PTP-3 phosphatase promotes intramolecular folding of SYD-2 to inactivate kinesin-3 UNC-104 in neurons

Muniesh Muthaiyan Shanmugam, Syed Barmaver, Hsin-Yi Huang, Odvogmed Bayansan, and Oliver Wagner

Corresponding author(s): Oliver Wagner, Institute of Molecular and Cellular Biology

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RE: E19-10-0591
TITLE: PTP-3(LAR PTPR) promotes intramolecular folding of SYD-2(liprin-α) to inactivate UNC-104(KIF1A) motors in neurons

Dear Dr. Wagner,
We are now in receipt of two reviews of your manuscript. As you can see, both reviewers find there to be interesting observations but that the manuscript will require major revisions in order to be acceptable for publication. If you decide to address the reviewer's comments, I would highlight the issues that pertain to Figures 1-4. Both reviewers expressed concerns and offered suggestions to bolster the conclusions from Figs. 2 and 3. From my own reading of the manuscript and the reviewers, I am particularly concerned with regard to the colocalization SYD-2/PTP-3B. At a minimum one would need to see a negative control. However, as indicated by Reviewer #2 the control would have to be two proteins that localize to the nerve ring, but do not associate with one another.
I hope that you find these reviews constructive in your decision to move forward with the manuscript.
Sincerely,
Kerry Bloom

Dear Prof. Wagner:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision online please use the link below, and include a cover letter that details, point-by-point, how the Monitoring Editor's and reviewers comments have been addressed. When entering the author names online, enter them exactly as they appear on the manuscript title page. Please send only the latest revised manuscript. DO NOT resend any previous versions. Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

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Resolution and Color Mode.
All images should be submitted at a minimum of 300dpi.
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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

RE: PTP-3(LAR PTPR) promotes intramolecular folding of SYD-2(liprin-α) to inactivate UNC-104(KIF1A) motors in neurons by Shanmugam et al

In this paper, the authors investigated the role of the PTP-3 phosphatase in SYD-2 mediated UNC-104 motor activation. The authors report that PTP-3 functions upstream of SYD-2 to regulate its intramolecular folding, presumable by dephosphorylation of Y741. Also, they report increased interaction between UNC-104 and SYD-2 in ptp-3 knockout worms. Based on intramolecular FRET analysis in living nematodes, the authors conclude that SYD-2 predominantly exists in an open conformation in ptp-3 mutants. Also, the authors demonstrate that in ptp-3 mutants there is an increased clustering of UNC-104 motor on vesicles, and that motor velocities increased, with affects the distribution of vesicles within the axons. In principle, these are interesting findings that can potentially broaden our understanding of how kinesin motors are regulated in an intracellular function context. However, there are major flaws in data analysis and presentation, several conclusions are not substantiated by the data. The following points should be addressed prior to publication.

Major points:
1. At the end of introduction, references to regulation of kinesin motors by Cdk1 should be added to the list of kinases that regulate kinesin functions (Blangy et al., 1995; Goldstein et al., 2019; Goldstein et al., 2017)

2. In Fig. 2, authors perform Intensity Correlation Analysis to calculate the Intensity correlation quotient (ICQ) as a measure of SYD-2/PTP-3B colocalization. Although the localization of the two proteins is scattered, the authors conclude (based on their analysis) that the SYD-2 and PTP-3 do co-localize. To substantiate this this conclusion, the authors should perform a negative control experiment to show the ICQ values for two proteins that do not co-localize.

3. On page 6: "Because SYD-2 exists in functional folded states" - it is not clear what is this statement based on. How many states? References should be added to backup this statement.

4. Fig. 3: If phosphorylation of SYS-2 facilitates open conformation (and lower FRET efficiency), how come in the ptp-mutant cells, the phospho-deficient SYD-2Y741F exhibits higher FRET than wt SYD-2?

5. Page 7: "Because unc-104 gene expression is reduced in mu256 mutants (Figure 1D), we may explain the cargo retention

phenotype partially with that finding." The authors should clarify what "that finding" mean.

6. The terms "integrated density" and "particle density" are confusing. It is not clear how the different densities are determined.

7. Page 7, last paragraph 2nd sentence starts with "as a result, ...", it is not clear as a result of what?

8. Fig. 5A, explanation of the different headings should be added. Fig. 5B and C: it is not clear what the different labels mean; also, there is no significance is indicated between the mutants and the wild-type controls. Thus, the various conclusions based on this figure are not substantiated.

9. Same comment as for Fig. 5, the different labels should be clearly explained. The acronyms are not clear.

Minor points:
1. The title is too convoluted; I suggest changing to: The PTP-3 phosphatase promotes intramolecular folding of SYD-2 to inactivate the kinesin-3 UNC-104 motors in neurons

2. In Fig. 3B change "Accepter" to "Acceptor"

3. Page 9 line 5, from the top: the phrase "are very consistent" should be rewritten.

Reviewer #2 (Remarks to the Author):

The C. elegans kinesin-3 UNC-104 is the major anterograde motor for synaptic vesicles in axons. SYD-2 (liprin-alpha), which interacts with the LAR protein tyrosine phosphatase PTP-3, is known to be a positive regulator of UNC-104. In this study Shanmugam and colleagues present in vivo evidence that PTP-3 acts in the SYD-2 pathway to negatively regulate UNC-104.

The authors find that the ptp-3 loss-of-function allele mu256 increases anterograde velocities, run length, and frequency of bi-directional movement of UNC-104 and SNB-1 (a synaptic vesicle marker), and that this effect depends on syd-2. Ptp-3(mu256) also increases the density of UNC-104 clusters in axons in a syd-2-dependent manner. Since there is evidence from a phospho-proteomic study that PTP-3 may de-phosphorylate SYD-2 at Y741, the authors examine the effect of SYD-2 phospho-mutants. The phospho-mimetic mutant Y741E, but not non-phosphorylatable Y741F, increases the density of axonal UNC-104 clusters. Using a Cypet::SYD-2::Ypet probe in vivo, decreased FRET signal is observed in the SYD-2(Y741E) mutant and in ptp-3(mu256), whereas SYD-2(Y741F) rescues the FRET signal in ptp-3(mu256). The authors propose that phosphorylation of SYD-2 at Y741, which is antagonized by PTP-3, stabilizes an open conformation of SYD-2 that results in enhanced SYD-2 binding to UNC-104 and therefore stimulates UNC-104 motility.

The data in Figures 5 and 6 support the authors' conclusion that PTP-3 acts via SYD-2 to down-regulate UNC-104 activity and that SYD-2 residue Y741 is involved. This is an interesting finding. However, the data in Figures 1-4 fails to provide sufficient evidence for the molecular mechanism proposed in Figure 7. In particular, controls are missing for the experiments in Figures 2 and 3, which complicates the interpretation of the data.

Figure 1: a key prediction of the proposed regulatory mechanism is that phosphorylation of SYD-2 at Y741 should strengthen the interaction between SYD-2 and UNC-104. The only evidence in support of this idea is a ~20% increase in the amount of SYD-2 that co-immunoprecipitates with UNC-104::GFP from ptp-3(mu256) worm lysate. The authors should make an effort to test this aspect of the model more rigorously. One approach would be to determine whether the phosphomimetic mutant SYD-2(Y741E) has increased affinity for UNC-104, either in binding assays with purified proteins, in a yeast 2-hybrid system, or in co-immunoprecipitation assays from worm lysate.

