JCB

Glial TGFβ signaling promotes neuronal survival in peripheral nerves

Alexandria Lassetter, Megan Corty, Romina Barria, Amy Sheehan, Jo Hill, Sue Aicher, A. Nicole Fox, and Marc Freeman

Corresponding Author(s): Marc Freeman, Oregon Health & Science University

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December 15, 2021

Re: JCB manuscript #202111053

Dr. Marc R Freeman
Oregon Health & Science University
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3181 SW Sam Jackson Park Road
Portland, OR 97239-3098

Dear Marc,

Thank you for submitting your manuscript entitled "Glial TGFβ signaling promotes axon survival in peripheral nerves" to JCB. I am attaching the evaluations of three reviewers to this letter. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB. I have also gone over the manuscript while comparing it to the reviewers' comments. My assessment after reading the reviews together with the manuscript is that you have an exciting story, but it needs some more mechanistic insight, i.e., mechanisms through which the wrapping glia control axonal and neuronal survival, to have the interest appropriate for the cell biological readership of this journal. For example, in present form, the results do not even include epistasis data where the ligand is mutated or knocked down in wrapping glia and the receptor and/or its effectors are mutated/knocked down in the affected neurons. I am also left with the impression that clues to mechanism could be rapidly obtained by consideration of the most plausible of the 60-odd hits not described in any detail through some simple epistasis experiments. To provide some guidance, I will go through the reviewers' comments and provide my assessments of their importance from the JCB perspective.

Review #1: This reviewer was obviously extremely impressed by the creativity of your system.

Major point #1: I need a response regarding this reviewer's first sentence. I do not share this reviewer's concern about the order of data in your manuscript, but hope that you will consider the reviewer's perspective.

Major point #2: I think this is largely a discussion item for you to consider.

Major point #3: I agree completely with the importance of this set of experiments recommended by the reviewer.

Major point #4:
Fig. 3. I agree with the reviewer's assessment of the images in this figure. I also could not see real differences.
Fig. 4F. I agree but very easy for you to fix.
Fig. 6. Figure 6 clearly need some expansion. At the level of resolution data is visualized in this figure, there do appear to be differences between the glia. I would agree that there are not obvious differences in glial coverage, but the images do not have the resolution to make statements about glial ensheathment. Perhaps you can provide some more details on your quantification methodology. It is not obvious to the reviewer or me how you obtained the data on the "wrapping index". Higher power images might help.

Major point #5 (concerning Figure 5): I hope you can consider this reviewer's assessment of your assertions. I agree that the labelling of the figure panels could be improved, perhaps by using A to refer to controls; B to refer to babo-Gal4? This is not a major issue.

Major point #6: This concern does not resonate with me.

Major point #7 (re. abstract): I agree that "cell autonomously" is over-stated in the abstract. It is certainly plausible, but short of definitive.

Of the minor points, I agree with each of the four, but assume you can address these. They are intended to be helpful.

Reviewer #2: This reviewer is obviously also very impressed but notices the lack of mechanism. This reviewer's key assessment is the final sentence of the second paragraph.

Major point #1: I think the final two sentences accurately reflect both the reviewer's and my own assessment that some more mechanistic insight is needed.

Major point #2: Much of this paragraph is really asking for discussion. Any thoughts you have on the mechanistic interactions between glial TGFbeta, WldS activity and the neuronal cell body would be welcome. The suggested use of Sarm mutants would

obviously be interesting, but is not something the JCB would insist on.

Additional points: Both of these seem quite reasonable. I was also struck by the small differences in Figure 4F. Why wasn't this panel extended to 28 days? It might have been more compelling.

Reviewer #3: This reviewer, while a bit less charitable, also shares the assessment with the second reviewer that more mechanistic insight is needed.

Major points:
1. While I do not know of evidence that Babo regulates vglut, is there evidence that rules this out in the literature? It is a reasonable question.

2. This could be provided in a supplemental table. I think the major item to provide would be summary data on the 70 hits. What other pathways may be implicated? Are there clues to TGF-beta's mechanism of action on glia and/or neurons from this screen?

3. I agree but assume you can easily make appropriate textual modifications.

4. (nothing from reviewer).

5. Please consider this.

6. Please consider this perspective.

7. This comment is reasonable, but I am guessing that the difference is due to levels of Wlds expression. Perhaps you can include a response for the reviewers in a letter accompanying a revision.

Minor points:
Please consider each of these minor comments. They are generally helpful for clarifying the manuscript.

To summarize, the consensus is that this is a start to a very important study, but some mechanistic insight needs to be included. In particular, I would encourage you to consider the many hits not discussed and determine whether one or more of them does not provide a rapid avenue to provide this insight.

I do encourage a revision of a revised manuscript and will provide leeway on timing. Please do not do this prematurely, since the JCB policy does not permit two revisions in instances where there are still major issues in the initial revised submission. If you want to discuss this letter or the reviewers' comments, please contact me by email (louis.reichardt@ucsf.edu) and we can find a mutually convenient time.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count 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: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript 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.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots

should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, 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. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

With best holiday wishes,

Sincerely yours,

Louis F. Reichardt, PhD
Monitoring Editor
Journal of Cell Biology

Andrea L. Marat, PhD
Senior Scientific Editor
Journal of Cell Biology

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

This manuscript by Lassetter et al explores neuron-glia interactions during axon maintenance. Using a very cool system, also developed in the Freeman lab, they can visualize single axons in the drosophila wing and manipulate wrapping glia genetically. Following a clever screen, they find and demonstrate that glial TGFbeta signaling is required for axonal maintenance during aging and also after injury. As the topic of neuron-glia interaction is still largly unstudied mechanistically, I think this manuscript should be accepted in JCB. However, I do believe that several aspects of the study should be strengthened before acceptance can be warranted.

Major points:

* The screen was conducted with Repo-Gal4, which is not specific for wrapping glia (WG). that is OK, but I would expect that they would KD the positive hits using the WG-specific Gal4 (more than just the babo-RNAi, which they do). Knocking down genes with Repo and looking at the outcome in the adult aged wing could be problematic since they manipulate glia thought development. The order of the manuscript is a bit weird in my opinion (but this is totally up to the authors) as some of the experiments that show no developmental defect (figure 6, for example) come up very late.

