Endogenously expressed Ranbp2 is not at the axon initial segment

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Original submission

First decision letter

MS ID#: JOCES/2020/256180

MS TITLE: Endogenously expressed Ranbp2 (Nup358) is not at the axon initial segment

AUTHORS: Yuki Ogawa and Matthew N Rasband
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, two reviewers praise the efforts of this paper in clarifying the contrasting evidence on the localisation of Ranbp2 at the AIS. This is indeed a worthy aim, which would help the field to move forward on solid grounds. However, as mentioned by the second reviewers, there are still gaps, which need to be fixed (and explanations to be given) to make this manuscript ready for publication in JCS. I will be delighted to see a revised version of this manuscript addressing these concerns.

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

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

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

Reviewer 1

Advance summary and potential significance to field

The manuscript shows that Ranbp2 is not at the axon initial segment. Previous studies by the same group (Hamdan et al., 2020, Nat. Commun.) and other group (Khalaf et al., 2019, J. Cell. Sci.) described the expression of Ranbp2 (Nup358) at the axon initial segment. A thorough experimental analysis in this manuscript concludes that Ranbp2 antibody used in these manuscripts do not recognizes Ranbp2, but Neurofascin, known to be a component of the axon initial segment. While complete exogenously expressed Ranbp2 construct is not localized at the axon initial segment, Khalaf et al and this manuscript describe that a Ranbp2 N-terminal fragment is localized at the axon initial segment. The present manuscript demonstrate that this construct is detected at the axon initial segment due to its interaction with stable microtubules, a characteristic of axon initial segment microtubules. The manuscript clarifies why Ranbp2 was thought to be an AIS component and discard its expression at the AIS.

Comments for the author

Authors clearly demonstrate that axon initial segment staining of Ranbp2 antibody (A301-796A), used in both previous studies, is actually recognizing neurofascin expression at the AIS. Moreover, authors demonstrate that the expression at AIS of a Ranbp2 N-terminal construct is due to its affinity for stable microtubules. Authors analyze endogenous Ranbp2 expression using inserted tags and do not find Ranbp2 expression at the AIS, as previously shown by Hamdan et al. and Khalaf et al.
Authors also discuss the need of further validation of proteomic studies and conclude that Ranbp2 identification at the AIS in their previous proteomic studies. Their conclusions are solidly supported by their experiments.

Reviewer 2

Advance summary and potential significance to field

This study does not contribute to the advancement of current knowledge.

Comments for the author

In the manuscript “Endogenously expressed RanBP2……..” by Ogawa and Rasband, the authors claims that the endogenous RanBP2 is not localized to axon initial segment (AIS). They have used different antibodies against RanBP2 and endogenous epitope tagging experiments to support their claim.
However, the results do not rule out the possibility of RanBP2 being a component of the AIS, for the reasons discussed below. Therefore, it does not add much to advancement of the current knowledge.
1. The authors claim that the RanBP2 antibody (A301-796A), used in this and earlier studies, stains AIS due to cross reaction with NF186, through ‘KPLQG’ sequence (which is present in both RanBP2 and NF186). In other words, A301-796A, still recognizes RanBP2, and also detects NF. So delineating the signal arising due to RanBP2 and NF at AIS is rather difficult. The result that depletion of NF leads to decrease in AIS staining at AIS (Fig. 1F), could also be explained if NF186 is required for the recruitment of RanBP2 to AIS. Moreover, the kinetics of localization of AnkhyrinG, NF186 and RanBP2 accumulation is different; AnkyrinG and NF186 marks AIS at 3-5 DIV, much before the accumulation of RanBP2 (as analyzed by A301-796A antibody) at AIS (7 DIV) (Khalaf et al., 2019). If the staining of A301-796A would have been exclusively due to cross reaction with NF, and RanBP2

