Deacetylation of Miro1 by HDAC6 blocks mitochondrial transport and mediates axon growth inhibition

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March 7, 2017

Re: JCB manuscript #201702187

Dr. Jeffery L Twiss
Univ South Carolina
Department of Biological Sciences
Columbia, SC 19104

Dear Dr. Twiss,

Thank you for submitting your Article manuscript entitled "CNS growth inhibitory substrates decrease axonal mitochondria transport through HDAC6" to The Journal of Cell Biology. As part of our normal reviewing procedure, your paper has been evaluated by at least two editors, including Zu-Hang Sheng, and an editorial statement is provided below. You will see that, in the consensus opinion of our editors, although we are interested in the concepts presented in this study, the manuscript is too preliminary for external review. We have thus decided not to subject your manuscript to a lengthy review process. We would be willing to consider a revised manuscript containing data addressing the detailed editorial comments below, assuming the novelty of the findings has not been compromised in the interim.

Because The Journal of Cell Biology addresses a wide and diverse audience of cell biologists, we must give priority to manuscripts that provide a substantial advance of broad appeal to the cell biology community, even though many others also present interesting and important advances for researchers in a particular field.

I am sorry that our answer on this occasion is not more positive, and I hope that this outcome will not dissuade you from submitting other manuscripts to us in the future.

Thank you for your interest in The Journal of Cell Biology.

With kind regards,

Jodi Nunnari, PhD
Editor-in-Chief
The Journal of Cell Biology

Editorial Statement:
HDAC6 is known to act as an α-tubulin deacteylase and inhibition of HDAC6 has been shown to promote neurite/axonal growth in the presence of CNS growth inhibitors like MAG and the chondroitin sulfate proteogylcans (CSPGs). However, the mechanism of HDAC6's action in the axon is still not fully understood. In this study, the authors provide evidence to suggest that inhibition of HDAC6 alters tubulin acetylation primarily in the distal axon and that this influences mitochondrial transport leading (somehow) to increased axonal growth in the presence of inhibitors like MAG and CSPGs. In addition, they suggest that HDAC6 may act downstream of Ca2+ flux and RhoA/ROCK signaling.

The authors' labs and several other groups previously reported that HDAC6 inhibition leads to increased axonal growth/regeneration on inhibitory substrates (for example Rivieccio et al., PNAS 2009). HDAC6 inhibition has also been shown to regulate mitochondrial transport/trafficking in axons (Biochim Biophys Acta. 2015 Nov;1852(11):2484-93; Mol Brain. 2016 Aug 17;9(1):79; and PLoS One. 2012;7(8):e42983) while the roles of Ca2+ flux and RhoA/ROCK signaling are well-established in axonal growth inhibition. Thus, the main novelty of the current study is to link these well-characterized pathways together. Given recent three reports consistently showing the importance of mitochondrial transport in promoting axonal growth and regeneration (Zhou et al., JCB 2016; Cartoni et al., Neuron 2016; Han et al., Neuron 2016), the current study seems rather limited to advance the field. In addition, the authors mainly examined axonal retraction in correlation of mitochondrial recovery in the distal axons in the presence of CNS growth inhibitors. The study did not show whether axon growth or regeneration could be rescued on non-permissive substrates by inhibiting HDAC6 and by recovering mitochondrial transport. The actual mechanistic links between Ca2+ flux, RhoA/ROCK activity, HDAC6 activity, and mitochondrial motility is still not fully elucidated. Therefore, it is questionable whether this study provides a substantial advance of broad appeal to the cell biology community. However, both editors including Zu-Hang Sheng made consensus recommendation that JCB would reconsider this paper if the authors can expand their study by (1) showing rescued axonal growth (not only retraction / expansion of growth cones) on non-permissive substrates by inhibiting HDAC6 and by recruiting mitochondria into growth cones, and (2) providing mechanistic links of these proposed pathways in regulating mitochondrial transport.

Please be assured that this decision is not a reflection of a lack of interest in the topic nor does it reflect any concerns about the technical aspects of the work, and we hope that this decision will not dissuade you from submitting your work to the JCB in the future.

March 22, 2018

Re: JCB manuscript #201702187R-A

Dr. Jeffery L Twiss
Univ South Carolina
Department of Biological Sciences
Columbia, SC 19104

Dear Jeff,

Thank you for submitting your manuscript entitled "Deacetylation of Miro1 attenuates mitochondrial trafficking in response to CNS growth inhibitors". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express considerable interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

We encourage you to address all concerns/issues raised by both reviewers. In particular, you should:
(1) Reassess mitochondrial transport using classical transport assays. Reviewer 1 has major concerns on the approach in assessing mitochondrial transport with FRAP (point 1). (ref #1, point #1). The suggested study of characterizing mitochondrial motility (anterograde versus retrograde; motile versus stationary) will clarify whether increased capture, reduced degradation, or altered photochemistry of mitochondria contribute to local mitochondrial abundance. This new measurement would also address the reviewer 2's question regarding relative number of motile and stationary mitochondria.
(2) Address issues raised by both reviewers on Figs 4 and 5 to support your claims of mitochondrial ablation and decreased mitochondrial transport but not recovery of mitochondria in your imaging systems.
(3) Focus on the central idea of Miro acetylation by expanding Fig 8 (ref #1, point 9);
(4) Provide evidence of the efficacy and specificity of HDAC6 inhibition/knockdown (ref #2, first two bullet points);
(5) Provide explanation for why HDAC6 inhibition does not influence retrograde transport (ref #2 third bullet point);
(6) Test other concentrations of the Tubastatin A compound (ref #2).

You should also strive to address and/or rebut all of the other reviewer comments.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

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, http://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.

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.

Sincerely,

Zu-Hang Sheng, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology
ORCiD: 0000-0003-0716-9936

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

Summary:
In this manuscript, Kalinski et al. suggest that HDAC6 via modulation of Miro1 acetylation would mediate the blocking effects of various growth inhibitors on mitochondrial transport in axons. For this the authors depend on various imaging assays (most notable a FRAP recovery assay that uses

either mito-Killer Red or mito-GFP as organelle label) and pharmacological, as well as genetic modulation of HDAC activity or Miro acetylation in dorsal root ganglionic (DRG) neuron cell cultures.
Evaluation:
The idea to examine the effects of axonal growth inhibitors on mitochondrial trafficking is timely, given the series of recent papers that suggest an essential role for mitochondrial transport in allowing axonal growth in various systems. As such, I found this study's starting point interesting and believe that it addresses an important question - also, the results presented towards the end of the paper are quite interesting; however, in my view the central FRAP assay is not a reliable measure of mitochondrial transport - precise description of the results would require talking of 'recovery of mitochondrially targeted fluorescence', which seems like very weak evidence given that mitochondria are amongst the few organelle that can actually be quantitatively accounted for by light microscopy. On this basis, I cannot recommend this manuscript for publication in a leading cell biology journal in its present form, and revisions to address my concerns would have to be fairly extensive.