* They discover that almost all components of the TGF-b pathway are important in glia - ligands, receptors, signaling moieties and so on, and mention that this could be the result of an autocrine signaling in glia, but they don't discuss this much in their discussion. Are there known cases of autocrine signaling in glia? I would expect they would elaborate on the conventional TGF-b in glia, them being the ligand-secreting cells, and discuss why this pathway acts differently in WG to protect axons.

* They mention that babo is also expressed in other glia and also in the sensory neurons. What happens if babo is KD in these neurons? do they degenerate after 28 days? that could hint towards a classic role of TGF-b secretion from glia to neurons. Additionally, what will happen if they over-express babo-ca in the sensory neurons, at the background of some TGF-b KD in WG? could they rescue the degeneration phenotype?

* Some data is not well presented -
Fig 3: This is a key figure and I don't get it. I cannot see real differences so please convince me and any random reader that there are real differences.

Fig 4F is not well annotated - figure or legend does not mention Gal80ts.
Fig 6: They examine the effect of TGF-b KD on the morphology of WG, but it's hard to see something in this resolution. How is this quantified? To me, SmoxRNAi using WG at 4 and 28days actually do seem to affect glia.

* Figure 5 focuses on babo-gal4. All Gal4, including babo-gal4s are not really great reporters of gene expression. Thus, either prove that babo is indeed expressed there (endogenous KI of GFP or another reporter, or Gal being transcribed within the same transcript as babo - such as in the mimic lines; or antibody - yes, I know they are shitty). Alternatively, I would greatly tone down claims here. Also - usually naming panels A' and A' means they are different channels of the same experiment. It is confusing and unnecessary to use the labeling system here.

* I find the last section about Wlds confusing (figure 7). The entire screen was done on a Wlds background so what is the logic in even doing this experiment and what does it really show?

* The abstract claims that TGFbeta is required in glia cell-autonomously. I am not sure that data is convincing in this regards.

Minor comments:

* Discussion is extremely long, ideally should be shortened and sub-divided it into sub-sections with strengthened logics.

* a table containing full genotypes would be of great help, especially since the annotation is often not great.

* figure 3: why not conclude the screen results in the table?

* figure2B - I think there is a type - reaper does not seem relevant here...

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

What is the role of glia in neuronal survival? The authors address this important topic in a Drosophila model of nerve injury that offers several clear experimental advantages including the ability to clearly visualize individual neuronal cell bodies and their axons. That glia can provide pro-survival cues to axons has been established in other studies. Here the authors use their model to make an important conceptual advance is in showing that the ability of WldS (a transgene thought to protect axons via its NAD-boosting NMNAT activity) to protect axons is diminished by genetic ablation of wrapping glia. In other words, even a supposedly axon-intrinsic protective mechanism requires a cue from glia. This is similar in theme to a manuscript on BioRxiv from Chun Han's group showing that neighboring glia are required for fly dendrite degeneration following injury. The key question then becomes identifying this cue. The authors make use of several new genetic tools to perform an RNAi screen of factors expressed in wrapping glia that, when deleted, impair survival of the axons that they ensheath.

This manuscript contains several exciting observations which summarized below, that once fleshed out, will represent important contributions to the fields of neuron-glia interactions and axon degeneration. In its current form, however the lack of mechanistic insights into these observations makes this work seem preliminary.

Major points:

-What is the mechanism of TGFB in glia affecting neuron survival? Other groups have found that glial TNFa can promote neuron death, and in this case there is a clear ligand (glia)-receptor (neuron) pair that simplifies the interpretation. Here the authors have found something fascinating: many components of the TGFb pathway (ligands, receptors, downstream effectors) all have the same loss-of-function phenotype when deleted from the glia: loss of axon survival. Is this autocrine/juxtaparacrine signaling within the glial cell? The authors compellingly rule out the most obvious explanation that TGFb defects lead to loss of glial ensheathment of axons. What remains unanswered is whether TGFb itself is involved in glia-axon signaling, or whether loss of TGFb signaling impairs some other aspect of glial function that indirectly supports neuronal survival, for example the ability of glia to provide metabolites to axons. At a minimum the authors should delete these same TGFb protein in neurons to test whether these manipulations phenocopy glial deletion with respect to axon survival.

- The authors identify a fascinating paradox: WldS, long-known to protect isolated axons from Wallerian degeneration, fails to protect cut axons in the wing if wrapping glia, or various components of the TGFbeta pathway within these glia, are deleted. These findings point to a requirement for a glial-derived factor(s) to maintain axonal integrity in conjunction with NAD-mediated mechanisms. However, if these same axons are not cut (their cells bodies are present), glial TGFbeta impairment has a dramatically different outcome. Here, cell bodies are killed and an increasing proportion of axons die (see below for point on axon death quantification), and both of these events are rescued by WldS expression. Together these data, as the authors point out in their discussion, suggest a genetic interaction between glial TGFbeta, WldS activity, and the neuronal cell body. What is the mechanistic basis of this interaction between the cell body and axons? While this is undoubtedly the subject of its own study the authors should provide some characterization of why WldS can only protect when the neuron is intact. Relatedly, does the

same genetic interaction hold if the authors use Sarm mutants instead of WldS over-expressors?

Additional points:
-The quantification of axon degeneration in response to glial ablation throughout lacks statistical analysis, which is present for somatic death. This is a key point in the paper and should be addressed.

-In Figure 4F, the effect of Smox and Babo knockdown on Vglut+ neuron number is quite small, and this is notable because the effect is only measured at 14 days, versus later (28day) timepoints for the adjacent Figure 4E. The authors should investigate the magnitude of the phenotype at this 28day point.

