

Combinatorial mechanisms specify cellular location and neurotransmitter identity during planarian neurogenesis

Corresponding Author: Dr Rachel Roberts-Galbraith

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript by Clay et al., the authors characterize how planarian stem cells are specified into dopaminergic neurons during both regeneration and in homeostatic cell turnover. In contrast to most animals, including mammals, planarians are able to constantly make new dopaminergic neurons from a population of adult pluripotent stem cells, called neoblasts. It is well accepted in the planarian field that pluripotent neoblasts give rise to specific lineage-committed progenitors that finally will end up differentiating into all the adult mature cell types. For the distinct neuronal cell types the specific progenitor populations have been characterized by the expression of specific transcription factors (TFs) for each of the neuronal lineages. However, it is not known which factors regulate the correct positioning of the newly generated neurons. In this sense, the authors use here the paradigm of dopaminergic neurons which are located in specific regions along the central and peripheral nervous system. Thus, dopaminergic neurons are found within the cephalic ganglia (CNS) as well as in the peripheral nervous system (probably within submuscular plexus) and within the pharynx (also peripheral nervous system). Remarkably, the authors find that different specific TFs direct the specification and differentiation of the spatially distinct populations of dopaminergic neurons. To my knowledge, this is the first example where it is described how a specific planarian neural cell type is specified not only in terms of its function (in this case the kind of neurotransmitter produced), but also in terms of its location along the body. These results are well supported and are of high interest not only in the planarian field to understand how regeneration is orchestrated, but also for the regenerative medicine field, as the authors state. Therefore, I recommend this manuscript to be accepted for publication.

Before final acceptance, the authors could consider the following points:

1.- The authors might consider to show the differences in the dorsal and ventral distribution of *irx4-6* and *lil-2* dopaminergic neurons in histological cross-sections to better characterize the normal pattern of distribution of such neurons. In the same line, can they exclude that dopaminergic neurons reside not only in the body-wall submuscular plexus but also in the neural plexus around the digestive system?

2.- In figure 3B the authors quantify the results of single gene RNAi and in figure 4B of double knockdowns (KD). But the bar showing the quantification of the controls is the same as I guess they check single and double RNAi on the same experiment. I would recommend to combine both graphs to show in one graph the results of single and double KD. Also, here the authors claim that the double KD of *irx-like* and *irx-4/6* causes a more severe abnormal phenotype compared to single *irx-4/6* RNAi. However, from the graphs it doesn't to be the case. They should clarify this point.

Minor points:

1.- On page 6 (pages are not numbered, though), in the first paragraph the authors write about TPH+ but I guess they refer to TBH+ cells

2.- For figure 2C it is not said to how many days after the last feeding of dsRNA correspond those animals.

3.- In figure 3A it is written "tyrosine hydroxylase" when in most figures it is written "TH".

4.- In the main text (on page 13), when describing figure 5B the authors refer to single *flii-2* and *irx-4/6* RNAi but in the legend of the panel shown in figure 5B it seems to correspond to double RNAi *flii1-2;flii1-2* (here it is probably also misspelled) and *irx4/6;irx-like*.

5.- On page 13, in the second paragraph, the authors refer to TH+/slc6a3+ cells but it gives the impression that they refer to cells co-expressing both genes, which is not what they really mean.

Reviewer #2

(Remarks to the Author)

The study by Clay et al investigates transcriptional regulation of dopaminergic (DA) neuron specification and regeneration in planaria, a powerful model system for studying adult neurogenesis due to its abundant pluripotent stem cells and regenerative capacity. The authors focus on a group of transcription factors (TFs), including *lmo1/3-1*, *app-L1*, *fli1-1/2*, and *irx4/6*, analyzing their expression in TH+ neurons and performing functional studies via RNAi-mediated knockdown. An experiment using a ventral flipping assay shows that TH+ neurons in the brain are required for efficient flipping when the worm is flipped ventral side up. Finally, the authors then perform an RNA-seq analysis following *soxB1-2*, *fli1-1/fli1-2*, and *irx-like/irx4* knockdown and identify genes with deregulated and overlapping expression, including, in some cases, deregulation of each other's expression.

Overall, while the study is nicely done and provides insight into planaria DA neuronal development, it lacks mechanistic insight. The idea that a core set of TFs act cell-autonomously to specify DA neuron identity, while regional or context-specific TFs modulate subtype or positional identity, is conceptually established in vertebrate models. However, this framework is not well studied in planaria or other regenerating systems, and the extension of these principles to an invertebrate regeneration model is potentially novel and valuable. The idea that identity and location decisions can influence the development of different regions, as suggested in Fig. 7 of the manuscript, is interesting; however, the data supporting this are limited and would require further studies to definitively demonstrate.

Specific comments:

1. The RNA-seq analyses following knockdown suggest some regulatory interdependence, however, the data do not resolve a clear regulatory network or pathway. Clarifying these points through additional temporal, cell fate, or rescue experiments would substantially strengthen the manuscript. If the authors could define how regional TFs modulate or gate core DA TF activity—or show distinct TF combinations for CNS vs. PNS DA neurons—the manuscript would significantly advance the field.
2. If the authors are claiming that the combined knockdown of IRX or FLI pairs leads to an enhanced phenotype, the individual and double knockdowns should be plotted in the same graph, and a statistical analysis performed. It was not obvious from the figures cited (Fig. 4A–B, Extended Fig. 7A–D) that there is an enhanced phenotype, as the single and double knockdown data are not shown in the same graph.
3. In the double knockdown experiments, a control should be performed that includes 5 µg of GFP dsRNA together with *irx4/6* dsRNA to control for non-specific effects caused by the amount of dsRNA. For example, the additional 5 µg of dsRNA against *irx-like* may stabilize the *irx4/6* dsRNA, enabling it to be more effective. In Extended Fig. 3F, *irx4/6* is reduced by only 50%, meaning that anything that stabilizes *irx4/6* dsRNA could lead to a greater loss of *irx4/6* and a more robust phenotype.
4. The authors describe *irx-like* as being restricted to the ventral side, but this data is not shown in Fig. 3. The figure should be revised to show this clearly. More clarity should also be included in the methods regarding how ventral and dorsal neurons are distinguished, as it is not clear how this is done during imaging analysis. Are all the neurons in the ventral and dorsal halves imaged, or only the neurons within a single focal plane?
5. What happens to TH+ cells that are lost in the knockdown experiments? Are they dying? Adopting a different fate? The RNAi-mediated decreases in TH+ cells in the regeneration experiments could also be due to a role in maintenance. For example, TH+ neuronal specification may be normal during regeneration, but cells may not be maintained and could die soon after they are generated. Lineage tracing (if possible) or early time-point analysis would help distinguish between specification and maintenance defects.
6. Non-neuronal cells can also synthesize dopamine. Are all the TH+ cells in planaria, especially those in this paper, neurons that co-localize with pan-neuronal markers? If so, what is the evidence? Some additional comment in the text would be helpful.
7. If *foxA* is expressed in a widespread manner pharyngeally and is involved in pharyngeal regeneration, could the loss of TH+ cells in regenerating pharynges after *foxA* knockdown be indirect and due to an earlier role for *foxA* in establishing peripheral fate? For example, are other pharyngeal cell types lost in the *foxA* knockdown (there appear to be many *foxA*-expressing cells that are TH-negative), or are TH+ cells specifically targeted?
8. Behavioral relevance (e.g., flipping assay): This provides functional validation, though more tightly linking molecular and

behavioral phenotypes would improve the impact.

