Regulation of UNC-40/DCC and UNC-6/Netrin by DAF-16 promotes functional rewiring of the injured axon

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MS TITLE: Regulation of UNC-40/DCC and UNC-6/Netrin by DAF-16 promotes functional rewiring of the injured axon

AUTHORS: Anindya Ghosh-Roy, Atrayee Basu, Sibaram Behera, and Shirshendu Dey

I have now received the reports of three referees on your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they request that you link insulin signaling and netrin/DCC functionally, e.g. by providing data showing that the increase in regeneration induced by daf-16 upregulation is due to DCC/netrin signaling. They also comment on the need to improve clarity of the writing and to add key references. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Basu et al. studies the functional regeneration of axons in C. elegans. They study a specific form of regeneration that leads to functional recovery when the neurites regrow to the ventral nerve cord. Moreover, they demonstrate that UNC-40/UNC-6 and DAF-16 are important for promoting this form of regeneration. This is an interesting study in the important field of functional neuronal regeneration.

Comments for the author

However, I do not think that this manuscript contains sufficient novelty to be considered by Development. The capacity of these neurons to functionally recover after axotomy has previously been established, as has the decline in recovery in older animals, the role of DAF-16 in regeneration, and the role of UNC-6/UNC-40 in axonal guidance. Although this manuscript focuses on a different means of functional recovery, there is a lack of mechanistic insight into how this occurs instead of axonal fusion, how and if synaptic reinnervation occurs, and how DAF-16 and UNC-6 function from the muscle to regulate the regeneration of the PLM neurite that is embedded in the hypodermis.

There is also an absence of discussion on previous findings that have shown that it is the electrical, not chemical, synapses that control touch sensation in these neurons. How the authors reconcile these previous findings with their own needs to be discussed.

Finally, the grammar and sentence structure need to be improved to help with the clarity and flow of the document. Currently, it is difficult to read in parts.

Reviewer 2

Advance summary and potential significance to field

The authors have assembled a compelling data set linking insulin signaling to declining regeneration in C. elegans adults. Moreover they show that a key aspect of declining regeneration is failure to target regenerating axons to ventral muscles. They link insulin signaling to transcriptional control of netrin and DCC and suggest that this guidance pathway is the key output of insulin signaling that is reduced in adults. However, the authors stop short of linking insulin signaling and netrin/DCC functionally.

Comments for the author

To make this story complete, it would be extremely helpful to pair genetic manipulation of insulin signaling with netrin or DCC mutants. If their model is correct, the increase in regeneration seen with elevated daf-16 should be reduced when DCC or netrin is absent. For example, if neuronal overexpression of daf-16 is paired with the unc-40 mutant is the increase in regeneration eliminated?

The other major issue is the writing. It is fairly opaque throughout and the grammar and wording needs substantial work.

A more minor issue is that it is unclear how the Rab-3 piece fits with guidance via netrin pathway. The weakness of the Rab3 data (see specific comments below) perhaps indicates that one solution would be to remove it.

Specific comments:
A little more info about PLM up front would help- where is sensory end, what does it synapse onto, maybe description of whole circuit near the beginning so it is clearer what functional recovery might entail.

In Fig 1B looks like there is some recovery at 24h. Are there synapses already formed in some cases at this timepoint?

Fig 2: the reduction in recovery in adult stages shown in A is linked to failure to target in the text. To distinguish between a failure to grow and a failure to target it would be useful to have the outgrowth quantitated as well as recovery and ventral targeting.

Fig2 D and E: I am having some trouble understanding this data. First, is there any reason to think that Rab3 intensity would be linked to synapse function? It looks from the images in D like there are still Rab3 puncta present in adult regenerating neurons. Is it clear what it means if they have less Rab3? The fact that Rab3 intensity goes down in uninjured neurons between L4 and A3 indicates that intensity levels do not reflect function, as I assume the cells are still functioning in uninjured A3? Also, what is the DS condition 24h after axotomy? How can severed neurons have developmental synapses? And in the ventral targeting A3 condition it looks like the n is only two. How can there be conclusions drawn from this?

Could they explain better what is meant by a developmental synapse?

The second section in the results focuses on adulthood. It would be helpful to mention in the first results section what life stage is being assayed there. It would also be helpful to define terms and ideas when they first appear. For example what is the A3 stage?