Major concern:
1) While I have little doubt that the authors successfully monitor and manipulate the deacetylase function of HDAC6 in their system, I have severe reservation with regards to their approach to measuring mitochondrial trafficking. I believe the appropriate way to do so is illustrated in Figure 3. Here there are no effects of the TubA treatment detectable, even though the authors write "the HDAC6-inhibited cultures showed a trend for more anterograde than retrograde mitochondrial trafficking (Figure 3C)." Perhaps I am misunderstanding something fundamental here, but my reading of the third bar graph in Fig. 3C is that there is no robust effect on transport at all - not even a 'trend' (I am not sure what this term means). Similarly, the imaging sequence and kymograph are not convincing and in any case only the basis for the quantitation that shows no effect. At the same time, there are strong effects of the same treatment on mitochondrial abundance in growth cones (Fig 3D) and on FRAP recovery (Fig 4D, some ROIs of Fig 5C). So in my view the paper provides good evidence of an effect of TubA treatment on some aspect of mitochondrial abundance and FRAP recovery, but also good evidence that this is NOT primarily mediated by altered transport - but perhaps increased capture, reduced degradation, altered photochemistry, local fluorescence protein diffusion or fluorescence protein import into mitochondria etc.. It is this disconnect that - in my view - the authors have to resolve before this paper can be further considered. I would strongly suggest using classical transport assays, rather than FRAP; as an alternative, the FRAP assay needs to be characterized in detail to understand what it measures (e.g. using conditions in which different aspects of transport, mitochondrial potential etc. are changed in a controlled manner - e.g. using the well-established Miro/Syntaphilin tool kit to modulate transport and titrated pharmacology to disrupt the cytoskeleton and mitochondrial respiration - to see how this affects the KillerRed and FRAP assays). In my estimate the latter is more effort than just doing the experiments with the straight forward particle-based analysis tools that are standard in the field since the ground-breaking work of Hollenbeck, Reynolds and colleagues.

2) Overall, Figure 8 contains the bulk of the most interesting data - so I feel, if the fundamental problem of the assay can be overcome, still the paper needs to be refocused on the central idea of Miro acetylation; probably by expanding Fig 8 into several, bringing some of these data forward in the flow of the manuscript, and removing or compressing some of the non-essential data (Fig 1 and 2, e.g. could be supplemental). At the same time, the authors have to strive to present results in a consistent format - the FRAP assay is variously reported by recovery curves or end point bar graphs, making comparison across experiments difficult (which, when done, shows substantial variability in the assay, see below).

Detailed comments:
3) Fig 3D: Here I am a bit confused - does the number of mitochondria not simply scale with growth cone size? I am pretty sure this would be true in any of the populations (i.e. if mitochondria number were plotted against growth cone area) - and it is not evidence of more transport, as doubling of mitochondrial content could probably be achieved in steady-state just by transiently increased growth cone capture or slightly altered mitochondrial life-time (after all, the DMSO-treated growth cone in SFig 1 only contains a handful of mitochondria - ten on average, SFig 3E; the figure overall is not representative of a two-fold increase). The mechanism of this increase could easily be ascertained by time-lapse imaging.

4) Fig S2: I am doubtful about the in vivo experiment (the only in vivo evidence of the paper) - how would a 2 hour local treatment with an HDAC inhibitor result in a measurable increase in mitochondria locally via a transport mechanism? Where are these mitochondria coming from (mitochondrial life time in axons probably measures in many hours)? Even if there was a retrograde signaling pathway from mid-axon to the soma, it would take more than 2 hours for new mitochondria to arrive at the site of analysis. The subsequent microfluidic system analysis (SFig 4) suggests an axonal origin of the added mitochondria also in vitro; so this could be addition due to local biogenesis or redistribution due to local capture - the latter would actually predict a distal depletion in the axon away from the treatment site, which in principle would be testable. But how does this chime with the suggested Miro-mechanisms that the paper concludes with - which in essence suggests acetylation-based regulation of Miro's calcium sensitivity, which few researchers would argue regulates mitochondrial density in mid-axon away from calcium-hotspots such as synapses?

5) Fig 4: In principle this is an impressive result - and off-target effects of the drug on the cytoskeleton are well controlled by siHDAC; my greater worry, however, are off-target effects of the CALI on the cytoskeleton, i.e. that the induced retraction is not at all due to loss of mitochondria, but to cytoskeletal damage. How is this ruled out? Can the authors target the killer red to another organelle or the cytoplasm and show that then no retraction is induced? Also, how is the problem compensated that the size of the growth cones are so different to start with and hence surface-volume ratios change - could this simply allow for more ROS-defense in the TubA and siHDAC cases, so that CALI is simply less efficient? As a side note - in Fig 4A, it seems that in the retracted DMSO case, there is almost no axon inside the ROI, because of the retraction - I assume this was taken into consideration in measuring recovery (i.e. the ROI was moved contrary to what the figure shows or only non-retracted axons were used. Also, the example for TubA here shows no recovery of mitochondria inside the ROI, so if it is representative it reinforces the idea that FRAP recovery does not equal mitochondrial repopulation.

6) Fig 5: Here again, I might not be understanding something - I believe the green CALI-recovery trace in Fig 4D (DMSO) to be essentially equivalent to the blue curve in Fig 5A (DMSO); while the imaging frequency seems to have changed, the recovery should be comparable - but in Fig. 4D it maxes out at 5% (and a decrease could be hardly measured), whereas in Fig 5A it reaches 25% (and even the 'decreased' recovery in the MAG-Fc treated group is well above the control in Fig. 4D). Were different ROIs used? In Fig 5B the control recovery value now reaches almost 75% after 16 min - suggesting that in one of these experiments at least, recovery is not recovery of mitochondrial numbers, reinforcing my doubt that this is a genuine assay of mitochondrial transport. The same fundamental variability of the assay is also apparent in Fig. 6 (compare "Cntl/DMSO" in Fig 6D and 6E). Fig 5C shows a slightly unusual analysis and lacks statistical analysis - and I believe, if a differential effect on anterograde vs. retrograde delivery is the aim, there is no alternative to

particle-resolved analysis, either using time-lapse or photo-conversion.

7) Fig 7: I would dispute the claim that the BAPTA-AM loading "does not affect mitochondrial motility under basal conditions" - the brown and blue curves clearly deviate for most of the 16 min, the effect is only gone after 10 minutes, when the statistical analysis is done.

8) JC-1 analysis: As the Sheetz and Miller paper is somewhat debated, and a correlation in any case would not necessarily imply causation, the authors should be very clear how they see the relationship between mitochondrial potential and FRAP recovery - is in their view the change in mitochondrial potential the primary effect and changes in FRAP secondary, or do they believe the increased mitochondrial potential to be the consequence of increased transport/ capture? I, at least, got lost in what the idea was - and also the discussion, which in other respects is very clear, is mute here.

9) Fig 8: In this Figure, after 7 rather leisurely figures, now a substantial amount of important data is crammed here that need to be better explained and expanded. Fig. 8A: This is just an overexpression experiment, right? - So which percentage of tubulin is "non-acetylatable or acetyl-mimetic alpha-tubulin" in this setting? This is a critical experiment, as it is central to the idea that HDAC6 effects here are not primarily on the cytoskeleton. So this analysis needs to be expanded, e.g. using the staining tools established in Fig. 1/2 and more quantitative biochemical analyses (Western blots etc.). The Western blot in Fig 8B does not seem very clear to me - the authors should explain the black smear over the AcL-Lys-IP band. How was the specificity of the two bands ascertained? Fig 8F-H: Why are only acetylation-mimics and not the acetylation-blocking mutants used in functional experiments? In any case, the basic effects of Miro acetylation on calcium sensitivity needs to be established at baseline, i.e. comparing transport between Mito-WT, Miro-KK and Miro-Q/A mutations with and without calcium release. Overall, this is the essential figure of this work, and all the previous controls and permutations (e.g. effect on the TubA and siHDAC experiments without thapsigargin) should be used with the Miro mutants that prevent and mimic acetylation - probably this figure should be expanded and split at the expense of some of the previous, less essential figures.

