend their own published works reporting functions of TRIM9 and TRIM67. TRIM67 is crucial for the netrin-dependent expansion of the growth cones, filopodial formation and elongation. They further study the molecular machinery of netrin-1- and TRIM67-dependent growth cone development. Unexpectedly, TRIM9-dependent ubiquitination of VASP is antagonized by TRIM67 in its ligase activity independent manner. I appreciate their live imaging and immunohisto/cytochemistry results, and EM data in Figures 1, 2, 3, and 4. Characterization of TRIM67f/f and TRIM67-/- neurons is thorough and deep in this regard. However, biochemistry and mouse genetics are not designed well enough and the results do not always support their conclusions. Overall, although this manuscript has a good potential to be an excellent work, it is too preliminary to be published in a highly appreciated journal such as JCB in the current form.

Major comments;
1) Details of quantification and statistics should be described in individual figure legends or as tables. Numbers of cells, animals, and/or culture experiments as well as statistics (e.g. t-test, one-way or two-way ANOVA, post-hoc tests) need to be documented for each individual figure. Some quantifications are shown as box-dot-plots others are as bar diagrams. They should always use the same way to present their data if they don't have particular reasons. In some bar diagrams, authors don't provide error bars in the control (e.g. the first bars in Fig 5F, 5H, 6D, and 7B). This should be corrected too (See Fig. 1B, Blanco-Suarez et al., Neuron, 100,1116, 2018 for example).
To improve the transparency of our data, we have added a supplemental table including the statistical tests and parameters of each experiment, as well as the means and deviation of data shown in each figure. The following sentences have been added to the results section at the end of the first paragraph, and in the statistics section of the methods, respectively:

"A table summarizing population size, mean, and deviance and statistical tests for all panels in this figure and subsequent figures is included (**Table 1**)."

"The data shown graphically in all figures is summarized in **Table 1**, with all numbers of samples, population mean and deviance, statistical tests used and p values listed."

We have also changed our bar charts in fig.2B, fig.5H/K, and fig.7A (now 8B) to box-and-dot plots, which should clarify confusion about our controls. However, the chart in figure 2E would be inappropriate as a box-and-dot plot as the presence of a somewhat small population of very high values makes the box region of the plot virtually impossible to see, while the presence of 0 values prohibits a logarithmic transformation. Additionally, the graphs in figure 7 have fewer than 5 samples per group and therefore would require interpolation to generate the 5 measures for a box plot. The explanations for these bar-charts have been added to their respective results sections for clarification. The experiments referred to have each been normalized to their respective control in each replicate to account for different staining intensities between blots. Therefore control values are 1 for each replicate and will have no deviation as these represent fold change from control. The axis labels have been updated to include (fold change) to indicate this.

2) Do they use littermate controls for experiments using mutant mice? For instance, TRIM67-/-, TRIM9-/- double KO should be compared with TRIM67+/+, TRIM9+/+ from the same litter in the most ideal case. However, I realize that it is unreasonable to request authors to repeat experiments with the littermate control, especially using TRIM67-/-, TRIM9-/- double KO. The chance to have these two genotypes (i.e. WT and double KO) in the same litter is extremely low. If they used the non-littermate control, authors should state this point in the legend or in Methods.

Anatomical experiments in Figure 1 were performed using littermate controls, however all other experiments used embryos from homozygous crosses. For endogenous ubiquitination assays, several pups of each genotype were necessary per condition, and as such, particularly with +/- netrin, individual litters from heterozygote crosses would never be large enough. In the case of transfected cultures, transfecting after the delay for genotyping would

considerably reduce cell viability, and transfecting before genotyping would be prohibitively expensive. We have clarified this in the Methods section as follows:

"*Trim67*$^{-/-}$ *and Trim67*$^{+/+}$ littermates from *Trim67*$^{+/-}$ crosses were used for neonatal corpus callosum measurements (**Fig.1**). Separate homozygous litters were used for culture-based assays, due to the number of embryos needed and the low number of homozygous knockouts per litter. "

3) In Fig 3C, TRIM67 KO neurons show increases in growth cone size and number of filopodia/growth cone in mock treated cells, while FigS1E showed no such phenotype. They need to explain the discrepancy. I am afraid that changes in growth cone/filipodia morphology in FGF-treated neurons might be due to relatively small values in mock-treated cells in Fig.S1E.