not being there, one would have seen a similar AIS localization kinetics of RanBP2 to that of NF (5 DIV) (Khalaf et al., 2019).
2. The authors' lab has recently identified RanBP2 in the AIS using shown by mass spectrometric analysis of AIS proteins through proximity biotinylation
(Hamdan et al., Nat. Commun. 2020). This was an independent way of identification of RanBP2 at AIS (without involving the cross-reacting antibodies).
3. Localization of ectopically expressed N-terminal region of RanBP2, but not full length, to AIS was already shown in Khalaf et al., 2019. The current paper supports the same conclusion. However, the authors claim that the localization is due to an indirect effect of this fragment associating with stabilized microtubules at AIS. Contrary to this, rather it has already been shown by two groups that expression of RanBP2 N-terminal fragment (1-900 aa) leads to remodelling and stabilization of the microtubules (Joseph and Dasso, FEBS Lett, 2006, Wu et al., J Cell Sci., 2018). Moreover, the mechanism of RanBP2-mediated stability is through acetylation of alpha-
tubulin by the GCN5L1-TAT1 axis (Wu et al., J Cell Sci., 2018). In other words, RanBP2-N localizes to and stabilizes microtubules - even without taxol (Joseph and Dasso, FEBS Lett, 2006, Wu et al., J Cell Sci., 2018).
4. It is possible that a splice variant of RanBP2 predominantly localizes to AIS and may be missed out by analysing the recruitment of the endogenous protein that was HA-tagged at different locations (Fig. 5). This could also be the reason for all the other antibodies raised against two other regions of RanBP2 used in this study (sc-74518 and ABN1385) not staining the AIS
(Fig. 1).

Reviewer 3

Advance summary and potential significance to field

The paper from Ogawa and Rasband provides a welcome clarification for previously published inconsistent results regarding the supposed AIS localization of Nup358/Ranbp2 (Khalaf et al., 2019; Hamdan et al., 2020),
a member of the nucleoporin family.
The authors convincingly demonstrate using different approaches that endogenous Ranbp2 is actually not expressed at the AIS but only in the nucleus and that its previously reported AIS localization was due to the fact that the only anti-Ranbp2 antibody that was used, actually cross-reacts with NF186, an AIS protein which shares a KPLQG sequence with Ranbp2. They nonetheless observe (as Khalaf et al., 2019) that an exogenously (over-)expressed N-terminal fragment of Ranbp2 localizes at the AIS, but they provide a mechanistic explanation for this surprising result, based on Ranbp2's intrinsic affinity for stabilized microtubules.

Comments for the author

This paper is an interesting study well suited for this journal.
Only minor comments:
- In the Discussion: it would be important to clarify why such an artifactual AIS candidate protein was identified in two proteomics approaches (Hamdan et, 2020; Torii et al., 2020), which may help reconsider other/future potential artifacts.
- Fig.1D: individual labelings should be shown (in addition to the merge panel) to better evaluate the extent of Ranbp2 labeling compared to that of NFasc: Ranbp2 labeling seems to extend beyond the paranode. Why? Paranodal labeling (where NF186 is not expressed) could also be discussed.
- In Materials and Methods, it would help the reader to have the epitope region recognized by each of the Ranbp2 antibodies used.

First revision

Author response to reviewers' comments

Reviewer 1. This reviewer had no suggestions for corrections and agrees with all of our conclusions. They state: “The manuscript shows that Ranbp2 is not at the axon initial segment” and “Their conclusions are solidly supported by the experiments.”