Figure 2: from the images in Figure 2A-D, it is evident that PTP-3B::CFP and GFP::SYD-2 are both present in the nerve ring, but what does this really mean? If the authors want to claim that the 'intensity correlation analysis' in 2F is indicative of close physical proximity, they should perform the assay with a negative control, i.e. with a CFP-tagged protein that is present in the nerve ring but is unlikely to closely associate with GFP::SYD-2 (or simply CFP on its own expressed in the nerve ring). The same caveat applies to the bimolecular fluorescent complementation assay in 2G and J: a control is needed in which the split Venus is present on a protein that is unlikely to directly interact with GFP::SYD-2.

Figure 3: the FRET approach to assess the conformation of SYD-2 is potentially insightful, but important controls appear to be missing: 1) another protein in which Cypet and Ypet are present in tandem to show that FRET for this probe is unaffected in the different genetic backgrounds, and 2) SYD-2 singly tagged with either Cypet or Ypet to measure the extent of fluorescence spillover into the FRET channel. Without the latter control experiments, how can the authors be sure that they are actually measuring FRET and not just spillover?

Figure 4: as the authors point out, the effect of ptp-3(mu256) on SNB-1 distribution is what would be predicted from the lower expression levels of UNC-104 shown in Figure 1, so the data is not informative with regards to the proposed activation of UNC-104 in the absence of PTP-3. As such, this figure has little relevance and could be moved to the supplement.

Minor issues:

Figure 2: the signal in 2E and 2G is very dim, and the cyan signal is virtually invisible in the merged images in 2C and 2E. Image brightness should be adjusted and a more appropriate color combination should be used (for example green and magenta).

Figure 3: the microscopy set up and the experimental protocol for the FRET analysis need to be described in more detail in the materials and methods section, including the filter sets used and the exact imaging protocol. From the current description, it is unclear what the 'Donor Cypet' and 'Acceptor Ypet' images in Figure 3B represent.

Figure 3B: the insets showing the FRET efficiency scale are too small.

Figures 5 and 6: what is the fluorescent tag on UNC-104, and what is the rationale for using the e1265 allele as the control? Were the other conditions also imaged in the e1265 background?

Figure 6: the authors should determine the effect of SYD-2(Y741E) expression on UNC-104 and/or SNB-1 motility. According to their model, the effect should be similar to that observed with the ptp-3(mu256) allele.

Reviewer #1 (Remarks to the Author):

RE: PTP-3(LAR PTPR) promotes intramolecular folding of SYD-2(liprin-α) to inactivate UNC-104(KIF1A) motors in neurons by Shanmugam et al

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We thank the reviewer for valuable suggestions that largely assist to improve the manuscript.

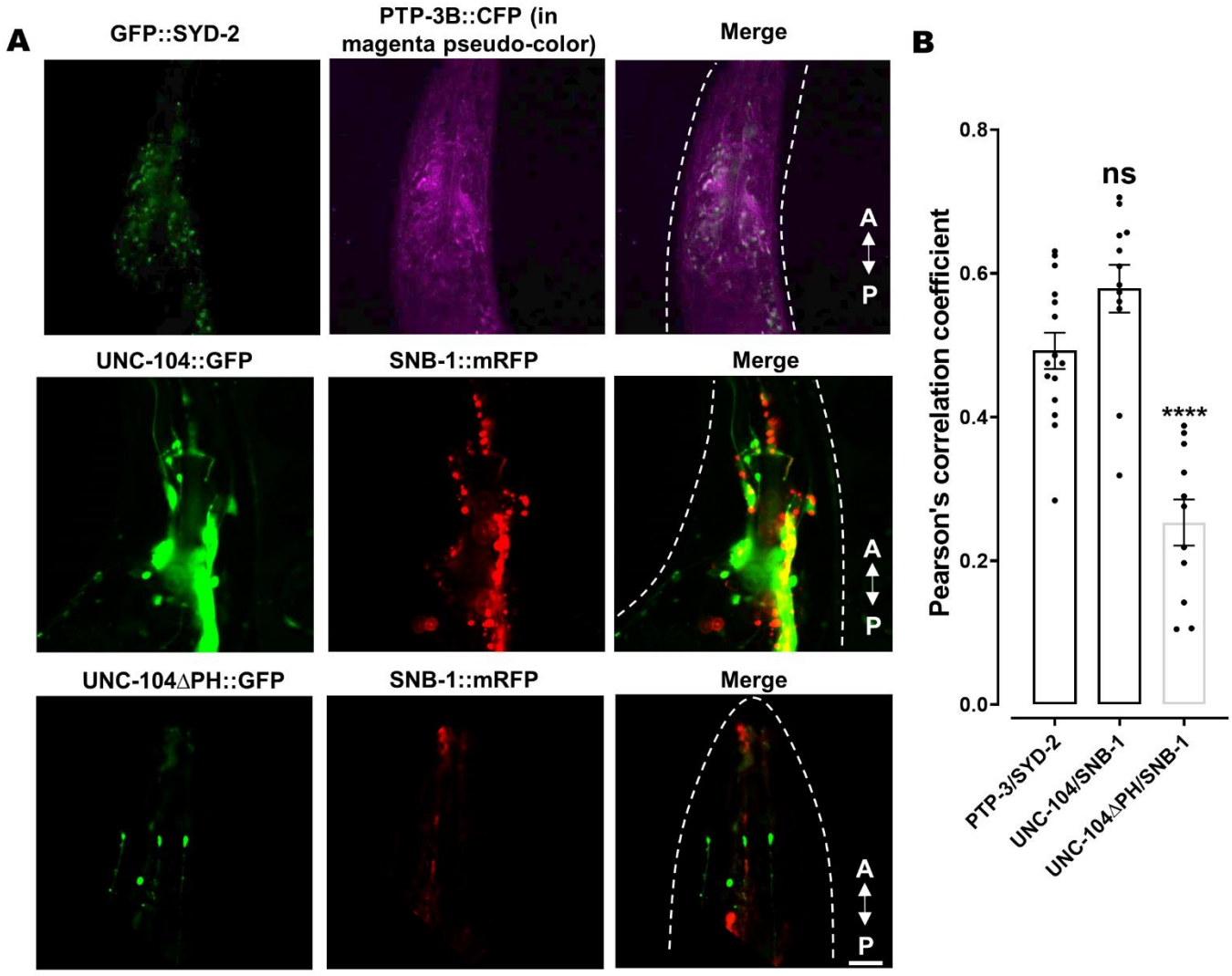
Major points:

1. At the end of introduction, references to regulation of kinesin motors by Cdk1 should be added to the list of kinases that regulate kinesin functions (Blangy et al., 1995; Goldstein et al., 2019; Goldstein et al., 2017)

Author's response: Above mentioned citations have been now included in the manuscript on page 3.

2. In Fig. 2, authors perform Intensity Correlation Analysis to calculate the Intensity correlation quotient (ICQ) as a measure of SYD-2/PTP-3B colocalization. Although the localization of the two proteins is scattered, the authors conclude (based on their analysis) that the SYD-2 and PTP-3 do co-localize. To substantiate this this conclusion, the authors should perform a negative control experiment to show the ICQ values for two proteins that do not co-localize.

Author's response: We now include a positive as well as a negative control (new Suppl. Figure 3A+B) in which we quantified colocalization of UNC-104/SNB-1 (positive control) and UNC-104ΔPH::GFP/SNB-1::mRFP (negative control). The negative control is based on the notion that the motor's PH domain is essential to bind to synaptic vesicles (e.g., Kumar, Choudhary et al. 2010). From this experiment it is evident that deletion of UNC-104's PH domain results in reduced interactions with SNB-1-containing synaptic vesicles, if comparing to the UNC-104/SNB-1 or SYD-2/PTP-3 protein pair (see below). Note that during the revision process, we decided to replace the colocalization analysis method "ICQ" by "Pearsons's" such as the latter method seems to be more robust due to its independency of relative changes in intensities of fluorophores. Also this method only calculates the coexistence of both fluorophores at a given pixel (Adler and Parmryd 2010, Sanderson 2019).