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

The manuscript by Lassetter et al investigates the function of glia in the prevention of axonal Wallerian degeneration (WD). Severed axons with Wlds degenerate more slowly in vivo than in culture, perhaps because of glial support. The authors claim that genetic ablation of wrapping glia results in age dependent degeneration of peripheral axons and neuronal loss, and that severed wing axons that over-express Wlds degenerate more if wrapping glia are ablated. To identify genes that might be expressed in glia to protect axons from WD, they used genetics to over-express and visualise neurons with vGlutQF2>QUASmyrGFP, Wlds and concomitantly knock-down candidate transmembrane and secreted genes in glia with repoGAL4>UAS-RNAi (testing 2,000 lines and identifying 70 candidates and 141 lethals). Knockdown in glia of TGFb signalling components, including the receptor babo and downstream effector smox, caused axon degeneration 10 days post-axotomy. Babo gives a stronger effect, and they confirm these data over-expressing various RNAi and dominant negative lines in glia, both during development and in the adult. They claim that babo is expressed in wing margin glia, but babo knockdown does not affect glial morphology. Lastly, they claim that Wlds over-expression rescues the degeneration caused by babo RNAi. Unfortunately, this is a preliminary candidate genetic screen, that has not developed into uncovering a mechanistic explanation for the biological process they had set to solve. For example, there is no explanation for how knocking down in glia genes with cell-autonomous functions (TGFb receptors and effectors) and signalling within glia can influence axonal or neuronal survival. Some attention has been given to babo, but none to how its function in glia can protect neurons. Also, the work suffers from technical problems, inaccuracies and a circular argument.

Major experimental points

1. All claims of cell death must be accompanied with evidence of cell death, using apoptotic markers such as anti-Dcp1: Figure 1H, Figure 4A, Figure 7C (and counts of neuronal number when referred to in text as neuronal death Figure 4,B,D,E,F): "Neuron cell death" graphs: showing number of vGlut+ neurons, as visualised with QF2/QUAS system, is not evidence of neuron cell death. Here, cells are visualised with a promoter-based QUAS. This could be evidence of cell loss or it could mean that vGlut expression in neurons depends on signals from glia. To demonstrate and claim neuronal death, the authors must demonstrate an increased incidence of apoptosis with markers, eg DCP1.
This is particularly important because the authors say that "babo is expressed in glutamatergic neurons (Figure S5), which indicates that TGFb signals may also be received by neurons". Conceivably, expression of vGlut in neurons could depend on TGFb ligands originating from glia, and this could be revealed with glial knock-down.

Figure 1H, Figure 4A,B,D,E,F, Figure 7C: "neuron cell bodies" not shown. The authors must provide images of the neuronal cell bodies they count as evidence.

"We found that ablating WG resulted in age-dependent degeneration of axons within the L1 nerve of the wing (Figure 1D-G)... much larger proportion exhibiting mild or severe...". However, this is not shown in images in Figure 1 D,E,F: first, figure 1E is labelled with UASDronc-GFP, whereas control in D and F are labelled with vGlut-QF2>QUASmyrGFP. Thus, they are not comparable. Second, the authors must present representative images for "mild and severe" phenotypes in Figure 1G and same argument for Figure 2B.

2. Complete list of the candidate RNAi genetic screen of the 2000 lines tested must be provided with the result for each line tested, including the names of the 70 hits and the 141 lethals.

3. All constant manipulations reflect the consequences of knockdown in development and how cells adapt to those earlier challenges. They do not reflect adult functions and therefore claims cannot be made of adult gene functions from experiments in which genes were manipulated constantly throughout development. Authors need to use the language accurately and tone down claims to reflect this. To make claims specifically on age-dependent neurodegeneration and its rescue, they need to manipulate Wlds and babo only from the adult stage with tubGAL80ts for experiments in Figure 7.

5. Figure 5 does not demonstrate that babo is expressed in WG along the wing margin. To demonstrate that babo+ cells are glia, babo and glial markers must be co-detected and co-localised in the same preparation. They could do baboGAL4 x UASnuclear GFP with nuclear anti-Repo. This needs to be done in the adult wing, not larva, to match the rest of the data.

The authors use a babo-CRIMIC line that still bears 3xP3-RFP. 3xP3-RFP is expressed in glia and the authors show that it is also expressed along the wing marking. Thus, this babo-GAL4 is inappropriate in this form and the authors must remove the fluorescence data using baboGAL4 (Figure 5), remove the 3xP3-RFP from the fly stock and provide clean data.

6. Figure 6A and 6B show different effects of baboRNAi: there seem to be more axons than in control with repo>babo RNAi and fewer with WG>baboRNAi. This does not look like neurodegeneration.

7. On Figure 7A,B: "Interestingly, blocking WD rescued axon degeneration in babo knockdown animals at 28 days.... Indicating that loss of babo in glia leads to activation of the Wlds-sensitive axon death pathway in sensory neurons". However, the same genotype was used in Figures 2 and 3 (vGlutQF2-GFP, QUAS-Wlds and repoGAL4>UASbabo-RNAi), where authors claimed that after axotomy, babo RNAi caused axonal degeneration in the presence of Wlds. What is true?

Minor points:

- Abstract: data don't argue, humans argue.
Significance: "Glia a believed...". Please revise English and typos throughout.
- The term "neurodegeneration" is used rather loosely. Sometimes it is used to mean axonal degeneration without neuronal death and sometimes with cell death. This generates confusion. The authors should make more explicit the cellular processes in each case and eliminate ambiguity.
- Similarly, the term "neuronal survival" is used rather loosely, sometimes to mean cell survival and sometimes to mean prevention of axonal degeneration, or axonal survival, instead. This ambiguity should be avoided.
- "Babo-GAL4 wings also labelled nuclei within the WG membrane" is logically unsound, ie nuclei are not found in membranes.
- Should the Cajal et al 1991 citation be checked? Santiago Ramony Cajal died in 1934. Is this another person with the same (rather unusual) name?
- "Loss of glial TGFb signalling activates Wlds-sensitive axon death signalling cascade...". This sentence is logically unsound: loss cannot activate. It is not "loss" that does the activating.
- Figure 3B says "RNAi lines shown" but those in the list are not shown, ie there are no images for all of these lines.
- Data in Figure6A are a repetition of Figure 4E and should be shown only once.
- Images in Figure 6D are not representative of data shown in graphs.
- Genotypes are not written neither in text nor in figures, and this makes following the paper rather difficult. Full genotypes must be provided.
- Discussion is far too long, and not always relevant to the findings from this paper. Some aspects would have been best mentioned in the introduction instead.

1st Revision - Authors' Response to Reviewers: September 6, 2022

We thank the reviewers (and you) for the thoughtful and constructive feedback on our manuscript. We have considered all of the comments provided to us regarding the manuscript, performed many new experiments, extended our mechanistic insights, and we have included an explanation of all edits and clarifications below.

Our responses are in red.