Reviewer 2.
1. *This study does not contribute to the advancement of current knowledge.* We respectfully and strongly disagree with this statement. I quote from a recent editorial published by Dr. Bernd Pulverer, Chief Editor at EMBO Journal: “Over one million peer-reviewed papers are published each year in the biosciences…. Unless we make it a virtue to correct literature diligently, we risk getting lost in this information flood.” (EMBO J 34:2483-2485, 2015). This paper is an effort to correct the scientific literature. Scientific progress depends on scientists being willing to re- evaluate and correct current accepted knowledge as new information and experiments are performed. That is exactly what we have done here. We performed new experiments that clarify why Ranbp2 is not at the axon initial segment (AIS). We do not claim that either we (Hamdan et al., 2020) or Khalaf et al. (2019) were negligent or fraudulent in previous publications. We simply performed new experiments that further our understanding of the molecular composition of the AIS. There are several examples in the literature where proteins have been reported at the AIS but the results were based on the use of spurious antibodies. For example, phosphorylated I□b□ was previously reported at the AIS and nodes of Ranvier (Sanchez-Ponce et al., Mol Cell Neurosci 2008; Politi et al., Neuropathol Appl Neurobiol, 2007). This was a very exciting observation and suggesting very interesting signaling at the AIS. Many investigators began to build models for AIS function based on the belief that phosphorylated I□b□ is at the AIS and is required for AIS assembly. It began to enter the literature and ‘muddy’ our understanding of AIS assembly (see for example Grubb and Burrone, Curr Opin Neurobiol, 2010). Investigators began designing experiments and writing grants based on this idea. However, the conclusion was based entirely on the use of antibodies. Our laboratory performed careful control experiments and found that the antibodies used do not actually label phosphorylated I□b□, but some other phosphorylated antigen that still remains unidentified (Buffington et al., Mol Cell Neurosci 2013). Similarly, MDM2 has been reported at the AIS (Zhao et al., Sheng Li Xue Bao 2014). However, we found this antibody cross-reacts with Trim46, a well-recognized AIS protein (Van Beuningen et al., Neuron 2015). Finally, 5HT1A serotonin receptors have also been reported at the AIS (Cruz et al., Am J Psychiatry, 2004), but again, this is based on antibody staining. We obtained 5HT1A knockouts and found that the immunosignal is still located at the AIS. Thus, Ranbp2, MDM2, phospho-I□b□, and 5HT1A were all false- positives for AIS proteins based on cross-reactivity among antibodies. My laboratory has spent considerable time, effort, and money chasing proteins that are not really at the AIS. Thus, we are highly sensitive to using extensive controls. I strongly believe that this study contributes to the advancement of current knowledge because it removes errors in our understanding of AIS composition. Correction is just as important as expansion of knowledge.
2. *A301-796A recognizes both Ranbp2 and NF186, making it difficult to distinguish between the two.* We agree, and for this reason we performed diverse experiments to support the conclusion that the immunostaining at the AIS simply reflects cross-reactivity with NF186.
a. We mapped the antibody binding site and show it is present in both NF186 and Ranbp2, and the 5 amino-acid sequence is both necessary and sufficient for antibody binding.
b. We show other Ranbp2 antibodies do not label the AIS.
c. We show that A301-796 antibodies label every structure (including paranodes of myelinating glia) where neurofascin gene products are found.
d. We used CRISPR-dependent tagging of the endogenous Ranbp2 to show it is not found at the AIS – this method does NOT depend on the use of A301-796 or knockdown of NF186. We consider this experiment to be very strong evidence that Ranbp2 is not at the AIS.
e. We show, using loss of function experiments, that loss of NF186 by knockdown blocks AIS immunostaining using A301-796 antibodies, but the nuclear signal remains unaffected.
3. *The depletion of NF186 leading to a decrease in AIS staining could be explained by the idea that NF186 is required for Ranbp2 localization.* We agree with the reviewer that this is a formal possibility. However, we consider it to be an exceedingly unlikely possibility since this would mean that NF186 and Ranbp2 are both at the AIS, AND recognized by the same antibody, AND

Ranbp2's localization depends on NF186. This would be an

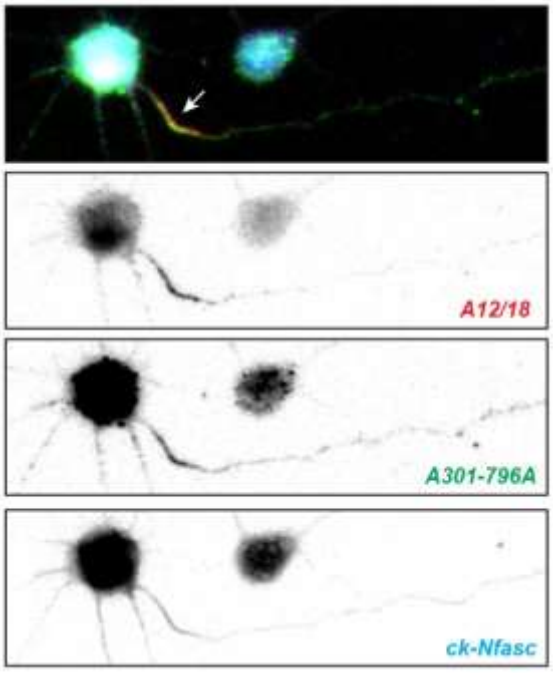

Figure A. DIV3 cultured hippocampal neuron labeled using the indicated antibodies. Note: the cell body signal is high due to the young age of the neurons and the long exposure times.

amazing coincidence. However, ALL of the results presented in our paper, in Hamdan et al. and in Khalaf et al. can be explained by the simple conclusion that the A301-796A antibody cross- reacts with NF186. The simplest conclusion is the best conclusion.