9. It is difficult to determine the extent of co-expression in the immunofluorescence panels, as the channels are not separated and greyscale was used to visualize nuclei (DAPI). This is problematic because green and magenta are being used for RNA/protein detection. Since co-localization of magenta and green appears white, it blends in with and is obscured by the DAPI greyscale channel. While magenta and green are preferred colors to accommodate those with color blindness, this is only useful if those are the only two channels in the image. For three-color imaging, a different color scheme needs to be used. Additionally, the color channels should be separated into individual panels.

Reviewer #3

(Remarks to the Author)

This manuscript from Clay et al describes a screen for genes required to specify dopaminergic neurons during regeneration. Regenerative neurogenesis is a challenge to study in animals with limited adult neurogenesis, so here the authors use planarians, which have robust and constant neurogenesis. In planarians, dopaminergic neurons occupy three standard domains, which the authors define as central, peripheral, and pharyngeal nervous systems. Mining single cell RNA-seq datasets for genes expressed in cells expressing tyrosine hydroxylase, a standard marker of dopaminergic neurons, the authors identify 73 genes to test. To assess the function of these genes in regeneration, they were individually knocked down with RNAi. Several appear to be required for the regeneration or maintenance of different populations of dopaminergic neurons. Subsequent analysis measures the abundance of dopaminergic neurons in animals, finding that some genes appear to be required for the maintenance or regeneration of distinct, spatially-localized portions of these neurons.

The novelty of the planarian system offers an opportunity to determine new gene regulatory networks that act in adult neurogenesis, and the authors take advantage of the ease of determining gene function in this system to identify new regulators of neurogenesis. Overall, the data presented are carefully analyzed. The addition of HPLC to verify the decrease in dopamine after knockdown is important. Although the broad conclusion that transcription factors are required for specification of neurons will not be surprising to most, this paper nicely integrates the function of several factors in reconstituting the nervous system during regeneration. However, there are some issues with data presentation and conclusions that should be addressed, as enumerated below.

1. The title suggests that combinatorial mechanisms are important to specify dopaminergic neurons, yet these mechanisms are not clearly described. Either the title should be modified to more accurately reflect the conclusions from the data in the paper (that transcription factors are required for specification of dopaminergic neurons), or experiments that demonstrate what the authors mean by combinatorial mechanisms should be included.

2. Double knockdowns are used because they are shown to have stronger phenotypes than single knockdowns. However, a statistical comparison between single and double knockdowns (*fli1-1*;*fli1-2* and *irx-like*;*irx-4/6*) should be verified side-by-side with single knockdowns.

3. The conclusion in Fig 5 that dopaminergic neurons in the CNS are required for dorsal-ventral flipping is not well supported as presented. The number of animals tested is not indicated. In Fig 5B, controls seem to be significantly slower than in A. The text describes single knockdowns, whereas the figure indicates double knockdown. Single knockdowns should be included along with TH as a positive control in a single assay, along with biological replicates. Because *fli1-2* knockdown animals show a decrease in the number of TH+ cells in the CNS (Fig 4G), the conclusion that peripheral neurons play a role in righting behavior should be toned down.

4. The specificity of gene knockdowns should be strengthened for the primary phenotypes in the paper (*lmo1/3-1*, *app-L1*, *fli-1*, *irx* homologs). ChAT and *cpp-1* are shown in Ext Fig 6 for *lmo1/3-1*, *app-L1*, suggesting that the defects are not specific to dopaminergic neurons, but analysis of other important knockdowns are not shown. Anything quantifiable would be informative (e.g. *cintillo*, other neuropeptides, or serotonergic neurons) and allow the authors to state whether these phenotypes are specific at all. For example, knockdown of *FoxA* prevents differentiation of many kinds of neurons in the pharynx (PMID: 40376085).

5. The concept of hierarchy should be supported with additional data and explanation (Fig 7). Although the authors hypothesize that these transcription factors operate in a hierarchy, the rationale underlying this hypothesis should be explained better.

- The qualitative yes/no in 7E should be supported by a concrete readout like RPKM along with statistics.

- The data in C and D, if understood correctly, are used to support the notion that these five transcription factors (*soxB1-2*, the 2 *fli*'s, and the 2 *irx* homologs) together regulate TH and *slc6a3*. However, the legend indicates that the genes represented by the circles include both upregulated and downregulated genes. Why have these been combined? Are AADCα, TH, and *slc6a3* all downregulated in the three data sets as expected? Please clarify.

- RNA-seq of *soxB1-2*(RNAi) has been previously reported in PMID: 30399335, but the numbers of genes differ significantly from those presented here (3278 vs 1192 downregulated, 2575 vs 1054 upregulated). I'm not sure that the number of genes is necessarily relevant, but can the authors explain this discrepancy, or provide evidence that similar genes are detected in both data sets as a positive control?

6. Some sections would benefit from revised writing to clarify the narrative.

- Rather than starting paragraphs with phrases like ‘we decided to follow up on fli1-2 and irx-4/6’ or ‘We next returned to...’, it would be better to rephrase topic sentences with hypotheses that connect the upcoming results to the overall goals of the paper.
- From the conclusion: “we confirm that fli1-2 and irx4/6 are expressed in G0 neural progenitors and that both fli1-2 and soxB1-2 are co-expressed with smedwi-1” comes as a surprise because there is not any data in the paper that shows this information.
- Confusing conclusion in this sentence: “We determined through both ISH and RNA-sequencing that soxB1-2(RNAi) caused reduction in irx-like and fli1-2 transcripts (Fig. 7E-G), which could support the idea that neural location is determined upstream of neurotransmitter identity.”
- Unclear what data was used to reach the following: “We conclude that there does not seem to be a definitive hierarchy in the creation of peripheral dopaminergic neurons.”

