Piecemeal regulation of convergent neuronal lineages by bHLH transcription factors in *Caenorhabditis elegans*

Neda Masoudi, Eviatar Yemini, Ralf Schnabel and Oliver Hobert
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MS TITLE: Piecemeal regulation of convergent neuronal lineages by bHLH transcription factors in C. elegans

AUTHORS: Neda Masoudi, Eviatar Yemini, Ralf Schnabel, and Oliver Hobert

I have now received two of the referees' reports on the above manuscript, and 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, the referees express considerable interest in your work, but have concerns regarding data interpretation that will need to be addressed before we can consider publication. For example, reviewer 1 points out that the current analysis relies entirely on NeuroPAL for determination of neuronal identity, however, in some situations since the determination is based on GFP expression, it is possible, for example, that the cells lose the GFP expression, rather than lose cell fate. If you are able to address this, and similar comments, with further textual detail or through a complementary analysis and revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript.

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

In presenting manuscript by Masoudi et.al., authors described two novel roles of well-examined bHLH transcription factor LIN-32/Ato in the C. elegans nervous system. Authors questioned that how neuronal cells in distinct lineages are phenotypically convergent to develop into functionally

symmetric cells. Authors focused on the lin-32/Ato and carefully examined its expression pattern through the cell lineage in the C. elegans embryonic development. Authors showed that two right/left or four radially symmetric neurons originated from distinct lineages differentially expressed lin-32 in their cell lineages: one lineage expressed it from earlier stage but the other later or not. Interestingly another bHLH transcription factor hlh-14 showed the mirror image of expression pattern against to lin-32. Authors concluded that these mirror imaged differential expressions of two TFs are probably required to regulate the expression of terminal selector genes for phenotypic convergence in symmetrical neuron pairs. The other role of LIN-32 is the regulation of terminal neuronal identity in cells from a lineage expressing lin-32 early and uniformly. The manuscript itself is a little bit descriptive and does not navigate us to a clear understanding of the molecular mechanisms by LIN-32 activities. However the scope and aim of the manuscript are novel, and the facts described in the results seem to be the first finding on cellular convergence if the data interpretation is reliable.

Comments for the author

As for data interpretation, several concerns exist which should be addressed before its publication. Especially, the reliability of cell identification should be exactly described in the case of TF mutant animals. I also recommend that a few figures can be modified as visually understandable figures for any readers.

Major concerns:
1. I am wondering how authors confirmed cells lacking expressions of terminal selector genes. For example, in Fig. 4A, authors showed the expression patterns of ceh-43::gfp transgene both in wild-type and lin-32 mutant animals. Some cells exactly lost ceh-43 expression, but for me, it is quite uncertain which cells really lacked gfp expression in the lin-32 mutant background. Did authors identify the cells by using their positions only in animals? How did authors say that cells expressing gfp are not IL1D or IL1V, even though gfp-expressing cells located at similar position compared to Wild-type? Same concern exists in the Fig. 8E and Fig. 9F analyses. Authors should indicate the method for cell identification without using NeuroPAL.

2. Related to above concern, I am strongly curious about applying NeuroPAL system to identify cells in TF mutants. I understand the principle and advantage of this system to identify each neuron in worms, and based on the color in each neuron, authors have tried to identify cells which are existing or lost in the TF mutant animals. However, in the lin-32 mutant animals, it seems that the color pattern was largely altered in a lot of neurons (for example, Fig 3A, Fig. 8B and Fig. 9A). Importantly, the NeuroPAL colors were altered in the cells closely positioned around the lin-32-expressing cell lineages. Presumably, the loss of a transcriptional factor activity affected expression patterns of some of marker genes in the NeuroPAL system and altered the color of cells. Thus, I suspect that identification of cells losing their original NeuroPAL colors are reliable in the lin-32 mutant animals. Authors should indicate the exact method for cell identification or discuss the reliability of their approach. In addition, I am also wondering whether cells whose colors were changed in the lin-32 mutants were generated from the lin-32-expressing cell lineages.

