Snazarus and its human ortholog SNX25 modulate autophagic flux

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MS TITLE: Snazarus and its human ortholog SNX25 regulate autophagic flux by affecting VAMP8 endocytosis

AUTHORS: Annie Lauzier, Marie-France Bossanyi, Rupali Ugrankar, Mike Henne, and Steve Jean
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 raise a number of concerns that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. Specifically, both reviewers indicate that the flow of the manuscript was difficult to follow, possibly due to references to large amounts of supplementary data, and the manuscript could benefit from some careful re-organization. In addition, reviewer comments request additional clarification, controls, and quantification for several experiments. My feeling is that addressing these comments will strengthen the manuscript. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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 paper by Lauzier et al. relates the functions of SNX25 as an autophagy-regulating sorting nexin to its proposed role in VAMP7(8) trafficking. Hence, when SNX25 is targeted, autophagosome-lysosome fusion is impaired. This is of potential interest to the autophagy and trafficking fields in general, but the manuscript is weak on functional data. It is also difficult to follow, as it leans so heavily on the supplemental data and meanders along without clear focus/direction. There is plenty here for a decent story, but I wonder whether a major order change would be beneficial to place the autophagy focused experiments at the end. As it stands it falls a bit flat. Otherwise, the authors would need to come up with experiments that link the final data on VAMP8 trafficking with autophagy. At the very least, this would need to be full autophagy flux analysis in the SNX25 rescue setting.

Comments for the author

Abstract:
1. Autophagy is not strictly speaking “essential”.
2. Needs to be “Macroautophagy”

Introduction:
1. The authors suggest that the molecular machinery for sealing, maturation and fusion remain unclear –
there is a large body of literature on these areas.
2. Please note that the role of SNX4 during mammalian autophagy was first described by Anton et al. (2020) jcs.246306 doi: 10.1242/jcs.246306, and this paper should be cited instead of (or alongside) Ravussin et al. 2021.

Results:
1. In the figures (several), what do the dotted lines signify? Cell boundaries or RNAi/transgene expression?
2. Fluorescence brightness – how well is this controlled? For example, there is a clear dip in LyTr fluorescence at 3 hours starvation in the snz:GFP expression experiment (Fig. S1E), but opbjects are still visible. Are they dimmer, or have the acquisition settings been altered. Fig. S1E is not a great representative image of the data in Fig. S1F. This is true for other figures also.
3. Fig. S1C does indeed show increased ref(2)P in Snz overexpressing tissue. However this is not a flux assay as it disregards expression. There should be a lysosomal inhibition control for flux assessment. Line 140 – where is the data for the increased ref(2)P puncta size? The GFP-mCherry-LC3 data look very nice.
4. The traffic light flux assay readout for the CRISPR null line is comparatively weak – is there compensation happening here? Could the authors please comment.
5. Lysotracker does not exclusively report autolysosomes (line 117/118).
6. Snz RNAi led to altered Lysotracker puncta as a suggestion of altered autophagy – weak link.
7. Fig. 2: What cells? Needs to be mentioned in the legend and text. For the LC3 analysis, the authors need to confirm that they are measuring LC3-II. Why is there so little LC3-I? Is this basal expression or treated with e.g. BafA1? Legend needs to mention that C/D are CRISPR edited. There are outliers in the data in 2D – is this because of the very poor tubulin transfer shown in the example? I am concerned if this is the best blot the authors have. Examples fluorescence images of cells stained with anti-LC3 should be shown. Did the authors generate multiple KO clones and did they all behave the same?
8. Line 153: this should read “…snz is necessary but not sufficient for efficient autophagic flux.”

9. The flux analysis in SNX13/14/25 suppressed cells is poor. There are clearly no real differences in baseline or BafA1-induced LC3-II, but the scramble siRNA also does not show increased LC3-II with BafA1. So to conclude, by implication, that this is evidence of a flux impairment in the SNX13/14/25 suppressed cells is not supportable. That said, the LC3 puncta data in Fig. 2 look clear. Based on the large range of data points in Fig. S2C, I wonder how well the BafA1 was working?
10. The PLA assay looks good, but needs to be quantitated.
11. The VAMP8 internalisation data look quite nice, but have any statistics been carried out on the data in Fig 5C? If not, they should be.