7. In general, the figures would benefit from polishing and/or reorganizing to improve clarity of the main take-home points. As is, the repetition of the assays obscures the conclusions. A lot of space is dedicated to bar graphs of co-expression which are not especially informative. Consider reorganizing figures to emphasize the take-home messages, and consolidating these supporting graphs in Extended Figures.

- Make font sizes and cartoon animals consistent across all panels. Figure panels within individual figures should be consistent (e.g. 5A/B)
- Do not use default brackets/y-axes from GraphPad to better reflect the data.
- Indicate on figures themselves what is being quantified: PNS/CNS, dorsal/ventral, what kinds of genes are represented by the two apparently similar diagrams in Fig 7C/D to help with readability
- Colors in fluorescent images are muted. Sometimes this happens because the figures were generated using CMYK instead of RGB.
- Modify cartoons to indicate more clearly newly regenerated vs homeostatic tissues.
- Refer to slc6a3 as DAT to help make it clear to readers what this gene’s function is.
- Order of ‘regions’ in Fig 2 should be kept consistent with those in Fig 1, and color-coded to match schematics in Fig 1.
- Why is celsr-1(RNAi) included in extended Fig. 2 and 3 but not mentioned in the text? Consider deleting it if it isn’t important to the message of the manuscript.

Minor comments:

Y-axis label, ext Fig 6H: ‘arean’, numbers are misaligned, and it could help to make the axis consistent with F and G.

Suggest adding page numbers/line numbers to help reviewers note the position of text.

Move repetitive information about quantification (e.g. 10000 μm^2 boxes) to methods to consolidate.

Fig 4M/N: rather than calling this experiment an ‘alternate RNAi paradigm’, it should be referred to as an ‘alternate regeneration paradigm’.

In 1L, maybe make the predictions more obvious with a chart instead of subtle changes in the number of dots

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I feel fully satisfied with all the revisions and improvements done and I strongly recommend it for publication

Reviewer #2

(Remarks to the Author)

The authors have made substantial revisions to the manuscript and with the exception of one minor point noted below, my concerns have been adequately addressed. I have no further major concerns regarding the manuscript.

In particular, the authors have done a good job addressing my questions regarding the fate of the TH⁺ cells lost in the knockdown experiments. These additional experiments provide mechanistic insight into the roles of these genes in dopaminergic neuron neurogenesis and/or survival. More broadly, this study provides new insight into how transcriptional programs coordinate neuronal identity, spatial patterning, and regeneration, highlighting the utility of planarians as a model for understanding mechanisms of nervous system repair. Overall, this is an interesting and well-executed study, and I recommend that it be accepted for publication.

(Original reviewer comment) 2. If the authors are claiming that the combined knockdown of IRX or FLI pairs leads to an enhanced phenotype, the individual and double knockdowns should be plotted in the same graph, and a statistical analysis performed. It was not obvious from the figures cited (Fig. 4A–B, Extended Fig. 7A–D) that there is an enhanced phenotype, as the single and double knockdown

data are not shown in the same graph.

(Author response) This has been corrected in Figure 3A-B and Extended Figure 9A-D to show both the single and double knockdowns together in the same experiment and graph. Additionally, the text has been corrected to cite Figure 3A-B instead of Figure 4A-B.

(Reviewer response to revision) In the revised manuscript, Figure 3B presents the single and double FLI/IRX knockdown conditions within the same experimental framework; however, the statistical analysis employs Dunnett's multiple comparisons test, which assesses each RNAi condition only against the control group. While this approach is appropriate for identifying differences relative to control, it does not test whether the double knockdown produces a significantly stronger phenotype than either single knockdown condition. Since the interpretation relies on functional redundancy between paralogs, the analysis should include direct statistical comparisons between single and double knockdown conditions (e.g., Tukey's multiple comparisons test following one-way ANOVA). Alternatively, a two-way ANOVA including an interaction term between paralog knockdowns would provide a more direct and informative assessment of genetic interaction and redundancy.

Reviewer #3

(Remarks to the Author)

The revised version of this manuscript is significantly improved in the figure presentation and in the writing. In particular, the inclusion of new data perturbing apoptosis and lineage tracing with EdU strengthens the authors conclusions about the role of these transcription factors in neurogenesis. Behavior and double RNAi experiments are better controlled. All other experimental concerns have been addressed.

Some very minor comments follow:

Figure 1F, text says that 64 expression patterns were determined but figure says 63.

Line 139, no figure number

Line 144 is garbled.

Suggest including some indication of the mechanism of action of M50054 rather than simply referring to its catalog number. Line 208-211 references Extended Figure 9 but it should be Extended Figure 6.

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We want to start by thanking the reviewers for their constructive feedback and encouragement. In response to reviews, we completed quite a number of new experiments and summarize the main additions to the manuscript below.

1. Previously, the reviewers had wanted to understand whether the differences in dopaminergic cell numbers that we saw after knocking down candidate genes happened because of differences in cell birth. We optimized an F-ara-EdU pulse-chase and fluorescent *in situ* hybridization (FISH) protocol so that we could definitively answer this question. Results from these experiments are now in Fig. 2F-G and Fig 5A-D. We find that *app-L1*, *lmo1/3-1*, *fli1-2*, and *irx4/6* all impact cell birth of dopaminergic neurons. The reviewers also wondered if the differences in dopaminergic cell numbers occurred because of cell death. This experiments required to test this idea were a bit trickier (and new to us), but we found two different ways to inhibit cell death: *bax-1/2*(RNAi) (which has not been shown before) and treatment with M50054 (which had been shown by the Beane lab previously PMID: 23250205). We were then able to combine RNAi treatment of candidate genes with these perturbations of cell death to understand the contribution of cell death to maintenance of dopaminergic neurons. Interestingly, we found that *app-L1* is also important for dopaminergic cell maintenance while *lmo1/3-1*'s role seems to be through neurogenesis alone. Our data indicate that *fli1-2* and *irx4/6* also influence dopaminergic cell maintenance. Interestingly (and counterintuitively), our perturbations of apoptosis by *bax-1/2*(RNAi) decreased the number of *TH*⁺ cells (while overall decreasing cell death), so our experiments may have also uncovered an apoptosis-independent role for *bax* in neuronal development that we plan to follow up on! (This isn't totally unprecedented, because there are non-apoptotic or non-canonical roles for BCL family members in neurons in the literature, as nicely reviewed in Gross and Katz in *Cell Death and Differentiation* 2017).
2. The reviewers were keen to understand how dopaminergic neuron identity factors and spatial regulators work together to direct cell fate. This ended up being possible to do based on our work with *fli1-2*, *irx4/6*, and *soxB1-2*. However, it was not easy! All three markers are expressed in *smedwi-1*⁺ cells, indicating that they are all expressed in the pool of neuronal progenitors that still have maintain stem cell markers. We were able to work around this by doing an F-ara-EdU pulse and then killing and fixing immediately after or after short chases. This allowed us to determine that *fli1-2* and *irx4/6* are expressed very shortly before *soxB1-2*. We can conclude that the transcription factors that promote neurotransmitter identity are present shortly before the place-specific transcription factor is turned on, at least for this cell type. This allowed us to put these factors together in a model that reflects sequential but near-simultaneous expression and co-dependence of these factors.
3. We identified some unexpected complexity of the *TH* neuron population, since *TH* had historically been used as a marker of dopaminergic neurons in our field. We find that there are also norepinephrine-producing neurons in planarians that express *TH* and *TBH* (counter to prior reports). Importantly, we also validated *DAT* as a dopaminergic neuron-specific marker so that we have independent confirmation of all our conclusions

