Src acts with WNT/FGFRL signaling to pattern the planarian anteroposterior axis

Nicolle A. Bonar, David I. Gittin and Christian P. Petersen
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MS TITLE: Src acts with WNT/FGFRL signaling to pattern the planarian anteroposterior axis

AUTHORS: Nicolle A. Bonar, David I. Gittin, and Christian P. Petersen

I have now received all 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 some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' 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 ms submitted for publication in Development by Bonar et al. related to the role of src-1 in anterior patterning is an extremely interesting descriptive paper that tries to ascertain the complex interactions in planarian axial determination and maintenance.

Comments for the author

. To get a more complete descriptive analysis I will appreciate the presentation of the 2 fragments head and trunk during regeneration and intacts in all the studies performed to get a more deep view of remodeling versus regeneration.
To main point of view, the results of src-1 RNAi supports a new function on modulating the anterior axis rather determining it. One of the evidences is the fact that the phenotype of anterior expansion is done in the head fragments that need to remodel than in the tail that generates a head the novo.
At the end of the chapter of “src-1 is Broadly expressed in both muscle and non-muscle cells” The authors considered muscle cell bodies- present normal distribution (pp230), according figures supplementary 2D and 2E the pattern of control and src-1 RNAi is different for both markers.
In the chapter of “src-1 can pattern axis independently from pole identity”
Although the authors already comment in supplementary figure 3, I could not see the midline expression of notum in src RNAi at 72h and 14d. to my point of view, it could not be considered a delay, but a real disturbance of Notum anterior pole formation. Such possibility is not discussed At the end of Figure 4 the % of each phenotype are summarized, the % in the text and in the figure do not coincide in ndk.
A graph summarizing the results will be appreciated I really appreciate a deep discussion on the relationship between notum and src-1. From the ms I understand some type of inter-relation between notum and src-1, but which one?

Minor comments:
Line 29: Highlights, there are 4 not 5, please renumber Line 401: Figure7 should be Figure 6

Reviewer 2

Advance summary and potential significance to field

The manuscript identifies tyrosine kinase src1 as a novel component of positional control system in planarians. RNAi experiments show that src1 has a role in maintaining positional information along the body axis (both in the head and in the trunk). By a series of elegant experiments combining RNAi on different genes, the authors next demonstrate that src1 acts in parallel to the well known positional signaling systems such as wnt and fgfrl. Fascinatingly, src1 does not appear to be expressed in a gradient-like fashion, instead being expressed along the axis in a broad range of cell types. While the exact mechanism of action of src1 in planarian body plan patterning remains unknown, its importance is clear and will warrant further investigations in the future.

Comments for the author

None, the manuscript is well written and beautifully illustrated.

Reviewer 3

Advance summary and potential significance to field

Planarian regeneration is a fascinating and important model system for the understanding of morphogenetic mechanisms. This paper provides novel insights by showing the function of a new gene that regulates the patterning of a primary axis, including the number of eyes and setting territories of gene expression. The novel roles of SRC and its relationship to the Wnt pathway are a

solid addition to the field and appropriate for Development. The highlights accurately summarize the new advances:
1. Src-1 suppresses head and trunk identity 2. Src-1 can regulate positional control gene domains 4. Src-1 likely acts independently of notum/Wnt and FGFRL signals 5. Src-1 inhibition broadly sensitizes animals to AP pattern disruption