We determined the number of growth cones needed for the FGF2/Slit2N experiments based on previously published experiments in the literature (Szebenyi 2001), in which sample sizes were 50-52 growth cones per group. As the FGF2 and Slit2N effects on growth cones are both quite robust, the estimated number of samples required is considerably less than the sample size predicted to be necessary to confidently detect a statistically significant difference between untreated *Trim67*$^{+/+}$ and *Trim67*$^{-/-}$ growth cone areas. This is due to the small effect size of TRIM67 knockout on growth cone area coupled with the inherently large deviation of these measurements. This information has been added to the methods (see red) to clarify this potential confusing factor. Since the difference between *Trim67*$^{+/+}$ and *Trim67*$^{-/-}$ growth cones was already established in figure 3, we did not increase the biological replicates in Figure s1.

4) CC is required for TRIM67's binding to VASP (Fig 5B) and deltaCC cannot rescue the phenotypes (Fig S2 and S3), indicating that TRIM67-binding to VASP is crucial (Fig 7F). However, other CC containing mutant cannot rescue the phenotype either, e.g. LD, deltaRING, deltaSPRY in Fig S2C. This result is confusing. What puzzles me more is the rescue of filopodial density by expressing Nterm (Fig S2D). This region has nothing to do with VASP-binding of TRIM67. I suggest to remove rescue data, except for the ones using FL, LD and deltaCC mutants, from the manuscript. FL and LD as well as deltaCC should stay in the manuscript since they include very important messages (i.e. catalytic activity is needed for the role of TRIM67).

We have removed several of the mutants from the rescue assay (ΔFN3, ΔCOS and ΔSPRY), as the function of these domains in TRIM67 is currently speculative. However, the ΔRING and LD mutants suggest that ligase activity is necessary for the rescue of filopodia response, and correlate with later VASPub assay results (TRIM67-LD does not rescue inhibition of VASP ubiquitination). Additionally, the N-terminus construct contains the ligase and coiled-coil domains, further supporting the conclusion that these are critical regions regulating netrin-1 response. We have clarified the nature of and rationale for each mutant in the text, and have added a section to the Discussion regarding the possible role of TRIM67 ligase activity in this regulation.

"The results of our structure-function VASP ubiquitination assay in HEK293 cells suggest that TRIM67 has other functions in addition to competitively inhibiting TRIM9-dependent VASP ubiquitination. The ligase-dead TRIM67 mutant was unable to reduce levels of VASP ubiquitination, suggesting that either the ligase function of TRIM67 is necessary to inhibit TRIM9 or another unknown pathway that regulates VASP-Ub. Our structure-function growth cone assay indicates that TRIM67 ligase function is also necessary for filopodial responses to netrin-1, as neither the ligase-dead nor RING domain deleted mutants were able to rescue the filopodial phenotype of *Trim67*$^{-/-}$ neurons. Determining the function and substrates of TRIM67 ligase activity will require further studies."

5) Authors should rescue TRIM67 KO by expressing only CC. If it does not work, they should discuss the reason in DISCUSSION, for instance by showing the disturbed localization of CC in the cells. In some cases, truncated mutant of TRIM proteins seem to be unstable in cells (Fig. 6 in Reymond et al., 2001, EMBOJ).
While we do agree with the reviewer that this could be an interesting experiment, based on the results of our TRIM67 structure-function rescue assay we have found that multiple domains of the protein are necessary for rescue of the knockout phenotype, as described in the previous response above. We also share concerns raised by the editor that mislocalization of such a truncated protein could significantly confound interpretation of this experiment.

6) The straightforward explanation of the lack of rescue by LD mutant in Fig.S2 could be the lack of TRIM67-mediated ubiquination and degradation of TRIM9. TRIM9 regulation by TRIM67 could be done in two ways; competing TRINM9/VASP interaction and degradation of TRIM9. Authors' arguments that regulations of VASP and of TRIM9 by TRIM67 are proteasome/degradation-independent are weak in Fig. S4I, S4J, S4K, and S4L. They used homogenates for the quantification. They showed that the regulations are local at the growth cone in immunocytoechemistry. If the majorities of VASP and TRIM9 are in the cell body and if only small fractions of VASP and TRIM9 is regulation at the growth cone, their local changes in the TRIM67 KO at the growth cone could be masked by the unchanged levels in the

cell body. To circumvent this, authors should show the level of VASP and TRIM9 in TRIM67 KO by immunocytochemistry at the growth cone. They have good antibodies for this purpose.