New Suppl. Figure 3A+B: Comparison of colocalization between UNC-104/SNB-1, UNC-104ΔPH/SNB-1 and SYD-2/PTP-3. Scale bar 10 µm. One-way ANOVA with Dunnett's multiple comparisons test, **** p<0.0001.

3. On page 6: "Because SYD-2 exists in functional folded states" - it is not clear what is this statement based on. How many states? References should be added to backup this statement.

Author's response: SYD-2 is proposed to exist in two structural states: 1) inactive folded state and 2) active unfolded state (Chia, Patel et al. 2013). We clarified this in the manuscript on page 6.

4. Fig. 3: If phosphorylation of SYD-2 facilitates open conformation (and lower FRET efficiency), how come in the ptp-mutant cells, the phospho-deficient SYD-2Y741F exhibits higher FRET than wt SYD-2?

Author's response: In a population of N2 worms, a certain population of (endogenous, wild type) SYD-2 will be phosphorylated while a remaining portion of (endogenous, wild type) SYD-2 remains unphosphorylated resulting in a FRET ratio of 1.8 (Figure 3). Irrespective of the availability of kinases, the phospho-deficient SYD-2Y741F cannot be phosphorylated resulting in folded conformations only. This folded phospho-deficient SYD-2Y741F protein cannot serve as a substrate for the PTP-3

phosphatase and resulting FRET ratios are (insignificantly) high whether PTP-3 is absent or present. Examples are N2[Syd-2 Y741F] with a 1.87 ratio and mu256[SYD-2 Y741F] with a 1.78 ratio. Note that the kinase that phosphorylates SYD-2 at Y741 is still unknown.

5. Page 7: "Because unc-104 gene expression is reduced in mu256 mutants (Figure 1D), we may explain the cargo retention phenotype partially with that finding." The authors should clarify what "that finding" mean.

Author's response: The expression "that finding" represents the reduced unc-104 gene expression in mu256 mutants. We clarified this issue on page 7.

6. The terms "integrated density" and "particle density" are confusing. It is not clear how the different densities are determined.

Author's response: The term "integrated density" is taken from an ImageJ option that allows for the quantification of intensities of fluorescent signals in images. The term "particle density" represents the number of fluorescent UNC-104 clusters in a measured neurite region. We clarified this issue on page 21.

7. Page 7, last paragraph 2nd sentence starts with "as a result, ...", it is not clear as a result of what?

Author's response: The phrase "As a result" indicates "As a result of our analysis". The sentence has been modified on page 7.

8. Fig. 5A, explanation of the different headings should be added. Fig. 5B and C: it is not clear what the different labels mean; also, there is no significance is indicated between the mutants and the wild-type controls. Thus, the various conclusions based on this figure are not substantiated.

Author's response: Regarding Fig. 5A, we add more details now in the figure legend. Regarding 5B and C we add "ns" to those experimental groups if comparisons with the control group are not significant (instead of no wildcard). Specific comparisons with guided lines are clearly marked with respective wildcards.

9. Same comment as for Fig. 5, the different labels should be clearly explained. The acronyms are not clear.

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Minor points:
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Author's response: The title has been modified as per this reviewer's suggestion.

2. In Fig. 3B change "Accepter" to "Acceptor"

Author's response: Figure 3B has been modified accordingly.

3. Page 9 line 5, from the top: the phrase "are very consistent" should be rewritten.

Author's response: We modified this sentence on page 9.

Reviewer #2 (Remarks to the Author):

We thank the reviewer for valuable suggestions that largely assist to improve the manuscript.

The C. elegans kinesin-3 UNC-104 is the major anterograde motor for synaptic vesicles in axons. SYD-2 (liprin-alpha), which interacts with the LAR protein tyrosine phosphatase PTP-3, is known to be a positive regulator of UNC-104. In this study Shanmugam and colleagues present in vivo evidence that PTP-3 acts in the SYD-2 pathway to negatively regulate UNC-104.

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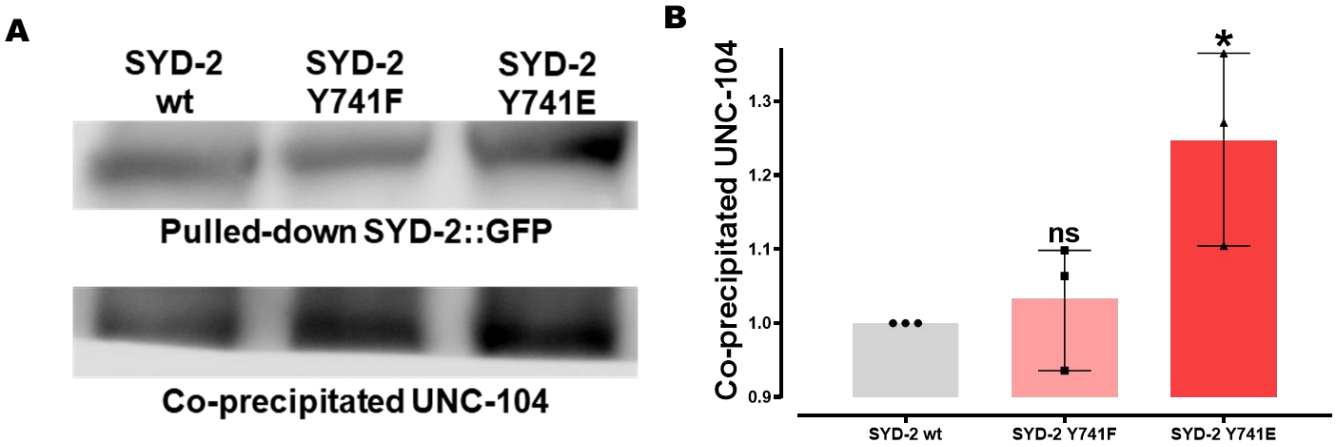
Figure 1: a key prediction of the proposed regulatory mechanism is that phosphorylation of SYD-2 at Y741 should strengthen the interaction between SYD-2 and UNC-104. The only evidence in support of this idea is a ~20% increase in the amount of SYD-2 that co-immunoprecipitates with UNC-104::GFP from ptp-3(mu256) worm lysate. The authors should make an effort to test this aspect of the model more rigorously. One approach would be to determine whether the phosphomimetic mutant SYD-2(Y741E) has increased affinity for UNC-104, either in binding assays with purified proteins, in a yeast 2-hybrid system, or in co-immunoprecipitation assays from worm lysate.

Author's response: We now add co-immunoprecipitation assays using lysates from worms expressing either phosphodeficient or phosphomimicking SYD-2. These new results demonstrate that interactions between phosphomimicking SYD-2 and UNC-104 are more robust as opposed to the phosphodeficient variant:



New Figure 5D+E: Interactions between phosphomimicking SYD-2 and UNC-104 are more robust as opposed to the phosphodeficient variant. (A) Representative blots from co-immunoprecipitation experiments. (B) Quantification of co-precipitated UNC-104. One-way ANOVA with Fishers's LSD multiple comparisons test. $p < 0.05$.