regarding the cell type. Though this finding is a bit niche to those working in our field, it is an important bit of correction of the literature on planarian neuroscience.

4. We also note that while many of our data indicate that *app-L1* is not required for dopaminergic neuronal maintenance, we did see a non-significant decrease in *TH*⁺ cell numbers after *app-L1* in one maintenance experiment (Fig. 2I). We therefore opted to remove the claim that the role of *app-L1* is regeneration-specific, pending further research.

There are several other additions and changes requested and incorporated, but these major changes each constitute a significant improvement in the paper. These changes also challenged us to innovate new approaches and apply new ones in our lab, so we are excited to share the results of all this effort!

Reviewer #1 (Remarks to the Author):

In this manuscript by Clay et al., the authors characterize how planarian stem cells are specified into dopaminergic neurons during both regeneration and in homeostatic cell turnover. In contrast to most animals, including mammals, planarians are able to constantly make new dopaminergic neurons from a population of adult pluripotent stem cells, called neoblasts. It is well accepted in the planarian field that pluripotent neoblasts give rise to specific lineage-committed progenitors that finally will end up differentiating into all the adult mature cell types. For the distinct neuronal cell types the specific progenitor populations have been characterized by the expression of specific transcription factors (TFs) for each of the neuronal lineages. However, it is not known which factors regulate the correct positioning of the newly generated neurons. In this sense, the authors use here the paradigm of dopaminergic neurons which are located in specific regions along the central and peripheral nervous system. Thus, dopaminergic neurons are found within the cephalic ganglia (CNS) as well as in the peripheral nervous system (probably within submuscular plexus) and within the pharynx (also peripheral nervous system). Remarkably, the authors find that different specific TFs direct the specification and differentiation of the spatially distinct populations of dopaminergic neurons. To my knowledge, this is the first example where it is described how a specific planarian neural cell type is specified not only in terms of its function (in this case the kind of neurotransmitter produced), but also in terms of its location along the body. These results are well supported and are of high interest not only in the planarian field to understand how regeneration is orchestrated, but also for the regenerative medicine field, as the authors state. Therefore, I recommend this manuscript to be accepted for publication.

Thank you for your positive feedback on our work and for your thoughtful questions and suggestions.

Before final acceptance, the authors could consider the following points:

- 1.- The authors might consider to show the differences in the dorsal and ventral distribution of *irx4-6* and *flil-2* dopaminergic neurons in histological cross-sections to better characterize the normal pattern of distribution of such neurons. In the same line, can they exclude that

dopaminergic neurons reside not only in the body-wall submuscular plexus but also in the neural plexus around the digestive system?

Thank you for posing this question. To assess the dorsal and ventral position of peripheral dopaminergic neurons, we assessed the location of *TH*⁺ neurons with respect to a pool of two muscle markers: *mhc* and *myoD*. This enabled us to use optical cross sections to show the position of dopaminergic neurons near the body-wall submuscular plexus. We did *not* note *TH*⁺ cell bodies near the intestine, though it is still possible that dopaminergic neurons project to this organ. These images have been added to Extended Figure 1E.

2.- In figure 3B the authors quantify the results of single gene RNAi and in figure 4B of double knockdowns (KD). But the bar showing the quantification of the controls is the same as I guess they check single and double RNAi on the same experiment. I would recommend to combine both graphs to show in one graph the results of single and double KD. Also, here the authors claim that the double KD of *irx-like* and *irx-4/6* causes a more severe abnormal phenotype compared to single *irx-4/6* RNAi. However, from the graphs it doesn't to be the case. They should clarify this point.

We thank you for pointing this out. We modified the figures (3A-B, Extended Figure 9A-D) to show the single and double KDs together with controls and we also detailed this approach more carefully in the methods.

Minor points:

1.- On page 6 (pages are not numbered, though), in the first paragraph the authors write about TPH⁺ but I guess they refer to TBH⁺ cells

We thank the reviewer for pointing out this typo, and we made the necessary correction.

2.- For figure 2C it is not said to how many days after the last feeding of dsRNA correspond those animals.

Thank you – this has been addressed in the figure caption for figure 2.

3.- In figure 3A it is written “tyrosine hydroxylase” when in most figures it is written “TH”.

Thank you – this has been changed to *TH*.

4.- In the main text (on page 13), when describing figure 5B the authors refer to single *flil-2* and *irx-4/6* RNAi but in the legend of the panel shown in figure 5B it seems to correspond to double RNAi *flil1-2;flil1-2* (here it is probably also misspelled) and *irx4/6;irx-like*.

Thank you – this has been addressed via amended data in Figure 6.

5.- On page 13, in the second paragraph, the authors refer to TH⁺/slc6a3⁺ cells but it gives the impression that they refer to cells co-expressing both genes, which is not what they really mean.

Thank you – we have corrected in text to refer to both *TH*⁺ cells and *DAT*⁺ cells separately. (*slc6a3* has been changed to *DAT* throughout in response to a reviewer comment below).

Reviewer #2 (Remarks to the Author):

The study by Clay et al investigates transcriptional regulation of dopaminergic (DA) neuron specification and regeneration in planaria, a powerful model system for studying adult neurogenesis due to its abundant pluripotent stem cells and regenerative capacity. The authors focus on a group of transcription factors (TFs), including *lmo1/3-1*, *app-L1*, *fli1-1/2*, and *irx4/6*, analyzing their expression in TH⁺ neurons and performing functional studies via RNAi-mediated knockdown. An experiment using a ventral flipping assay shows that TH⁺ neurons in the brain are required for efficient flipping when the worm is flipped ventral side up. Finally, the authors then perform an RNA-seq analysis following *soxB1-2*, *fli1-1/fli1-2*, and *irx-like/irx4/* knockdown and identify genes with deregulated and overlapping expression, including, in some cases, deregulation of each other's expression.