To address this comment, we used an antibody that recognized endogenous VASP, to compare VASP protein levels in the growth cones and growth cone filopodia of *Trim67*$^{+/+}$ *and Trim67*$^{-/-}$ neurons, and found no change in VASP levels. These results are included in Fig. S4, and described in the results as such

"Changes in levels of VASP protein were not detected in *Trim67*$^{-/-}$ *or Trim9*$^{-/-}$ brain lysates (**Fig.S4I,J**), consistent with no degradation of VASP. However, the small proportion of total VASP that was ubiquitinated might impair detection of loss of protein. To examine if loss of *Trim67* and the associated increase in VASP-Ub caused local changes to VASP protein levels in the growth cone, we analyzed endogenous VASP localization by immunocytochemistry. This demonstrated no change in VASP protein levels of *Trim67*$^{+/+}$ *and Trim67*$^{-/-}$ growth cones or filopodia (**Fig.S4K,L**)."

Unfortunately, our TRIM9 antibody demonstrates high levels of background fluorescence, as documented In Winkle et al., 2014 JCB, so we cannot confirm TRIM9 levels in the growth cone. As such, we have offered both possibilities in the discussion.

"Although the mechanism by which TRIM67 antagonizes VASP ubiquitination is not known, several possibilities are consistent with our data. First, we find that TRIM67 outcompetes TRIM9 for interaction with VASP. Second, the requirement of TRIM67 ligase activity for inhibition of VASP ubiquitination suggests additional levels of regulation. This could occur potentially via TRIM67-mediated ubiquitination of TRIM9, leading to either TRIM9 degradation or altered ligase activity. Although we observed no change in total TRIM9 protein following deletion of *Trim67,* whether degradation of the local pool of TRIM9 in the growth cone occurs is not known, due to insufficient quality of TRIM9 antibodies for immunocytochemistry (Winkle et al., 2014). Further, whether an unidentified substrate of TRIM67 regulates TRIM9-mediated ubiquitination of VASP is unknown."

7) If they think that this ubiquitination is proteasome independent, western blot using anti-K6, ati-K11, and anti-K63 antibodies is interesting. At least some of them should be tried with proper positive controls (i.e. purified polyubiquitin chains of each type). Antibodies and positive controls are available commercially (e.g. from BostonBiochem). If they are negative, authors should also discuss the possibility of multiubiquitination.

We thank the reviewer for this suggestion, which offered a potentially rich area of research for the lab. Prior to investing in these numerous expensive reagents, we wanted to convince ourselves that VASP was polyubiquitinated, since we had not observed high molecular weight smears of VASP consistent with poly-ubiquitination and our results were potentially more consistent with mono or multi-monoubiquitination. To do so, we acquired ubiquitin expression constructs harboring specific lysine mutations, and performed Myc-VASP ubiquitination assays in HEK293 cells. We include a representative ubiquitination assay of Myc-VASP with either wildtype HA-ubiquitin or an HA-ubiquitin mutant that precludes ubiquitin chain formation (HA-ubiquitin^{K0}). A single predominant species of VASP-Ub was apparent in both conditions (**Fig.6I**), further supporting that VASP is not polyubiquitinated, but rather is mono- or multi-monoubiqutinated. However, in light of VASP's peculiar apparently molecular weight shift after a simple phosphorylation (~4 kDa)[1, 2], we were hesitant to suppose the number of ubiquitin moieties ligated to VASP, as mentioned in new text included in the results and discussion (see red text). Interestingly, mono or multi-monoubiquitination suggests only a few (or even one) ubiquitin molecules would be ligated to each VASP protein. This low number of ubiquitin proteins may partially explain why detection of endogenous VASP-Ub from neurons was difficult, but we do not include this possible explanation in the revised manuscript.

[1]**Reinhard, M., Halbrügge, M., Scheer, U., Wiegand, C., Jockusch, B. M. and Walter, U.** (1992). The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. *EMBO J.* **11**, 2063–2070.
[2]**Butt, E., Abels, K., Kriegers, M., Palmn, D., Hoppen, V., Hoppen, J. and Walter, U.** (1994). CAMP-and cGMP-dependent Protein Kinase Phosphorylation Sites of the Focal Adhesion Vasodilator-stimulated Phosphoprotein (VASP) in Vitro and in Intact Human Platelets*. *J. Biol. Chem.* **269**, 14509–14517.