Figure 2: from the images in Figure 2A-D, it is evident that PTP-3B::CFP and GFP::SYD-2 are both present in the nerve ring, but what does this really mean? If the authors want to claim that the 'intensity correlation analysis' in 2F is indicative of close physical proximity, they should perform the assay with a negative control, i.e. with a CFP-tagged protein that is present in the nerve ring but is unlikely to closely associate with GFP::SYD-2 (or simply CFP on its own expressed in the nerve ring). The same caveat applies to the bimolecular fluorescent complementation assay in 2G and J: a control is needed in which the split Venus is present on a protein that is unlikely to directly interact with GFP::SYD-2.

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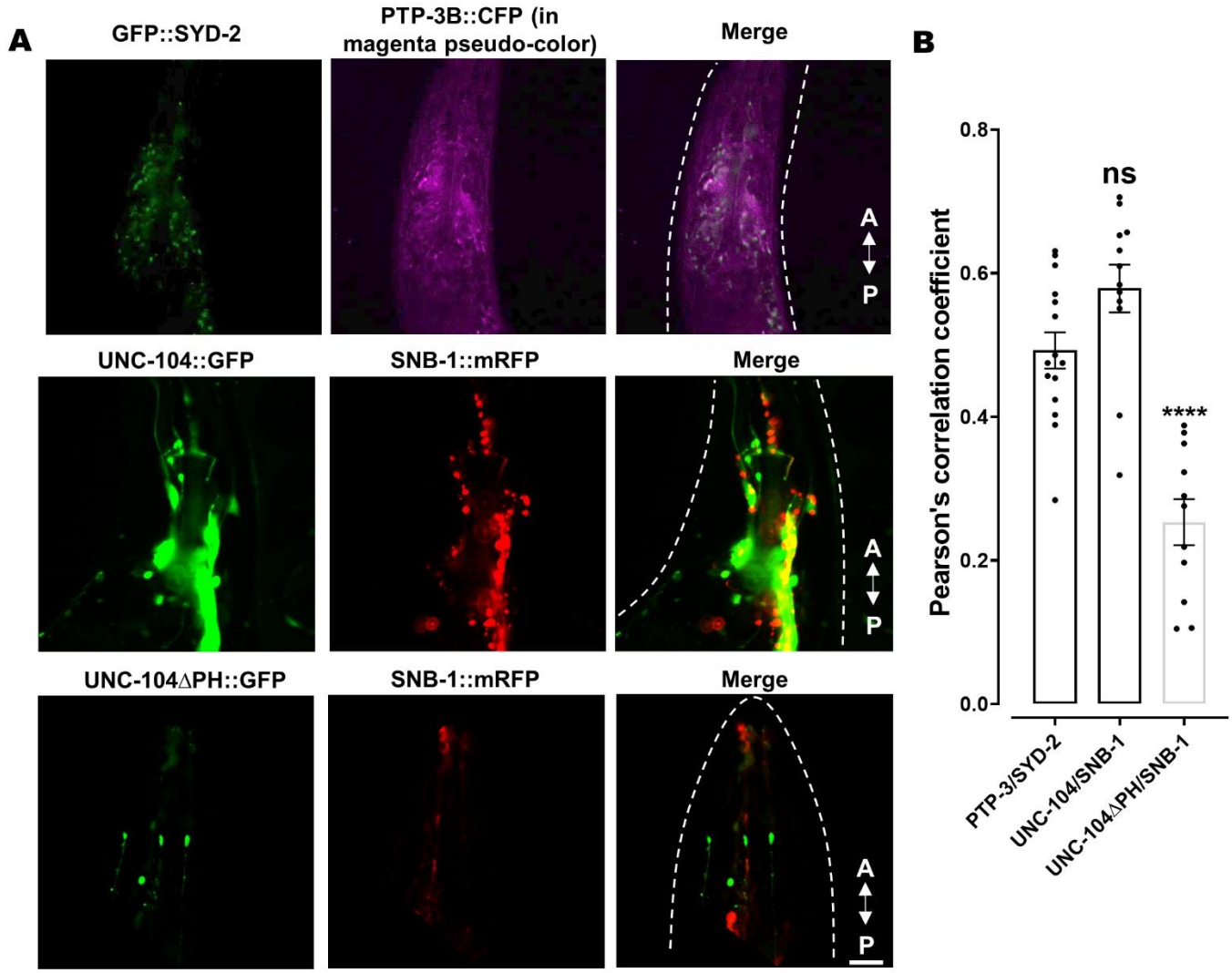
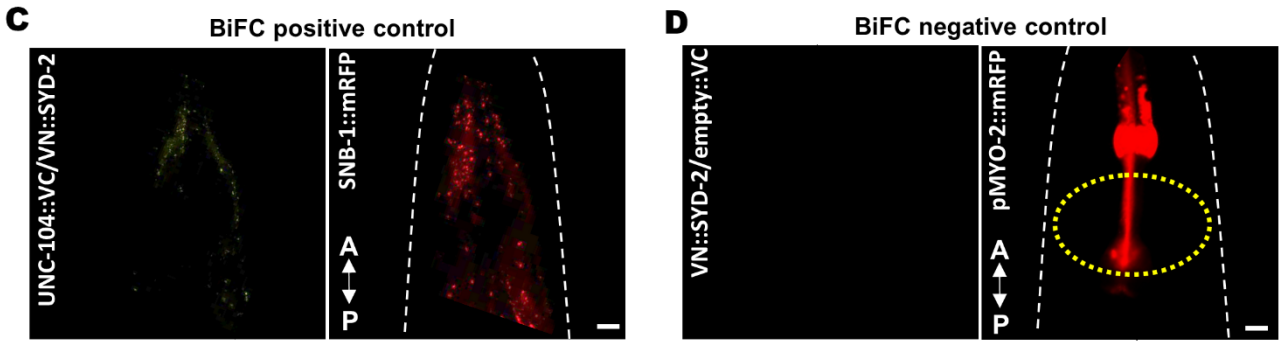


New Suppl. Figure 3A+B: Comparison of colocalization between UNC-104/SNB-1, UNC-104ΔPH/SNB-1 and SYD-2/PTP-3. Scale bar 10 µm. One-way ANOVA with Dunnett's multiple comparisons test, **** p<0.0001.

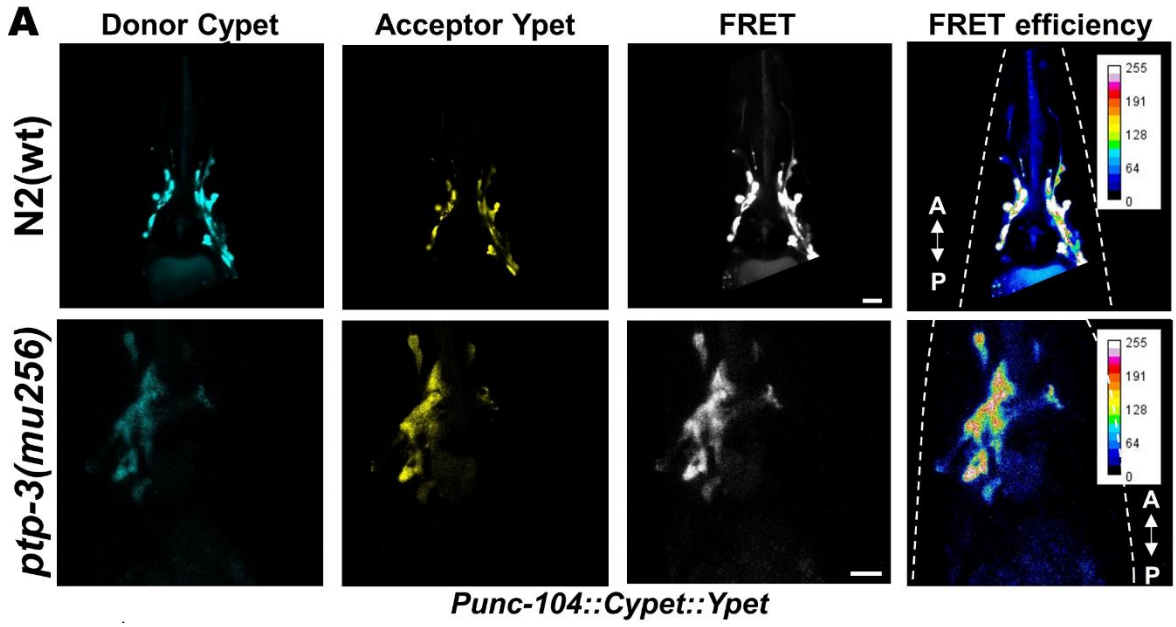
We also now add a BiFC positive as well as negative control. The positive control is based on well-described UNC-104/SYD-2 interactions (see Introduction in manuscript and (Wagner, Esposito et al. 2009)) and the negative control is VN::SYD-2/empty::VC. Note that to avoid bleed through artifacts in negative control, we refrained from overexpressing any (red) fluorescent axonal marker. Instead, we used a pharyngeal marker (Pmyo-2::mrfp) to locate the position of the nerve ring (yellow, dotted line) and screened through all confocal planes for BiFC signal at that location.