Overall, while the study is nicely done and provides insight into planaria DA neuronal development, it lacks mechanistic insight. The idea that a core set of TFs act cell-autonomously to specify DA neuron identity, while regional or context-specific TFs modulate subtype or positional identity, is conceptually established in vertebrate models. However, this framework is not well studied in planaria or other regenerating systems, and the extension of these principles to an invertebrate regeneration model is potentially novel and valuable. The idea that identity and location decisions can influence the development of different regions, as suggested in Fig. 7 of the manuscript, is interesting; however, the data supporting this are limited and would require further studies to definitively demonstrate.

We appreciate the feedback and we worked hard to address the main points raised in this thoughtful review. Details on our changes are shown below.

Specific comments:

1. The RNA-seq analyses following knockdown suggest some regulatory interdependence, however, the data do not resolve a clear regulatory network or pathway. Clarifying these points through additional temporal, cell fate, or rescue experiments would substantially strengthen the manuscript. If the authors could define how regional TFs modulate or gate core DA TF activity—or show distinct TF combinations for CNS vs. PNS DA neurons—the manuscript would significantly advance the field.

Thank you for this idea. We included images of expression patterns for core DA TFs after knockdown of the regional TF *soxB1-2* (Fig. 8F-G), and showed expression patterns of TFs after either *fli* or *irx* knockdown (Extended Fig 12I-K). It was more challenging than expected to address the hierarchy of these TFs, but we have made progress through the several experiments.

We determined that all three TFs are co-expressed with *smedwi-1*, indicating they all play a role in early specification of progenitor cells. Assessing the proportions of cells co-expressing combinations of *soxB1-2*, *fli1-2*, and *smedwi-1* revealed that there are similar proportions of co-positive and single positive cells, indicating there are always examples of one TF being expressed without the other (Fig 8H). This might be because each factor plays additional roles in addition to regulating dopaminergic neurons.

Since these approaches were not sufficient, we went to an additional round of experiments. First, we completed co-expression analysis of *smedwi-1* and either *soxB1-2*, *fli1-2*, or *irx-4/6*. Second, we completed F-ara-EdU pulse or short pulse/chase during regeneration (Fig 8I-J). We assessed the percentage of F-ara-EdU+ cells that expressed *smedwi-1* and the aforementioned transcription factors immediately after the pulse and after 12 hour, 24 hour, or 36 hour chases. This allowed us to ascertain a timeline of when during or after S phase stem cells started to express the TFs. Based on our data, we conclude that core DA TFs (*fli1-2* and *irx-4/6*) begin to be expressed during or immediately after S phase, whereas the regional factor (*soxB1-2*) is not expressed in stem cells until 12 hours after labeling. This indicated that the core DA TF program is activated slightly before region-specific regulators, showing that identity is specified first and that location is specified very shortly thereafter (see Fig 8L diagram).

2. If the authors are claiming that the combined knockdown of IRX or FLI pairs leads to an enhanced phenotype, the individual and double knockdowns should be plotted in the same graph, and a statistical analysis performed. It was not obvious from the figures cited (Fig. 4A–B, Extended Fig. 7A–D) that there is an enhanced phenotype, as the single and double knockdown data are not shown in the same graph.

This has been corrected in Figure 3A-B and Extended Figure 9A-D to show both the single and double knockdowns together in the same experiment and graph. Additionally, the text has been corrected to cite Figure 3A-B instead of Figure 4A-B.

3. In the double knockdown experiments, a control should be performed that includes 5 µg of GFP dsRNA together with *irx4/6* dsRNA to control for non-specific effects caused by the amount of dsRNA. For example, the additional 5 µg of dsRNA against *irx*-like may stabilize the *irx4/6* dsRNA, enabling it to be more effective. In Extended Fig. 3F, *irx4/6* is reduced by only 50%, meaning that anything that stabilizes *irx4/6* dsRNA could lead to a greater loss of *irx4/6* and a more robust phenotype.

Thank you for pointing this out. We performed this experiment as described with the additional 5 µg of dsRNA against GFP to control for non-specific effects in the single KD vs double KD paradigm. This has been clarified in the methods and is shown in Figure 3A-B and Extended Figure 9A-D.

4. The authors describe *irx*-like as being restricted to the ventral side, but this data is not shown in Fig. 3. The figure should be revised to show this clearly. More clarity should also be included in the methods regarding how ventral and dorsal neurons are distinguished, as it is not clear

how this is done during imaging analysis. Are all the neurons in the ventral and dorsal halves imaged, or only the neurons within a single focal plane?

Thank you for this suggestion. We clarified in the methods how we determine the dorsal and ventral side of the animal during imaging. For PNS imaging, animals are mounted in PBSTx without clearing, which allows us to use the opacity of animal to obscure color from the opposite side of the body. This allowed us to definitively see the dorsal vs ventral sides. During colorimetric ISH imaging, ventral and dorsal sides are imaged separately, leading to a single image of each side. The dorsal view of *irx-like* is shown in Extended Fig 9G.

5. What happens to TH⁺ cells that are lost in the knockdown experiments? Are they dying? Adopting a different fate? The RNAi-mediated decreases in TH⁺ cells in the regeneration experiments could also be due to a role in maintenance. For example, TH⁺ neuronal specification may be normal during regeneration, but cells may not be maintained and could die soon after they are generated. Lineage tracing (if possible) or early time-point analysis would help distinguish between specification and maintenance defects.

Thank you for posing these questions. To address the mechanism of TH⁺ cell loss after knockdowns, we conducted several experiments. First, to assess the possibility that each gene plays a role in cell birth, we repeated RNAi experiments and labeled cells in S phase using F-ara-EdU. After labeling, we chased for 7 days before completing F-ara-EdU labeling with FISH using a TH probe (Fig 2F-G, Fig 5A-D). Second, to assess the possibility that our genes play a role in cell death, we blocked cell death in our knockdowns using two methods. We first knocked down homologs of planarian *bax* and separately we administered the anti-apoptotic drug, M50054 (Fig 2H-J, Fig 5E-H, Extended Fig 6H-M, Extended Fig 10L-M). While the M50054 method has been used previously (PMID: 23250205), we developed a *bax* knockdown approach for this paper. We also attempted TUNEL staining with FISH using the TH probe, but that was ultimately not successful in our hands. Through our efforts, we showed that *lmo1/3-1*, *app-L1*, *fli1-2*, and *irx-4/6* each act to promote cell birth of dopaminergic neurons in the brain. *app-L1*, *fli1-2*, and *irx-4/6* also act to prevent cell death of TH⁺ cells in the brain. *fli1-2* and *irx-4/6* further promote survival of TH⁺ cells in the PNS and promote cell birth of TH⁺ cells in the pharynx. These results have been integrated into our model. Our results also raise the idea that many pro-neurogenic factors in planarians continue to be expressed in mature neurons where they protect cells from apoptosis.