Reviewer 2

Advance summary and potential significance to field

In this article, the authors have identified a sorting nexin in Drosophila, snazarus (snz) that when knockdown or depleted from fat body cells result in defective autophagy flux. At least 2 human homologous, SNX14 and SNX25 also have similar autophagy defects when ablated. The authors, further discover Vamp7 also accumulates in these mutant cells and hypothesize, trafficking of Vamp7/VAMP8 via a snz/SNX25 pathway is the underlying defect causing the autophagy fusion defect and not endocytosis. They also find the PX domain of SNX25 is critical to mediate it's role in trafficking Vamp8, and not it's transmembrane domain, suggesting functional and spatial pools of SNX25 that might balance lipid homeostasis and protein trafficking/autophagy. There is also a nice correlation between various SNX25 splice variants that lack the ER-anchoring transmembrane (TM) domain and this TM lacking isoform was more abundant in cancer cell lines. The manuscript is bit hard to follow, as the narrative exchanges between a large amount of supplemental information but overall is a nicely executed study that provides some insights into sorting nexin function during autophagy. However, some additional information and experiments outlined below would increase the study's impact.

Comments for the author

Major Points:
1. The authors make a point snz has 4 human orthologs, SNX13, 14, 19 and 25. Why was SNX19 not tested in any of the experiments? Moreover, it's unclear why SNX14 wasn't included in subsequent experiments, especially since it appeared to accumulate more LC3 than SNX25. Can overexpression of any of the other orthologs complement?
2. While I agree, it appears the SNX25 PX domain is required for Vamp8 trafficking, that doesn't necessarily mean it's required for autophagy function. What does LC3 look like in a PX mutants?
3. There have been reports in budding yeast that the sorting nexins are supplying specific lipids to the lysosome/vacuole (Ma et al 2018) that show similar fusion defects. Could SNX25 also be trafficking lipids to lysosome membrane? Can you rescue with a lipid supplement? Given SNX14 connection to lipid droplets it seems reasonable, a combination of lipid and VAMP8 maybe needed.
4. Likewise, can you rescue delta-snz with overexpressing Vamp7? Or VAMP8 in SNX25 knock-out cells? This would show some specificity.

Minor Points:
1. Figure 4F: SNX25-202 (deltaTM) cartoon still has a TM domain?
2. In many of the presented figures or figure legends, it is unclear whether starvation conditions are needed for these defects.

First revision

Author response to reviewers' comments

Response to referee comments :

Referee #1 :

Major points :

- *Referee comment #1: The manuscript is of potential interest to the autophagy field and trafficking field in general, but the manuscript is weak on functional data and difficult to follow given its heavy reliance on supplemental data. Moreover, either a major order change or a full autophagy flux analysis in the SNX25 rescue setting would benefit the manuscript.*

Author response #1: We first wish to thank the referee for acknowledging the strength and weaknesses of our study and for suggesting specific modifications to improve our manuscript. As suggested, we have reorganized the data presentation and increased the amount of data presented in the main figures. Directly modifying the order and general flow of the manuscript proved difficult, and since referee #2 also suggested assessing autophagy in a SNX25 rescue setting, we opted to perform these analyses. Interestingly, we found that SNX25's roles in autophagy were rescuable by ectopic expression of either SNX14 or SNX25. Moreover, although endocytosis of VAMP8 was dependent on SNX25's PX domain and not its TM region, autophagy was rescued by both mutants. These results highlight that SNX25's requirement for autophagy is not solely linked to VAMP8 but also modulated by domains involved in lipid metabolism. Furthermore, we were able to rescue SNX25 autophagy defects by the addition of exogenous ethanolamine, which indicates that lipid imbalances could lead to the autophagy defect observed. Given these new findings, the title along with multiple other sections of the manuscript were modified. We believe that these additions and revisions added throughout the manuscript improve our understanding of SNX25 and its paralogues in autophagy and trafficking and hope that they address the referee's concerns.