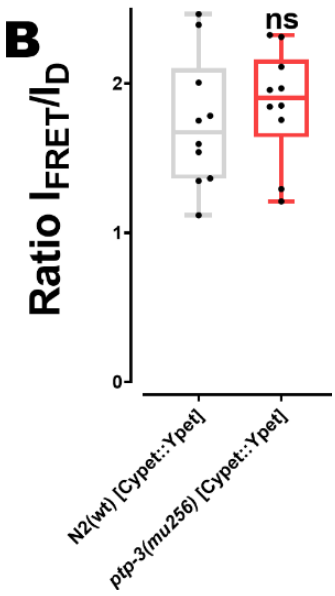


New Suppl. Figure 3 C and D: BiFC positive control (interaction between UNC-104::VC and VN::SYD-2; Punc-104::snb-1::mrfp as neuronal marker) and negative control (lack of interaction between VN::SYD-2 and VC; Pmyo-2::mrfp as pharyngeal marker). Scale bar 10 µm.

Figure 3: the FRET approach to assess the conformation of SYD-2 is potentially insightful, but important controls appear to be missing: 1) another protein in which Cypet and Ypet are present in tandem to show that FRET for this probe is unaffected in the different genetic backgrounds, and 2) SYD-2 singly tagged with either Cypet or Ypet to measure the extent of fluorescence spillover into the FRET channel. Without the latter control experiments, how can the authors be sure that they are actually measuring FRET and not just spillover?

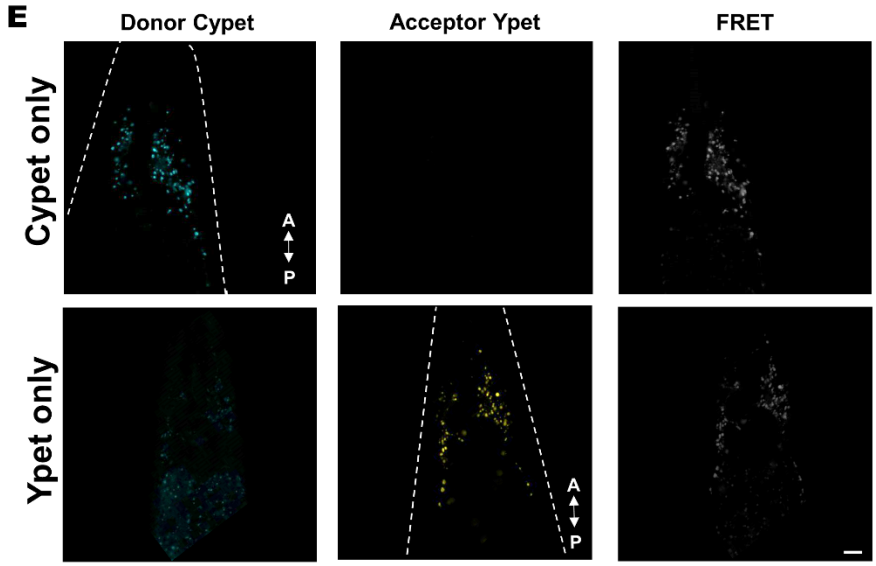
Author's response: We performed the following new control experiments. (1) We generated a new strain pUNC-104::Cypet::Ypet where Cypet and Ypet proteins are directly linked together. Here, FRET ratios should remain the same in N2 and mu256 genetic backgrounds. Indeed, the new data below demonstrate that FRET ratios did not significantly change between N2(wt) and mu256 mutants:





New Suppl. Figure 4: Cypet::Ypet FRET efficiency control experiment. (A) Representative images of worms expressing Punc-104::Cypet::Ypet in either N2(wt) or in mu256 mutant background. (B) Quantification of FRET ratio reveals no significant difference between N2(wt) and mu256 mutant. Student t-test with Welch's correction. Scale bar 10 µm.

(2) We also generated new bleed-through control worms either expressing Cypet::SYD-2 or SYD-2::Ypet. Images below reveal the proportion of bleed-through in the respective FRET channels. Please note that subtraction of bleed-through signals is recommended only for intermolecular FRET where the ratio of Cypet-tagged protein to Ypet-tagged protein is not equal to 1 and varies from cell to cell. In the case of intramolecular FRET where the donor and acceptor are tagged to same protein and their ratio is always 1, subtraction of bleed-through is not recommended (Fred S. Wouters, Peter J. Verveer et al. 2001, Kalab, Pralle et al. 2006, Spiering, Bravo-Cordero et al. 2013).



New Suppl. Figure 4E: Bleed-through control experiments using worms either expressing Cypet::SYD-2 or SYD-2::Ypet. Images reveal the proportion of bleed-through in the respective FRET channels. Scale bar 10 µm.

Figure 4: as the authors point out, the effect of ptp-3(mu256) on SNB-1 distribution is what would be predicted from the lower expression levels of UNC-104 shown in Figure 1, so the data is not informative with regards to the proposed activation of UNC-104 in the absence of PTP-3. As such, this figure has little relevance and could be moved to the supplement.

Author's response: Though this result is indeed as expected, we still need to experimentally prove it based on the hypothesis-driven approach of our study. Therefore, we believe it deserves presentation in the main body of the manuscript. Also, accumulation of SNB-1 containing synaptic vesicles in soma (related to axonal transport deficits) is often observed in neurological diseases. Furthermore, observed egg retention phenotypes (Fig. S2) may point to direct physiological consequences from these effects. A collective understanding from Figs. 1, 4 and 6 is that mu256 genetic background may modulate critical cellular signaling pathways resulting in unique consequences of cellular functions. For example, the involvement of Wnd/DLK MAP kinase pathway in modulating expression levels of synaptic proteins as discussed on page 12. Overall, we hope this reviewer doesn't mind we keep these data in the main text.

Minor issues:

Figure 2: the signal in 2E and 2G is very dim, and the cyan signal is virtually invisible in the merged images in 2C and 2E. Image brightness should be adjusted and a more appropriate color combination should be used (for example green and magenta).