Comments for the author

This is a very solid study - high-quality data which support the main claims. Beautiful figures, clear text, direct mention of the unknowns. It is appropriate for Development with minor edits only. A few suggestions to increase value for readers:
1) there is a prior paper from E. Salo's group on a SRC-related gene in planaria (https://www.ncbi.nlm.nih.gov/pubmed/7510865) - the current manuscript is certainly an advance over that work, but shouldn't it be at least mentioned?
2) role of src in morphogenesis: p. 18 discusses some molecular facts known about src and its relationship to other relevant genes, but it would be helpful to say a sentence or two about whether (and how) src participates in morphogenetic phenotypes in other model systems (and possible connection to its better-known role in cancer?)
3) The following sentence provides in text definitions of AP and DV axis but not mediolateral: 92 – 95 “PCGs include signaling molecules in the Wnt, FGF, and BMP pathways that control tissue identity along the anteroposterior (AP) from head to tail, the dorsoventral (DV) axis from back to belly and the mediolateral axis.”
4) 289 – 314 (Figure 3)
What do the authors make of the very weak presence of the ectopic posterior eyes that form in double RNAi experiments with notum+src1? Src1 alone makes far more relevant photoreceptors posterior to the native photoreceptors then it can do when notum is knock downed. From the images it appears that it also does not achieve duality and can only form these ectopic eyes on a given side. Are there any examples for dual ectopic photoreceptors in src1+notum as there are with src1 or notum alone? This perhaps suggests an interesting interaction with notum signaling in the ability to create duality across the medio-lateral axis.
5) Figure 5 These are remarkable findings; is there any quantification for this phenotypic sensitization data? It is plainly obvious from the images provided but quantification of a given phenotype, e.g. 3 times as many eyes, would be helpful. The spatial pattern of the locations of the ectopic eyes is also most interesting.
6) The authors make the argument that dual-RNAi of trunk PCGs with src1 and their effect on cintillo expression reinforces the independent nature of the tissues in which they exert dominion. They also note, however, that dual RNAi of anterior PCGs with Src1 does lead to an increase of pharynx structures. These data were used to suggest that src1 396 – 397
“helps to channel regionalized factors into controlling the identity of specific anterior territories”
However, the argument can be made that if these new pharynx(s) are formed posterior to the native pharynx in regenerates may be an indication of a one-way dependence between the anterior and posterior PCG network in which trunk features have dependent signaling from the anterior to help delineate feature landmarks. Dual RNAi of Src1 and these anterior PCGs may exacerbate this crosstalk. Or it would suggest that src1 plays a role in landmarking features in the trunk in a manner that also depends on anterior signaling since src1 RNAi alone leads to minor occurrences of ectopic pharynx formation. It seems the conclusion drawn in lines 396 – 397 is too broad and may not touch on the nuances that the data exhibit?
7) Discussion:
467 – 468
“However, inhibition of FGF or FGFRs in planarians have not resulted in patterning phenotypes.”
Is the above quote derived from in-house data or from literature? Please clarify by either stating that lab data shows this result or provide citation to relevant literature.
8) Did the authors explore any possible connection of src1 to nr4A (elife 2019 Dayan J Li) which is expressed predominantly in muscle and produces similarly placed posterior ectopic photoreceptors and posterior broadening of the anterior pole?
9) Figure Design Comments:
- figure 1:
The use of the illustrated planaria and its amputation (red dotted line) is not very contextually informative to the data it is supporting to its right. In the text, the experiment is described as one where the head and tail are amputated and the group measures regenerative phenotypes in head

fragments, trunk fragments, and tail fragments. These phenotypes discussed and then scored across the different regenerating fragments after ~14days. It may be more informative to use the illustration to show that the animals were cut into these 3 fragments, display the scores for the phenotypes being displayed to its right via FISH, and then circle the fragment in which the photographic data is referring too. As it stands, the illustration can be understood two ways, either A) the head is amputated and these images represent the anterior portion that regenerated or B) the head was amputated, it regrew the posterior, and these images focus on the anterior tissue of this regenerated animal (which I believe is the intended understanding). Even though it would make sense to design these figures based solely on anterior fragments as they represent the highest score, if indeed these data are derived from animals only being from head amputations, then this should be emphasized in the text or figure legend.
10) Grammar:
113 - 120
“At later times in regeneration (by 24-72hours) and throughout homeostasis, stem cell-dependent processes (Hayashi et al., 2011; Currie and Pearson, 2013; Marz et al., 2013; Scimone et al., 2014; Vasquez-Doorman and Petersen, 2014; Vogg et al., 2014; Tejada-Romero et al., 2015; Schad and Petersen, 2020) generate**s** cells expressing wnt1 and notum in muscle cells at the posterior and anterior midline termini respectively (termed poles) where they may function to control region-specific patterning or act at the tip of a hierarchy of AP regulatory factors (Adell et al., 2009; Petersen and Reddien, 2009; Gurley et al., 2010; Stuckemann et al., 2017; Schad and Petersen, 2020).”
184 - 185
“By contrast, src-1(RNAi) animals formed ectopic posterior eyes in addition ectopic eyes posterior to their normal eyes (Figure 1A).” - the words "posterior eyes in addition ectopic" should be deleted