Editor: Thank you for submitting your manuscript entitled "Glial TGFβ signaling promotes axon survival in peripheral nerves" to JCB. I am attaching the evaluations of three reviewers to this letter. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB. I have also gone over the manuscript while comparing it to the reviewers' comments. My assessment after reading the reviews together with the manuscript is that you have an exciting story, but it needs some more mechanistic insight, i.e., mechanisms through which the wrapping glia control axonal and neuronal survival, to have the interest appropriate for the cell biological readership of this journal. For example, in present form, the results do not even include epistasis data where the ligand is mutated or knocked down in wrapping glia and the receptor and/or its effectors are mutated/knocked down in the affected neurons. I am also left with the impression that clues to mechanism could be rapidly obtained by consideration of the most plausible of the 60-odd hits not described in any detail through some simple epistasis experiments. To provide some guidance, I will go through the reviewers' comments and provide my assessments of their importance from the JCB perspective.

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

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Editor: Review #1: This reviewer was obviously extremely impressed by the creativity of your system.

Major points:

* The screen was conducted with Repo-Gal4, which is not specific for wrapping glia (WG). that is OK, but I would expect that they would KD the positive hits using the WG-specific Gal4 (more than just the babo-RNAi, which they do). Knocking down genes with Repo and looking at the outcome in the adult aged wing could be problematic since they manipulate glia thought development. The order of the manuscript is a bit weird in my opinion (but this is totally up to the authors) as some of the experiments that show no developmental defect (figure 6, for example) come up very late.
Editor: I need a response regarding this reviewer's first sentence. I do not share this reviewer's

concern about the order of data in your manuscript, but hope that you will consider the reviewer's perspective.

Response: This is an excellent suggestion. We have addressed this comment by performing the proposed experiment: knocking down each of the TGFbeta superfamily genes in only WG. This new data, which supports our original conclusions, is included in the revised manuscript.

* They discover that almost all components of the TGF-b pathway are important in glia - ligands, receptors, signaling moieties and so on, and mention that this could be the result of an autocrine signaling in glia, but they don't discuss this much in their discussion. Are there known cases of autocrine signaling in glia? I would expect they would elaborate on the conventional TGF-b in glia, them being the ligand-secreting cells, and discuss why this pathway acts differently in WG to protect axons.
Editor: I think this is largely a discussion item for you to consider.

Response: We have expanded our discussion on the context for our findings regarding TGFbeta pathway acting differently in glia. Please see also below our discussion regarding roles in neurons.

* They mention that babo is also expressed in other glia and also in the sensory neurons. What happens if babo is KD in these neurons? do they degenerate after 28 days? that could hint towards a classic role of TGF-b secretion from glia to neurons. Additionally, what will happen if they over-express babo-ca in the sensory neurons, at the background of some TGF-b KD in WG? could they rescue the degeneration phenotype?
Editor: I agree completely with the importance of this set of experiments recommended by the reviewer.

Response: We have knocked down TGFbeta components in neurons instead of glia and added these results to the manuscript. The only condition that caused significant neuron loss was Smox knockdown and these results are included in Fig. S2D We agree that overexpression of a babo-CA in neurons while knocking down the ligand in WG would be an excellent experiment, this is not possible with the current tools available. Both of these tools are UAS constructs and would therefore both be expressed in any cell with Gal4 present. We would need to make a LexA/LexAop or QF/QUAS version to do this experiment.

* Some data is not well presented -
Fig 3: This is a key figure and I don't get it. I cannot see real differences so please convince me and any random reader that there are real differences.

Editor: I agree with the reviewer's assessment of the images in this figure. I also could not see real differences.

Response: We have re-arranged the figure and chose a different representation method for the images (single slice instead of compressed stack) to make the phenotypes clear.

Fig 4F is not well annotated - figure or legend does not mention Gal80ts.

Editor: I agree but very easy for you to fix.

Response: We have clarified where Gal80ts is used.

Fig 6: They examine the effect of TGF-b KD on the morphology of WG, but it's hard to see something in this resolution. How is this quantified? To me, SmoxRNAi using WG at 4 and 28days actually do seem to affect glia.

Editor: Figure 6 clearly need some expansion. At the level of resolution data is visualized in this figure, there do appear to be differences between the glia. I would agree that there are not obvious differences in glial coverage, but the images do not have the resolution to make statements about glial ensheathment. Perhaps you can provide some more details on your quantification methodology. It is not obvious to the reviewer or me how you obtained the data on the "wrapping index". Higher power images might help.

Response: We have clarified our wording about the status of glial ensheathment and provided further detail as to how we calculated the wrapping index of the electron micrographs.

*Figure 5 focuses on babo-gal4. All Gal4, including babo-gal4s are not really great reporters of gene expression. Thus, either prove that babo is indeed expressed there (endogenous KI of GFP or another reporter, or Gal being transcribed within the same transcript as babo - such as in the mimic lines; or antibody - yes, I know they are shitty). Alternatively, I would greatly tone down claims here. Also - usually naming panels A' and A' means they are different channels of the same experiment. It is confusing and unnecessary to use the labeling system here.
Editor: (concerning Figure 5): I hope you can consider this reviewer's assessment of your assertions. I agree that the labelling of the figure panels could be improved, perhaps by using A to refer to controls; B to refer to babo-Gal4? This is not a major issue.

Response: We apologize for the confusion. We realize that we were unclear in as to the nature of the *babo-Gal4* that we used. The reviewer rightly points out that some Gal4 reporters are less reliable than others in representing a given gene's expression pattern. We indeed used a Gal4 that was inserted into the *babo* gene (CRIMiC) as the reviewer suggested, but we have provided more detail for clarity. Additionally, the included supplemental table of all genotypes corresponding to each figure will help this to be clearer to readers. We have also modified our wording and conclusions from these results, and changed the panel labeling of the figure to be more straightforward.

* I find the last section about Wlds confusing (figure 7). The entire screen was done on a Wlds background so what is the logic in even doing this experiment and what does it really show?
Editor: This concern does not resonate with me.

Response: We have tried to explain more clearly our rational for these experiments and the discussion of these results. In short, an underlying notion is that any molecule required for the long-term survival of axons in a WldS background might also play a role in normal maintenance in wild type as a general glial-derived axon support mechanism. This is what prompted us to

assay neuronal preservation during normal aging. This notion seems to be borne out by our work on TGFbeta signaling.