Specific comments:

- *Referee comment #2: Abstract:*
  1. *Autophagy is not strictly speaking "essential"*
  2. *Needs to be "Macroautophagy"*

  Introduction:
  1. *The authors suggest that the molecular machinery for sealing, maturation and fusion remain unclear - there is a large body of literature on these areas.*
  2. *Please note that the role of SNX4 during mammalian autophagy was first described by Anton et al. (2020) jcs.246306 doi: 10.1242/jcs.246306, and this paper should be cited instead of (or alongside) Ravussin et al. 2021.*

Author response #2: Thank you for pointing these oversights. We have modified the text accordingly and added the appropriate reference.

- *Referee comment #3: In the figures (several), what do the dotted lines signify? Cell boundaries or RNAi/transgene expression?*

Author response #3: Thank you for pointing out this omission. The dotted lines signify cell boundaries. We have added this statement to the applicable figure legends.

- *Referee comment #4: Fluorescence brightness - how well is this controlled? For example, there is a clear dip in LyTr fluorescence at 3 hours starvation in the snz:GFP expression experiment (Fig. S1E), but opbjects are still visible. Are they dimmer, or have the acquisition settings been altered. Fig. S1E is not a great representative image of the data in Fig. S1F. This is true for other figures also.*

Author response #4: We apologize for not appropriately describing our imaging setup in our methods section in the original submission and thank the referee for pointing out the issue. For conditions where staining intensities or the number of puncta were compared, we kept all acquisition settings on the microscope constant for all conditions in a replicate. Specifically, the condition yielding the strongest signal was imaged first (e.g., starved wild type fat bodies for LysoTracker) to ensure that images are acquired over the whole intensity spectrum and avoid saturated signals for strong staining. Once acquisition settings were set, all images were acquired using the same settings, as we have done previously (Jean et al. *EMBO Reports*, 2015). For most fat body experiments, four independent experiments were performed, with small setting modifications between replicates to ensure optimal image quality and alleviate staining differences between replicates. Finally, image processing was performed similarly for each displayed image. This information has been added to the revised methods section.

As observed by the referee, in some instances dimmer LyTr signals could still be observed, as is sometimes the case for LyTr staining in fat bodies. Settings used for automated object counting were set to quantify objects with strong and intermediate intensities, with weak objects excluded. These criteria were used throughout experimental sets. We have modified specific images to better represent the quantifications provided throughout the manuscript.

- *Referee comment #5: Fig. S1C does indeed show increased ref(2)P in Snz overexpressing tissue. However this is not a flux assay as it disregards expression. There should be a lysosomal inhibition control for flux assessment. Line 140 - where is the data for the increased ref(2)P puncta size? The GFP-mCherry-LC3 data look very nice.*

Author response #5: We apologize for the lack of clarity linked to Fig S1C. This figure did not assess ref(2)p upon snz overexpression. Instead, it is a control experiment showing that the RNAi hairpin used in the manuscript efficiently decreases ectopic snz-GFP expression. Hence, the green puncta are snz-GFP puncta mostly localized close to the cell edges, as shown before (Ugrankar et al., 2019). However, upon co-expression of the snz RNAi construct used in this study, a striking decrease in snz-GFP level can be observed, validating the RNAi construct.

We thank the reviewer for the positive comment on the GFP-mCherry-LC3 flux reporter.

- *Referee comment #6: The traffic light flux assay readout for the CRISPR null line is comparatively weak - is there compensation happening here?*

Author response #6: We thank the reviewer for pointing this out and agree with the statement. Unfortunately, we cannot conclude whether there is compensation by other genes or pathways. However, this is likely, since it is often observed that upon full knockout of a given gene, an organism can compensate by upregulating other pathways partially/completely rescuing a specific phenotype. This is also consistent with the fact that we can mostly rescue *SNX25* KO in HeLa cells by supplementing ETA for 24 h. We have now added a sentence in the manuscript discussing this possibility (line 172-173).