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

The paper by Kalinski et al. investigates the underlying molecular mechanism responsible for the effect of HDAC6 inhibition on the axon growth on non-permissive substrates. This effect seems to be related to the anterograde transport of axonal mitochondria into the growth cone. The non-permissive substrates activate RhoA activity, subsequently this leads to the release of calcium and the activation of the HDAC6 which deacetylates Miro1. The Miro/Milton complex is needed for mitochondrial transport and an acetylation mimicking form of Miro prevents the stimulus-dependent decline in mitochondrial trafficking and supports the growth on non-permissive substrates. Overall, this manuscript contains a solid piece of work using the appropriate techniques to address a very interesting and relevant question.

Major comments
- The authors use a relatively high concentration of Tubastatin (10 µM) throughout their manuscript. Despite the fact that Tubastatin is a selective inhibitor, this selectivity is concentration dependent and at 10 µM it cannot be excluded that also other HDACs will be inhibited. Did the

authors check histone acetylation after an overnight treatment with HDAC6?
- The issue of selectivity (and the possibility of off-target effects) is now tackled by downregulating HDAC6 using siRNA-based depletion of HDAC6. Before concluding that the effects observed are indeed due to HDAC6 depletion, it should be shown in the first place that the siRNA is doing what it is supposed to do. Therefore the downregulation of HDAC6 by the siRNAs should be confirmed. Moreover, this strategy is only used in the context of one aspect of the study (growth cone retraction after ablation of mitochondria). It would be interesting to see whether this siRNA strategy also confirms the other effects of Tubastatin A.
- The authors should provide a reasonable explanation why they do not see increased retrograde transport after HDAC6 inhibition (Figure 3). As HDAC6 inhibition increased acetyl-α-Tubulin and recruits dynein/dynactin, one would also expect an increase in retrograde transport. Moreover, this effect has been repeatedly reported before by different groups. Although it is suggested that Miro deacetylation possibly underlies these previously reported benefits on axonal transport, this does not clarify why in study no effects on retrograde transport are observed.

Minor comments
- The introduction/discussion should be updated and recent publications on the therapeutic role of HDAC6 inhibition should be included.
- In several cases, data are normalised to controls. It should be explained in more detail how the variation on these controls is obtained and how this normalisation influences the statistical analysis.
- Figure 1: If possible, colocalisation of HDAC6 with a growth cone marker (Panel E) should be added.
- Figure 1: It would be interesting to know what the effect is of the non-permissive substrates on HDAC6 activity and localisation.
- It is indicated that transfected HDA6 localizes to distal axons (p.6). As this is an important observation, I would suggest to include these data in the supplementary material (instead of data not shown)
- Figure 2: Although the quantification is not shown, β-tubulin immunofluorescence appears to be reduced in TubaA treated cells. This would be unexpected since HDAC6-mediated deacetylation mainly occurs on polymerized microtubules.
- Figure 3: The authors should increase the number of neurons studied in order to obtain statistically significant differences (now it is only a trend). The depicted kymographs also show a higher effect compared to the quantification. Could it be that the concentration of Tubastatin A used in this study is too high? If possible, it should be tested whether lower doses have an effect (also on retrograde transport), while it could be that 10 µM is high enough to reduce microtubule dynamics.
- Figure 3: It would be interesting to know the actual numbers of moving and stationary mitochondria.
- Figure 4: While the technique used in this figure is very nice, it appears that there are less mitochondria in the growth cones of non-retracting axons. Since the ROS damage induced with this technique requires the presence of mitochondria in the growth cone, the authors should assure that there are indeed equal amounts of mitochondria in the growth cone at the start of their experiment. This to exclude that the observed effects of Tubastatin A are due to the treatment and not because there are less mitochondria present to induce ROS damage in the first place. When looking at the images of figure S3, you do see a lot of mitochondria in the growth cone at pre-CALI, and still have retraction. Including the siRNA results in fig 4 or the use of more suitable images with enough mitochondria at pre-CALI could solve this problem.
- Figure 5 & S4: Using microfluidic chambers is it elegantly shown that MAG exposure to the axons causes mitochondrial loss in the distal part. It would be interesting to see whether MAG/TubaA exposure to the cell body affects/restores mitochondrial mobilisation from the cell body to the distal

axon.
- Figure 5: The authors show a decrease in energized mitochondria in the axon shaft. Is this effect consistent along the axon (similarly as the observed effects on recovery of mito-GFP).
- Figure 6: Panel E shows a low recovery in control conditions compared to other experiments. (~25% compared to 60% in panel D and 75% in other figures). What is the explanation for these large differences
- Figure 7. The authors should be careful with their interpretation stating HDAC6 inhibition is upstream of RhoA signalling, since their conclusions are based on the measurements of only 9 axons. Perhaps additional effects of ROCK inhibitors or Ca2+ chelators might become apparent when increasing the number of axons.
- Figure 8. There is a typo in the legend: "HDAC6-dpeendent" should be "HDAC6-dependent"
...to attenuate and mitochondrial... should be ...to attenuate mitochondrial...
- Figure 8: The quality of the blot shown in panel B should be improved as the bands in the AcLys condition is hardly visible. Why is the background so high in this last IP condition?
- Figure 8. Would it be possible to include the data on Miro-KK and mitochondrial membrane potential.
- Figure 8. The authors should attempt to show an interaction between HDAC6 and Miro1 by means of IP. (detection of HDAC6 in myc-miro1 IP and/or vice versa)

JCB manuscript #201702187RR
Kalinski et al.

Response to Reviewers

We sincerely appreciate the reviewers' and associate editor's consideration of our manuscript. We have substantially changed the manuscript to address concerns raised by adding new experiments, modifying presentation of the original data, and editing the text. We fully agree with the suggestion to expand the data on Miro1 included in the original submission. So the bulk of the new data is reflective of this, with 2 figures and 2 supplemental figures. This effort took us longer than anticipated, as we had to develop an acetyl (Ac) K105 specific antibody for Miro1. Given comments on specificity from our original submission, we have painstakingly validated this new reagent. We think that these changes together have strengthened the manuscript and we hope that the reviewers and editor will agree. We want to emphasize that although acetylation of Miro1 is reported in acetylome studies in non-neuronal cells, the possibility that this occurs in neurons and the functional consequences of Miro1 acetylation have not been reported. Consequently, we believe that these findings remain quite novel and warrant further consideration by the Journal.

We outline the changes below in reference to the Editor's and individual Reviewers' comments shown in italics.

EDITOR COMMENTS:

We encourage you to address all concerns/issues raised by both reviewers. In particular, you should:

(1) Reassess mitochondrial transport using classical transport assays. Reviewer 1 has major concerns on the approach in assessing mitochondrial transport with FRAP (point 1). (ref #1, point #1). The suggested study of characterizing mitochondrial motility (anterograde versus retrograde; motile versus stationary) will clarify whether increased capture, reduced degradation, or altered photochemistry of mitochondria contribute to local mitochondrial abundance. This new measurement would also address the reviewer 2's question regarding relative number of motile and stationary mitochondria.

We had concentrated on the FRAP assays to correlate with the initial CALI experiments. However, we do see your points to incorporate classic transport assays into the manuscript. We have now done so for the original work with *in vitro* MitoTracker labeling (new Suppl. Fig. S3), new *in vivo* analyses of mitochondrial motility in sciatic nerve axons with TMRM labeling (new Fig. 2 and Suppl. Fig. S3), and Miro1 mutant protein expression (new Fig. 7F-I and Suppl. Fig. S7A-B).

(2) Address issues raised by both reviewers on Figs 4 and 5 to support your claims of mitochondrial ablation and decreased mitochondrial transport but not recovery of mitochondria in your imaging systems.