The abstract claims that TGFbeta is required in glia cell-autonomously. I am not sure that data is convincing in this regards.
Editor: (re. abstract): I agree that "cell autonomously" is over-stated in the abstract. It is certainly plausible, but short of definitive.

Response: With regards to cell-autonomy, our intent was to convey the requirement for the TGFbeta receptor and transcription factor in glia specifically for axon maintenance. However, we agree this could be autocrine signaling or autocrine/paracrine. Our new data with neuronal loss of Smox is in line with the latter notion, but both remain possible. We have clarified our wording in the abstract as requested and throughout the text.

Minor comments:

* Discussion is extremely long, ideally should be shortened and sub-divided it into sub-sections with strengthened logics.

Response: We have trimmed down the discussion and divided into sub-sections for clarity.

* a table containing full genotypes would be of great help, especially since the annotation is often not great.

Response: We have added a supplementary table with all genotypes corresponding to figures.

* figure 3: why not conclude the screen results in the table?

Response: We have re-worked figure 3 and filled in the table.

* figure2B - I think there is a type - reaper does not seem relevant here...
Editor: Of the minor points, I agree with each of the four, but assume you can address these. They are intended to be helpful.

Response: In Figure 2B “WG-ablated” refers to animals expressing UAS-Reaper in WG using the split Gal4 expression system. We have re-labelled this figure to focus on the manipulation rather than the specific genotypes and have included the genotypes in the supplemental table of genotypes for clarity.

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

What is the role of glia in neuronal survival? The authors address this important topic in a Drosophila model of nerve injury that offers several clear experimental advantages including the ability to clearly visualize individual neuronal cell bodies and their axons. That glia can provide pro-survival cues to axons has been established in other studies. Here the authors use their model to make an important conceptual advance is in showing that the ability of WldS (a transgene thought to protect axons via its NAD-boosting NMNAT activity) to protect axons is diminished by genetic ablation of wrapping glia. In other words, even a supposedly axon-intrinsic protective mechanism requires a cue from glia. This is similar in theme to a manuscript on BioRxiv from Chun Han's group showing that neighboring glia are required for fly dendrite degeneration following injury. The key question then becomes identifying this cue. The authors make use of several new genetic tools to perform an RNAi screen of factors expressed in wrapping glia that, when deleted, impair survival of the axons that they ensheath.

This manuscript contains several exciting observations which summarized below, that once fleshed out, will represent important contributions to the fields of neuron-glia interactions and axon degeneration. In its current form, however the lack of mechanistic insights into these observations makes this work seem preliminary.
Editor: Reviewer #2: This reviewer is obviously also very impressed but notices the lack of mechanism. This reviewer's key assessment is the final sentence of the second paragraph.

Major points:

-What is the mechanism of TGFB in glia affecting neuron survival? Other groups have found that glial TNFa can promote neuron death, and in this case there is a clear ligand (glia)-receptor (neuron) pair that simplifies the interpretation. Here the authors have found something fascinating: many components of the TGFb pathway (ligands, receptors, downstream effectors) all have the same loss-of-function phenotype when deleted from the glia: loss of axon survival. Is this autocrine/juxtaparacrine signaling within the glial cell? The authors compellingly rule out the most obvious explanation that TGFb defects lead to loss of glial ensheathment of axons. What remains unanswered is whether TGFb itself is involved in glia-axon signaling, or whether loss of TGFb signaling impairs some other aspect of glial function that indirectly supports neuronal survival, for example the ability of glia to provide metabolites to axons. At a minimum the authors should delete these same TGFb protein in neurons to test whether these manipulations phenocopy glial deletion with respect to axon survival.
Editor: I think the final two sentences accurately reflect both the reviewer's and my own assessment that some more mechanistic insight is needed.

Response: It was indeed unclear from the data we presented in the initial submission whether TGFbeta activity in glia directly impacts neuron survival via direct downstream signaling or rather by impacting glial function in some way that indirectly impacts support of axon maintenance. We would argue that both are of great interest to the field, but a more direct connection is more meaningful. The reviewer suggested that we knockdown the same components in the neurons to see whether it phenocopies. We have since done this and found that knockdown of *Smox* in neurons led to a decreased neuron survival in aged animals compared to controls. This indeed argues for a neuronal role for TGFbeta signaling

components, or at least the transcription factor downstream, in neuronal survival. We have added these results and our interpretations in the manuscript.

More importantly, at this reviewer's suggestion we have investigated how loss of babo in glia may impact their metabolic support of neurons, and observed some interesting changes in metabolic function. Previous work in the *Drosophila* fat body has demonstrated that babo/smox loss in fat body resulted in increased TCA cycle function and ATP, increased mitochondrial biogenesis, which were thought to results from babo/smox regulation of a battery of metabolic genes (Ghosh & O'Connor, 2014). If acting in glia in the context of metabolic support of neurons, we predicted that loss of glial babo would lead to increased ATP and that enhancing mitochondrial biogenesis would also lead to increased glial ATP. Perhaps more importantly, we show that increasing mitochondrial biogenesis can lead to neuronal loss as animals age, similar to the babo phenotype. Finally, to provide additional evidence that neuronal loss can be caused by decreased glial support of neurons, we knocked down the monocarboxylate transporter (MCT) chaperone BSG in glia and found increased glial ATP and age-dependent neuronal loss. All of this data is included in our revised manuscript. Together these experiments support the model that TGFbeta signaling in the nerve helps balance glial metabolic support for axons.

- The authors identify a fascinating paradox: WldS, long-known to protect isolated axons from Wallerian degeneration, fails to protect cut axons in the wing if wrapping glia, or various components of the TGFbeta pathway within these glia, are deleted. These findings point to a requirement for a glial-derived factor(s) to maintain axonal integrity in conjunction with NAD-mediated mechanisms. However, if these same axons are not cut (their cells bodies are present), glial TGFbeta impairment has a dramatically different outcome. Here, cell bodies are killed and an increasing proportion of axons die (see below for point on axon death quantification), and both of these events are rescued by WldS expression. Together these data, as the authors point out in their discussion, suggest a genetic interaction between glial TGFbeta, WldS activity, and the neuronal cell body. What is the mechanistic basis of this interaction between the cell body and axons? While this is undoubtedly the subject of its own study the authors should provide some characterization of why WldS can only protect when the neuron is intact. Relatedly, does the same genetic interaction hold if the authors use Sarm mutants instead of WldS over-expressors?
Editor: Much of this paragraph is really asking for discussion. Any thoughts you have on the mechanistic interactions between glial TGFbeta, WldS activity and the neuronal cell body would be welcome. The suggested use of Sarm mutants would obviously be interesting, but is not something the JCB would insist on.