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First revision

Author response to reviewers' comments

We appreciate the reviewer’s careful attention to the work and their helpful suggestions. Below, we have written our responses in red, with revised text from the manuscript included in blue for convenience.

Reviewer 1 Advance Summary and Potential Significance to Field:
The ms submitted for publication in Development by Bonar et al. related to the role of src-1 in anterior patterning is an extremely interesting descriptive paper that tries to ascertain the complex interactions in planarian axial determination and maintenance.

Reviewer 1 Comments for the Author:

- To get a more complete descriptive analysis I will appreciate the presentation of the 2 fragments head and trunk during regeneration and intacts in all the studies performed to get a more deep view of remodeling versus regeneration. To main point of view, the results of src-1 RNAi supports a new function on modulating the anterior axis rather determining it. One of the evidences is the fact that the phenotype of anterior expansion is done in the head fragments that need to remodel than in the tail that generates a head the novo.

We completely agree that the effect of src-1 RNAi appears to vary by axial position and appreciate the suggestion that src-1 modulates the anterior axis. In light of this very useful suggestion, we now present additional data and analysis to test the whether src-1 RNAi exerts qualitatively distinct effects dependent on the mode of regeneration (epimorphosis or morphallaxis with respect to head, or via continual homeostasis). We noted several phenotypes from src-1 RNAi: extra eyes, enlarged brain, and extra pharynx. We find that extra eye phenotypes and enlarged brain emerge in all regeneration conditions after src-1 RNAi. For example, both src-1(RNAi) head fragments undergoing head morphallaxis (Fig 1A) and trunk fragments undergoing head epimorphosis (Fig 1C) form extra eyes. In addition, we now also show that homeostatic animals generate extra posterior eyes (Figure S1C). Together these

results suggest that src-1 has a generalizable function in suppressing eye formation at more posterior locations. Brain expansion phenotypes were also observed in both head fragments undergoing morphallaxis (Figure 1B, 3B, Fig S5) and trunk fragments undergoing epimorphosis (Fig 1C,Fig. S1D) and also in homeostasis (Fig S1C and also Figure 2, ndk staining of the brain). We could only detect the extra pharynx defect in trunk fragments (Figure 1C) and not in regeneration head fragments (Figure 1D). However, this specificity is similar to other reported posterior pharynx duplications after wntP-2, ptk7, or ndl-3 RNAi, suggesting it is intrinsic to that region, for example reflective of a shift to the position of the pharynx that only results in duplication when a pre-existing pharynx is near (Scimone and Reddien 2016, Lander and Petersen 2016). Together, these observations now strongly indicate that the primary functions of src-1 to suppress anterior identity are not specific to particular modes of regeneration. This suggests that analysis of these phenotypes in one regeneration contexts can be generalized to several contexts. Instead, we favor a model that src-1 acts homeostatically and also in regeneration in order to modulate anterior regionalization.

- At the end of the chapter of "src-1 is Broadly expressed in both muscle and non-muscle cells" The authors considered muscle cell bodies- present normal distribution (pp230), according figures supplementary 2D and 2E the pattern of control and src-1 RNAi is different for both markers.