We moved from Mito-KR to Mito-GFP specifically to avoid toxicity from the Mito-KR and possible effects for exactly the reasons raised by the reviewers. We have now included transport assays with the wild type and mutant Miro1 protein and see alterations in mitochondrial transport – these data are consistent with the recovery from FRAP and CALI. Notably, we have used different reagents to visualize mitochondria across the manuscript (5 if including the ψ_M studies). Though we cannot completely exclude differences in recovery, it seems unlikely given that the sum of the data are

consistent with our conclusion that TubA increases mitochondrial transport. This is also consistent with previous publications that we reference herein.

(3) Focus on the central idea of Miro acetylation by expanding Fig 8 (ref #1, point 9);

We agree HDAC6-dependent Miro acetylation and its effects are the most novel aspects of this work, so we have extensively edited the manuscript to focus on this issue. As noted above, we have expanded the data on Miro1 acetylation with new Figs. 6 & 7 and Suppl. Figs. S6 & S7. We also include new data from a Miro1-AcK105 specific antibody that we generated since submission of this manuscript. Both generation and validation of this antibody took substantial effort, but we believe that these new data are compelling and biochemically validate our original findings with mutant Miro1 constructs.

(4) Provide evidence of the efficacy and specificity of HDAC6 inhibition/knockdown (ref #2, first two bullet points);

We now include immunoblot data to validate efficacy and specificity of the HDAC6 knockdowns (new Suppl. Fig. S4C-D). For the inhibition, we had previously shown that 10 µM TubA inhibits HDAC6 but not other HDACs (Rivieccio *et al*., 2009, PNAS *106*, 19599-604). However, to be certain that this dose is specific in the adult PNS neurons used here, we tested effects on α-Tubulin and Histone H4 acetylation for 1-100 µM TubA (new Suppl. Fig S1B-C). The 10 µM TubA used herein does not alter histone acetylation in the primary DRG cultures (or in cortical cultures as reported in Rivieccio *et al*., 2009).

(5) Provide explanation for why HDAC6 inhibition does not influence retrograde transport (ref #2 third bullet point);

I think that we confused the reviewers and editor with our description of these data. We consistently see a decrease in number of retrogradely moving mitochondria after HDAC6 inhibition both *in vitro* (new Suppl. Fig. S3E) and *in vivo* (new Fig. 2D). We reanalyzed the *in vitro* data and now report modestly significant changes in anterograde relative to retrograde movement with TubA treatment (Suppl. Fig. S3E). Axons *in vivo* show more anterogradely than retrogradely moving mitochondria under basal conditions, but there is also an increase in anterograde and decrease in retrograde moving mitochondria with TubA treatment in these new in vivo data that approaches significance with a p value of 0.052 (new Fig. 2E). Moreover, these in vivo kinetic analyses show a significant increase in pausing of retrogradely moving mitochondria (new Fig. 2F). So we think that the sum of the data point to decreased retrograde mitochondrial movement in addition to increased anterograde movement, which is consistent with the effects on Miro1 function that we show deacetylation has in new Figs. 6-7 and Suppl. Fig. S6-7. We have emphasized this point in the revised results and discussion.

(6) Test other concentrations of the Tubastatin A compound (ref #2).

We had used 10 µM TubA in our original submission as Rivieccio et al. (2009, PNAS *106*, 19599-604) had shown that this concentration inhibits HDAC6 but not other HDACs. We now include data in Suppl. Fig. S1B-C for testing other concentrations. 10 µM is indeed specific for HDAC6.

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

The idea to examine the effects of axonal growth inhibitors on mitochondrial trafficking is timely, given the series of recent papers that suggest an essential role for mitochondrial transport in allowing axonal growth in various systems. As such, I found this study's starting point interesting and believe that it addresses an important question - also, the results presented towards the end of the paper are quite interesting; however, in my view the central FRAP assay is not a reliable measure of mitochondrial transport - precise description of the results would require talking of 'recovery of mitochondrially targeted fluorescence', which seems like very weak evidence given that mitochondria are amongst the few organelle that can actually be quantitatively accounted for by light microscopy. On this basis, I cannot recommend this manuscript for publication in a leading cell biology journal in its present form, and revisions to address my concerns would have to be fairly extensive.

We appreciate the Reviewer's comments on the timeliness of and interest in our work. We hope that the reviewer will agree that we have made extensive revisions to this manuscript to address their comments, and they will now have even more interest in our findings.

Major concerns:

1) While I have little doubt that the authors successfully monitor and manipulate the deacetylase function of HDAC6 in their system, I have severe reservation with regards to their approach to measuring mitochondrial trafficking. I believe the appropriate way to do so is illustrated in Figure 3. Here there are no effects of the TubA treatment detectable, even though the authors write "the HDAC6-inhibited cultures showed a trend for more anterograde than retrograde mitochondrial trafficking (Figure 3C)." Perhaps I am misunderstanding something fundamental here, but my reading of the third bar graph in Fig. 3C is that there is no robust effect on transport at all - not even a 'trend' (I am not sure what this term means). Similarly, the imaging sequence and kymograph are not convincing and in any case only the basis for the quantitation that shows no effect. At the same time, there are strong effects of the same treatment on mitochondrial abundance in growth cones (Fig 3D) and on FRAP recovery (Fig 4D, some ROIs of Fig 5C). So in my view the paper provides good evidence of an effect of TubA treatment on some aspect of mitochondrial abundance and FRAP recovery, but also good evidence that this is NOT primarily mediated by altered transport - but perhaps increased capture, reduced degradation, altered photochemistry, local fluorescence protein diffusion or fluorescence protein import into mitochondria etc.. It is this disconnect that - in my view - the authors have to resolve before this paper can be further considered. I would strongly suggest using classical transport assays, rather than FRAP; as an alternative, the FRAP assay needs to be characterized in detail to understand what it measures (e.g. using conditions in which different aspects of transport, mitochondrial potential etc. are changed in a controlled manner - e.g. using the well-established Miro/Syntaphilin tool kit to modulate transport and titrated pharmacology to disrupt the cytoskeleton and mitochondrial respiration - to see how this affects the KillerRed and FRAP assays). In my estimate the latter is more effort than just doing the experiments with the straight forward particle-based analysis tools that are standard in the field since the ground-breaking work of Hollenbeck, Reynolds and colleagues.

The kymograph and image sequences shown now in Suppl. Fig. S3 are individual axons. Admittedly, the differences are not marked, so we regard the quantitative analyses across multiple neurons and culture preparations as much more rigorous than these snapshots of individual axons. We have reanalyzed the *in vitro* kinetic studies for axonal mitochondria that were originally shown in Fig. 3C (now Suppl. Fig. S3E). There is no significant difference between percentages of anterogradely and retrogradely moving mitochondria under control conditions (vehicle treated); however, TubA results

in a significant difference in percent anterograde vs. retrograde movement (reported by paired T test; ANOVA with Tukey's post-hoc shows even more significance at p = 0.0008).

We also include new *in vivo* imaging data for mitochondrial trafficking in sciatic nerve axons using TMRM to label mitochondria (Fig. 2D-F and Suppl. Fig. S3F-G). These data similarly show that an increase in anterograde mitochondrial movement that approaches statistical significance (p = 0.052). There is significantly increased pausing for retrogradely moving mitochondria for the *in vivo* transport studies (new Fig. 2F). With these changes plus the significant increase in growth cone mitochondria with TubA treatment (new Fig. 2A), we reasoned that the FRAP approach would allow us to dissect subtle changes in net anterograde/retrograde movement beyond the classical transport assays.