4. *The kinetics of AnkyrinG, NF186, and Ranbp2 is different*. Indeed, Khalaf et al. (2019) claim different kinetics of localization. However, this is based on the use of different antibodies. Unfortunately, one cannot make definitive conclusions about the kinetics of localization based on the use of antibodies because 1) each antibody has different affinities, and 2) different antibody concentrations will yield different fluorescence intensities. Thus, the signal at the AIS depends on antibody affinity and concentration. To illustrate the challenge of making definitive conclusions based on antibody staining, we performed immunostaining at DIV3 using antibodies against NF186 and Ranbp2. We used 2 different well-characterized and validated anti-NF186 antibodies (A12/18 from neuromab and chicken anti-Neurofascin from R&D systems) and the A301-796 antibody (Figure A). At DIV3 we found that A12/18 and A301-796A colocalized, while the chicken anti-Neurofascin antibodies labeled the AIS more weakly than the other two. This illustrates how one cannot make absolute conclusions about the kinetics of protein localization based solely on the use of antibodies.

5. *The authors' own lab identified Ranbp2 as an AIS protein using mass spectrometry and proximity biotinylation. This is an independent way of confirming that Ranbp2 is at the AIS (without involving antibodies)*. The author is correct that we did identify Ranbp2 in Hamdan et al. (2020). However, in our proximity biotinylation study we identified many proteins – some of which are not at the AIS but were purified based on endogenous biotinylation. We were very hopeful and encouraged that Ranbp2 would be at the AIS. The whole point of the present paper under consideration is to illustrate how important it is to follow up these mass-spectrometry experiments with the use of very rigorous control experiments. In unpublished experiments (paper in progress) we performed similar proximity biotinylation, but this time we performed these studies *in vivo* and in triplicate. Although we found excellent agreement between the results of Hamdan et al. (2020) and our *in vivo* experiments, one notable protein missing from the *in vivo* studies was Ranbp2. Thus, the identification of Ranbp2 may reflect different biochemical conditions used to purify biotinylated proteins from cultured neurons compared to brain homogenates. Although these studies are still in progress, we are happy to provide the mass-spectrometry results from our *in vivo* study to the editors and reviewers, but request they be kept confidential.

6. *Localization of ectopically expressed N-terminal region of Ranbp2, but not full-length,*

to the AIS was already shown by Khalaf et al. 2019. Yes, we agree this is not an original observation by us and we cite the paper by Khalaf et al. We considered their observation to be the most perplexing (and strongest) argument for AIS Ranbp2 and struggled to understand the apparent discrepancy between our antibody experiments and the exogenous expression. If the introduction of the exogenous N-terminal region of Ranbp2 had not localized at the AIS, then this would have been a very, very simple case of cross-reactivity by an antibody. We consider it remarkable that the exogenously expressed Ranbp2 fragment goes to the AIS. We are grateful to the reviewer for pointing out several references that support the idea that this localization reflects the ability of Ranbp2 to bind to stable microtubules. We have added these references to our manuscript. We agree that Ranbp2 can bind to stable microtubules without the use of taxol – indeed we show this in neurons. However, our experiments in HeLa cells required the addition of taxol to induce strong localization of Ranbp2 to microtubules. In future experiments, it would be interesting to determine if the addition of Trim46, the AIS microtubule bundling protein, can also promote the association and AIS localization.

7. *It is possible that a splice variant of Ranbp2 predominantly localizes to the AIS and may be missed by analyzing the recruitment of endogenous protein that was HA tagged at various locations.* Yes, this is possible, but unlikely for three reasons:

a. We used CRISPR-based methods to tag endogenous Ranbp2 at N-, C, and internal sites (3 different locations).

b. We used three different antibodies that recognize Ranbp2, all of which bind to different parts of Ranbp2 (see Figure 1E from the manuscript).

c. We performed extensive analyses of truncation and internal deletion mutants (see Figure 3) and find the entire N-terminal domain is necessary for AIS localization of exogenous Ranbp2.

We conclude the simplest explanation is that Ranbp2 is not at the AIS, rather than invoking the existence of some unknown splice variant never before seen.

Reviewer 3. This reviewer thought our paper is a 'welcome clarification', 'interesting…and well suited for this journal.' The reviewer had two suggestions for improvement.