Fig 3: the phenotypes seem very strong and convincing. It would be helpful to see some example images of L3 animals with enhanced regeneration.

The title of the figure suggests that upstream kinase mutants would be paired with daf-16 (lf) to show dependence of the phenotype on daf-16, however this data is not included. It would be better to change the title to better match the experiments.

Fig 4: example images of the enhanced ventral targeting to go with graphs in A and B would be helpful to see. Is dlk required for the increased recovery induced by loss of daf-16? This seems at least as important to determine as the effect of overexpression of dlk.

Fig 5: Identifying a guidance pathway regulated by daf-16 really strengthens this story. In Figure 5, many of the images used to quantitate ratios of unc-40 to mScarlet look completely saturated. I hope this is only how the authors are displaying the data in the figure and that the images used for quantitation were not saturated, but showing the saturated examples makes them difficult to interpret.

The key thing missing in figures 5 and 6 is functional data showing that the increase in regeneration induced by daf-16 upregulation is due to DCC/netrin signaling. Pairing several of the tools they already have should allow this to be tested relatively easily.

Reviewer 3

Advance summary and potential significance to field

Basu et al present their finding that daf-16 regulates guidance of regenerating ventral branches PLM mechanosensory neurons. The question of how regenerating axons are reguided to their targets in an adult animal is an important one. The authors convincingly show that daf-16 determines guidance by regulating unc-6 and unc-40 in the muscle and neurons, respectively. It has been previously shown that unc-6 directs guidance of regenerating ventral branches of another class of neuron, that daf-16 regulates guidance of amphid neurons, and that daf-16 regulates axon regeneration. However, the finding that daf-16 directs axon guidance by regulating expression of unc-6 and unc-40 in separate tissues is interesting and adds to our understanding of how regenerating axons are guided.
While it has to be said that the paper does not address a developmental process it does focus on developmental mechanisms such as netrin signaling that are repurposed in adults.

Comments for the author

It seems important to discuss whether the longitudinal position of the regrowing ventral branch varies and whether that influences the regenerating axon's ability to make a functional connection? The question of whether a lack of ventral targeting and therefore recovery (Figure 2) is a result of lack of outgrowth with increased age should be addressed. Even though an axon has reached the ventral cord, could recovery be affected if the axons do not grow longitudinally once they reach the ventral cord?

There is a significant amount of variability in the recovery index. It would be helpful to know whether that reflects variability in PLM function before injury.

Key references are missing from the text.

For example, Gabel et al. found that unc-6 is required to guide regeneration of the ventral branch of the AVM mechanosensory neuron. This finding should be mentioned and cited. The Chang group also found that slt-1 and sax-3 repel the ventral branch from the dorsal aspect of the worm. The overlap with this dataset should be mentioned.

Given the parallel nature of the Byrne et al. paper, it should be mentioned in the introduction that the Hammarlund group found regeneration is inhibited in aging neurons by daf-2 and daf-16. The conclusion at the end of the tissue-specific rescue section should specify that the findings apply to the PLM neurons and that the Byrne group looked at GABA neurons.

Grossman et al. found that daf-16 regulates amphid axon guidance. The previously identified role of daf-16 in C. elegans axon guidance should be cited.

First revision

Author response to reviewers' comments

A point-wise response to the queries of the reviewers

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript by Basu et al. studies the functional regeneration of axons in *C. elegans*. They study a specific form of regeneration that leads to functional recovery when the neurites regrow to the ventral nerve cord. Moreover, they demonstrate that UNC-40/UNC-6 and DAF-16 are important for promoting this form of regeneration. This is an interesting study in the important field of functional neuronal regeneration.

Reviewer 1 Comments for the Author
However, I do not think that this manuscript contains sufficient novelty to be considered by Development. The capacity of these neurons to functionally recover after axotomy has previously been established, as has the decline in recovery in older animals, the role of DAF-16 in regeneration, and the role of UNC-6/UNC-40 in axonal guidance.

Response:

Thank you for mentioning both the strength and weaknesses of the manuscript. We are highlighting novel points that this manuscript brings to this field.