Though we cannot completely exclude altered photochemistry, please note that we have used four different methods to label mitochondria in this manuscript, with all pointing to the same conclusion.

We now also include analyses of mitochondrial kinetics for neurons expressing Miro1 mutant proteins (new Fig. 7F-G and Suppl. Fig. S7B-C).

2) Overall, Figure 8 contains the bulk of the most interesting data - so I feel, if the fundamental problem of the assay can be overcome, still the paper needs to be refocused on the central idea of Miro acetylation; probably by expanding Fig 8 into several, bringing some of these data forward in the flow of the manuscript, and removing or compressing some of the non-essential data (Fig 1 and 2, e.g. could be supplemental). At the same time, the authors have to strive to present results in a consistent format - the FRAP assay is variously reported by recovery curves or end point bar graphs, making comparison across experiments difficult (which, when done, shows substantial variability in the assay, see below).

We have refocused the manuscript on the Miro acetylation aspects now. We have specifically expanded old Fig. 8 into two main figures and two supplemental figures (Figs. 6-7 and Suppl. Figs. S6-7). Although we see the reviewer's point on the FRAP data presented, we are concerned that we would excessively fatigue readers and reviewers by reporting every FRAP curve – moreover, many of these experiments include 4-6 variables that we have found extremely difficult to present on a single graph where the reviewer and reader can appropriately compare between conditions without major frustration. As a compromise, we have included all of the FRAP curves where conditions can easily be compared (*i.e.*, 2-3 conditions), first test for recovery of mito-GFP (new Suppl. Fig. S5A), initial CSPG exposures (new Fig. 4C), and initial BAPTA-AM experiments (new Fig. 5A). We are happy to include all of the FRAP curves in supplemental data if the reviewers feel this is needed and the Journal will permit.

Detailed comments:

3) Fig 3D: Here I am a bit confused - does the number of mitochondria not simply scale with growth cone size? I am pretty sure this would be true in any of the populations (i.e., if mitochondria number were plotted against growth cone area) - and it is not evidence of more transport, as doubling of mitochondrial content could probably be achieved in steady-state just by transiently increased growth cone capture or slightly altered mitochondrial life-time (after all, the DMSO-treated growth cone in SFig 1 only contains a handful of mitochondria - ten on average, SFig 3E; the figure overall is not representative of a two-fold increase). The mechanism of this increase could easily be ascertained by time-lapse imaging.

In new Suppl. Fig. S4A-B, we provide quantitation of growth cone area and numbers of mitochondria per growth cone at start of the CALI sequences. We fully agree that if mitochondria numbers scale with growth cone size, then the increase in growth cone size with TubA treatment would naturally correlate with more mitochondria. Thus, we strove to match initial growth cone areas at the beginning of each time lapse for these CALI experiments. It is comforting to see no statistical difference in growth cone area or mitochondrial content. We also provide more representative image sequences for the CALI experiments now (new Fig. 3A). Along with these CALI experiments, we interpret the sum of the FRAP and mitochondrial transport data to support our conclusion that the increase is driven by alterations in transport with inhibition of HDAC6 and after exposure to MAG or CSPGs.

4) Fig S2: I am doubtful about the in vivo experiment (the only in vivo evidence of the paper) - how would a 2 hour local treatment with an HDAC inhibitor result in a measurable increase in mitochondria locally via a transport mechanism? Where are these mitochondria coming from (mitochondrial life time in axons probably measures in many hours)? Even if there was a retrograde signaling pathway from mid-axon to the soma, it would take more than 2 hours for new mitochondria to arrive at the site of analysis. The subsequent microfluidic system analysis (SFig 4) suggests an axonal origin of the added mitochondria also in vitro; so this could be addition due to local biogenesis or redistribution due to local capture - the latter would actually predict a distal depletion in the axon away from the treatment site, which in principle would be testable. But how does this chime with the suggested Miro-mechanisms that the paper concludes with - which in essence suggests acetylation-based regulation of Miro's calcium sensitivity, which few researchers would argue regulates mitochondrial density in mid-axon away from calcium-hotspots such as synapses?

Admittedly, the EM data are a snapshot. In contrast to the culture preparations, the TubA is applied directly to the nerve in these studies. So we interpret the effects as occurring locally in the region or TubA exposure rather than a retrograde signaling event to the soma – i.e., we are altering mitochondrial dynamics in the region of the axons exposed to TubA. This interpretation is consistent with the localized effects seen in new Suppl. Fig. 4C-D and data now presented in new Fig. 7G and Suppl. Fig. S7D.

In terms of interpreting the effects locally in the nerve, we would capture more mitochondria in the EMs whether TubA alters anterograde transport or retrograde transport (or both), or increases stalling of mitochondria in that area. So we appreciate the reviewer pushing us to do more definitive experiments. With help of the Schiavo lab (included as authors on this revised manuscript), we directly analyzed axonal mitochondrial dynamics in vivo in the sciatic nerve in response to vehicle vs. TubA treatments. These data match our in vitro work (Fig. 2D-I & Suppl. Fig. S3F-G), so together all point to a net shift in transport to favor anterograde movement of mitochondria when HDAC6 is inhibited.

5) Fig 4: In principle this is an impressive result - and off-target effects of the drug on the cytoskeleton are well controlled by siHDAC; my greater worry, however, are off-target effects of the CALI on the cytoskeleton, i.e. that the induced retraction is not at all due to loss of mitochondria, but to cytoskeletal damage. How is this ruled out? Can the authors target the killer red to another organelle or the cytoplasm and show that then no retraction is induced? Also, how is the problem compensated that the size of the growth cones are so different to start with and hence surface-volume ratios change - could this simply allow for more ROS-defense in the TubA and siHDAC cases, so that CALI is simply less efficient? As a side note - in Fig 4A, it seems that in the retracted DMSO case, there is almost no axon

inside the ROI, because of the retraction - I assume this was taken into consideration in measuring recovery (i.e. the ROI was moved contrary to what the figure shows or only non-retracted axons were used. Also, the example for TubA here shows no recovery of mitochondria inside the ROI, so if it is representative it reinforces the idea that FRAP recovery does not equal mitochondrial repopulation.

The reviewer makes an excellent point here, and we cannot fully exclude contributions of microtubules or other cytoskeletal components for the growth cone stabilization seen with HDAC6 inhibition and depletion. Recent work from co-authors Wong, Picci, Willis and Langley also show effects of HDAC6 that support the conclusion for microtubule stabilization by HDAC6 inhibition (Wong et al., 2018 referenced herein). However, we were struck by the rapid replenishment of mitochondria in the post-ablation phase for the HDAC6 inhibited and depleted cultures. As the reviewer notes, the retraction complicates assessment of recovery, which is why we moved to using mito-GFP for subsequent studies. Mito-GFP excitation does not ablate the mitochondria, so the ROS-defense issues mentioned by the reviewer are much less of a concern with this reagent.

6) Fig 5: Here again, I might not be understanding something - I believe the green CALI-recovery trace in Fig 4D (DMSO) to be essentially equivalent to the blue curve in Fig 5A (DMSO); while the imaging frequency seems to have changed, the recovery should be comparable - but in Fig. 4D it maxes out at 5% (and a decrease could be hardly measured), whereas in Fig 5A it reaches 25% (and even the 'decreased' recovery in the MAG-Fc treated group is well above the control in Fig. 4D). Were different ROIs used? In Fig 5B the control recovery value now reaches almost 75% after 16 min - suggesting that in one of these experiments at least, recovery is not recovery of mitochondrial numbers, reinforcing my doubt that this is a genuine assay of mitochondrial transport. The same fundamental variability of the assay is also apparent in Fig. 6 (compare "Cntl/DMSO" in Fig 6D and 6E). Fig 5C shows a slightly unusual analysis and lacks statistical analysis - and I believe, if a differential effect on anterograde vs. retrograde delivery is the aim, there is no alternative to particle-resolved analysis, either using time-lapse or photo-conversion.