1. *Clarify why such an artifactual AIS candidate protein was identified in two proteomics studies.* We agree that this is an important concern and have addressed this point in the final paragraph of the discussion. We do not know why Ranbp2 appeared in both independent studies, although as described above (Reviewer 2, point 5) Ranbp2 does not appear in our subsequent and even more rigorous experiments *in vivo.* As we point out, these apparent contradictory results warrant careful controls. This was the original rationale for the paper under consideration here. This is also the major message we hope our paper gives. That when using proteomic data sets, and antibodies, careful and extensive control experiments must be performed to confirm a protein is truly at the AIS.

2. We performed new immunostaining experiments in both optic and sciatic nerve. For clarity, we now show colocalization at nodes and paranodes between neurofascin and A301-796 immunostaining in sciatic nerve with individual channels. We revised Fig. 1D accordingly.

3. We added the requested information in the methods about the Ranpb2 antibodies used. Please note, we also independently mapped the binding regions for the different Ranbp2 antibodies used here in Figure 1E of the manuscript. The binding domains for each of the Ranbp2 antibodies used here is also included in Fig. 2C.

Second decision letter

MS ID#: JOCES/2020/256180

MS TITLE: Endogenously expressed Ranbp2 (Nup358) is not at the axon initial segment

AUTHORS: Yuki Ogawa and Matthew N Rasband
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers gave favourable reports. The most critical reviewer however raised some final points that you may take into consideration. Please let me know if you decide to alter your manuscript in light of these comments.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. Instead please use yellow shading or different colour font to highlight changes in your revised manuscript

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

Reviewer 1

Advance summary and potential significance to field

After appropiate review, reviewers comments discussion and inclusion of new data, it is clear that previous reports on Ranbp2 expression at the AIS can not be further supported.

Comments for the author

The revised manuscript has added new data and authors have clearly discussed reviewers comments to support their conclusions.

Reviewer 2

Advance summary and potential significance to field

One needs to be cautious about making conclusions only based on data generated using any antibody or mass spectrometry data.

Comments for the author

This reviewer agrees with the authors in that ‘correction is a part of accumulating the right knowledge and some reports in the past have diluted the scientific progress’. The authors also may agree that the contrary is true as well; there are discoveries that were neglected due to lack of further evidence for decades (often due to lack of right experimental tools/for not rigorously followed upon) that delayed progress in a specific scientific area - one of the many being ‘the presence of ribosomes and active translation in axons’. This is the reason one needs to take a balanced view on a particular finding. When one makes a statement - Endogenous RanBP2 is not present at the AIS’- one needs to analyse and discuss the potential limitations of the study based on which the conclusion is made.
The authors have agreed to most of the different possibilities pointed out by this reviewer that could explain the results (however remote they may seem to be). Their response to the specific queries raised by the reviewer can be included in the discussion to achieve the balance. While this publication will add to the cautiousness the scientists should observe, the other possibilities should not be completely ruled out.
The last point, sorry to have missed in the previous review comments, is that the evidence for expression of intact full length endogenous HA-RanBP2 (tagged at N-, middle and C-term regions) in neurons, after the CRISPR editing, is not shown. This is an important point that needs be addressed before any conclusion is drawn. If western blotting and identification of the intactness (based on molecular weight) is technically challenging, the antibodies detecting different regions of RanBP2 can be used in co-

immunostaining to show that the full length protein is expressed (for example, N-terminally HA-tagged RanBP2 can be co-stained with HA and an antibody raised to other regions of RanBP2 - the middle and C-terminal region- and look for co-localization; likewise, C-terminally HA-tagged version can be checked with an N-terminal and middle antibody; and the middle HA-tagged version can be checked with N- and C-terminal antibodies etc.).

Reviewer 3

Advance summary and potential significance to field

The paper from Ogawa and Rasband provides a welcome clarification for previously published inconsistent results regarding the supposed AIS localization of Nup358/Ranbp2 (Khalaf et al., 2019; Hamdan et al., 2020),
a member of the nucleoporin family.
The authors convincingly demonstrate using different approaches that endogenous Ranbp2 is actually not expressed at the AIS but only in the nucleus and that its previously reported AIS localization was due to the fact that the only anti-Ranbp2 antibody that was used, actually cross-reacts with NF186, an AIS protein which shares a KPLQG sequence with Ranbp2. They nonetheless observe (as Khalaf et al., 2019) that an exogenously (over-)expressed N-terminal fragment of Ranbp2 localizes at the AIS, but they provide a mechanistic explanation for this surprising result, based on Ranbp2's intrinsic affinity for stabilized microtubules.