3. The authors have shown that lin-32 and hlh-14 were expressed as a mirror image in symmetric left/right neuron pairs. However, the phenotypic penetrance in each mutant seem to be largely distinct. The lack of lin-32 activity strongly affected the expression of terminal selector genes (Fig. 4), but animals lacking hlh-14 activity showed quite mild defects in the expression of terminal selector genes (ceh-10 expression in CAN and hlh-34 expression in AVH, Fig. 7). Why did one lineage cause stronger defects in daughter cells but the other not? In addition, authors showed that cells at ventral side were not strongly affected by the loss of LIN-32 activity, compared to the cells at dorsal side. Why?
Discussing the regulatory mechanism of these interesting phenomena would improve the manuscript.

Minor concerns:
1. Several miswriting are found in all through the manuscript. Please check text carefully and should be revised suitably. For example, at P8 line 1, ‘Even tough’ will be ‘Even though’, and at line 10, ‘We Fig.9’ will be ‘We found’, I guess.

2. Add scale bars in microscopic images. It would be enough to show those in the first appeared images.

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5. In Fig. 4, defects of the terminal selector gene expression were milder in ventral side compared to dorsal side. Why? And IL1V neurons are also generated in distinct lineage but affected stronger than IL2V cells. Are the differences in phenotypic penetrance dependent on lineages generating each cell?

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Reviewer 2

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This paper studies the regulation of lineage convergence in several neuronal lineages by the bHLH transcription factors lin-32/Ato and hlh-14. Many phenotypically similar neurons in C. elegans as well as other organisms emerge from distinct lineages, but there little known about the mechanisms underlying such lineage convergence. The authors demonstrate that different transcription factors such as lin-32/Ato and hlh-14 can function on the terminal selector level in different lineages to give rise to phenotypically identical neurons. The use of the neuroPAL tool to study the mutants also allows for more specific assessment of what happens to specific neurons and whether they actually lose their neuronal identities in the mutants analyzed.

Overall, the study is comprehensive and the experiments are well done, and the manuscript is clear and logically-written. The finding that lin-32/Ato and hlh-14 exhibit mirrored expression patterns is intriguing, and provides a potential mechanism at the genetic level of how neurons of the same class are generated from asymmetric lineages. The data generated is detailed and convincing, and publication would definitely benefit the field.

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First revision

Author response to reviewers' comments

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Apologies for not being clear in the manuscript and thanks for catching this, it is a critical point. We actually HAVE used NeuroPAL for the identification of the expressing cells, we just did not show the additional color channels and only showed the gene under investigation. In addition, for some neurons we used Neuropal-independent terminal identity markers such as eat-4 prom 8, nlp-45, ttl-

9, klp-6 and flp-3 which indeed confirm our neuropal IDs. We have now added a statement in the figure legend that describes this. If the reviewers feels that we need to show primary images for this, we are happy to do this, but felt it was not necessary.

2. Related to above concern, I am strongly curious about applying NeuroPAL system to identify cells in TF mutants. I understand the principle and advantage of this system to identify each neuron in worms, and based on the color in each neuron, authors have tried to identify cells which are existing or lost in the TF mutant animals. However, in the lin-32 mutant animals, it seems that the color pattern was largely altered in a lot of neurons (for example, Fig 3A, Fig. 8B and Fig. 9A). Importantly, the NeuroPAL colors were altered in the cells closely positioned around the lin-32-expressing cell lineages. Presumably, the loss of a transcriptional factor activity affected expression patterns of some of marker genes in the NeuroPAL system and altered the color of cells. Thus, I suspect that identification of cells losing their original NeuroPAL colors are reliable in the lin-32 mutant animals. Authors should indicate the exact method for cell identification or discuss the reliability of their approach. In addition, I am also wondering whether cells whose colors were changed in the lin-32 mutants were generated from the lin-32-expressing cell lineages.
Very good points, thank you. First of all, in reference to the last point, all color changes were restricted to those cells (or lineages) that express lin-32. We found no evidence for cell-non-autonomous defects.
This being said, we very much understand why the reviewer raises the question – at first sight, it appears that color codes of surrounding cells have changed, but this is not the case because (1) cells have subtly changed their relative position; (2) there are important issues with single-plane-of-focus images: If a neighboring, unaffected cell is not in the same plane of focus into wt vs mutant, it may appear like a color change, but this is simply because different color in the composite color image are more affected than others – we now illustrate this nice in an added Supp Fig.S1. We have now added a detailed description of these issues in the Methods (and in the added Supp. Fig), we have added more description to the figure legend and, lastly, we have numbers to the color neurons to facilitate the proper matching of wt vs. mutant images.