This is the culmination of several years of work. In 2013, we purchased a new confocal microscope shortly after the GASP detectors had become available. The detectors were replaced in mid 2014 with newer version that were much more sensitive in our hands and our lasers were modified. Consequently, different wavelengths were used for both photo-activation and excitation of the recovery phase in the CALI experiments (now Figs. 3 & Suppl. Fig. S4E-H) compared to those in the FRAP image sequences. The methods of our previous submission did not appropriately reflect this difference. We have now better described the parameters used for CALI and photobleaching, as well as acquisition of emissions in the post-CALI and post-bleaching phases of these imaging sequences on pages 29-30. Admittedly, there was also a bit of us gaining more skills with this new microscope over time that is likely reflected in the progression of these studies.

We have now include statistics for the ‘slightly unusual analysis’ that was shown in Fig. 5C of the previous submission (now included as Suppl. Fig. S5B-C). Note that there is greater variability in these values with the subdivision of the bleached ROI into 4 smaller ROIs where recovery was monitored. Nonetheless, for the control Fc + DMSO treated neurons, there is statically greater recovery in the most distal than most proximal ROI. The MAG-Fc + DMSO treated neurons show significantly less recovery in both proximal and distal ROIs than the Fc + DMSO treated neurons (so lower overall transport). TubA treatment reverses MAG effect for the recovery in the proximal ROI, with MAG + TubA neurons showing significantly more recovery in the most proximal ROI than the MAG + DMSO neurons.

As requested, we have included more standard particle-resolved analysis in Figs. 2D-F & 7F-G, and Suppl. Figs. S3C-G & S7A-D where we distinguish anterogradely and retrogradely moving mitochondria as tracked particles. Notably those in Figs. 7F-G & S7A-D include data on Miro1 acetylation that both the editor and reviewers suggested to expand.

7) Fig 7: I would dispute the claim that the BAPTA-AM loading "does not affect mitochondrial motility under basal conditions" - the brown and blue curves clearly deviate for most of the 16 min, the effect is only gone after 10 minutes, when the statistical analysis is done.

The reviewer is correct. We have adjusted the text on page 10 to reflect that initial difference in recovery with BAPTA-AM but that the curves overlap after 5 min.

8) JC-1 analysis: As the Sheetz and Miller paper is somewhat debated, and a correlation in any case would not necessarily imply causation, the authors should be very clear how they see the relationship between mitochondrial potential and FRAP recovery - is in their view the change in mitochondrial potential the primary effect and changes in FRAP secondary, or do they believe the increased mitochondrial potential to be the consequence of increased transport/ capture? I, at least, got lost in what the idea was - and also the discussion, which in other respects is very clear, is mute here.

This is an excellent point that we struggled with while interpreting these experiments. We do not know which comes first. With the revised focus on Miro1 here, we report the changes in ψ_M and do not attempt to conclude primary vs. secondary effects. Notably, the FRAP analyses were done with soluble ligands and the ψ_M analyses were a combination of soluble and substrate bound MAG-Fc and CSPGs. So the reviewer is quite correct that we should not over interpret any causation here. We now mention the Sheetz & Miller work as justification to test ψ_M, but we have not tested nor discussed causality. We hope with the substantial addition of new data on Miro1 to the manuscript that the reviewer will agree that dissecting the mitochondrial energetics is beyond the scope of this work.

9) Fig 8: In this Figure, after 7 rather leisurely figures, now a substantial amount of important data is crammed here that need to be better explained and expanded. Fig. 8A: This is just an overexpression experiment, right? - So which percentage of tubulin is "non-acetylatable or acetyl-mimetic alpha-tubulin" in this setting? This is a critical experiment, as it is central to the idea that HDAC6 effects here are not primarily on the cytoskeleton. So this analysis needs to be expanded, e.g. using the staining tools established in Fig. 1/2 and more quantitative biochemical analyses (Western blots etc.). The Western blot in Fig 8B does not seem very clear to me - the authors should explain the black smear over the Ac-Lys-IP band. How was the specificity of the two bands ascertained? Fig 8F-H: Why are only acetylation-mimics and not the acetylation-blocking mutants used in functional experiments? In any case, the basic effects of Miro acetylation on calcium sensitivity needs to be established at baseline, i.e. comparing transport between Mito-WT, Miro-KK and Miro-Q/A mutations with and without calcium release. Overall, this is the essential figure of this work, and all the previous controls and permutations (e.g. effect on the TubA and siHDAC experiments without thapsigargin) should be used with the Miro mutants that prevent and mimic acetylation - probably this figure should be expanded and split at the expense of some of the previous, less essential figures.

We apologize for cramming so much data into the previous Fig. 8. We worry that the new Figs. 5 & 6 are only somewhat less dense, but we felt it was important to group these thematic data together.

We have moved what we interpret as non-essential data to the supplemental files, and we hope that Suppl. Figs. 6 & 7 will help to ease some of the density here that the reviewer refers to.

We agree with the critical point for lack of effects for the α-tubulin mutant expression. The data for mitochondrial transport with α-tubulin mutant expression are shown in Fig. 6A in the revised manuscript. We now include immunofluorescence and immunoblots for the α-tubulin mutants as Suppl. Fig. S6A-B. The immunofluorescence shows that α-tubulin-mCherry is incorporated into the axonal microtubules for wild type, K40A, and K40Q variants expressed here (Suppl. Fig. S6A). Immunoblots show relatively equivalent expression of these α-tubulin-mCherry proteins in the DRG cultures (Suppl. Fig. S6B). We hope that the reviewer will agree with this approach.

The black smear in old Fig. 8B resulted from the best available Ac-Lys antibodies being a cocktail of mouse and rabbit IgGs (please note, that we have tested many different commercial anti-Ac-Lys antibodies). Unfortunately, this combination of mouse and rabbit antibodies wreaks havoc on subsequent immunoblot signals. I appreciate the reviewer help in pushing my lab to find a better approach. We moved to a using anti-Ac-Lys antibodies covalently coupled to magnetic beads for the precipitations. We think that this cleared up the issue (see new Fig. 6B). Further, we have included work with a new anti-Miro1-AcK105 antibody that we developed herein (new Fig. 6F-J and Suppl. Fig. S6C), and we show that HDAC6 and Miro1 co-immunoprecipitate (Fig. 6C).

We now include functional data for both acetyl-mimetic and non-acetylatable Miro1 mutants (Fig. 7F and Suppl. Fig. S7B-C). Further, we directly compare basal transport for the Miro1 wild type, KK, K105Q, K105A, K629Q, and K629A in Suppl. Fig. S7B-C (i.e., mitochondrial kinetics for cultures grown on the permissive substrate laminin).

Reviewer #2:

The paper by Kalinski et al. investigates the underlying molecular mechanism responsible for the effect of HDAC6 inhibition on the axon growth on non-permissive substrates. This effect seems to be related to the anterograde transport of axonal mitochondria into the growth cone. The non-permissive substrates activate RhoA activity, subsequently this leads to the release of calcium and the activation of the HDAC6 which deacetylates Miro1. The Miro/Milton complex is needed for mitochondrial transport and an acetylation mimicking form of Miro prevents the stimulus-dependent decline in mitochondrial trafficking and supports the growth on non-permissive substrates. Overall, this manuscript contains a solid piece of work using the appropriate techniques to address a very interesting and relevant question.