Response: We agree with the reviewer that this is a very interesting observation. To further explore this biology, we have performed additional experiments to assess whether directly promoting cell survival (i.e. by inhibiting caspase activity, see Fig. 8) would reciprocally rescue axon survival. To our surprise, it did. Our revised working model is that loss of TGFbeta signaling in glia results in decreased neuronal support causing neurons to be more susceptible to degeneration with age. Either the axon can degenerate first (and be rescued by WldS) or the cell body can (and be rescued by caspase blockade), and these cellular compartments seem interdependent—if one compartment degenerates the other follows.

Examining this phenomenon in dSarm mutants would be an excellent experiment, and we would anticipate this to phenocopy the Wlds rescue. Due to technical limitations, however, this is not

feasible due to the complex genetic backgrounds that would be necessary. Specifically, dSarm mutant animals are homozygous lethal in *Drosophila* so we would have to incorporate MARCM or another clonal strategy in combination with the glial RNAi and titrate the clone numbers to be sufficiently high enough to detect a difference in neuron survival at 28 days. This was not possible technically, but we thank the reviewer for the thoughtful suggestion.

Additional points:
-The quantification of axon degeneration in response to glial ablation throughout lacks statistical analysis, which is present for somatic death. This is a key point in the paper and should be addressed.

Response: We have revised our quantification of axon degeneration and used Fisher's exact or Chi-square tests to statistically analyze differences between conditions.

-In Figure 4F, the effect of Smox and Babo knockdown on Vglut+ neuron number is quite small, and this is notable because the effect is only measured at 14 days, versus later (28day) timepoints for the adjacent Figure 4E. The authors should investigate the magnitude of the phenotype at this 28day point.
Editor: Additional points: Both of these seem quite reasonable. I was also struck by the small differences in Figure 4F. Why wasn't this panel extended to 28 days? It might have been more compelling.

Response: The experiments presented in Figure 4F were performed in a Gal80ts background to achieve adult-specific knockdown which requires shifting the animals to 31C as adults. The combination of genetic background and the high temperatures made it so that nearly all animals died before ~21 days post-eclosion. This prevented us from examining nerves at 28 days as we had tried to do which is why we used the 14-day timepoint for this experiment. We have made a note of this technical limitation in the text.

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

The manuscript by Lassetter et al investigates the function of glia in the prevention of axonal Wallerian degeneration (WD). Severed axons with Wlds degenerate more slowly in vivo than in culture, perhaps because of glial support. The authors claim that genetic ablation of wrapping glia results in age dependent degeneration of peripheral axons and neuronal loss, and that severed wing axons that over-express Wlds degenerate more if wrapping glia are ablated. To identify genes that might be expressed in glia to protect axons from WD they used genetics to over-express and visualise neurons with vGlutQF2>QUASmyrGFP, Wlds and concomitantly knock-down candidate transmembrane and secreted genes in glia with repoGAL4>UAS-RNAi (testing 2,000 lines and identifying 70 candidates and 141 lethals). Knockdown in glia of TGFb signalling components, including the receptor babo and downstream effector smox, caused axon degeneration 10 days post-axotomy. Babo gives a stronger effect, and they confirm these data over-expressing various RNAi and dominant negative lines in glia, both during development and in the adult. They claim that babo is expressed in wing margin glia, but babo knockdown does not affect glial morphology. Lastly, they claim that Wlds over-expression rescues the degeneration caused by babo RNAi. Unfortunately, this is a preliminary candidate genetic screen, that has not developed into uncovering a mechanistic explanation for the biological process they had set to solve. For example, there is no explanation for how knocking down in glia genes with cell-autonomous functions (TGFb receptors and effectors) and signalling within glia can influence axonal or neuronal survival. Some attention has been given to babo, but none to how its function in glia can protect neurons. Also, the work suffers from technical problems, inaccuracies and a circular argument.
Editor: Reviewer #3: This reviewer, while a bit less charitable, also shares the assessment with the second reviewer that more mechanistic insight is needed.

Major experimental points

1. All claims of cell death must be accompanied with evidence of cell death, using apoptotic markers such as anti-Dcp1: Figure 1H, Figure 4A, Figure 7C (and counts of neuronal number when referred to in text as neuronal death Figure 4,B,D,E,F): "Neuron cell death" graphs: showing number of vGlut+ neurons, as visualised with QF2/QUAS system, is not evidence of neuron cell death. Here, cells are visualised with a promoter-based QUAS. This could be evidence of cell loss or it could mean that vGlut expression in neurons depends on signals from glia. To demonstrate and claim neuronal death, the authors must demonstrate an increased incidence of apoptosis with markers, eg DCP1.
This is particularly important because the authors say that "babo is expressed in glutamatergic neurons (Figure S5), which indicates that TGFb signals may also be received by neurons". Conceivably, expression of vGlut in neurons could depend on TGFb ligands originating from glia, and this could be revealed with glial knock-down.
Editor: 1. While I do not know of evidence that Babo regulates vglut, is there evidence that rules this out in the literature? It is a reasonable question.

Figure 1H, Figure 4A,B,D,E,F, Figure 7C: "neuron cell bodies" not shown. The authors must provide images of the neuronal cell bodies they count as evidence.

"We found that ablating WG resulted in age-dependent degeneration of axons within the L1 nerve of the wing (Figure 1D-G)... much larger proportion exhibiting mild or severe...". However, this is not shown in images in Figure 1 D,E,F: first, figure 1E is labelled with UASDronc-GFP, whereas control in D and F are labelled with vGlut-QF2>QUASmyrGFP. Thus, they are not comparable. Second, the authors must present representative images for "mild and severe" phenotypes in Figure 1G and same argument for Figure 2B.

Response: Dcp1 staining would be the ideal way to address this reviewer's concern, however we would need to stain the distal region where the neuron cell bodies reside, and we have not been successful in doing so in that region thus far. Antibody staining in the wing is extremely poor. We have gotten it to work for some robust antibodies like anti-Repo but only in the region proximal to the thorax of the wing nerve very close to the cut site (see Fig. 5C-D).