Comments for the author

The authors have satisfactorily addressed my concerns in their revised manuscript.

Second revision

Author response to reviewers' comments

1. The authors have agreed to most of the different possibilities pointed out by this reviewer that could explain the results (however remote they may seem to be). Their response to the specific queries raised by the reviewer can be included in the discussion to achieve the balance. While this publication will add to the cautiousness the scientists should observe, the other possibilities should not be completely ruled out.

This reviewer is concerned about the strongly worded conclusion (title, abstract, etc.) that Ranbp2 is not at the AIS. As shown in the results and reiterated in the discussion, the conclusion is based on 5 independent lines of very compelling evidence. Nevertheless, Reviewer #2 suggests that in the interest of balance we should not discount the very remote possibilities that 1) Ranbp2's expression depends on NF186, and 2) Ranbp2 exists at the AIS as a splice variant not detected by any of the methods used in our study. We respectfully disagree and argue that giving additional discussion to possibilities that are exceedingly remote is not balanced, but rather elevates these to a level of credibility that is not warranted. Therefore, we prefer not to include alternatives that are so unlikely that they strain the imagination. Nevertheless, if the editor believes it is important to discuss these, we are prepared to include in the discussion the following sentences: "The preponderance of data provided strongly support the conclusion that Ranbp2 is not located at the AIS. Nevertheless, it remains a formal possibility, albeit extremely unlikely, that a unique, previously unknown splice variant of Ranbp2 is located at the AIS, its localization depends on NF186, and the only antibody that detects this splice variant of Ranbp2 coincidentally also recognizes a small epitope found in NF186."

2. The last point, sorry to have missed in the previous review comments, is that the evidence for expression of intact full length endogenous HA-RanBP2 (tagged at N-, middle and C-term regions) in neurons, after the CRISPR editing, is not shown. This is an important point that needs to be addressed before any conclusion is drawn. If western blotting and identification of the

intactness (based on molecular weight) is technically challenging, the antibodies detecting different regions of RanBP2 can be used in co-immunostaining to show that the full length protein is expressed (for example, N-terminally HA-tagged RanBP2 can be co-stained with HA and an antibody raised to other regions of RanBP2 - the middle and C-terminal region- and look for co-localization; likewise, C-terminally HA-tagged version can be checked with an N-terminal and middle antibody; and the middle HA-tagged version can be checked with N- and C-terminal antibodies, etc.).

Respectfully, the purpose of this experiment was not to provide evidence for the expression of full-length, endogenous Ranbp2, but rather to investigate whether there are unique, previously unknown Ranbp2 splice variants that localize to the AIS. In our experiments we show that no matter where we tag or label the endogenous Ranbp2, it is located at the nuclear envelope, not the AIS. This reviewer is correct that immunoblots to detect the tagged protein are technically extremely challenging due to the low transduction efficiency of neurons. Recognizing this, Reviewer #2 suggests that instead we use antibodies against different domains of Ranbp2 to show colocalization with the tag. We have essentially already done this. In Fig. 1 we showed different Ranbp2 antibodies recognize different domains of Ranbp2 and that all label the nuclear envelope. In Fig. 5 we show the tagging of endogenous protein at N-, C- and internal sites also only labels the nuclear envelope. Thus, the antibody and tagging based approaches reveal identical localization for Ranbp2 at the nuclear envelope and not at the AIS. This strongly argues against the existence of some splice variant that somehow has escaped all of our efforts to detect it.

Third decision letter

MS ID#: JOCES/2020/256180

MS TITLE: Endogenously expressed Ranbp2 (Nup358) is not at the axon initial segment

AUTHORS: Yuki Ogawa and Matthew N Rasband
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Thank you for agreeing to include in the Discussion the sentence:

'The preponderance of data provided strongly support the conclusion that Ranbp2 is not located at the AIS. Nevertheless, it remains a formal possibility, albeit extremely unlikely, that a unique, previously unknown splice variant of Ranbp2 is located at the AIS, its localization depends on NF186, and the only antibody that detects this splice variant of Ranbp2 coincidentally also recognizes a small epitope found in NF186.'

However, we feel that this sentence will not help the reader to reach their own conclusions based on the evidence presented. Henceforth, we will be happy to publish this manuscript without its inclusion.