8) I realize that quantification of ubiquitination level is often challenging. To support the estimation of ubiquitin western blots in Fig. 5E, 5G, and 7A, authors should show entire gel images with and without regions of interest (ROIs). The result of quantification of ubiquitin western blot depends on how one chooses ROIs.
As mentioned to reviewer 1 above, and we have taken multiple steps to address this concern, discussed at the end of this paragraph. As recognized by reviewer 3 and the field, endogenous ubiquitination of proteins is notoriously difficult to detect and quantify, particularly when the substrate is not modified by multiple ubiquitins (poly-ubiquitinated). Further,

acquiring sufficient material from timed pregnant litters of multiple genotypes is limiting. The quantification we show in Figure 5 and 8 (previously 7) are from multiple experiments (multiple litters each) and show the overall consistency of our data, but perfectly representative blots are difficult to come by. For this reason, and in response to this concern and those of reviewer three, we now include additional example ubiquitination assay blots in figures 5 and 8 (previously figure 7), and supplemental figures 4 and 5, and show larger fields of view, pointing out comigrating bands of VASP and Ubiquitin (Red arrowheads), unmodified VASP (black arrowheads) and spurious background bands in the ubiquitin channel (*). Further we more clearly describe the analysis of VASP-ub levels in the methods. We clarify our language regarding the results and observations in the results and draw clearer comparisons between difficult to see endogenous ubiquitination and more easily detectable ubiquitination in HEK293 cells. In addition, we provide more transparency in the text regarding the limitations of this assay.

9) Ubiquitination experiment in Fig. 5G is very nice since they use TRIM67 KO HEK293 cells. However, GFP is not the best tag for the substrate. This tag has several lysine residues and could be conjugated by K48-linked polyubiquitin chains. Indeed, estimation of GFP variant turnover (decay time) is often used to monitor the proteasome activity in the live cell imaging. Authors should repeat this experiment using a small tag, such as myc, flag or HA. Also the difference of the molecular weights of ubiquitinated and non-ubiquitinated GFP-VASP is smaller than 24 kDa for me. Using a small tag will solve this problem too.

Thank you for pointing out this very important point. To address this concern, we performed several additional ubiquitination assays with Myc-VASP, and a variety of tagRFP-TRIM67 constructs, these are now included in Fig.S5B,C, and the following text has been added to the results section:

“GFP contains twenty lysine residues and ubiquitin-dependent degradation of GFP is documented (Dantuma et al., 2000). Therefore, we repeated ubiquitination assays with myc-VASP and tagRFP-TRIM67 constructs (**Fig.S5B,C**). Ubiquitination of myc-VASP was similarly reduced by expression of TRIM67 and increased by expression of TRIM67ΔCC, indicating ubiquitination of GFP-VASP is a reliable readout. ”
And to figure legend:

“**F)** Ubiquitination-precipitation assays of Myc-VASP expressed in HEK293T cells lacking *TRIM67* expressing indicated tagRFP-TRIM67 constructs, along with FLAG-ubiquitin. A FLAG-Ub migrates at a heavier molecular weight (red arrowhead) than unmodified VASP (black arrowhead). **G)** Individual data points and box and whisker plots showing the data spread in the interquartile range (box) and min and max (whiskers) of of VASP-Ub, quantified from FLAG signal relative to total Myc-VASP, normalized to the RFP control condition.”

Further, we have decided to remove numerical interpretation of the molecular weight shift, due to the curious behavior of VASP molecular weight changes caused by phosphorylation, as mentioned above.

10) I found images used for the first, second, seventh, and eighth lanes in Fig.5E and first four lanes in Fig.7A seem to be identical. If they are indeed same and if data used for the wild type in quantifications in Fig.5F and Fig.7B are same, it should be stated in the legends. If they are same, did authors perform experiments for Fig.5F and 7B at the same time as a single set of experiments? It is not allowed to compare the values from a control and from a KO taken in two independent experiments.
These blots have been replaced with more illustrative examples. In what is now figure 6 and 8. Now blots shown in 8A’ are extended regions of blots shown in Figure 6C, this has been clarified in the figure legend of both figures.