We believe that rescue of decreased neuron cell numbers in aged babo knockdown animals with Wlds and (now in the revised manuscript) the caspase inhibitor P35 overexpression (see Fig 7-8) indicates that pro-degenerative pathways are activated in these neurons. That P35 suppresses this phenotype argues for a direct role for caspases. Additionally, we see evidence of cell corpses as pictured in S1 that appear morphologically identical to dying neurons when NMNAT is knocked down in neurons from other projects in the lab. We have not positively identified this as apoptotic cell death specifically, which is why we had chosen to refer to it more generally as cell death. We feel that reasonably addresses the concern, but are also open to the possibility of using the phrase "cell loss" rather than cell death.

To the possibility of vGlut expression being regulated by TGFbeta ligands from glia, we have not seen evidence to suggest this. Nevertheless, we have noted this possibility in the results. We think this is unlikely, given the fact that loss of the ligands and receptors selectively in glia, lead to the neurodegenerative phenotype (i.e. not just the ligands).

Figure 1H corresponds to Figure 1D-F where the cell bodies are shown in box 2 on the right. We have modified the labeling to make this clearer. Figure 7C corresponds to Figure 7A and the cell bodies are shown to the left of the axons.

The labeling for Figure 1 was confusing and we have revised this labeling to reflect what is shown. Figure 1D-F are all showing WG>tdTomato and VGlut>mCD8::GFP. This is also clarified in the included table of full genotypes for each figure. Figure 1G corresponds to Figure 1D-F box 1 showing the axons that were classified. S2 shows the variety of phenotypes used for classification into intact, mild, or severe.

2. Complete list of the candidate RNAi genetic screen of the 2000 lines tested must be provided with the result for each line tested, including the names of the 70 hits and the 141 lethals.
Editor: 2. This could be provided in a supplemental table. I think the major item to provide would be summary data on the 70 hits. What other pathways may be implicated? Are there clues to TGF-beta's mechanism of action on glia and/or neurons from this screen?

Response: We failed to upload the complete list of RNAis used for the genetic screen during our original submission. Our apologies for that oversight. This information is included as a spreadsheet in the resubmission and all screen results are included. We hope this will be a useful resource for the field. We also understand that *Drosophila* genotypes can get quite

complicated, but the details are crucial. In the revised manuscript we included a supplemental table of all genotypes corresponding to each figure.

3. All constant manipulations reflect the consequences of knockdown in development and how cells adapt to those earlier challenges. They do not reflect adult functions and therefore claims cannot be made of adult gene functions from experiments in which genes were manipulated constantly throughout development. Authors need to use the language accurately and tone down claims to reflect this. To make claims specifically on age-dependent neurodegeneration and its rescue, they need to manipulate Wlds and babo only from the adult stage with tubGAL80ts for experiments in Figure 7.
Editor: 3. I agree but assume you can easily make appropriate textual modifications.

Response: We have clarified the wording and our meaning by “age-dependent” throughout as opposed to “adult-specific.” The reviewer rightly points out that knockdown occurs throughout development and in the mature fly. Our meaning here by “age-dependent” refers to the degeneration that occurs only in animals that have also been aged. Because Wlds and the RNAi are driven by different expression systems (QF vs Gal4), we do not have a way to temporally control both simultaneously. We agree that this would be the ideal experiment. Nonetheless, the combination of babo knockdown and aging of the animals is what results in neuron loss, hence our use of the term “age-dependent degeneration.” We are open to suggestions as to different wording to convey this result if requested.

5. Figure 5 does not demonstrate that babo is expressed in WG along the wing margin. To demonstrate that babo+ cells are glia, babo and glial markers must be co-detected and co-localised in the same preparation. They could do baboGAL4 x UASnuclear GFP with nuclear anti-Repo. This needs to be done in the adult wing, not larva, to match the rest of the data.

Response: Although, as mentioned above, this is a very difficult staining protocol, we were able to detect co-localization of anti-Repo with a nuclear-mCherry driven by babo-Gal4CRIMIC (no antibody and 3xP3-RFP was excised) as proposed by the reviewer in the proximal region of the L1 nerve. This data is included in Figure 5C-D. The immunofluorescence is somewhat messy due to technical limitations of the tissue (the nerve is embedded within an extremely thick cuticle), however, co-localization is evident.

The authors use a babo-CRIMIC line that still bears 3xP3-RFP. 3xP3-RFP is expressed in glia and the authors show that it is also expressed along the wing marking. Thus, this babo-GAL4 is inappropriate in this form and the authors must remove the fluorescence data using baboGAL4 (Figure 5), remove the 3xP3-RFP from the fly stock and provide clean data.
Editor: 5. Please consider this.

Response: We removed the LoxP flanked 3xP3-RFP using Cre and repeated the nuclear-mCherry expression experiments in the wing. Figure 5 and S3 have been updated with the new images and the 3xP3-RFP supplemental figure has been removed as it is no longer relevant. Thank you for pointing this out.

6. Figure 6A and 6B show different effects of baboRNAi: there seem to be more axons than in control with repo>babo RNAi and fewer with WG>baboRNAi. This does not look like neurodegeneration.
Editor: 6. Please consider this perspective.

Response: Figure 6A the image for babo-RNAi the fluorescence was particularly bright and this obscured the debris in the flattened stack. We used a different image that is more representative of the observed phenotype.

7. On Figure 7A,B: "Interestingly, blocking WD rescued axon degeneration in babo knockdown animals at 28 days.... Indicating that loss of babo in glia leads to activation of the Wlds-sensitive axon death pathway in sensory neurons". However, the same genotype was used in Figures 2 and 3 (vGlutQF2-GFP, QUAS-Wlds and repoGAL4>UASbabo-RNAi), where authors claimed that after axotomy, babo RNAi caused axonal degeneration in the presence of Wlds. What is true?
Editor: 7. This comment is reasonable, but I am guessing that the difference is due to levels of Wlds expression. Perhaps you can include a response for the reviewers in a letter accompanying a revision.

