# Pbx4 limits heart size and fosters arch artery formation by partitioning second heart field progenitors and restricting proliferation

Andrew Holowiecki, Kelsey Linstrum, Padmapriyadarshini Ravisankar, Kashish Chetal, Nathan Salomonis and Joshua S. Waxman
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**Review timeline**

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**Original submission**

First decision letter

MS ID#: DEVELOP/2019/185652

MS TITLE: Pbx4 limits heart size and fosters arch artery formation through partitioning second heart field progenitors and restricting proliferation

AUTHORS: Andrew Holowiecki, Kelsey Linstrum, Padmapriyadarshini Ravisankar, Kashish Chetal, Nathan Salomonis, and Joshua Waxman

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 BenchPressand 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. Important concerns were raised about redundancy of some results relative to previous publications, but enthusiasm for the expanded analysis and new experiments presented. Other considerations on specific points are outlined in thereviewer comments. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by 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.

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

Reviewer 1

*Advance summary and potential significance to field*

In this manuscript, Waxman and colleagues examine the role of Pbx4 (zebrafish homologue of mammalian Pbx1) in second heart field (SHF) development. Zebrafish lazarus (pbx4) mutants have previously demonstrated cardiac defects, whereas in mice deletion of the related Pbx1 causes defects in outflow tract (OFT) development, supporting an association between PBX1 mutations and congenital heart disease in humans. Despite these observations, the precise function of Pbx1/4 in cardiac development remain to be elucidated.

The authors show two apparently distinct phenotypes in lazarus mutants: an absence of partitioning of posterior pharyngeal arch progenitors (and their subsequent differentiation), and a later increased number of ventricular CMs and OFT smooth muscle cells. The increased chamber size evident in lazarus mutants is due to increased SHF-mediated contribution of ventricular cardiomyocytes (CMs) and smooth muscle, supporting a role in OFT development. Via elegant lineage tracing and single cell RNAseq analysis, the authors demonstrate that the expanded SHF progenitor pool in lazarus mutants is due to a loss in posterior pharyngeal arch artery (pPAA) fate.

This work provides a detailed analysis of cardiac defects arising from Pbx4, in particular based on the lineage tracing and scRNAseq methodologies used. The scRNAseq analysis of the Nkx2.5 population at 28hpf provides a novel and detailed initial examination of heterogeneity in the SHF progenitor population, which will be of interest to the cardiac development field in general. Overall, the quality and rigour of this work is of a high level. I have only a few minor comments/points to be addressed.

*Comments for the author*

Minor comments:

1. Figure S3: in the images shown, it is not clear what area of nkx2.5 expression is being counted.

2. Line 123: I don't believe it is correct to state that mef2cb is a marker of "differentiating CM progenitors" at 20 somite stage. Mef2cb does mark arterial pole SHF progenitors (as well as ventricular CMs) at later heart tube stages.

3. Figure S8: many of the cNC-derived cardiac cells shown are in the area of the AVC or atrial chamber. Can contribution to the OFT and SHF-serived CM population be specifically assessed/quantified? Is a CM-specific floxed reporter available to use for these experiments?

4. The data for Figure 8F-J is described in lines 257-9 as showing contribution of anterior nkx+ cells to the OFT and ventricular CMs extending to the AVC, however 8J shows 100% ventricular contribution. This discrepancy should be addressed.

5. Lines 371-2: is Pbx4 specifically limiting OFT size? The data would appear to argue it limits overall SHF contribution.

Reviewer 2

*Advance summary and potential significance to field*

The manuscript "Pbx4 limits heart size and fosters arch artery formation through partitioning second heart field progenitors and restricting proliferation" is a very interesting paper from the lab of Dr. Waxman. In this manuscript, the authors detail their discovery that Pbx4 regulates cardiac morphogenesis in zebrafish. The authors show that the increase in the number of ventricular cardiomyocytes arises from a surplus of SHF progenitors which are later added to the arterial pole of the zebrafish heart. The authors show that this defect could be due to increased proliferation of Nkx2.5 SHF cells and to a potential switch in fate.