Author's response: We now use magenta pseudo-color instead of cyan and adjusted brightness/contrast of images in this Figure. Generally, we believe that PDM images are a better visualization method (Figure 2D). Also note that Pearson's analysis now indicates clear coexistence between PTP-3 and SYD-2 (Figure 2F).

Figure 3: the microscopy set up and the experimental protocol for the FRET analysis need to be described in more detail in the materials and methods section, including the filter sets used and the exact imaging protocol. From the current description, it is unclear what the 'Donor Cypet' and 'Acceptor Ypet' images in Figure 3B represent.

Author's response: The requested information has been added in Materials & Methods on page 20 and 21.

Figure 3B: the insets showing the FRET efficiency scale are too small.

Author's response: We increased the size of the insets in the FRET efficiency images.

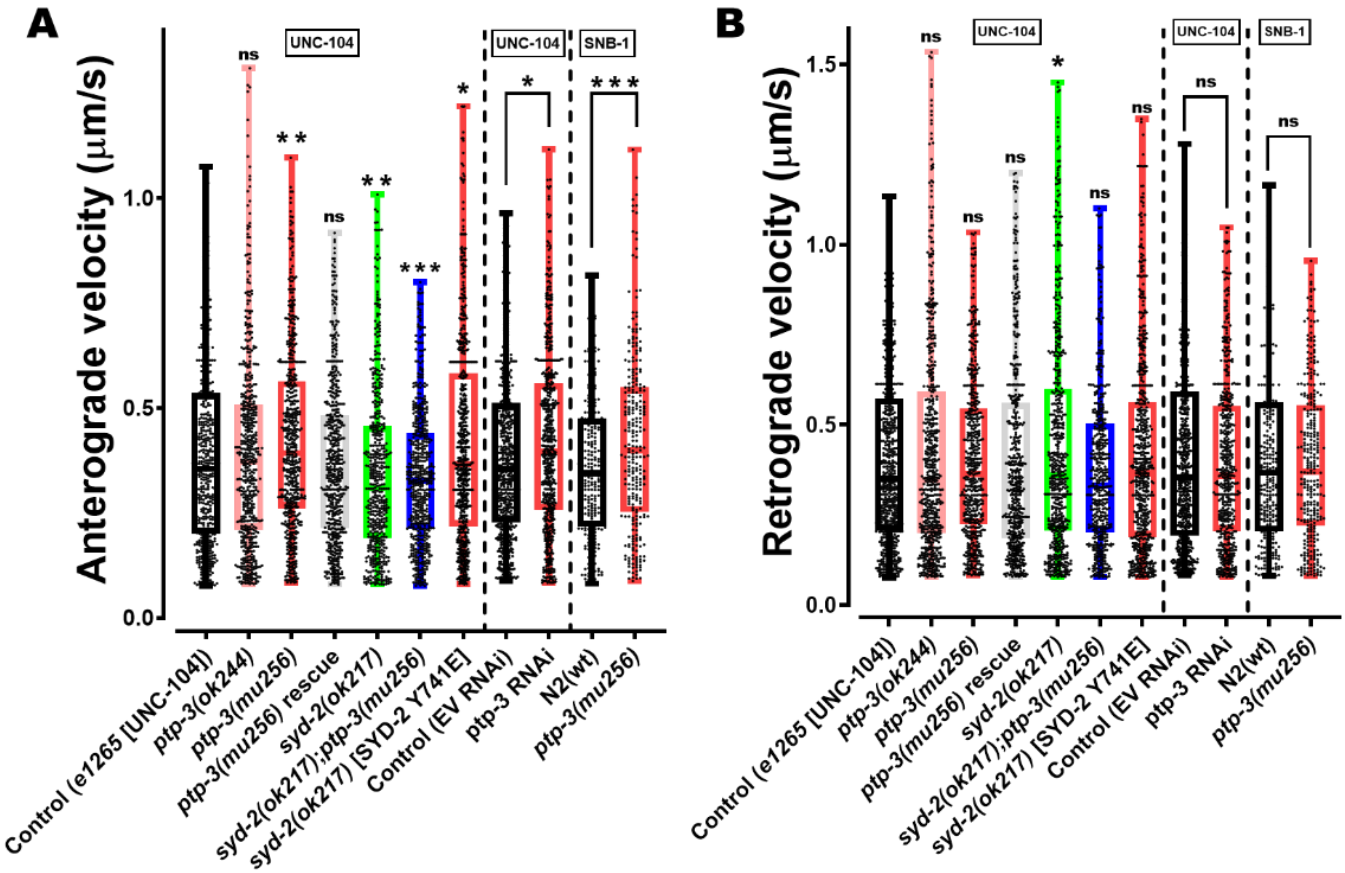
Figures 5 and 6: what is the fluorescent tag on UNC-104, and what is the rationale for using the e1265 allele as the control? Were the other conditions also imaged in the e1265 background?

Author's response: The e1265 allele encodes for a point mutation in the PH domain of the motor, therefore disrupting its interactions with synaptic vesicles which results in uncoordinated movements and paralyzed phenotypes (Hall and Hedgecock 1991, Kumar, Choudhary et al. 2010). We usually rescue these strains by injecting UNC-104::mRFP plasmids at 75 ng/µl which is just enough

to reverse the uncoordinated phenotype to typical wildtype movements (Wagner, Esposito et al. 2009, Tien, Wu et al. 2011, Wu, Muthaiyan Shanmugam et al. 2016, Bhan Prerana, Muniesh Muthaiyan Shanmugam et al. 2020). Subsequently we name these rescue worms "wild type animals". Other genetic backgrounds do not include the e1265 background.

Figure 6: the authors should determine the effect of SYD-2(Y741E) expression on UNC-104 and/or SNB-1 motility. According to their model, the effect should be similar to that observed with the ptp-3(mu256) allele.

Author's response: Below we present velocity data obtained from a newly generated strain UNC-104::mRFP(ok217) co-expressing GFP::SYD-2 Y741E. It is evident from these new data that GFP::SYD-2 Y741E phosphomimicking regulates UNC-104 motor velocity similar to hyperphosphorylated SYD-2 in mu256 mutants.



New Figure 6A+B: Anterograde (A) and Retrograde (B) velocity of UNC-104 and SNB-1 from various genetic backgrounds.

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Adler, J. and I. Parmryd (2010). "Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient." Cytometry A **77**(8): 733-742.

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Wagner, O. I., A. Esposito, B. Kohler, C. W. Chen, C. P. Shen, G. H. Wu, E. Butkevich, S. Mandalapu, D. Wenzel, F. S. Wouters and D. R. Klopfenstein (2009). "Synaptic scaffolding protein SYD-2 clusters and activates kinesin-3 UNC-104 in C. elegans." Proc Natl Acad Sci U S A **106**(46): 19605-19610.

Wu, G. H., M. Muthaiyan Shanmugam, P. Bhan, Y. H. Huang and O. I. Wagner (2016). "Identification and Characterization of LIN-2(CASK) as a Regulator of Kinesin-3 UNC-104(KIF1A) Motility and Clustering in Neurons." Traffic **17**(8): 891-907.

RE: Manuscript #E19-10-0591R
TITLE: "PTP-3 phosphatase promotes intramolecular folding of SYD-2 to inactivate kinesin-3 UNC-104 in neurons"

Dear Dr. Wagner,

I am pleased to inform you that the reviewers found your revised manuscript to be significantly improved. Reviewer 2 had a few remaining questions that I am sure you can readily address, and I think will be useful for the readership. I look forward to the final version of this work. Thank you for submitting this work to the Molecular Biology of the Cell,

Kerry Bloom

Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Wagner,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The authorst have adiquatly addressed my comments. I have no further remarks.