11) In discussion, authors argument that 'netrin responses in a Trim67:Trim9 double knockout neuron resemble that of a Trim9-/- neuron. This suggests that TRIM9 acts downstream of TRIM67.' This argument is weak. If they want to show that TRIM67 functions as an upstream inhibitor of TRIM9, TRIM67 KO phenotype should be restored by additional knocking out or knocking down of TRIM9. They should change this part of DISCUSSION.
We thank the reviewer for this suggestion. We have edited the discussion extensively, removing all language suggesting that TRIM67 acts upstream of TRIM9, and alternatively suggest that TRIM67-mediated regulation of VASP involves antagonizing the function of TRIM9, as detailed above also to reviewer 1, and in the discussion in red labeled text.

12) Details of FRAP estimation are not documented in Methods section. It seems to be incorrect, judging based on the top image in Fig 5I. If a part of fluorescence remains after photobleaching (t=0' in Fig.5I), they need to subtract the residual fluorescence intensity from intensities at all imaging time-points. Do they take the background signals for the estimation in account? This should be also subtracted. They should re-estimate using the existing dataset based on a

detailed method paper (McNally, Methods in Cell Biology, 85, 329, for instance). If they want to estimate the turn over of the protein (i.e. protein degradation), half-time recovery should be estimated. If they aim to study the local exchange rate of VASP, which is dependent on protein-protein interactions, they should try to fit the FRAP curve to single- and multi-exponential curves. If fitting works the best with double exponential curve for instance, they could discuss that the exchange of VASP is possibly dependent on two protein-protein interactions.

The appearance of incomplete bleaching was due to our selection of a poor example filopodium and only circling the tip in the before-bleach frame, along with the illustration of a FRAP curve not reaching 0 fluorescence (to indicate unbleachable background/noise). We apologize for the confusion and have included an appropriate curve in which background signal has been subtracted, as well as circled the tip region in each frame of the bleached filopodia tip. We did subtract background signal (dark noise of the camera, etc), and have detailed this is in the methods. We are not using this technique to estimate protein degradation, since we are only bleaching GFP-VASP at a single filopodia tip, but rather the local exchange rate. As such, we report the t1/2 or half-time of recovery. Indeed, our FRAP curves fit well to a single exponential curve, and we have included more description of our analysis in the Methods to clarify (see sections in red):

"The data was fit to a single exponential function as opposed to a higher-order exponential (eg. $F = A*(1 - e^{-\tau t} - e^{-\tau' t})$) as the R^2 value for a double exponential ($R^2 = 0.845$) was the same as for a single exponential ($R^2 = 0.845$) fit to the average of all control data."

Minor comments;

1) There are some inconsistency and mistakes in the manuscript. In page 14, 'mcherry' is used while 'mCherry' is used in page 19. In the second paragraph of page 6, 'Fig. 6D, E' should be 'Fig. 6C, D'. 'Fig. 6F' should be 'Fig. 6E'.
We have corrected these errors in the text.

2) Some abbreviations are used without explanation. For instance, in the third paragraph in page 6, '2XKO' appears without explanation. This abbreviation is not used afterwards. In the manuscript in JCB, abbreviations need to be explained in their first appearance and need to be used several times. '2XKO' appears only once.
This abbreviation has been replaced with the appropriate "*Trim67*$^{-/-}$*:Trim9*$^{-/-}$".

3) Fig S4K, why does each genotype have two lanes? Are they samples from different mice with same genotypes? Did authors load samples in a single gel? This should be stated in the legend.
These were indeed lysates from multiple animals run simultaneously. We have clarified this in the legends, no S5B:

"Representative western blot of VASP in embryonic cortical lysate from two animals of each indicated genotype."

4) They need to describe more precise molecular weight in their western blot results. Tick marks on the right side of the molecular weight number are necessary.
We have added tick marks to indicate the position of molecular weight ladder bands alongside the corresponding numbers in all figures.

5) They should explain what PR-619 is in the main text. Readers do not always have a strong background of ubiquitination biochemistry.
We have included the description of PR-619 along with a reference for its function in the text:

"... PR-619, a broad inhibitor of deubiquitinating enzymes (Sieberlich et al., 2012) which we have previously shown increases ubiquitination of VASP (Menon et al., 2015)."