Intriguingly, the authors demonstrate that Pbx4 mutant fish lack posterior pharyngeal arch arteries (PAAs). Since the endothelium of these vessels arises from the SHF, the authors explore an interesting idea that Pbx4 regulates the stratification of Nkx2.5+ SHF progenitors into cells fated to be CMs and PAA ECs. To examine this idea, the authors have performed single cell RNAseq and discovered that Nkx2.5+ cells at 28 dpf are stratified into at least 14 distinct populations, and that Pbx4 mutants have increased numbers of less differentiated SHF cells. Although, authors' data suggest that there is a decrease in the number of cells with endothelial cell fate, that decrease appears to be minor, or not significant. It is also interesting that there were no Nkx2.5 cells identified as endothelial progenitors in scRNAseq, and that the difference in cells with EC differentiation potential was not altered in the mutants. Given the presence of extra Elnb+ cells, it is surprising the single cell RNAseq did not identify differences in SMC vs EC fate between control and mutant embryos. Experiments in this manuscript are incredibly well-done and well-controlled. The authors have carefully addressed multiple possibilities prior to arriving to their conclusions. However, the concluding sentence of the abstract is not entirely and unequivocally backed up by the data.

*Comments for the author*

Major comments:
The lzr mutants appear to have dysmorphic hearts and problems with cardiac looping, and may be convergent extension. Heart defects are already apparent at 20s (Fig. S4) with seemingly delayed fusion of cardiac primordia. This defect together with the presence of Elnb+ cells outside the heart suggest defects in migration.

While the idea that Pbx4 mutation results in fate alteration of SHF cells is extremely interesting, the data in this manuscript are not entirely supportive of this idea. Since Pbx4 is mutated globally, these data do not rule out defect in migration.

It may be interesting to quantify numbers of endocardial cells to assay a possibility that Nkx2.5 cells with EC potential may be midirected to form endocardial cells.

Minor comments:
Kao et al 2015 used similar quantification methods and fish lines to quantify CM numbers and atria and ventricles in Pbx4 MO and came up with a different conclusion about their numbers. Could the authors comment on the differences between their and the current study, and why they think their quantification is right and the one in the previous manuscript is wrong?

Despite the claim that heart cells appear to fuse anteriorly at 20s in Pbx4 mutants, the heart morphology does not appear to be consistent among mutants at this stage. For example, while Fig. S2 panel E indeed show that the Mef2cb+ cells are coming together anteriorly prematurely, the fusion of cardiac primordia appears to be delayed in Fig. S3B compared with the control in S3A. Similarly, the fusion of VMHC+ cardiac primordia appears to be delayed in the lzr mutants in Fig. 2B compared with Fig. 2A.

Foxa2 ISH in FIG S4 does not contribute to the argument that endoderm formation is not affected in the mutants; Foxa2 staining in the mutant is more extensive than in control. Is this a consistent difference?

Fig. 3 It is not clear whether or not the hearts are shown in the same orientation in Fig. 3A and 3B. It would be helpful to understand the data if embryonic axis were marked in each figure.

Surplus of Elnb+ cells appear to reside outside of the heart, suggesting defective migration or ectopic differentiation into smooth muscle lineage

It is not clear how Elnb+ nuclei were quantified, the yellow dots do not mark Elnb+ cells in Fig. 3G' - H', as stated in the legend

Add a side bar to Fig. 3G – H' to indicate which panels are WT and which are lzr;

If the stars on bar graphs are meant to indicate statistical significance, please add the p value and the test(s) used.

Please add scale bars to all figures

Reviewer 3

*Advance summary and potential significance to field*

The work by Holowiecki et al., investigates the role of Pbx4 in the development of the heart and pharyngeal arches in the zebrafish model. Using a pbx4 mutant and, at times, morphants, the authors describe that pbx4 is required to restrict and segregate the heart field (specifically the ventricle and outflow tract region/OFT) and permit posterior pharyngeal arch artery (pPAA) development. The scRNAseq data is interesting, exciting, and supports the phenotypes. Unfortunately, I find several flaws with other parts of the work that make it unsuitable for publication. These include:

1. A large proportion of 4 figures in this manuscript have been reported elsewhere (specifically figures 1, 3, 5 and 7). Whilst there are some discrepancies between the two studies, these are relatively minor and the major phenotypes (the expansion of the OFT, increased ventricular cell number, failure to segregate heart field) has already been described.

2. Several of the images are unclear and difficult to see. This includes the lineage tracing (which appears to contribute to the atrium as well as the ventricle) and the Elnb and pHH3 staining.