Reviewer #2 (Remarks to the Author):

The revised version contains additional controls for the FRAP and co-localization experiments, as well as new experiments as suggested by this reviewer. I find the manuscript improved, but a number of concerns remain. The following issues require clarification before publication (original comments are included along with my response):

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Original reviewer comment:

Figure 1: a key prediction of the proposed regulatory mechanism is that phosphorylation of SYD-2 at Y741 should strengthen the interaction between SYD-2 and UNC-104. The only evidence in support of this idea is a ~20% increase in the amount of SYD-2 that co-immunoprecipitates with UNC- 104::GFP from ptp-3(mu256) worm lysate. The authors should make an effort to test this aspect of the model more rigorously. One approach would be to determine whether the phosphomimetic mutant SYD-2(Y741E) has increased affinity for UNC-104, either in binding assays with purified proteins, in a yeast 2-hybrid system, or in co-immunoprecipitation assays from worm lysate.

Author's response:

We now add co-immunoprecipitation assays using lysates from worms express- ing either phosphodeficient or phosphomimicking SYD-2. These new results demonstrate that interactions between phosphomimicking SYD-2 and UNC-104 are more robust as opposed to the phospho-deficient variant.

Reviewer comment on revision:

As with the other co-IP experiment in Figure 1, the effect here is very subtle. From the description of this experiment in the materials and methods section, it is not clear whether the amount of co-immunoprecipitated UNC-104 was normalized to the amount of immunoprecipitated SYD-2::GFP (it looks like there is slightly more GFP::SYD-2 Y741E in the IP fraction compared to GFP::SYD-2 WT and Y741F).

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2)

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Figure 2: from the images in Figure 2A-D, it is evident that PTP-3B::CFP and GFP::SYD-2 are both present in the nerve ring, but what does this really mean? If the authors want to claim that the 'intensity correlation analysis' in 2F is indicative of close physical proximity, they should perform the assay with a negative control, i.e. with a CFP-tagged protein that is present in the nerve ring but is unlikely to closely associate with GFP::SYD-2 (or simply CFP on its own expressed in the nerve ring). The same caveat applies to the bimolecular fluorescent complementation assay in 2G and J: a control is needed in which the split Venus is present on a protein that is unlikely to directly interact with GFP::SYD-2.

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We now include a positive as well as a negative control (new Suppl. Figure 3A+B) in which we quantified colocalization of UNC-104/SNB-1 (positive control) and UNC- 104ΔPH::GFP/SNB-1::mRFP (negative control). The negative control is based on the notion that the motor's PH domain is essential to bind to synaptic vesicles (e.g., Kumar, Choudhary et al. 2010). From this experiment it is evident that deletion of UNC-104's PH domain results in reduced interac- tions with SNB-1-containing synaptic vesicles, if comparing to the UNC-104/SNB-1 or SYD-2/PTP-3 protein pair (see below). Note that during the revision process, we decided to replace the colocaliza- tion analysis method "ICQ" by "Pearsons's" such as the latter method seems to be more robust due to its independency of relative changes in intensities of fluorophores. Also this method only calcu- lates the coexistence of both fluorophores at a given pixel (Adler and Parmryd 2010, Sanderson 2019).

Revision reviewer comment:

I accept the validity of the positive and negative controls, but I wonder why the authors did not choose controls that use the same fluorescent tag pair as in the actual experiment, i.e. GFP and CFP.

---

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Original reviewer comment:

Figures 5 and 6: what is the fluorescent tag on UNC-104, and what is the rationale for using the e1265 allele as the control? Were the other conditions also imaged in the e1265 background?

Author's response:

The e1265 allele encodes for a point mutation in the PH domain of the motor, therefore disrupting its interactions with synaptic vesicles which results in uncoordinated movements and paralyzed phenotypes (Hall and Hedgecock 1991, Kumar, Choudhary et al. 2010). We usually rescue these strains by injecting UNC-104::mRFP plasmids at 75 ng/µl which is just enough to reverse the uncoordinated phenotype to typical wildtype movements (Wagner, Esposito et al. 2009, Tien, Wu et al. 2011, Wu, Muthaiyan Shanmugam et al. 2016, Bhan Prerana, Muniesh Muthaiyan Shanmugam et al. 2020). Subsequently we name these rescue worms "wild type animals". Other genetic backgrounds do not include the e1265 background.

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Answer: Indeed, we missed to describe in the Method section that in the new Figure 5D co-immunoprecipitated UNC-104 proteins were normalized to immunoprecipitated (pulled-down) GFP::SYD-2. This information has been added now. Regarding the “slightly more GFP::SYD-2 Y741E” in the representative gel in Figure 5D, we wish to remark that at the same time also UNC-104 is increased in this experimental group. Thus, together with these data and the other two repeats, co-precipitated UNC-104 was

significantly higher in this group as compared to the SYD-2 wt and SYD-2 Y741F groups. Generally, from our Co-IP quantification in Figure 5E, it is obvious that fluctuations in measurements exist reflected by the error bars. A representative gel can only reflect some of these data points.
In terms of the general remark that effects seen in Co-IPs in Figs. 1+5 are only subtle, we wish to emphasize that significant effects can be still quantified by standard methods. Also, please note that we do not use cell expression systems “optimized for best results”, but lysates from whole worms with more than 20,000 proteins all interacting with each other in intricated ways.

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Original reviewer comment:

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Revision reviewer comment:

I accept the validity of the positive and negative controls, but I wonder why the authors did not choose controls that use the same fluorescent tag pair as in the actual experiment, i.e. GFP and CFP.

Answer: The reason was that in order to save time (due to the large amount of revision requests form both reviewers), we decided to use existing worm strains expressing the aforementioned fluorophores.

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Answer: We now performed motility and cluster analysis in CB1265 (e1265)[UNC-104::mRFP] as well as in N2(*wt*)[UNC-104::mRFP] strains. We determined that the analyzed parameters are not significantly different between the groups. The Figure below has been added to the Supplementals as new Figure S7.