1) The Mechanism supporting the long-distance axon regrowth following injury has been the point of fundamental interest in the axon regeneration field as adult axons often fail to reach the correct destination. For the axon to reach the target cell/tissue, the injured axon must navigate through the adult environment. Our work shows that the regulation of guidance cues Netrin/DCC helps the

injured axon to reach the target area for making a functional connection. This raises hope to manipulate ventral guidance cues for successful axon regeneration.

2) Although we (Basu et al., 2017) and others (Abay et al., 2017) have shown that functional restoration occurs through axon fusion in the touch neuron system. This mechanism is only restricted to invertebrates and not seen in humans. Axonal fusion mechanism requires a protein EFF-1 which is absent in vertebrate models (Ghosh- Roy et al., 2010, Neumann et al., 2015, Basu et al., 2017).
Our new findings in this report that PLM axon can rewire and make new synapse-like structure opens up possibilities to use PLM neuron to study the functional rewiring relevant to the peripheral and central nervous system of higher models.

3) Previous work on IIS-DAF-16 on motor neuron regeneration showed that IIS leads to the age-dependent decline in axon regeneration ability (Byrne et al 2014).
Our work brings the novel idea that DAF-16 plays an active role in axon guidance and functional restoration irrespective of age (both in larval and adult stages). It plays a role regardless of changes in insulin receptor or downstream kinases. Since FOXO/DAF-16 is conserved across species, it will be an exciting direction to find relevant targets of this transcription factor that might promote intrinsic axon regeneration mechanisms

Although this manuscript focuses on a different means of functional recovery, there is a lack of mechanistic insight into how this occurs instead of axonal fusion, how and if synaptic reinnervation occurs, and how DAF- 16 and UNC-6 function from the muscle to regulate the regeneration of the PLM neurite that is embedded in the hypodermis.

Response:

For successful rewiring, the injured axon must be guided to the correct tissue or neuron. This work gives mechanistic insight involving proper guidance of regenerating axon. Our work shows that appropriate control of the Netrin/UNC-6 in muscle and DCC in neuron is required for functional regrowth of the PLM axon. DCC is upregulated by DAF-16 in the neuron after axon injury.
In muscle, DAF-16 is required to maintain the *unc-6* expression. Previous studies have indicated that the UNC-6 is secreted from muscle (Asakura et al., 2010, Genetics). Therefore, we have shown a cell-autonomous as well as non-autonomous roles of DAF-16 in controlling axon guidance during regeneration.
To address the comments of reviewer-2 and the editor, we have added new data that DAF-16 related guidance depends on *unc-40* and *unc-6* (Figure 5B and 5C) (Page 12, line 355-366). These additional data make the story more insightful and mechanistic.

There is also an absence of discussion on previous findings that have shown that it is the electrical, not chemical, synapses that control touch sensation in these neurons. How the authors reconcile these previous findings with their own needs to be discussed.

Response:

Thanks for this suggestion.
The PLM neuron has a gap junction connection with the interneuron PVC that initiates forward movement in response to a posterior touch to a backward moving worm. Similarly, PLM neuron makes a chemical connection with interneurons AVA and AVD, which is predicted to prevent the backward movement in response to a posterior touch to a backward moving worm. According to the ablation experiments done in **Chalfie et al.,1985**, the gap junction between PLM and PVC is sufficient to produce a posterior touch response. This conclusion is heavily dependent on the ablation of the neurons in larval stages. It was not supported by precise removal of either the synaptic connection or the gap junction

However, **Wicks and Rankin** showed in 1995, using the tap response assay, that PLMs have inhibitory chemical connections with AVA and AVD interneurons.
We have shown previously in **Basu et al., 2017** that when only the ventral synaptic branch of the PLM was removed using laser surgery, the posterior touch response index declined by ~30% (Figure 1E of Basu et al., 2017). This result infers that the chemical connection between the PLM and AVA

has a role in the generation posterior touch response. Therefore, making a new chemical synapse during regeneration is relevant for functional recovery in this neuron.

We have added these points in the discussion section (Page 15, line 452-464). We have also added the description of the circuitry in Figure 1A (Page 5, line 138-144).

Finally, the grammar and sentence structure need to be improved to help with the clarity and flow of the document. Currently, it is difficult to read in parts.