3. Line 169 “We found that at 28 hpf lzr mutants have an increased percentage of pHH3+/Nkx2.5+ progenitors (Fig. 4G).” compared to Line 172 “nor an increased rate of proliferation in differentiated CMs of lzr mutants at 28 hpf (Fig. S10)”. This directly contradicts

4. There are some experiments that appear underpowered. A “not significant” result is described but this may be due to insufficient numbers

5. There are several claims that are too strong

*Comments for the author*

1. The orientation of the heart is unclear in many images and is not always consistent
2. Several things are missing from figure legends, including descriptions of arrows and arrowheads, experimental details needed to interpret the data (sorted cells versus whole embryos), numbers of embryos in panels, nomenclature of transgenics is incorrect and italics of mutant, gene and mRNA names is absent

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**First revision**

Author response to reviewers' comments

Response to reviews - DEVELOP/2019/185652

We are very appreciative of the positive assessment and constructive comments of our original manuscript from all of the reviewers. Reviewers 1 and 2 were very positive and respectively stated that the “quality and rigour of this work is of a high level” and “experiments in this manuscript are incredibly well-done and well-controlled”. Reviewer 3 highly praised the scRNAseq data, stating that it is “interesting, exciting, and supports the phenotypes.” However, they were concerned about overlap with a previous study that proposed a different hypothesis for Pbx function in the zebrafish heart. The critiques were very helpful to us for identifying issues that needed to be

changed in order to improve the manuscript. In the revised manuscript, we have directly addressed all the issues brought up by the three reviewers. We have made all the changes that were specifically suggested for the text and figures and included an experiment that was requested by Reviewer 2. Our direct responses to each of the reviewers' specific comments are below and indicated in blue. Changes in the manuscript made in response to the critiques are highlighted in gray.

Reviewer 1 Advance summary and potential significance to field
In this manuscript, Waxman and colleagues examine the role of Pbx4 (zebrafish homologue of mammalian Pbx1) in second heart field (SHF) development. Zebrafish lazarus (pbx4) mutants have previously demonstrated cardiac defects, whereas in mice deletion of the related Pbx1 causes defects in outflow tract (OFT) development, supporting an association between PBX1 mutations and congenital heart disease in humans. Despite these observations, the precise function of Pbx1/4 in cardiac development remain to be elucidated.

The authors show two apparently distinct phenotypes in lazarus mutants: an absence of partitioning of posterior pharyngeal arch progenitors (and their subsequent differentiation), and a later increased number of ventricular CMs and OFT smooth muscle cells. The increased chamber size evident in lazarus mutants is due to increased SHF-mediated contribution of ventricular cardiomyocytes (CMs) and smooth muscle, supporting a role in OFT development. Via elegant lineage tracing and single cell RNAseq analysis, the authors demonstrate that the expanded SHF progenitor pool in lazarus mutants is due to a loss in posterior pharyngeal arch artery (pPAA) fate.

This work provides a detailed analysis of cardiac defects arising from Pbx4, in particular based on the lineage tracing and scRNAseq methodologies used. The scRNAseq analysis of the Nkx2.5 population at 28hpf provides a novel and detailed initial examination of heterogeneity in the SHF progenitor population, which will be of interest to the cardiac development field in general. Overall, the quality and rigour of this work is of a high level. I have only a few minor comments/points to be addressed.

Reviewer 1 Comments for the author
Minor comments:

1. Figure S3: in the images shown, it is not clear what area of nkx2.5 expression is being counted.

We counted all visible Nkx2.5+ cells/nuclei with the aid of Imaris as indicated in the Methods (line 491-495). We did not provide the individual channels or Imaris images used to count as it was not clear this information provided additional information beyond the quantification. Arrows have been added to show examples of Nkx2.5+ nuclei in the Figure.

2. Line 123: I don't believe it is correct to state that mef2cb is a marker of "differentiating CM progenitors" at 20 somite stage. Mef2cb does mark arterial pole SHF progenitors (as well as ventricular CMs) at later heart tube stages.

We have changed the statement (lines 124 and 125 of the revised manuscript).

3. Figure S8: many of the cNC-derived cardiac cells shown are in the area of the AVC or atrial chamber. Can contribution to the OFT and SHF-serived CM population be specifically assessed/quantified? Is a CM-specific floxed reporter available to use for these experiments?