Together, these data show mechanistically how each gene promotes neurogenesis and/or survival of dopaminergic neurons. Importantly, we also added valuable methods to the planarian toolkit that can be useful to other researchers.

6. Non-neuronal cells can also synthesize dopamine. Are all the TH⁺ cells in planaria, especially those in this paper, neurons that co-localize with pan-neuronal markers? If so, what is the evidence? Some additional comment in the text would be helpful.

According to transcriptomic evidence (PMID: 29674431 and 29674432), mRNAs for TH and AADC are enriched in neural clusters and not enriched in other cell types. To confirm these

results, we performed double fluorescent *in situ* hybridization with a pan-neuronal pool of markers and confirmed that all *TH*⁺ cells are also neural (Extended Fig 1C-D).

7. If *foxA* is expressed in a widespread manner pharyngeally and is involved in pharyngeal regeneration, could the loss of *TH*⁺ cells in regenerating pharynges after *foxA* knockdown be indirect and due to an earlier role for *foxA* in establishing peripheral fate? For example, are other pharyngeal cell types lost in the *foxA* knockdown (there appear to be many *foxA*-expressing cells that are *TH*-negative), or are *TH*⁺ cells specifically targeted?

Thank you for these questions. Many neural cell types are lost in the *foxA* knockdown as reported by PMID: 40376085. Adler and colleagues propose a model in which *foxA* is required for the specification of neural lineages within the pharynx, which is also supported by our data showing that *foxA* is required for the *TH*⁺ neural lineage within the pharynx. This information has been added to the discussion.

8. Behavioral relevance (e.g., flipping assay): This provides functional validation, though more tightly linking molecular and behavioral phenotypes would improve the impact.

Thank you for this comment. To more tightly link molecular and behavioral phenotypes, we knocked down our candidate genes alongside *TH* and *TBH* to assess for the roles of dopamine and norepinephrine. Behavioral data are shown in Figure 6. Our data indicate that optimal righting behavior requires dopaminergic neurons in the brain, and that *irx-4/6*⁺ and *TH*⁺ neurons contribute to gliding speed. Additionally, we performed HPLC on all knockdowns to assess neurotransmitter abundance. We confirm that DA levels are significantly reduced after *fli1-2* and *irx-4/6* knockdowns, which we show affect all regional populations of dopaminergic neurons (Extended Fig. 12E). However, global DA levels are not significantly changed after either *Imo1/3-1* or *app-L1* knockdowns (Extended Fig. 8E). This is not surprising given that these genes only impact a subset of dopaminergic neurons (those in the CNS) (Extended Fig. 4).

9. It is difficult to determine the extent of co-expression in the immunofluorescence panels, as the channels are not separated and greyscale was used to visualize nuclei (DAPI). This is problematic because green and magenta are being used for RNA/protein detection. Since co-localization of magenta and green appears white, it blends in with and is obscured by the DAPI greyscale channel. While magenta and green are preferred colors to accommodate those with color blindness, this is only useful if those are the only two channels in the image. For three-color imaging, a different color scheme needs to be used. Additionally, the color channels should be separated into individual panels.

Thank you for this comment. We addressed this throughout the manuscript to provide individual channels in grayscale where necessary and multicolor images with DAPI shown in blue. Additionally, three-color imaging has been colored using magenta, green, and yellow with DAPI shown in blue. We hope that this presentation of data is more understandable for our readers.

Reviewer #3 (Remarks to the Author):

This manuscript from Clay et al describes a screen for genes required to specify dopaminergic neurons during regeneration. Regenerative neurogenesis is a challenge to study in animals with limited adult neurogenesis, so here the authors use planarians, which have robust and constant neurogenesis. In planarians, dopaminergic neurons occupy three standard domains, which the authors define as central, peripheral, and pharyngeal nervous systems. Mining single cell RNA-seq datasets for genes expressed in cells expressing tyrosine hydroxylase, a standard marker of dopaminergic neurons, the authors identify 73 genes to test. To assess the function of these genes in regeneration, they were individually knocked down with RNAi. Several appear to be required for the regeneration or maintenance of different populations of dopaminergic neurons. Subsequent analysis measures the abundance of dopaminergic neurons in animals, finding that some genes appear to be required for the maintenance or regeneration of distinct, spatially-localized portions of these neurons.

The novelty of the planarian system offers an opportunity to determine new gene regulatory networks that act in adult neurogenesis, and the authors take advantage of the ease of determining gene function in this system to identify new regulators of neurogenesis. Overall, the data presented are carefully analyzed. The addition of HPLC to verify the decrease in dopamine after knockdown is important. Although the broad conclusion that transcription factors are required for specification of neurons will not be surprising to most, this paper nicely integrates the function of several factors in reconstituting the nervous system during regeneration. However, there are some issues with data presentation and conclusions that should be addressed, as enumerated below.

We appreciate the thoughtful feedback and suggestions very much. Please find below detailed responses to these points.

1. The title suggests that combinatorial mechanisms are important to specify dopaminergic neurons, yet these mechanisms are not clearly described. Either the title should be modified to more accurately reflect the conclusions from the data in the paper (that transcription factors are required for specification of dopaminergic neurons), or experiments that demonstrate what the authors mean by combinatorial mechanisms should be included.

With added data in Fig 8, we confirm that identity and location factors regulate each other and are turned on near-simultaneously, with identity factors coming slightly before the location factor. Additionally, our mechanistic studies show that the genes described regulate the birth of dopaminergic neurons (Fig. 2F-G; Fig. 5A-D). Therefore, we amended the title to “Combinatorial mechanisms specify cellular location and neurotransmitter identity during planarian neurogenesis”.

2. Double knockdowns are used because they are shown to have stronger phenotypes than single knockdowns. However, a statistical comparison between single and double knockdowns (fli1-1;fli1-2 and irx-like;irx-4/6) should be verified side-by-side with single knockdowns.