Response:

Yes, we have gone through the manuscript extensively and rewritten it to improve the language. We have taken help of colleagues and done multiple rounds of revision.

Reviewer 2 Comments for the Author:

Significance in the field:
The authors have assembled a compelling data set linking insulin signaling to declining regeneration in *C. elegans* adults. Moreover, they show that a key aspect of declining regeneration is failure to target regenerating axons to ventral muscles. They link insulin signaling to transcriptional control of netrin and DCC and suggest that this guidance pathway is the key output of insulin signaling that is reduced in adults. However, the authors stop short of linking insulin signaling and netrin/DCC functionally.

To make this story complete, it would be extremely helpful to pair genetic manipulation of insulin signalling with netrin or DCC mutants. If their model is correct, the increase in regeneration seen with elevated daf-16 should be reduced when DCC or netrin is absent. For example, if neuronal overexpression of daf-16 is paired with the unc-40 mutant is the increase in regeneration eliminated?

Response:

Thank you for the suggestion. To address this, we have constructed *daf-16* overexpressing strain in the background of both *unc-40* and *unc-6* mutants. We have presented these data in Figure 5B and 5C. This shows that enhanced ventral targeting in the *daf-16* -overactivated condition is dependent on the presence of *unc-40* and *unc-6* (Page 12, line 355-366).

The other major issue is the writing. It is fairly opaque throughout and the grammar and wording needs substantial work.

Response:

We have revised the manuscript extensively for grammatical errors and clarity. We have taken help from colleagues and done multiple rounds of revision.

A more minor issue is that it is unclear how the Rab-3 piece fits with guidance via netrin pathway. The weakness of the Rab3 data (see specific comments below) perhaps indicates that one solution would be to remove it.

Response:

Thanks for the specific suggestions on the RAB-3 piece of the data. Please see that how we addressed the specific points raised below. We felt that the RAB-3 enrichment nicely correlates to the functional recovery. Please see that in our final summary (Figure 7, Page 14, line 423-432), the functional restoration during the axon regeneration of PLM neuron involves guidance of the regenerating proximal stump towards the ventral cord followed by the formation of presynaptic structures in the ventral cord. Therefore, the RAB-3 assay is one major readout of functional rewiring.

Specific comments:

A little more info about PLM up front would help- where is sensory end, what does it synapse onto, may be description of whole circuit near the beginning so it is clearer what functional recovery might entail.

Response:

We have revised the Figure 1A panel and covered these points (Page 5, line 138- 144).

In Fig 1B looks like there is some recovery at 24h. Are there synapses already formed in some cases at this timepoint?

Response:

Yes, there are some events in the whole cohort of 'Non-fusion' events that are ventrally targeted and accumulation GFP::RAB-3 is also there (Figure 1D and 1E). The average recovery index of these events is above 1 (Figure S1A, C), which indicates functional recovery (Page 7, line 194-197).

Fig 2: the reduction in recovery in adult stages shown in A is linked to failure to target in the text. To distinguish between a failure to grow and a failure to target it would be useful to have the outgrowth quantitated as well as recovery and ventral targeting.

Response:

Thanks for raising this point. We have presented the comparison of the total regrowth values between the L4 and A3 stages. Although there is a slight decrease in the average value of regrowth at A3 stage, it is not significant (Figure 2 C). The axons which don't turn ventrally, not necessarily show reduced regrowth. We have presented the evidence that the total regrowth values in the two classes 'ventral' and 'non-ventral' are comparable. (Figure 2 C). Moreover, we found that number of ventral-turning events is significantly reduced at A3 stage (Figure 1B). These data strongly suggest that there is a specific downregulation of axon guidance at day 3 (A3) stage. See the description of these data and interpretation (Page 7-8, line 219-226).

Fig2 D and E: I am having some trouble understanding this data. First, is there any reason to think that Rab3 intensity would be linked to synapse function? It looks from the images in D like there are still Rab3 puncta present in adult regenerating neurons. Is it clear what it means if they have less Rab3? The fact that Rab3 intensity goes down in uninjured neurons between L4 and A3 indicates that intensity levels do not reflect function, as I assume the cells are still functioning in uninjured A3?