6) In page 5, line 10 from the bottom, "To assay effects of increased VASP ubiquitination on FRAP t1/2 we treated neurons with PR-619; in Trim67+/+ neurons PR-619 increased GFP-VASP FRAP t1/2, consistent with an increase in ubiquitination (Fig.5J)." They never showed an increase in ubiquitination of VASP upon PR-619 treatment in WT cells. The text needs to be changed.
We have edited this text to instead refer to our previous publication showing the effect of PR-619 on both VASP ubiquitination and FRAP t1/2. It now reads as follows:

"To assay effects of increased VASP-Ub on VASP dynamics, we treated neurons with PR-619, a broad inhibitor of deubiquitinating enzymes (Seiberlich et al., 2012), which increases VASP-Ub (Menon et al., 2015). In *Trim67*$^{+/+}$ neurons

PR-619 increased GFP-VASP FRAP $t_{1/2}$ (**Fig.6F**, p=.024), consistent with our previously published FRAP data (Menon et al., 2015)."

7) In general, methods are not well documented. It would be difficult to repeat experiments only with this description. Authors should document the detailed methods as other manuscripts published in JCB recently.
We have included additional details in our Methods section which should make replication easier.

8) In the method section, animal background is explained as C57BL/6J. However, in the recently published paper from authors' group (Boyer et al., eNeuro, 5, e0186, 2018), authors explain that TRIM67 KO animals have C57BL/7 background. If so, they should have crossed TRIM67 KO with C57BL/6J wild type several times. How many times they backcrossed needs to be explained. The phenotype of midline crossing is highly depends on the genetic background of mice.
We thank the reviewer for noting this discrepancy. There is in fact a typo in the eNeuro paper, in which we used the same strain of knockout mice on a C57Bl/6J background. We have submitted a corrigendum to eNeuro to correct this. You can find this published here: https://www.eneuro.org/content/6/4/ENEURO.0281-19.2019

9) Authors may want to acknowledge Dr. Simon Rothenfusser for his providing them a material.
We thank the reviewer for noting this oversight, and have added an acknowledgment to Dr. Rothenfuβer in the text:

"HEK293 cells (female) were obtained from Dr. Simon Rothenfusser (Klinikum der Universität München), as were HEK293 cells lacking *TRIM9*, which were described previously (Menon et al., 2015)."

10) In the section of Immunoblotting and precipication assay in page 19, they need to explain why they included MG132. As far as VASP ubiquitination is nothing to do with proteasomal degradation, MG132 increases the background. It does not make sense to include MG132. The rationale should be explained.
MG132 pretreatment was included to maintain consistency with previously published VASP ubiquitination assays. This has been clarified in the text:

"MG132 pretreatment was used to maintain consistency with previously published ubiquitination assays (Menon et al., 2015)."

11) In the section of Immunoblotting and precipication assay in page 19, 270 ul should be 270 µl.
This typo has been fixed.

12) In Figure 3B legend, "B) Growth cones from primary neuronal cultures stained for filamentous actin (phalloidin), β-III-tubulin, and TRIM67". However, they never showed staining of TRIM67 in the figure.
TRIM67 has been removed from this figure legend.

13) Figure 7A has four panels; the first and third ones shows a similar pattern and the second and fourth have a similar pattern. I assume that the first and third panels are from the anti-VASP and the second and fourth ones are from the anti-ubiquitin antibody. If I am right, authors should correct the labeling of the figure.

This is correct. The figure has been edited to fix this mislabeling.

October 21, 2019

RE: JCB Manuscript #201902088R

Prof. Stephanie L Gupton
University of North Carolina at Chapel Hill
111 Mason Farm Road 4332 MBRB, CB 7090
Chapel Hill, North Carolina 27599

Dear Prof. Gupton,

Thank you for submitting your revised manuscript entitled "A pair of E3 ubiquitin ligases compete to regulate filopodial dynamics and axon guidance". Thank you for your patience during the re-review process of the paper. You will see that Reviewer #1 now recommends publication. Reviewer #3 is also supportive of the work but is concerned about the nature of the controls used in the experiments using cells from the double and single KO animals.