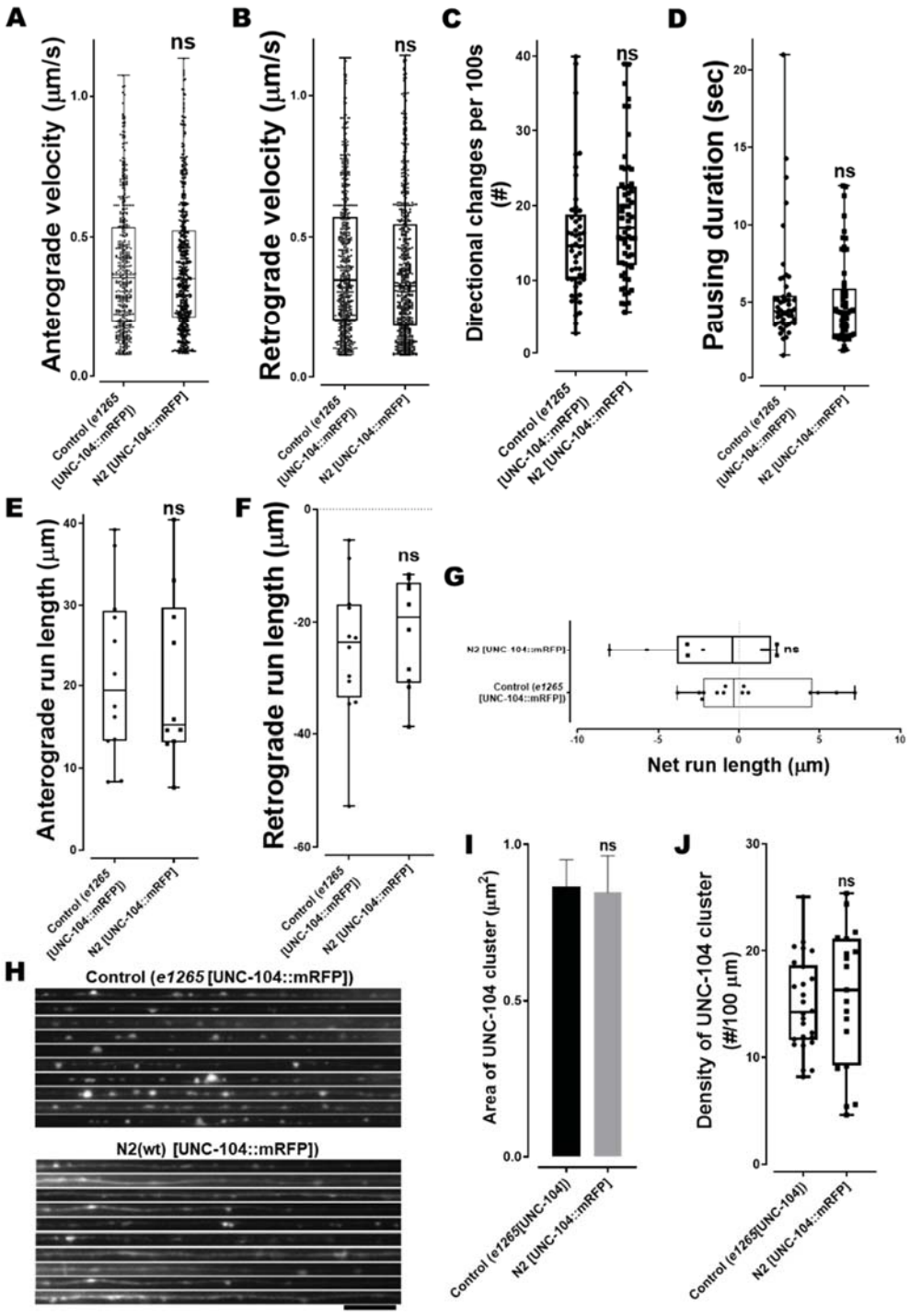

Figure S7 legend: Motility parameters and UNC-104 clustering in CB1265 (*e1265*)[UNC-104::mRFP] compared to N2(*wt*) [UNC-104::mRFP] worms. (A) Anterograde velocity, (B) retrograde velocity, (C) directional changes (motor reversals), (D) pausing duration, (E) anterograde run length, (F) retrograde run length, (G) net run length, (H) straightened sublateral neurites for cluster analysis, (I) area of UNC-104 particle in sublateral neurites, and (J) cluster density. Scale bar: 10 µm. Number of events in motility data > 1500 events. Unpaired Student's t-test.

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Figure 6: the authors should determine the effect of SYD-2(Y741E) expression on UNC-104 and/or SNB-1 motility. According to their model, the effect should be similar to that observed with the ptp- 3(mu256) allele.

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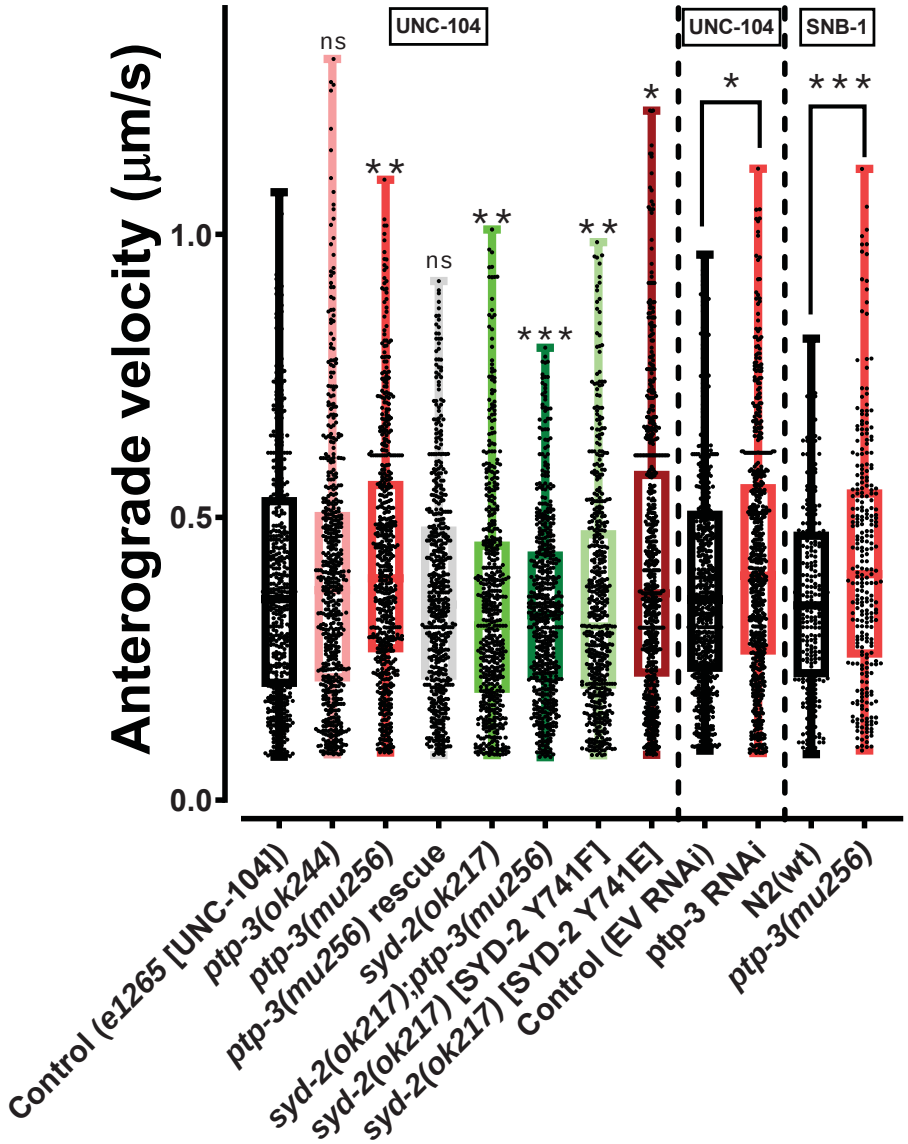
Answer: We addressed this issue by now including new motility data from *syd-2(ok217)* [UNC-104::mRFP; GFP::SYD-2 Y741F] worms. From the data below it is evident that SYD-2 Y741F does indeed not affect anterograde velocities of UNC-104.


Anterograde velocity (µm/s)
0.0
0.5
1.0
UNC-104
UNC-104
SNB-1
ns
**
ns
**
***
**
*
*
***
Control (e1265 [UNC-104])
ptp-3(ok244)
ptp-3(mu256)
ptp-3(mu256) rescue
syd-2(ok217)
syd-2(ok217);ptp-3(mu256)
syd-2(ok217) [SYD-2 Y741F]
syd-2(ok217) [SYD-2 Y741E]
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N2(wt)
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RE: Manuscript #E19-10-0591RR
TITLE: "PTP-3 phosphatase promotes intramolecular folding of SYD-2 to inactivate kinesin-3 UNC-104 in neurons"

Dear Dr. Wagner,

I am pleased to inform you that your manuscript is now suitable for publication in the Molecular Biology of the Cell. Thank you for submitting this work.

Kerry Bloom
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Wagner:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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