Response:

RAB-3 is a small exocytic RAB GTPase associated with presynaptic vesicular protein (Nonet et al.,1997 & Mahoney et al., 2006, Michael Nonet). So RAB-3 enrichment is an indicator for pre-synaptic density localization. We have seen that in early adulthood (L4 stage), the 'ventral targeting' events correlates to the accumulation of RAB-3 along the ventral cord (Fig. 1E,G) (Page 6-7, line 184-192). We agree that intensity is not an indicator of function. Here the 'intensity-index' is an indicator of synapse formation. The enrichment is correlated with a functional synapse at L4 stage. This data is presented in Figure S1C (Page 7, line 194-197).

Also, what is the DS condition 24h after axotomy? How can severed neurons have developmental synapses?

Response:

In 'Figure 1E,' the remnant of the old synapse after injury is still visible. GFP is frequently visible at the old synapse even after the axonal injury (yellow arrowheads, Fig. 1E), as the perdurance of GFP is very high. Similarly, the distal axon after injury remains often visible although they appear

fragmented (df, Fig. 1C).

And in the ventral targeting A3 condition it looks like the n is only two. How can there be conclusions drawn from this?

Response:

We have taken care of this issue. We now have n = 22 now for A3 condition (Fig 2E, G). We have also measured the longitudinal regrowth along the cord after axon has reached the ventral cord (as per the suggestion of the Reviewer 3). It seems that in A3-stage, there is a reduction of fasciculation /growth along the ventral cord along with reduction of RAB-3 enrichment (Figure 2E-G). Therefore, both the fasciculation and enrichment of synaptic machineries are perturbed in the A3 stage (Page-8 , line 227-234) .

Could they explain better what is meant by a developmental synapse?

Response:

Here we indicated the original chemical synapse of PLM with the AVA interneuron as the 'developmental synapse' (Figure 1A, 1F. Also, please see the explanation (Page 6-7, line 184-192, legend of Figure 1F, line 824-825).

The second section in the results focuses on adulthood. It would be helpful to mention in the first results section what life stage is being assayed there. It would also be helpful to define terms and ideas when they first appear. For example, what is the A3 stage?

Response:

The first result section (Figure 1) deals with experiments done at late larval (L4) stage (early adulthood). By this stage the PLM development is complete and most of the published work on PLM axon regeneration deals with L4 stage (Ghosh-Roy et al 2010, 2012, Yan et al 2009). At this stage, the injured PLM axons regenerates robustly.
We have now mentioned about the life-stages in all the figures, their legends and in the main text as suggested by the reviewer.

Fig 3: the phenotypes seem very strong and convincing. It would be helpful to see some example images of L3 animals with enhanced regeneration. The title of the figure suggests that upstream kinase mutants would be paired with *daf-16 (lf)* to show dependence of the phenotype on daf-16, however this data is not included. It would be better to change the title to better match the experiments.

Response:

We have included the images of ventral targeting events in *daf-2(lf)* mutant in Fig 3D.

The title of this figure was:

"Downregulation of Insulin receptor or downstream kinases enhances ventral targeting and functional restoration in a *daf-16* dependent manner"

We have changed this title to:

"Downregulation of Insulin receptor enhances ventral targeting and functional restoration in a *daf-16* dependent manner"

See (Page 28, line 852-853).

Fig 4: example images of the enhanced ventral targeting to go with graphs in A and B would be helpful to see. Is *dlk* required for the increased recovery induced by loss of daf-16? This seems at least as important to determine as the effect of overexpression of *dlk*.

Response:

We have included the images of 'ventral targeting' events in *daf-16* mutant expressing the *daf-16f* isoform (Fig 4C).

In Figure 4C panel, we have shown that the *dlk-1* is not enough to increase recovery index and ventral targeting in *daf-16* mutant. We have also presented the data in *dlk- 1(lf); daf-2(lf)* in Fig S4E-F background as suggested. We found that DLK-1 is required for the increased ventral targeting and functional recovery in *daf-2* mutant (DAF-16 overactivated). See (Page 11, line 324-325.