We have discussed these points and greatly appreciate that you could clarify the controls used when we reached out via email. We agree with your arguments. For example and beyond the question of the amount of biological materials, it is not that simple even with single mutation models to arrange transfection in culture experiments with genotyping. We consider that the design of the experiments is acceptable, the conclusions are solid, and thus do not feel that repeating some experiments/adding rescue experiments would be needed for publication. Nevertheless, we agree that it is important for the readers to precisely know about the conditions of the experiments, so please provide all details for all experiments via text edits. Please also attend to the final changes suggested by Rev#3 in their minor points. No further experimentation is needed.

We would be happy to publish your paper in JCB pending these revisions and final revisions necessary to meet our formatting guidelines (see details below).

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

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
- Please include an eTOC statement on the title page of the revised ms; it should start with "First author name(s) et al..." to match our preferred style.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 1G, 2C, 7A, S1A (magnification)

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly

described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: 1BDE, 2BE, 3CD, 4BDE, 5E, 6DFH, 7BDG, 8CE, S1CEG, S2CDE, S3B, S4JL, S5ACEFH

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- Please include the basic genetic features for all mouse lines, vectors, and cell lines, even if described in other published works/gifts from other researchers. Alternatively, database/vendor IDs (e.g., Jackson lab strain IDs, ATCC, Addgene, etc.) can be provided.
- Please include sequences for all CRISPR guides and oligos used, even negative controls.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

6) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include ~1 brief descriptive sentence per item.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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Sincerely,

Valerie Castellani, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

The authors have satisfactorily addressed all the reviewers' comments.

Reviewer #3 (Comments to the Authors (Required)):

I am very happy to know that authors took my comments seriously and improved the manuscript. I still have one major and a few minor concerns.

Major point;
I am now concerned about their way to perform the primary culture experiments. In response to my comment 2) in their rebuttal letter, authors documented "Separate homozygous litters were used for culture-based assays, due to the number of embryos needed and the low number of homozygous knockouts per litter." I disagree with them if mice have only one mutation; by crossing heterozygotes, the chance to have wild type and KO is 1/4 for each. Four to six pairs of mating should be enough to perform experiments. Do they compare neurons from non-littermate wild type and Trim9-/- (or Trim67-/-)? As I mentioned in the major point 2) in my previous comment, it is acceptable, - but not ideal -, to use non-littermate wild type mice as controls for double mutant mice (e.g. Trim9-/-;Trim67-/- double KO). It is just unrealistic to use littermate controls in such experiments. If mice have only one mutation, such as Trim9-/- or Trim67-/-, they need to be

compared with the littermate wild type mice. In this way, we can exclude the possibility of Trim9- or Trim67-unrelated effects (e.g. epigenetic effects). This is particularly important when authors have not rescued the phenotypes of KO by expressing recombinant Trim9/67 proteins. Rescue experiments have not been performed except for experiments in Figure S2 and S3. If authors have done other experiments (e.g. FRAP, biochemistry, EM studies of growth cones, filopodia lifetime, and live imaging of axon growth) by comparing non-littermate mice, they should repeat experiments using littermate control and single KO or rescue the phenotype by expressing recombinant proteins.

Minor points;
1) Regarding the data presentation in Figure 2E and Figure 7, authors should preset as scattered dot-plot or as dot-plot with bar diagrams if the sample number is small. Readers should be informed how variable the data are.

2) Some bands authors highlighted with red arrowheads can not be ubiquitinated VASP in Figure 6B and 8A. There are two bands of VASP-Ub in anti-VASP and anti-ubiquitin blots. In anti-VASP western blot, the top band is stronger than the bottom band, while they are almost equal (Figure 6B) or the bottom band is stronger (Figure 8A) in anti-ubiquitin western blot. Authors should point out actual VASP-Ub bands in blots. If they can not be sure, authors should highlight only the one that they can be sure.

3) I strongly recommend to delete Figure 6C and 6D, and move panels in Figure 8A and 8B (or 8A' and 8B) here from where they are now. Showing identical images twice would confuse readers. Indeed, I was very confused. The left bottom panel in Figure 8A should be aligned with the left top panel.

Together my comment 2), I would use only panels in Figure 8A and 8B as Figure 6C and 6D. Anti-ubiquitin blots in Figure 8A' and 6C (identical blots) are not convincing. The signal from bands is too weak. They do not represent the quantification shown in Figure 8B and 6D.