Thank you for this comment. We edited Figure 3A-B and Extended Figure 9A-D to better reflect the experiment in which single and double knockdowns were compared. Additionally, we completed a statistical test to verify this phenomenon shown in Extended Figure 9A and D.

3. The conclusion in Fig 5 that dopaminergic neurons in the CNS are required for dorsal-ventral flipping is not well supported as presented. The number of animals tested is not indicated. In Fig 5B, controls seem to be significantly slower than in A. The text describes single knockdowns, whereas the figure indicates double knockdown. Single knockdowns should be included along with TH as a positive control in a single assay, along with biological replicates. Because *fli1-2* knockdown animals show a decrease in the number of TH⁺ cells in the CNS (Fig 4G), the conclusion that peripheral neurons play a role in righting behavior should be toned down.

We have addressed this comment by repeating behavioral experiments for single knockdowns of the main hits from the paper. These experiments were done with one set of controls to minimize cross-experiment comparisons. However, during revisions, we found that an alternative dsRNA dosage and feeding paradigm provides a stronger knockdown of *lmo1/3-1*. Therefore, mechanistic experiments (Fig 2F-J) and behavior (Fig 6) were conducted with two separate paradigms. The controls between the *lmo1/3-1* experiment and the experiment with all the other genes show no statistically significant differences in curve kinetics, confirming that the two experiments are reliably similar. We also included *TBH* as another point of comparison to show that it is in fact the dopamine production that is important for the righting behavior as opposed to norepinephrine or other neurotransmitters produced in *TH⁺/TBH⁺* neurons.

4. The specificity of gene knockdowns should be strengthened for the primary phenotypes in the paper (*lmo1/3-1*, *app-L1*, *fli-1*, *irx* homologs). ChAT and *cpp-1* are shown in Ext Fig 6 for *lmo1/3-1*, *app-L1*, suggesting that the defects are not specific to dopaminergic neurons, but analysis of other important knockdowns are not shown. Anything quantifiable would be informative (e.g. *cintillo*, other neuropeptides, or serotonergic neurons) and allow the authors to state whether these phenotypes are specific at all. For example, knockdown of *FoxA* prevents differentiation of many kinds of neurons in the pharynx (PMID: 40376085).

Thank you for bringing this up. We addressed the lack of specificity of *foxA* in the text by citing PMID: 40376085. Additional data regarding *lmo1/3-1* and *app-L1* specificity have been included in Extended Figure 7-8, showing that *lmo1/3-1* affects a few neuron types beyond dopaminergic neurons. We also found that *app-L1* affects non-neuronal cell types, including epidermal cells and protonephridial cells. Additional data regarding *irx-4/6* and *fli1-2* specificity can be found in extended figure 12, where we show that *irx-4/6* is required for certain populations of sensory neurons. Also, our RNA-seq data can be mined by other researchers interested in additional cell types. We uploaded the RNA-seq data in NCBI (BioProject Accession: PRJNA1249448).

5. The concept of hierarchy should be supported with additional data and explanation (Fig 7). Although the authors hypothesize that these transcription factors operate in a hierarchy, the rationale underlying this hypothesis should be explained better.

REVIEWER COMMENTS ORIGINAL SUBMISSION TO NAT COMM

– The qualitative yes/no in 7E should be supported by a concrete readout like RPKM along with statistics.

Thank you. We corrected this table to provide fold change and the p-value, with targets represented by highlighted cells (Fig 8E).

– The data in C and D, if understood correctly, are used to support the notion that these five transcription factors (*soxB1-2*, the 2 *fli*'s, and the 2 *irx* homologs) together regulate TH and *slc6a3*. However, the legend indicates that the genes represented by the circles include both upregulated and downregulated genes. Why have these been combined? Are *AADCα*, TH, and *slc6a3* all downregulated in the three data sets as expected? Please clarify.

We changed these figures to indicate genes downregulated (Fig 8C) and upregulated (Fig 8D). However, *AADCα* was upregulated after *soxB1-2*(RNAi). We would like to note that this is also the case for *soxB1-2*(RNAi) dataset from PMID: 30399335; *AADCα* is significantly upregulated and *slc6a3* (*DAT*) is significantly downregulated in their six-feed paradigm.

– RNA-seq of *soxB1-2*(RNAi) has been previously reported in PMID: 30399335, but the numbers of genes differ significantly from those presented here (3278 vs 1192 downregulated, 2575 vs 1054 upregulated). I'm not sure that the number of genes is necessarily relevant, but can the authors explain this discrepancy, or provide evidence that similar genes are detected in both data sets as a positive control?

Thank you for this comment. In PMID: 30399335, the authors used three different paradigms relying on either 1, 4, or 6 feeds of dsRNA. They administered dsRNA by feeding bacteria induced to produce dsRNA constructs so it is possible that either of these differences may have impacted results. Further, in PMID: 30399335, the authors completed 3 biological replicates and we used 5 biological replicates for our sequencing analysis. Potentially for these reasons, the level of *soxB1-2* transcript in our RNAseq is a bit lower (fold change of -2.36 for our dataset, and a fold change of -0.24 in the single feed paradigm, -0.227 in the four-feed paradigm, and -0.162 in the six-feed paradigm for their dataset, all p-values <0.001).

6. Some sections would benefit from revised writing to clarify the narrative.

– Rather than starting paragraphs with phrases like 'we decided to follow up on *fli1-2* and *irx-4/6*' or 'We next returned to...', it would be better to rephrase topic sentences with hypotheses that connect the upcoming results to the overall goals of the paper.

Thank you for this suggestion. We edited our writing accordingly.

– From the conclusion: "we confirm that *fli1-2* and *irx4/6* are expressed in G0 neural progenitors and that both *fli1-2* and *soxB1-2* are co-expressed with *smedwi-1*" comes as a surprise because there is not any data in the paper that shows this information.

Thank you for pointing this out. We showed co-expression of *fli1-2* and *irx-4/6* with *smedwi-1* in Fig 8H.

REVIEWER COMMENTS ORIGINAL SUBMISSION TO NAT COMM

– Confusing conclusion in this sentence: “We determined through both ISH and RNA-sequencing that soxB1-2(RNAi) caused reduction in irx-like and fli1-2 transcripts (Fig. 7E-G), which could support the idea that neural location is determined upstream of neurotransmitter identity.”

Thank you - we have reworded this.

– Unclear what data was used to reach the following: “We conclude that there does not seem to be a definitive hierarchy in the creation of peripheral dopaminergic neurons.”

We added additional data which led us to the model that identity is specified very shortly before location, near simultaneously (Fig. 8). We updated the text accordingly.