We appreciate the reviewer noting the solid nature of our work and importance of the questions addressed here. We have expanded the analyses of Miro1 here and hope that the reviewer will agree that these additions further strengthen the manuscript.

Major comments

- The authors use a relatively high concentration of Tubastatin (10 µM) throughout their manuscript. Despite the fact that Tubastatin is a selective inhibitor, this selectivity is concentration dependent and at 10 µM it cannot be excluded that also other HDACs will be inhibited. Did the authors check histone acetylation after an overnight treatment with HDAC6?

We focused on 10 µM TubA because of previous work from Rivieccio et al. (2009, PNAS *106*, 19599-604) that included Willis, Twiss, and Langley as authors. 10 µM TubA was shown to be selective for HDAC6 in that publication. However, to be certain that this specificity holds for the adult DRG neurons used here, we have performed dose response experiments to test the TubA lots for specificity. These data are now included in Suppl. Fig. S1B-C.

- The issue of selectivity (and the possibility of off-target effects) is now tackled by down regulating HDAC6 using siRNA-based depletion of HDAC6. Before concluding that the effects observed are indeed due to HDAC6 depletion, it should be shown in the first place that the siRNA is doing what it is supposed to do. Therefore the downregulation of HDAC6 by the siRNAs should be confirmed. Moreover, this strategy is only used in the context of one aspect of the study (growth cone retraction after ablation of mitochondria). It would be interesting to see whether this siRNA strategy also confirms the other effects of Tubastatin A.

We include validation of the siRNA in Suppl. Fig. S4C-D.

- The authors should provide a reasonable explanation why they do not see increased retrograde transport after HDAC6 inhibition (Figure 3). As HDAC6 inhibition increased acetyl-α-Tubulin and recruits dynein/dynactin, one would also expect an increase in retrograde transport. Moreover, this effect has been repeatedly reported before by different groups. Although it is suggested that Miro deacetylation possibly underlies these previously reported benefits on axonal transport, this does not clarify why in study no effects on retrograde transport are observed.

See response to Editor's comment # 5 above. We consistently see a decrease in retrogradely moving mitochondria after HDAC6 inhibition both *in vitro* (new Suppl. Fig. S3E) and *in vivo* (new Fig. 2D). For the *in vitro* data, there is no statistical difference when comparing the percentage of anterogradely to retrogradely moving mitochondria under control condition (DMSO treated); however, the TubA treated cultures show significant difference between anterograde and retrograde percentages by paired T test. The *in vivo* data approach significance with P = 0.052 for TubA treatment as we note in the text. There is significantly increased pausing for retrogradely moving mitochondria *in vivo* (new Fig. 2F). Finally, the Miro1^{K105Q} mutant compared to wild type Miro1 and Miro1^{K629Q} mutant shows an increased anterograde and decreased retrograde mitochondrial transport for neurons cultured on CSPGs (new Fig. 7G).

Minor comments

- The introduction/discussion should be updated and recent publications on the therapeutic role of HDAC6 inhibition should be included.

We have updated the discussion as suggested.

- In several cases, data are normalised to controls. It should be explained in more detail how the variation on these controls is obtained and how this normalisation influences the statistical analysis.

We have included details in the methods. The variation for these controls represent technical error across multiple experiments, which we feel should be included for all control data. This represents the technical error of the experiments. Since variation in the control data are carried through the normalization for statistical comparisons, the normalization does not affect the statistical analyses.

- Figure 1: If possible, colocalisation of HDAC6 with a growth cone marker (Panel E) should be added.

We have not found a consistently good marker for growth cones in rodent DRGs. We use F-actin in new Fig 4G, but this varies with growth cone morphology. We have now included images for transfected HDAC6 in new Suppl. Fig. S1A that similarly localizes to the distal axon. We have revised the test to emphasize that HDAC6 is seen in the distal axon extending beyond the neurofilament signal.

- Figure 1: It would be interesting to know what the effect is of the non-permissive substrates on HDAC6 activity and localisation.

We agree that this would be an interesting experiment. Notably, co-authors Wong, Picci, Willis & Langley recently published data showing that CSPGs and MAG stabilize HDAC6 and decrease α-TAT in distal axons relative to laminin (Wong et al., 2018). We have noted this in the discussion and emphasized that we do not know which transferase is used to acetylate Miro1. We hope the reviewers will permit us to leave uncovering the Miro1 acetyl-transferase for future work.

- It is indicated that transfected HDA6 localizes to distal axons (p.6). As this is an important observation, I would suggest to include these data in the supplementary material (instead of data not shown)

We have now included images for transfected HDAC6 in new Suppl. Fig. S1A that similarly localizes to the distal axon.

- Figure 2: Although the quantification is not shown, β-tubulin immunofluorescence appears to be reduced in TubaA treated cells. This would be unexpected since HDAC6-mediated deacetylation mainly occurs on polymerized microtubules.

These images appear to have a slight decrease in β-tubulin, however that has not been consistent across multiple images. With the new focus on Ac-Miro1, the objective of showing these images (now Suppl. Fig. S2) is to validate the increase in axonal Ac-α-Tubulin signals with HDAC6 inhibition in our hands that others have shown with HDAC6 inhibition. So we hope the reviewer and editor will agree that with refocusing this manuscript on Miro1, a full characterization of microtubule makeup after HDAC6 inhibition is beyond the scope of this manuscript and will detract from the impact of the Miro1 changes.

- Figure 3: The authors should increase the number of neurons studied in order to obtain statistically significant differences (now it is only a trend). The depicted kymographs also show a higher effect compared to the quantification. Could it be that the concentration of Tubastatin A used in this study is too high? If possible, it should be tested whether lower doses have an effect (also on retrograde transport), while it could be that 10 µM is high enough to reduce microtubule dynamics.

We have revisited the statistical analyses for this figure and now show that there are statistical differences with TubA treatment (Suppl. Fig. S3). Please do note that the kymograph is analysis of a single axon, so we have more confidence in the quantitations shown in Fig. 2A and Suppl. Fig. S3C-E. We include data in Suppl. Fig. S1B-C showing specificity for this dose of TubA.

- Figure 3: It would be interesting to know the actual numbers of moving and stationary mitochondria.

We report these numbers now in the legend of new Suppl. Fig. S3.

- Figure 4: While the technique used in this figure is very nice, it appears that there are less mitochondria in the growth cones of non-retracting axons. Since the ROS damage induced with this technique requires the presence of mitochondria in the growth cone, the authors should assure that there are indeed equal amounts of mitochondria in the growth cone at the start of their experiment. This to exclude that the observed effects of Tubastatin A are due to the treatment and not because there are less mitochondria present to induce ROS damage in the first place. When looking at the images of figure S3, you do see a lot of mitochondria in the growth cone at pre-CALI, and still have retraction. Including the siRNA results in fig 4 or the use of more suitable images with enough mitochondria at pre-CALI could solve this problem.

We have included quantifications for growth cone area and mitochondrial content for the t=0 time points in these CALI experiments (Suppl. Fig. S4A-B). Please note that we made every attempt to match initial axon size and mitochondrial content for these time-lapse experiments and this is reflected in the quantitations shown in Suppl. Fig. S4A-B. Both growth cone area and mitochondrial number showed no statistical difference between the control and HDAC6 inhibited cultures at the onset of the CALI experiments.