  We appreciate this analysis, and we imaged collagen-stained animals at a higher magnification to examine this possibility (Fig S2E). We could not find any consistent difference in collagen staining between control and src-1 RNAi. The muscle fiber antibody stains are somewhat variable in our hands in general, and we also could not see any consistent differences after src-1 RNAi. However, because of the greater variability of that assay it could be that there are subtle differences from src-1 RNAi on muscle fiber structure, so we have modified the text to reflect this possibility:

  > Line 238: In addition, muscle cell bodies labeled by *collagen* mRNA were also presentin apparently normal distributions in regenerating *src-1(RNAi)* animals (Supplementary Figure 2E). Although it remains possible *src-1* influences muscle fiberorientation and/or muscle cell bodies in a subtle way, these suggests *src-1* regulatesanterior patterning not through affecting muscle formation but instead by signaling within muscle or in other cell types.

- In the chapter of "src-1 can pattern axis independently from pole identity" Although the authors already comment in supplementary figure 3, I could not see the midline expression of notum in src RNAi at 72h and 14d. to my point of view, it could not be considered a delay, but a real disturbance of Notum anterior pole formation. Such possibility is not discussed.

  We thank the reviewer for noticing this effect. We have now incorporated this observation into the text and verified that the pole eventually forms in regeneration as detected by *foxD* expression (Fig S3E):

  > Line 245: At 72-hours, *notum* was anteriorly but localized more broadly and with apparently reduced intensity along the midline after *src-1* RNAi, suggestive of an early disturbance in pole formation (Supplementary Figure 3B). By 14-days after amputations, however, all *src-1(RNAi)* animals had succeeded in regenerating a *notum*+ anterior pole which was mildly expanded laterally, and they regenerated pole-expressed *foxD* (Supplementary Figure 3B-E).

- At the end of Figure 4 the % of each phenotype are summarized, the % in the text and in the figure do not coincide in ndk. A graph summarizing the results will be appreciated. I really appreciate a deep discussion on the relationship between notum and src-1. From the ms I understand some type of inter-relation between notum and src-1, but which one?

We double checked the numbers in the figure and also figure legends and don't see a

discrepancy noted here. We think the issue might be the fact that in order to appropriately label each image with scoring information, some of the scorings shown in the figure indicate successful regeneration while some indicate unsuccessful regeneration. We added a graph to Figure 4 that now summarizes the table to provide further clarification.

We also clarified in the discussion our proposed model for the relationship between Src and notum. Our interpretation of the synthetic eye phenotype between Src and notum suggests Src can act independently of notum for that aspect of anterior patterning. Because notum appears to act fully through wnt11-6 in that context, this suggests Src can act independently of at least wnt11-6. We cannot rule out the possibility that Src acts in conjunction of other Wnts however. We now clarify this interpretation in the Discussion:

> Line 466: "In prior work, *wnt11-6* inhibition fully suppressed the *notum(RNAi)* ectopic eye phenotype, suggesting that *notum* primarily acts through *wnt11-6* for controlling eye placement (Hill and Petersen, 2015), and that *src-1* is unlikely to act primarily downstream of *wnt11-6*. However, we cannot rule out the possibility that *src-1* could act downstream of any Wnts that can act independently of *notum* and influence head regionalization or downstream of Wnts with involvement in other patterning roles."

Minor comments:
Line 29: Highlights, there are 4 not 5, please renumber
Line 401: Figure7 should be Figure 6

Thank you for identifying theses errors, which we now fixed.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript identifies tyrosine kinase src1 as a novel component of positional control system in planarians. RNAi experiments show that src1 has a role in maintaining positional information along the body axis (both in the head and in the trunk). By a series of elegant experiments combining RNAi on different genes, the authors next demonstrate that src1 acts in parallel to the well known positional signaling systems such as wnt and fgfrl. Fascinatingly, src1 does not appear to be expressed in a gradient-like fashion, instead being expressed along the axis in a broad range of cell types. While the exact mechanism of action of src1 in planarian body plan patterning remains unknown, its importance is clear and will warrant further investigations in the future.

Reviewer 2 Comments for the Author:
None, the manuscript is well written and beautifully illustrated.