- *Referee comment #7: Lysotracker does not exclusively report autolysosomes (line 117/118).*

Author response #7: This is indeed true in most cell types. However, in fed fly fat bodies, LysoTracker does not stain any acidic compartments, as these only appear upon autophagy induction and were shown to be mostly autolysosomes (Lőrincz et al., 2017; Mauvezin et al., 2014). We have added this information to the manuscript, along with additional references (Line 137-140).

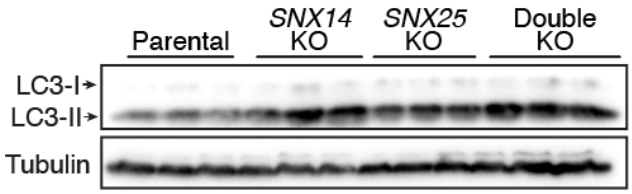
- *Referee comment #8: Snz RNAi led to altered Lysotracker puncta as a suggestion of altered autophagy - weak link.*

Author response #8: We agree with the referee that decreased LyTr staining is not sufficient to make conclusions regarding autophagy. However, given previous publications in the fly fat body, it suggests a direct or indirect effect on autophagy or lysosome (and autolysosome) acidification. To address this, we have modified the sentence in the text (Line 152-156).

- *Referee comment #9: Fig. 2: What cells? Needs to be mentioned in the legend and text. For the LC3 analysis, the authors need to confirm that they are measuring LC3-II. Why is there so little LC3-I? Is this basal expression or treated with e.g. BafA1? Legend needs to mention that C/D are CRISPR edited. There are outliers in the data in 2D - is this because of the very poor tubulin transfer shown in the example? I am concerned if this is the best blot the authors have. Examples fluorescence images of cells stained with anti-LC3 should be shown. Did the authors generate multiple KO clones and did they all behave the same?*

Author response #9: We thank the reviewer for pointing out multiple omissions, for which we apologize. The mammalian cells used throughout our study were HeLa cells and we did indeed measure LC3-II in the various densitometric analyses. In our hands, LC3-I levels in HeLa cells are low at steady-state and even lower under starvation. The levels observed are under basal conditions, unless stated, and LC3-I levels increase upon bafilomycin addition. All densitometric analyses were normalized to a housekeeping gene (either GAPDH or tubulin).

We have specified in the figures and figure legends if experiments were performed with siRNAs or on CRISPR/Cas9 KO cells. For transparency's sake, we are providing below a replicate blot that was performed on the KO cells to demonstrate the LC3 accumulation in the KO cell populations.



Response to referees, Fig.1: Replicate western blot analysis of LC3-I and -II levels in the various KO cell populations

Finally, we did not generate single KO clones. Instead, we worked on cell populations that were generated using the same gRNAs, but at different times. Hence, Figure 2F shows three independent populations for SNX14, SNX25 and SNX14/25, and their quantification is displayed in Figure 2G.

- *Referee comment #10: Line 153: this should read "…snz is necessary but not sufficient for efficient autophagic flux."*

Author response #10: We apologize for the mistake and have made this modification.

- *Referee comment #11: The flux analysis in SNX13/14/25 suppressed cells is poor. There are clearly no real differences in baseline or BafA1-induced LC3-II, but the scramble siRNA also does not show increased LC3-II with BafA1. So to conclude, by implication, that this is evidence of a flux impairment in the SNX13/14/25 suppressed cells is not supportable. That said, the LC3 puncta data in Fig. 2 look clear. Based on the large range of data points in Fig. S2C, I wonder how well the BafA1 was working?*

Author response #11: We thank the referee for this comment and apologize for the lack of clarity in our figure. Western blots of LC3-II levels are quite variable between experiments given the various steps required in these experiments. Although there is variability in the presented data, some degree of increase in LC3-II was consistently observed for SNX14 and SNX25 compared to the scramble siRNA in the basal condition.