3. The authors have shown that lin-32 and hlh-14 were expressed as a mirror image in symmetric left/right neuron pairs. However, the phenotypic penetrance in each mutant seem to be largely distinct. The lack of lin-32 activity strongly affected the expression of terminal selector genes (Fig. 4), but animals lacking hlh-14 activity showed quite mild defects in the expression of terminal selector genes (ceh-10 expression in CAN and hlh-34 expression in AVH, Fig. 7). Why did one lineage cause stronger defects in daughter cells but the other not? In addition, authors showed that cells at ventral side were not strongly affected by the loss of LIN-32 activity, compared to the cells at dorsal side. Why? Discussing the regulatory mechanism of these interesting phenomena would improve the manuscript.
Yes, there are indeed difference in penetrance. But, frankly, we do not have any interesting explanation that we could discuss. One can also always argue that incomplete penetrance is due to partial compensation by other factors, such as other ASc family members, but that's a trivial statement and we would prefer to not engage in such speculation.

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lin-32 mutant animals. Did you compare the difference in cell number expressing rab-3 between L1 and L4 stage? However, such description is not seen in anywhere in the figure?
Sample size added in legend. No, we did not do any comparisons between L1 and L4 stage. At each stage, we only compared wt and lin-32, as indicated.

5. In Fig. 4, defects of the terminal selector gene expression were milder in ventral side compared to dorsal side. Why? And IL1V neurons are also generated in distinct lineage but affected stronger than IL2V cells. Are the differences in phenotypic penetrance dependent on lineages generating each cell?
Good question – no, it's not dependent on lineage and we can not explain the differences in penetrance. As we stated in response to a question above, it's probably again due to compensatory mechanisms, i.e. other factors that are present (or NOT), that can partially compensate for loss of the gene under investigation. Since this is all speculation and not a really good answer, we refrain from discussing this in the text.

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Fixed.

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Reviewer 2 Advance Summary and Potential Significance to Field:
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This is a fair point and we fixed this in the manuscript, referring to the previous analysis of the expression – which was only done with a a promoter fusion (and also did not go as far down in the lineage) – which indeed revealed a very similar expression pattern.

2. On page 13, the authors mention that lin-32/Ato affects expression of "ceh-43/Dlx in CANL, but not CANR." While they show the expression of ceh-43::GFP in the head region in Fig. 4A, they do not show ceh-43 expression in CAN neurons.
Apologies for this oversight. Now added in Figure 5C.

3. The authors show how hlh-14 expression is a mirror of lin-32/Ato, and functions to help specify the counterpart neuron when examining CAN and AVH neurons. It would be interesting to see if either transcription factor can functionally substitute for the other to generate the proper neuron identity. Seeing if expression of hlh-14 in the lin-32/Ato expressing lineages of lin-32 mutants or vice versa can restore the missing CAN or AVH neuron may strengthen the idea of these transcription factors as regulators of convergent lineages.
To avoid overexpression artefacts (an issue if one deals with somewhat closely related proteins that may display distinct affinities to the same targets), we actually tried to do this experiment by a complex CRISPR experiment, in which we attempted to replace the exons of lin-32 with those of hlh-14 – but couldn't get this to work.

4. In Fig. 9A, the lineages that generate URY and OLQ neurons all express lin-32/Ato, but OLQ neurons apparently transform into URY and express ceh-32/Six3 in lin-32/Ato mutants. It would be helpful to address if and how lin-32/Ato functions differently for these neurons, but a discussion would suffice.
This is actually the same situation as in the anterior deirid lineage, where lin-32 is also expressed in multiple cells of the lineage, and in the mutant, all cells become ADE neuron-like – and like in the anterior lineage the OLQ/URY change is likely the result of changes in the expression of terminal selector. We're now making this cleaer in the manuscript.

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Fixed.

Second decision letter

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AUTHORS: Neda Masoudi, Eviatar Yemini, Ralf Schnabel, and Oliver Hobert
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

As I commented on the first review, authors focused on quite novel phenomena on the cellular convergence in the C. elegans nervous system. I feel that this manuscript is potentially suitable for the publication in the Development.

Comments for the author

Authors promptly responded to all the concerns I raised.

Reviewer 2

Advance summary and potential significance to field

The revised manuscript addressed all my questions. I recommend the manuscript for publication. Congratulations.

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