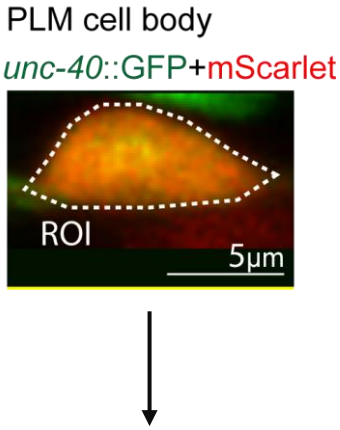
Fig 5: Identifying a guidance pathway regulated by DAF-16 really strengthens this story. In Figure 5, many of the images used to quantitate ratios of *unc-40* to mScarlet look completely saturated. I hope this is only how the authors are displaying the data in the figure and that the images used for quantitation were not saturated, but showing the saturated examples makes them difficult to interpret.

Response:

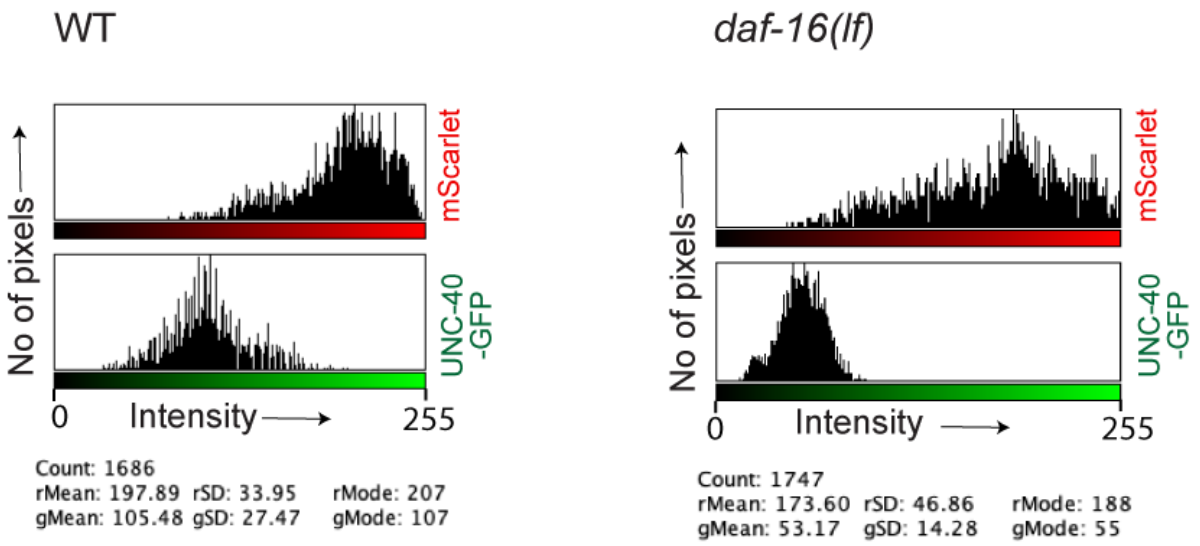
We apologise for displaying some of the images as the saturated ones. During acquisition of these images, we maintained the laser power below saturation limit for both 488 nm and 560 nm lasers. We have now revised the images in Figure 5D-E.

We have captured these images in 8 bits, and they are within the intensity saturation limit of 256 a.u. Please see the scatter plot of the mean intensity of UNC-40::GFP and mScarlet in Figure S5D-G. Please also see the "imaging of UNC-40::GFP in method section" (Page 23-24 Line 699-718). This shows that the intensity distribution in the region of our interest is below saturation.

We have provided here the intensity (grey values) distributions of 2 represented samples in Fig. 5D. For all other samples, the intensity range remain the same.



Example of Histogram (Intensity per pixel) plots drawn from ROI surrounding the cell body *WT* and *daf-16(lf)* before axotomy using Image J software.



The key thing missing in figures 5 and 6 is functional data showing that the increase in regeneration induced by daf-16 upregulation is due to DCC/netrin signaling. Pairing several of the tools they already have should allow this to be tested relatively easily.

Response:

We have performed these experiments and put the results in the revised panel Fig. 5B and Fig. 5C. The enhanced ventral targeting and recovery indices seen due to overexpression of DAF-16 in neuron or muscle is dependent on both guidance ligand (UNC-6) and receptors (UNC-40). Similarly, we have presented evidence in *akt-1* mutant background, In *akt-1* mutant, the DAF-16 is upregulated (Paradis & Ruvkun 1998). We saw that the enhanced ventral targeting and functional recovery in *akt-1* mutant is suppressed either by *unc-40* or *unc-6* mutant (Figure 5B-C). See (Page 12, line 355-366).