We very much appreciate this reviewer's assessment and comments.

Reviewer 3 Advance Summary and Potential Significance to Field:
Planarian regeneration is a fascinating and important model system for the understanding of morphogenetic mechanisms. This paper provides novel insights by showing the function of a new gene that regulates the patterning of a primary axis, including the number of eyes and setting territories of gene expression. The novel roles of SRC and its relationship to the Wnt pathway are a solid addition to the field and appropriate for Development. The highlights accurately summarize the new advances:

1. Src-1 suppresses head and trunk identity
2. Src-1 can regulate positional control gene domains
4. Src-1 likely acts independently of notum/Wnt and FGFRL signals
5. Src-1 inhibition broadly sensitizes animals to AP pattern disruption

Reviewer 3 Comments for the Author:
This is a very solid study - high-quality data which support the main claims. Beautiful figures, clear text, direct mention of the unknowns. It is appropriate for Development with minor edits only. A few suggestions to increase value for readers:

1) there is a prior paper from E. Salo's group on a SRC-related gene in planaria

(https://www.ncbi.nlm.nih.gov/pubmed/7510865) - the current manuscript is certainly an advance over that work, but shouldn't it be at least mentioned?

We thank the reviewer for identifying this additional citation and now include it:

> Line 178: A Src-like gene had been cloned from planaria previously but its functional roles in regeneration were unknown (Burgaya et al., 1994).

2) role of src in morphogenesis: p. 18 discusses some molecular facts known about src and its relationship to other relevant genes, but it would be helpful to say a sentence or two about whether (and how) src participates in morphogenetic phenotypes in other model systems (and possible connection to its better-known role in cancer?) –

We add to the discussion a summary of Src's broad roles in development and morphogenesis to help contextualize possible roles in planarian patterning:

> Line 412: "As tyrosine kinases activated my many upstream signals and capable of regulating many downstream factors, Src-related factors control both signaling and morphogenesis to regulate many aspects of tissue formation and maintenance, including cell proliferation, differentiation, migration, survival, polarity, and cell mechanical properties, with activating mutations to Src capable of driving cancer progression (Thomas and Brugge, 1997; Guarino, 2010; Kohlmaier et al., 2015; Espada and Martin-Perez, 2017; Anton et al., 2018; Tamada et al., 2021)."

3) The following sentence provides in text definitions of AP and DV axis but not mediolateral: 92 - 95

"PCGs include signaling molecules in the Wnt, FGF, and BMP pathways that control tissue identity along the anteroposterior (AP) from head to tail, the dorsoventral (DV) axis from back to belly and the mediolateral axis." –

We added an abbreviation for mediolateral (ML)

4) 289 – 314 (Figure 3)

What do the authors make of the very weak presence of the ectopic posterior eyes that form in double RNAi experiments with notum+src1? Src1 alone makes far more relevant photoreceptors posterior to the native photoreceptors then it can do when notum is knock downed. From the images it appears that it also does not achieve duality and can only form these ectopic eyes on a given side. Are there any examples for dual ectopic photoreceptors in src1+notum as there are with src1 or notum alone? This perhaps suggests an interesting interaction with notum signaling in the ability to create duality across the medio-lateral axis.

We noticed when studying the src-1 RNAi phenotype that cases of lower expressivity could sometimes result in only 1 rather than 2 ectopic photoreceptors forming. We have also seen this phenomenon when inhibiting wntA/wnt11-6 to generate ectopic posterior photoreceptors. Possibly, this reflects some latent asymmetry or an asymmetry generated through the process of pattern alteration. Alternatively, weaker pattern alteration could push the system just over some boundary limiting eye formation resulting in just 1 but not two eyes forming. In the epistasis tests between notum and src, the majority of animals showing both anterior and posterior phenotypes indeed had only 1 ectopic posterior photoreceptor, but we did find 1-2 animals with symmetric eyes. However, because this "symmetric" phenotype is less representative of the nature of the synthetic phenotypes in our analysis we prefer showing the asymmetric image as shown. In addition, in cases of only 1 extra posterior eye, we could not find a preference for left or right sides of the animal. We now modified the text to point out this more weakly expressive phenotype:

> Line 308: "In a majority of cases, the synthetic phenotypes occurred with only a single posterior ectopic eye but two anterior ectopic eyes as shown, but we identified a small number of cases of such animals in which two posterior eyes were symmetrically formed (2/42 animals)."