Regarding the bafilomycin treatment, it is not possible to compare the staining directly, since the western blots were performed on different gels to fit all the samples in the proper order. Also, we reasoned that it was best to show a non-overexposed band (shorter exposure time) for the bafilomycin experiment, which resulted in a similar-looking band compared to that of control cells not treated with bafilomycin. One indication that the bafilomycin treatment impacted the cells is that the LC3-I:LC3-II ratio is different between the non-treated and treated cells. Moreover, the same bafilomycin stock was used in the immunofluorescence experiments at the same concentration and for the same duration, indicating that the treatment is working. Having said that,

we agree with the referee that we can't strongly conclude this based on the provided data, and this is why we performed immunofluorescence experiments on LC3 and generated KO cells to strengthen this conclusion. Together, the various experiments indicate a role of SNX25 in modulating autophagic flux.

- *Referee comment #12: The PLA assay looks good, but needs to be quantitated.*

Author response #12: We thank the referee for this comment. The quantitated data now includes a statistical analysis.

- *Referee comment #13: The VAMP8 internalisation data look quite nice, but have any statistics been carried out on the data in Fig 5C? If not, they should be.*

Author response #12: We thank the referee for this comment. We have now added the statistical analysis.

Referee #2 :

Major Points :

- *Referee comment #1: The authors make a point snz has 4 human orthologs, SNX13, 14, 19 and 25. Why was SNX19 not tested in any of the experiments? Moreover, it's unclear why SNX14 wasn't included in subsequent experiments, especially since it appeared to accumulate more LC3 than SNX25. Can overexpression of any of the other orthologs complement?*

Author response #1: We thank the referee for these thoughtful comments. We did not pursue SNX19 originally, because it does not contain an RGS sequence like SNX13, SNX14, SNX25, and snz. However, we have now performed experiments on SNX19 using two siRNAs (although one had a minimal effect on the mRNA) and did not observe strong phenotypes, indicating that SNX19 has a weak effect on autophagy. Nonetheless, and considering a recent publication reporting a role for SNX19 as an ER/endolysosome tether, it could still indirectly affect autophagy by modulating lysosome movement. We have added this data and a sentence on a potential autophagic role for SNX19 (Line 198-203).

We have opted to mostly focus on SNX25 because it is the closest orthologue to snz and its PX domain shows a similar preference for diphosphorylated phosphoinositides. We have provided the reasoning behind this choice in more detail in the manuscript.

Finally, as suggested by the referee, we have performed rescue experiments and found that both SNX14 and SNX25 can rescue the loss of SNX25. These interesting new findings are now incorporated into the manuscript. Given these results, we have revisited the focus on VAMP8 to postulate a more general and differential role of SNX25 on VAMP8 endocytosis and lipid regulation.

- *Referee comment #2: While I agree, it appears the SNX25 PX domain is required for Vamp8 trafficking, that doesn't necessarily mean it's required for autophagy function. What does LC3 look like in a PX mutants?*

Author response #2: We thank the reviewer for this suggestion. We have performed a comparison between the ΔTM or the ΔPX/Nexin SNX25 constructs. Unexpectedly, SNX25 lacking its PX and Nexin domains was able to rescue the accumulation of LC3 puncta. This indicates, as suggested by the referee, that the SNX25 phenotype is broader than a simple effect on VAMP8 internalization. As such, it suggests multiple roles for SNX25 in modulating trafficking and autophagy since both constructs were able to rescue SNX25 depletion. We have now incorporated this novel data into the manuscript and modified multiple statements and conclusions in the text.

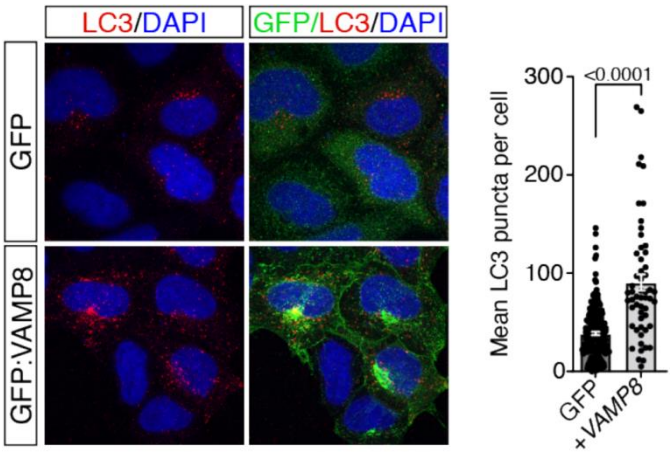
We are grateful for the suggestion, which lead to new experiments that strongly improved our manuscript and conclusions.