Reviewer 3

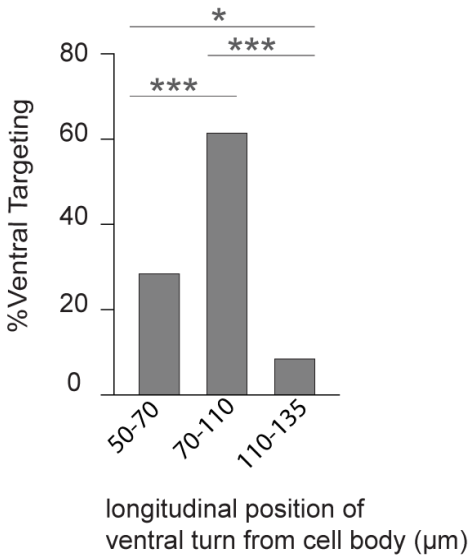
Advance Summary and Potential Significance to Field:

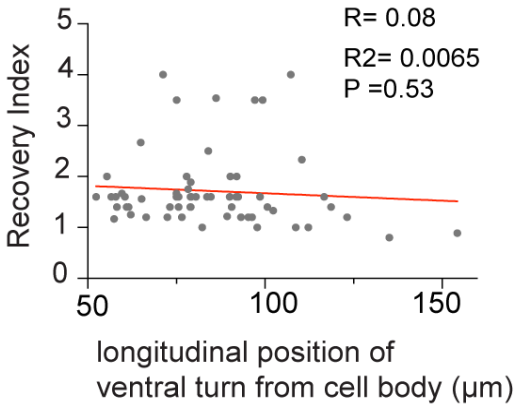
Basu et al present their finding that daf-16 regulates guidance of regenerating ventral branches PLM mechanosensory neurons. The question of how regenerating axons are re-guided to their targets in an adult animal is an important one. The authors convincingly show that daf-16 determines guidance by regulating unc-6 and unc-40 in the muscle and neurons, respectively. It has been previously shown that unc-6 directs guidance of regenerating ventral branches of another class of neuron, that daf-16 regulates guidance of amphid neurons, and that daf-16 regulates axon regeneration. However, the finding that daf-16 directs axon guidance by regulating expression of unc-6 and unc-40 in separate tissues is interesting and adds to our understanding of how regenerating axons are guided. While it has to be said that the paper does not address a developmental process, it does focus on developmental mechanisms such as netrin signalling that are repurposed in adults.

Reviewer 3 Comments for the Author:

It seems important to discuss whether the longitudinal position of the regrowing ventral branch varies and whether that influences the regenerating axon's ability to make a functional connection?

Response:
Thank you for this suggestion. We have looked at the confocal images of the regeneration events across various batches and found that longitudinal position of the ventral branching or turning varies from 60 µm to 120 µm from the PLM cell body. As suggested, we have done a correlation analysis between the longitudinal distance of the ventral turns and recovery index and could not find any correlation between the position of turning and the recovery index. Please see below.





The question of whether a lack of ventral targeting and therefore recovery (Figure 2) is a result of lack of outgrowth with increased age should be addressed.

Response:

We have presented the comparison of the total regrowth values between the L4 and the A3 stages. Although the average value of regrowth at the A3 stage showed a slight decrease, it is not significant (Fig. 2C). However, we found that number of ventral turn events is significantly reduced (Fig. 2B). Moreover, the total regrowth values in the two classes, 'ventral' and 'non-ventral' are comparable (Fig. 2C).
Overall, our conclusion that the ventral guidance is compromised at A3 stage is correct in the light of the above set of data. Also, please See (Page 7-8, line 219-226).

Even though an axon has reached the ventral cord, could recovery be affected if the axons do not grow longitudinally once they reach the ventral cord?