5) Figure 5

These are remarkable findings; is there any quantification for this phenotypic sensitization data? It is plainly obvious from the images provided but quantification of a given phenotype, e.g. 3 times as many eyes, would be helpful. The spatial pattern of the locations of the ectopic

eyes is also most interesting.

We appreciate the feedback and we now quantify the effects of the double-RNAi on eyes and graph the results in Figure 5A. Each of the treatments showing expanded phenotype showed a statistically significant increase in eye number compared to src-1 RNAi alone or RNAi of the corresponding tested positional control genes.

6) The authors make the argument that dual-RNAi of trunk PCGs with src1 and their effect on cintillo expression reinforces the independent nature of the tissues in which they exert dominion. They also note, however, that dual RNAi of anterior PCGs with Src1 does lead to an increase of pharynx structures. These data were used to suggest that src1, 396 – 397 "helps to channel regionalized factors into controlling the identity of specific anterior territories" However, the argument can be made that if these new pharynx(s) are formed posterior to the native pharynx in regenerates may be an indication of a one-way dependence between the anterior and posterior PCG network in which trunk features have dependent signaling from the anterior to help delineate feature landmarks. Dual RNAi of Src1 and these anterior PCGs may exacerbate this crosstalk. Or it would suggest that src1 plays a role in landmarking features in the trunk in a manner that also depends on anterior signaling since src1 RNAi alone leads to minor occurrences of ectopic pharynx formation. It seems the conclusion drawn in lines 396 – 397 is too broad and may not touch on the nuances that the data exhibit?

We really appreciate this insight and suggestion and have modified the discussion to incorporate further possibilities and nuances in interpretation, in particular the idea of "posterior prevalence" for these factors.

> Line 399: "These results point to an unexpected interplay of patterning signals otherwise known to be associated with distinct regions. This interaction could arise from a role for *src-1* in assigning PCGs to individual outputs. Alternatively, the PCG system might be set up in a way that allows region-to-region control such that any sufficiently strong head expansion could ultimately also push the trunk territory further posterior. These results identify *src-1* as a strong modifier of positional control used for whole-body regeneration."

7) Discussion: 467 – 468
"However, inhibition of FGF or FGFRs in planarians have not resulted in patterning phenotypes."
Is the above quote derived from in-house data or from literature? Please clarify by either stating that lab data shows this result or provide citation to relevant literature.

We appreciate the reviewer catching this error. We now cite Auwal 2020 for their work inhibiting FGF and Wagner et al 2012 for their work inhibiting selected FGFRs in planarians.

8) Did the authors explore any possible connection of src1 to nr4A (elife 2019 Dayan J Li) which is expressed predominantly in muscle and produces similarly placed posterior ectopic photoreceptors and posterior broadening of the anterior pole?

We appreciate this intriguing idea and now explore this possible connection through analysis and comparison of the src-1 and nr4A RNAi phenotypes. Nr4A RNAi caused several other defects like loss of muscle from the anterior and ultimately loss of the original eyes, and also modification of wnt1 expression in the posterior. We did not see these types of effects from src- 1 RNAi, so we conclude that src-1 and nr4A are unlikely to be directly upstream/downstream of each other in general, and we now describe this reasoning in the text.

> Line 457: "In principle *src-1* could have interactions with other genes reported to cause ectopic photoreceptor formation posteriorly when knocked down, for example the nuclear receptor *nr4A* (Li et al., 2019). However, *nr4A* RNAi causes additional defects such as loss of muscle from the anterior and a shift in the *wnt1* expression domain not detected in *src-1* RNAi, suggesting these factors likely do not obligately regulate each other in all situations. Instead, we suggest there may be multiple inputs

into eye patterning reflective of patterning as a multi-stepped process."