7. In general, the figures would benefit from polishing and/or reorganizing to improve clarity of the main take-home points. As is, the repetition of the assays obscures the conclusions. A lot of space is dedicated to bar graphs of co-expression which are not especially informative. Consider reorganizing figures to emphasize the take-home messages, and consolidating these supporting graphs in Extended Figures.

Thank you for this feedback. We moved the bar graphs to extended figures and reported the percentage of *TH*⁺ cells co-expressing the gene of interest in the insets.

– Make font sizes and cartoon animals consistent across all panels. Figure panels within individual figures should be consistent (e.g. 5A/B)

Thank you - this has been corrected throughout.

– Do not use default brackets/y-axes from GraphPad to better reflect the data.

This has also been corrected throughout.

– Indicate on figures themselves what is being quantified: PNS/CNS, dorsal/ventral, what kinds of genes are represented by the two apparently similar diagrams in Fig 7C/D to help with readability

This has also been corrected throughout.

– Colors in fluorescent images are muted. Sometimes this happens because the figures were generated using CMYK instead of RGB.

This has also been corrected throughout.

– Modify cartoons to indicate more clearly newly regenerated vs homeostatic tissues.

This has been changed throughout to provide better contrast.

– Refer to *slc6a3* as DAT to help make it clear to readers what this gene's function is.

REVIEWER COMMENTS ORIGINAL SUBMISSION TO NAT COMM

Thank you for this suggestion. We changed nomenclature throughout the text and figure captions and figures.

– Order of ‘regions’ in Fig 2 should be kept consistent with those in Fig 1, and color-coded to match schematics in Fig 1.

We have changed the order of the regions in Figure 2 and Extended Figure 3 to be consistent with Figure 1.

– Why is *celsr-1*(RNAi) included in extended Fig. 2 and 3 but not mentioned in the text? Consider deleting it if it isn’t important to the message of the manuscript.

Thank you for pointing this out. This has been added to the text, confirming that although *ce/sr-1* is co-expressed with *TH*, it is not at all required for *TH* expression.

Minor comments:

Thank you for noting these - they have all been addressed.

Y-axis label, ext Fig 6H: ‘arean’, numbers are misaligned, and it could help to make the axis consistent with F and G.

These data are now in Extended Figure 8.

Suggest adding page numbers/line numbers to help reviewers note the position of text.

Completed, thank you.

Move repetitive information about quantification (e.g. 10000 μm^2 boxes) to methods to consolidate.

Completed, thank you.

Fig 4M/N: rather than calling this experiment an ‘alternate RNAi paradigm’, it should be referred to as an ‘alternate regeneration paradigm’.

Completed, thank you.

In 1L, maybe make the predictions more obvious with a chart instead of subtle changes in the number of dots.

We did not change it (now Fig 1J) because as it is, it reflects the subtlety of the phenotype we observe. However, we did change the image to make the contrast more visible.

Overall, we have ensured all the references are cited within the text and reordered the numbering where necessary. Extended Figures and Supplementary Files have been made Supplementary Figures and renumbered as appropriate; figure titles have been removed from main figures. We have addressed the typos pointed out by reviewer 3 and changed the graphs in Supplementary Figure 13 (formerly Extended Figure 9) to show the two-way ANOVA data to highlight the genetic interaction and redundancy. Any quotations, italics, or underline not part of gene names or Latin phrases has been restored to standard text formatting. The sections of the manuscript have been reordered according to the provided guidelines. Funding information has been reworded and added to its own section per the example guidelines. Additional statistical information has been added to the figure legends (exact p-values, statistical tests, precise N's) and a source data file has been compiled for the main and supplementary figures.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I feel fully satisfied with all the revisions and improvements done and I strongly recommend it for publication

Reviewer #2 (Remarks to the Author):

The authors have made substantial revisions to the manuscript and with the exception of one minor point noted below, my concerns have been adequately addressed. I have no further major concerns regarding the manuscript.

In particular, the authors have done a good job addressing my questions regarding the fate of the TH⁺ cells lost in the knockdown experiments. These additional experiments provide mechanistic insight into the roles of these genes in dopaminergic neuron neurogenesis and/or survival. More broadly, this study provides new insight into how transcriptional programs coordinate neuronal identity, spatial patterning, and regeneration, highlighting the utility of planarians as a model for understanding mechanisms of nervous system repair. Overall, this is an interesting and well-executed study, and I recommend that it be accepted for publication.

(Original reviewer comment) 2. If the authors are claiming that the combined knockdown of IRX or FLI pairs leads to an enhanced phenotype, the individual and double knockdowns should be plotted in the same graph, and a statistical analysis performed. It was not obvious from the

figures cited (Fig. 4A–B, Extended Fig. 7A–D) that there is an enhanced phenotype, as the single and double knockdown data are not shown in the same graph.

(Author response) This has been corrected in Figure 3A-B and Extended Figure 9A-D to show both the single and double knockdowns together in the same experiment and graph. Additionally, the text has been corrected to cite Figure 3A-B instead of Figure 4A-B.

(Reviewer response to revision) In the revised manuscript, Figure 3B presents the single and double FLI/IRX knockdown conditions within the same experimental framework; however, the statistical analysis employs Dunnett's multiple comparisons test, which assesses each RNAi condition only against the control group. While this approach is appropriate for identifying differences relative to control, it does not test whether the double knockdown produces a significantly stronger phenotype than either single knockdown condition. Since the interpretation relies on functional redundancy between paralogs, the analysis should include direct statistical comparisons between single and double knockdown conditions (e.g., Tukey's multiple comparisons test following one-way ANOVA). Alternatively, a two-way ANOVA including an interaction term between paralog knockdowns would provide a more direct and informative assessment of genetic interaction and redundancy.

To address this point, we have revised Supplementary Figure 13 with a two-way ANOVA, also reporting the interaction term in the figure legend.

Reviewer #3 (Remarks to the Author):

The revised version of this manuscript is significantly improved in the figure presentation and in the writing. In particular, the inclusion of new data perturbing apoptosis and lineage tracing with EdU strengthens the authors conclusions about the role of these transcription factors in neurogenesis. Behavior and double RNAi experiments are better controlled. All other experimental concerns have been addressed.

Some very minor comments follow:

Figure 1F, text says that 64 expression patterns were determined but figure says 63.

Line 139, no figure number

Line 144 is garbled.

Suggest including some indication of the mechanism of action of M50054 rather than simply referring to its catalog number. Line 208-211 references Extended Figure 9 but it should be Extended Figure 6.

The minor comments have been addressed. A citation has been added to provide the mechanism of action of M50054, which works through inhibition of caspase-3.