- *Referee comment #3: There have been reports in budding yeast that the sorting nexins are supplying specific lipids to the lysosome/vacuole (Ma et al 2018) that show similar fusion defects. Could SNX25 also be trafficking lipids to lysosome membrane? Can you rescue with a lipid supplement? Given SNX14 connection to lipid droplets it seems reasonable, a combination of lipid and VAMP8 maybe needed.*

Author response #3: We are very thankful to the referee for this suggestion. We tested increasing concentrations of ethanolamine (new Fig. 7) since it was used at a very high concentration in Ma et al 2018 in yeast, and made the exciting observation that low concentrations of ETA rescued autophagosome accumulation in *SNX25* KO cells. This was ablated at the high ETA concentration and led to a striking LC3-II accumulation in both WT and KO cells. The rescue was not the result of decreased autophagosome synthesis, as LC3 puncta remained constant in ETA and BafA1- treated cells (Fig. S5). Moreover, the treatment did not fully rescue VAMP8 knockdown, as predicted from Ma et al. 2018. From these new data, we conclude that SNX25 affects autophagy in two independent ways, by modulating VAMP8 internalization and lipid homeostasis. These findings also open new research avenues to better characterize those differences. We have expanded our discussion to account for this new data.

- *Referee comment #4: Likewise, can you rescue delta-snz with overexpressing Vamp7? Or VAMP8 in SNX25 knock-out cells? This would show some specificity.*

Author response #4: We thank the referee for this suggestion. We have tried to perform the suggested rescue. However, before attempting to rescue of multiple mutants, we tested the effect of overexpressing VAMP8 in wild-type cells and noticed that VAMP8 led to LC3 accumulation, possibly by affecting various trafficking pathways. Therefore, we did not perform the full set of rescues since VAMP8 overexpression was affecting autophagic flux on its own. Moreover, since ETA and SNX25 ΔPX/Nexin can rescue LC3 accumulation in *SNX25* KO cells, it is clear that SNX25 affects autophagy in multiple ways, and we have thus adjusted the narrative of the manuscript along those lines.



Response to referees, Fig. 2: VAMP8 overexpression affects LC3-positive structures. Wild-type HeLa cells expressing GFP or GFP-VAMP8 were stained for endogenous LC3 (red). Quantification of LC3 puncta per cell, error bars represent the SEM. *n*=3 independent experiments.

Minor points:

- *Referee comment #5: 1. Figure 4F: SNX25-202 (deltaTM) cartoon still has a TM domain?*

Author response #5: We apologize for the lack of clarity in the displayed cartoon. The two isoforms do not start with the same exons and the TM domains are in the first large exon of transcript 208. The first coding exon in the 202 transcript is the second one. We have darkened the TM-encoding exon and added this information to the figure legend.

- *Referee comment #6: In many of the presented figures or figure legends, it is unclear whether starvation conditions are needed for these defects.*

Author response #6: We apologize for the lack of clarity. We have added the requested information. To summarize rapidly, in flies, all the effects were observed under starvation conditions apart from ref(2)p accumulation, which was monitored in fed larvae. In HeLa cells, all the presented findings are in normal growth conditions and were not amplified by starvation. We focused on normal growth conditions since the phenotype was readily observable in them, suggesting a general role for SNX25 in autophagy modulation.

Second decision letter

MS ID#: JOCES/2021/258733

MS TITLE: Snazarus and its human ortholog SNX25 modulate autophagic flux

AUTHORS: Annie Lauzier, Marie-France Bossanyi, Raphaelle Larcher, Sonya Nassari, Rupali Ugrankar, Mike Henne, and Steve Jean
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 submitting this interesting work to JCS. I appreciate your thoughtful responses to the reviewer comments, including the addition of new experimental data and modifications to the text.