9) Figure Design Comments:
- figure 1:
The use of the illustrated planaria and its amputation (red dotted line) is not very contextually informative to the data it is supporting to its right. In the text, the experiment is described as one where the head and tail are amputated and the group measures regenerative phenotypes in head fragments, trunk fragments, and tail fragments. These phenotypes discussed and then scored across the different regenerating fragments after ~14days. It may be more informative to use the illustration to show that the animals were cut into these 3 fragments, display the scores for the phenotypes being displayed to its right via FISH, and then circle the fragment in which the photographic data is referring too. As it stands, the illustration can be understood two ways, either A) the head is amputated and these images represent the anterior portion that regenerated or B) the head was amputated, it regrew the posterior, and these images focus on the anterior tissue of this regenerated animal (which I believe is the intended understanding).
Even though it would make sense to design these figures based solely on anterior fragments as they represent the highest score, if indeed these data are derived from animals only being from head amputations, then this should be emphasized in the text or figure legend.

We have made new cartoons following these suggestions which show the two-amputation scheme and highlight the fragment of focus for each panel. For additional clarity, the terms "head fragments" and "trunk fragments" are indicated by additional text in figure 1 underneath the cartoons.

10) Grammar:
113 - 120
"At later times in regeneration (by 24-72hours) and throughout homeostasis, stem cell-dependent processes (Hayashi et al., 2011; Currie and Pearson, 2013; Marz et al., 2013; Scimone et al., 2014; Vasquez-Doorman and Petersen, 2014; Vogg et al., 2014; Tejada- Romero et al., 2015; Schad and Petersen, 2020) generate**s** cells expressing wnt1 and notum in muscle cells at the posterior and anterior midline termini respectively (termed poles) where they may function to control region-specific patterning or act at the tip of a hierarchy of AP regulatory factors (Adell et al., 2009; Petersen and Reddien, 2009; Gurley et al., 2010; Stuckemann et al., 2017; Schad and Petersen, 2020)."
We fixed the grammar issue here

184 - 185
"By contrast, src-1(RNAi) animals formed ectopic posterior eyes in addition ectopic eyes posterior to their normal eyes (Figure 1A)." - the words "posterior eyes in addition ectopic" should be deleted
We fixed the grammar issue here

Second decision letter

MS ID#: DEVELOP/2021/200125

MS TITLE: Src acts with WNT/FGFRL signaling to pattern the planarian anteroposterior axis

AUTHORS: Nicolle A. Bonar, David I. Gittin, and Christian P. Petersen
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

The authors have discussed and or modified accordingly the comments in the first referee report. I considered the ms ready to be published in the present form.
Congratulations is a significant and complete piece of work.

Comments for the author

Minor comments:
In the reference list: BURGAYA, F., GARCIA-FERNANDEZ, J., RIUTORT, M., BAGUNA, J. & SALO, E. 1994. Structure and expression of Spk-1, an src-related gene product found in the planarian Dugesia (G) tigrina. Oncogene, 9, 1267-72.
BAGUNA is BAGUÑÀ
SALO is SALÓ

Reviewer 2

Advance summary and potential significance to field

Please see my previous review; the changes made in response to other comments make the paper even better.

Comments for the author

No suggestions to the scientific content; a minor issue only: the yellow/orange scheme of Fig. 6 is probably not very fortunate - please make it clearer.

Reviewer 3

Advance summary and potential significance to field

The authors show new information about a gene, SRC, in the context of regeneration in planaria. This is an important topic and new, high-quality information.The revision makes the prior data even clearer.

Comments for the author

The authors have done a great job addressing all of the comments, and the paper is now suitable for publication, I have no more suggestions.
One tiny thing: "a multi-stepped" - is that correct grammar? Maybe it should be "multi-step"?