- Figure 5 & S4: Using microfluidic chambers is it elegantly shown that MAG exposure to the axons causes mitochondrial loss in the distal part. It would be interesting to see whether MAG/TubaA exposure to the cell body affects/restores mitochondrial mobilisation from the cell body to the distal axon.

We appreciate the reviewer raising this very intriguing possibility. We struggled to run this experiment in microfluidic chambers. Consequently, we moved to aggrecan linked to polystyrene beads that we had previously used for both assessment of RNA transport and responses to growth inhibitory substrates (Willis et al., 2007, J Cell Biol *178,* 965-80 and Rivieccio et al., 2009, PNAS *106*, 19599-604, respectively). No change in axonal mitochondrial transport was seen when the aggrecan-linked beads were adjacent to the cell body of DRGs neurons (and not contacting axons). However, when the aggrecan-linked beads lay adjacent to the axons, axonal mitochondrial motility decreased in anterograde and increased in retrograde movement (new Suppl. Fig. S7D). Expression of the acetyl-mimetic Miro1 K105Q, but not K105A or wild type Miro1, prevented this alteration in axon transport seen with axonal aggrecan stimulation (new Fig. 7G).

- Figure 5: The authors show a decrease in energized mitochondria in the axon shaft. Is this effect consistent along the axon (similarly as the observed effects on recovery of mito-GFP).

The ψ_M data in new Figs. 4D, 5E, and 7B derive from measurements of JC1 signals across the length of axons using high content imaging. Based on this, we have not dissected effects relative to the length of the axons. The new data shown in Suppl. Fig. S7D emphasize that this experiment needs to be done with localized signaling from the CNS growth inhibitors (i.e., the bead experiments mentioned above). We agree that this is an important distinction to make, but we hope that the reviewer will agree that this is beyond the scope of the present manuscript and will allow us to address this issue in future submissions.

- Figure 6: Panel E shows a low recovery in control conditions compared to other experiments. (~25%

compared to 60% in panel D and 75% in other figures). What is the explanation for these large differences

The control conditions for this experiment (Fig. 4H in the revised manuscript) shows CSPG treated neurons. So the recovery seen here is comparable to the recovery seen for the CSPG + DMSO and MAG + DMSO curves in Figs. 4C and A, respectively. We have edited the graph to clarify that CSPG exposure was used for all conditions.

- Figure 7. The authors should be careful with their interpretation stating HDAC6 inhibition is upstream of RhoA signalling, since their conclusions are based on the measurements of only 9 axons. Perhaps additional effects of ROCK inhibitors or Ca2+ chelators might become apparent when increasing the number of axons.

We apparently were not as clear as we could have been here. Our data indicate that HDAC6 is downstream of RhoA/ROCK signaling. This is based on CSPG stimulation in the presence of ROCK inhibitor (new Fig. 4H), direct stimulation of RhoA activity with Rho Activator (new Figs. 5C, E-F), CSPG stimulation in presence of BAPTA-AM Ca^{2+}-chelator (new Figs. 5A, B, F), directly increasing cytoplasmic Ca^{2+} by SERCA inhibition (new Figs. 5D-F), and combined use of Rho activator plus Ca^{2+} chelation with BAPTA-AM (new Fig. 5F). In each case, the data show that HDAC6 dependent effects on mitochondria are downstream of RhoA/Rock and Ca^{2+}. The sum of these experiments incorporates over 70 neurons imaged over 19 separate culture preparations, and required more than 190 hours of imaging to accomplish. Obviously, we cannot exclude additional effects of these pharmacological agents beyond those tested here. However, when considering the sum of the experiments, we hope that the reviewer will agree that we have not over-interpreted these data.

- Figure 8. There is a typo in the legend: "HDAC6-dpeendent" should be "HDAC6-dependent"
...to attenuate and mitochondrial... should be ...to attenuate mitochondrial...

We have fixed this.

- Figure 8: The quality of the blot shown in panel B should be improved as the bands in the AcLys condition is hardly visible. Why is the background so high in this last IP condition?

We fully agree on the quality of the immunoprecipitation blot previously shown. The Ac-Lys antibodies are a mixture of mouse and rabbit IgGs that have been optimized for mass spec experiments – unfortunately, this preparation has the highest affinity of those we have tested. This combination of rabbit and mouse heavy and light chains brings an annoyingly high background for the immunoblots. We tried to use many other anti-Ac-Lys antibodies without luck. Covalently conjugating the anti-Ac-Lys antibody cocktail to magnetic beads with elution protocols to avoid denaturation of the bead bound antibodies solved the issue (see new Fig 6B). Further, our newly generated anti-Miro1-AcK105 antibody outlined above that provides high specificity for detection of Miro1-Ac-K105 .

- Figure 8. Would it be possible to include the data on Miro-KK and mitochondrial membrane potential.

These data are now included as new Fig. 7B.

- Figure 8. The authors should attempt to show an interaction between HDAC6 and Miro1 by means of IP. (detection of HDAC6 in myc-miro1 IP and/or vice versa)

We include co-immunoprecipitation data in new Fig. 6C. Miro1 immunoprecipitates with HDAC6 and HDAC6 immunoprecipitates with Miro1. The efficiency is not great, but this is not unexpected with an enzyme-substrate pair (i.e., the interaction could be relatively evanescent).

March 12, 2019

RE: JCB Manuscript #201702187RR

Dr. Jeffery L Twiss
Univ South Carolina
Department of Biological Sciences
Columbia, SC 19104

Dear Dr. Twiss:

Thank you for submitting your revised manuscript entitled "Miro1 Deacetylation by HDAC6 attenuates mitochondrial transport in response to CNS growth inhibitors". 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, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is ~40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends. You appear to be somewhat over this limit but we feel that shortening the text would undermine the clarity of the paper so we will be able to give you a bit more space this time. However, please try not to extend the word count any further in the final revision.

2) 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, including cropped blots - please add markers to the blots in Supplementary Figures 1B and 6C.

3) 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 (both in the figure legend itself and 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, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must

state something to the effect that "Data distribution was assumed to be normal but this was not formally tested." In addition, we noticed that you used t tests in several of your figures (e.g. Figure 6B and SFig 4A-D) but these tests were not described in the "Statistical Analysis" section of your methods. Please add this information and be sure that no other statistical information is missing.

4) Title: The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.
While your current title will be appreciated by the specialists, we do not feel that it will be accessible to a broader cell biology audience. Therefore we suggest the following title: "Deacetylation of Miro1 by HDAC6 blocks mitochondrial transport and mediates axon growth inhibition".

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts.

6) 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.

7) 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.).

8) 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.

9) Supplemental materials: There are usually strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. At the moment, you currently have 7 such figures. Once again, I think that we can allow you extra space to keep the 7 figures but please do not add to this total in the final revision. 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.

10) 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."

11) 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.

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 video files: See our detailed guidelines for preparing your production-ready images, http://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.

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

Sincerely,

Zu-Hang Sheng, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology
ORCiD: 0000-0003-0716-9936

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

Jeffrey Twiss and colleagues have resubmitted their paper on Miro acetylation and mitochondrial transport in axons. I find the manuscript much improved and suitable for the Journal, after the authors have made a comprehensive effort to respond to my co-reviewer's and my previous comments. They have added exciting new data (e.g. in vivo imaging) and new tools (the new antibody) plus addressed most of the open concerns, while discussing the remaining uncertainties openly. I want to thank the authors for their constructive response to my critique; I know such revisions can be a big hassle, and hope they feel it was not a wasted effort. I would be delighted to see this paper in print in JCB soon!

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

No further comments.