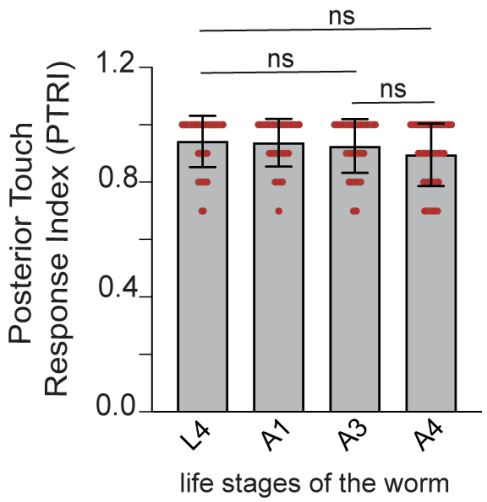
Response:

To address this, we have measured the longitudinal regrowth along the ventral nerve cord for the regrowing axons, which made it to the ventral nerve cord (yellow arrowhead traces, Figure 2E, 2F). We indeed found that the length covered in the cord gets decreased at A3 stage as predicted by this reviewer (Fig. 2F). Simultaneously, RAB-3 enrichment is also affected (Fig. 2G) (Page-8, line 227-234).

There is a significant amount of variability in the recovery index. It would be helpful to know whether that reflects variability in PLM function before injury.

Response:

We have previously shown that the PTRI value remains unaffected till the A4 stage (Basu et al 2017). Please see below that at L4 or A3 stage, the PTRI value ranges between 0.8 to 1. The PTRI values highly vary at 24 h postaxotomy in both L4 and A3 stages depending on the type of regeneration pattern. Due to this reason the Recovery Index (24h PTRI/3h PTRI) values also highly vary.



Key references are missing from the text. For example, Gabel et al. found that unc-6 is required to guide regeneration of the ventral branch of the AVM mechanosensory neuron. This finding should be mentioned and cited.
The Chang group also found that slt-1 and sax-3 repel the ventral branch from the dorsal aspect of the worm. The overlap with this dataset should be mentioned.

Response:

We have referred Gabel et al 2008 in the context of our finding in the discussion (Page- 16, line 483-485).

Response:

We have added the reference **"Distinct cellular and molecular mechanisms mediate initial axon development and adult-stage axon regeneration in *C. elegans*"** by Christopher V. Gabel, Faustine Antoine, Chiou-Fen Chuang, Aravinthan
D. T. Samuel, Chieh Chang. (Page-16, line 483-485)

Given the parallel nature of the Byrne et al. paper, it should be mentioned in the introduction that the Hammarlund group found regeneration is inhibited in aging neurons by daf-2 and daf-16. The conclusion at the end of the tissue-specific rescue section should specify that the findings apply to the PLM neurons and that the Byrne group looked at GABA neurons.

Response:

We have included the reference 'Byrne et al, 2014' in the introduction part (Page-4, line 108-110).

We found that *daf-16* is required in both touch neuron and muscle for regeneration of PLM neurons. Whereas, according to Byrne et al, the function of *daf-16* is neuron- specific for the regeneration of GABAergic D motor neurons. (Page-11, line 317-321).

Grossman et al. found that daf-16 regulates amphid axon guidance. The previously identified role of daf-16 in C. elegans axon guidance should be cited.

Response:

We have included the reference 'Grossman et al, 2013' in the discussion. We have indicated the previously reported roles of DAF-16 in axon growth and guidance (Page 16, line 483-489).

Second decision letter

MS ID#: DEVELOP/2020/198044

MS TITLE: Regulation of UNC-40/DCC and UNC-6/Netrin by DAF-16 promotes functional rewiring of the injured axon

AUTHORS: Atrayee Basu, Sibaram Behera, Smriti Bhardwaj, Shirshendu Dey, and Anindya Ghosh-Roy
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

previously provided

Comments for the author

Thank you to the authors for their thoughtful responses and additional data in the resubmission. The loss of function dlk data is helpful for context in the field and the example images make the figures easier to digest.
The wording is still awkward in places, and some of the explanations could still be clearer. For example, the difference between a developmental synapse and normal synapse is still not explained I think.

Reviewer 3

Advance summary and potential significance to field

Basu et al present their finding that daf-16 regulates guidance of regenerating ventral branches of PLM mechanosensory neurons. The question of how regenerating axons are reguided to their targets in an adult animal is an important one. Here the authors demonstrate that daf-16 regulates unc-40 mediated axon guidance of regenerating PLM axons.

Comments for the author

The authors have addressed my previous concerns.