Response: We understand that this was likely to cause some confusion. We discuss the differences of these experiments in the discussion to clarify these results, however, we have also refined our wording in the results section for clarity. Ultimately, the experimental insults to the neuron are different in these manipulations. The first was loss of babo in glia combined with loss of the entire neuron's cell body whereas in Fig. 7 the neuron still has a cell body to support its axon when babo is lost in glia and when Wlds is present this is sufficient to prevent axon and cell loss. However, when the axon is not attached to a cell body, WldS (which in control animals prevents axon degeneration) is insufficient to prevent degeneration if either the glia are gone, or if they lack babo. The primary factor that is different in these two experiments is the presence or absence of the neuron cell body (where it likely gets its supply of the pro-survival factor dNmnat).

Minor points:

- Abstract: data don't argue, humans argue.
Significance: "Glia a believed...". Please revise English and typos throughout.
- The term "neurodegeneration" is used rather loosely. Sometimes it is used to mean axonal degeneration without neuronal death and sometimes with cell death. This generates confusion. The authors should make more explicit the cellular processes in each case and eliminate ambiguity.
- Similarly, the term "neuronal survival" is used rather loosely, sometimes to mean cell survival and sometimes to mean prevention of axonal degeneration, or axonal survival, instead. This ambiguity should be avoided.
- "Babo-GAL4 wings also labelled nuclei within the WG membrane" is logically unsound, ie nuclei are not found in membranes.
- Should the Cajal et al 1991 citation be checked? Santiago Ramony Cajal died in 1934. Is this another person with the same (rather unusual) name?

- "Loss of glial TGFb signalling activates Wlds-sensitive axon death signalling cascade...". This sentence is logically unsound: loss cannot activate. It is not "loss" that does the activating.
- Figure 3B says "RNAi lines shown" but those in the list are not shown, ie there are no images for all of these lines.
- Data in Figure6A are a repetition of Figure 4E and should be shown only once.
- Images in Figure 6D are not representative of data shown in graphs.
- Genotypes are not written neither in text nor in figures, and this makes following the paper rather difficult. Full genotypes must be provided.
- Discussion is far too long, and not always relevant to the findings from this paper. Some aspects would have been best mentioned in the introduction instead.

Please consider each of these minor comments. They are generally helpful for clarifying the manuscript.

Response: each of these points have been addressed as described below.

- We have modified wording and fixed typos.

- We explicitly state axon versus cell death/loss rather than using neurodegeneration.

- We have changed the wording to “surrounded by GFP+ WG membrane”

- We have clarified that this is a translation of Cajal’s publication

- We have revised the wording regarding activation of a Wlds-sensitive cascade.

- We have reformatted this figure.

- Figure 6A and 4E are different datasets, this is clarified by the supplemental table of genotypes in figures.

- We have included a table of all genotypes corresponding to each figure.

- We have revised the discussion and moved some information to the introduction as seen fit.

Editor: To summarize, the consensus is that this is a start to a very important study, but some mechanistic insight needs to be included. In particular, I would encourage you to consider the many hits not discussed and determine whether one or more of them does not provide a rapid avenue to provide this insight.

I do encourage a revision of a revised manuscript and will provide leeway on timing. Please do not do this prematurely, since the JCB policy does not permit two revisions in instances where there are still major issues in the initial revised submission. If you want to discuss this letter or the reviewers' comments, please contact me by email (louis.reichardt@ucsf.edu) and we can find a mutually convenient time.

Summary:

We have worked to address all of the feedback in this revised submission. The revisions not only include clarifying language and specific conclusions from the data, but also significant new experimental data that furthers the mechanistic insights into how loss of TGFbeta activity in glia affects glial function and ultimately neuron maintenance. We provide evidence for neuron death specifically by rescue of neuron cell body loss by caspase inhibition. We knocked down TGFbeta superfamily components in WG specifically as well as in neurons and demonstrate roles in both cell types. As requested, we removed the 3xP3-RFP from the babo-Gal4 genetic background and repeated the expression experiments, finding that babo reporter expression was indeed present in WG in the adult nerve. We also confirmed Repo co-localization.

Perhaps most importantly, we investigated how inhibition of TGFbeta activity in glia altered their metabolic function. Loss of babo/smox in *Drosophila* fat body leads to changes in the expression of a suite of metabolic genes, increased TCA cycle function and ATP, and increased mitochondrial biogenesis. Intriguingly, we found that glial knockdown of babo also led to increased ATP, and that forcing increased mitochondrial biogenesis (by overexpression of Srl) also led to increased ATP and ultimately age-dependent neuronal loss similar to that observed when TGFbeta signaling is depleted from glia. Finally, we have shown that knockdown of the key MCT chaperone Bsg also leads to increased ATP and age-dependent neuronal loss. Given the well-known roles for MCTs in metabolically supporting axons/neurons (e.g. lactate delivery), we propose a model whereby neuron-glia TGFbeta signaling helps balance glial metabolic support of neurons/axons and thereby promotes long term survival.

October 5, 2022

RE: JCB Manuscript #202111053R

Dr. Marc R Freeman
Oregon Health & Science University
Vollum Institute, Oregon Health & Science University
3181 SW Sam Jackson Park Road
Portland, OR 97239-3098

Dear Marc,

Thank you for submitting your revised manuscript entitled "Glial TGFβ signaling promotes neuronal survival in peripheral nerves". We would be happy to publish your paper in JCB pending 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.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

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 also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) 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.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) 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.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-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.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Louis Reichardt
Monitoring Editor

Andrea L. Marat
Senior Scientific Editor

Journal of Cell Biology

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

I liked the study in its first version and the authors have nicely addressed my comments. The question raised by reviewers 2+3 about a deeper mechanistic analysis is understandable but is often extremely difficult in these in vivo systems especially given the involvement of two distinct cellular populations. I personally think that the paper presented sufficient novelty both in terms of tools and experimental strategy and also in terms of new biological findings - yes, with many open questions remaining. Nevertheless, given the requirement for better mechanistic understanding, the authors now present a new direction in which they show that the TGF-β perturbations result in increased glial ATP levels. They also show that increasing ATP levels in glia by other means also affects axonal health. How increased ATP levels in glia affect axon health remains still open. Obviously, glia are know to provide metabolic support to neurons but how increased glia ATP is deleterious to neurons is not trivial. Is neuronal ATP reduced? or do glia now generate increased ROS? The path to a better mechanistic understanding never ends. Which is why I would also be open with keeping this last figure for a subsequent, more mechanistic study.

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

The authors have nicely addressed all of my concerns. This works will complement a growing and exciting literature on glia-axon signaling.