We currently do not have a genetic tool that will specifically allow us to address this question. The ltbp3 (SHF) lines/promoter reported in Zhou et al. 2011 do not report expression until more than 3 days post-fertilization and unfortunately are not even available anymore. It is not clear what benefit using a myl7:Switch (cardiomyocyte specific lineage tracer) would provide over what we have already done, as it would label all cardiomyocytes. One could potentially restrict the cNC-derived ventricular cardiomyocytes using a vmhc:Switch, but we unfortunately currently do not have that tool and could not feasibly make the line and perform the experiment in a reasonable amount of time.

While we did not use a chamber-specific marker for the analysis, most of the cardiomyocytes were clearly within the ventricle, as was reported previously (Cavanaugh et al, 2015). Although the morphological boundary between the ventricle and atrium may not be as distinct in the Pbx4-depleted embryos as the controls, the overall number of cNC-derived cardiomyocytes did not change and for both were only ~10 per heart. Thus, they overall were a small percentage of the total cardiomyocytes in WT and Pbx4-depleted embryos. Given the low number of cNC-derived cardiomyocytes we found in the heart and that we do not observe an overall change in their number or distribution, it is presently not clear to us how quantification of the cNC-derived cardiomyocytes specifically in the OFT and ventricle would change the results and interpretation that the cNC-derived cardiomyocytes do not appear to be contributing to the increase in ventricular cardiomyocytes.

4. The data for Figure 8F-J is described in lines 257-9 as showing contribution of anterior nkx+ cells to the OFT and ventricular CMs extending to the AVC, however 8J shows 100% ventricular contribution. This discrepancy should be addressed.

The label in 8J reflects the observation that cells were labeled throughout the ventricles in lzr mutants, while in controls the labels were restricted to arterial pole of the OFT and ventral aorta. Although ventricular cardiomyocytes reside at the arterial pole of the heart, we did not have a way of distinguishing if Nkx2.5+ labeled cells within the OFT/arterial pole were ventricular or vascular at these stages other than by morphology and localization. We have removed OFT in the sentence to prevent any confusion (lines 258-260 of the revised manuscript).

5. Lines 371-2: is Pbx4 specifically limiting OFT size? The data would appear to argue it limits overall SHF contribution.

Thank you. We have changed the sentence to reflect this correct statement (lines 373 of the revised manuscript).

Reviewer 2 Advance summary and potential significance to field
The manuscript “Pbx4 limits heart size and fosters arch artery formation through partitioning second heart field progenitors and restricting proliferation” is a very interesting paper from the lab of Dr. Waxman. In this manuscript, the authors detail their discovery that Pbx4 regulates cardiac morphogenesis in zebrafish. The authors show that the increase in the number of ventricular cardiomyocytes arises from a surplus of SHF progenitors which are later added to the arterial pole of the zebrafish heart. The authors show that this defect could be due to increased proliferation of Nkx2.5 SHF cells and to a potential switch in fate. Intriguingly, the authors demonstrate that Pbx4 mutant fish lack posterior pharyngeal arch arteries (PAAs). Since the endothelium of these vessels arises from the SHF, the authors explore an interesting idea that Pbx4 regulates the stratification of Nkx2.5+ SHF progenitors into cells fated to be CMs and PAA ECs. To examine this idea, the authors have performed single cell RNAseq and discovered that Nkx2.5+ cells at 28 dpf are stratified into at least 14 distinct populations, and that Pbx4 mutants have increased numbers of less differentiated SHF cells. Although, authors’ data suggest that there is a decrease in the number of cells with endothelial cell fate, that decrease appears to be minor, or not significant. It is also interesting that there were no Nkx2.5 cells identified as endothelial progenitors in scRNAseq, and that the difference in cells with EC differentiation potential was not altered in the mutants. Given the presence of extra Elnb+ cells, it is surprising the single cell RNAseq did not identify differences in SMC vs EC fate between control and mutant embryos. Experiments in this manuscript are incredibly well-done and well-controlled. The authors have carefully addressed multiple possibilities prior to arriving to their conclusions. However, the concluding sentence of the abstract is not entirely and unequivocally backed up by the data.

Reviewer 2 Comments for the author
Major comments:
The lzr mutants appear to have dysmorphic hearts and problems with cardiac looping, and may be convergent extension. Heart defects are already apparent at 20s (Fig. S4) with seemingly delayed fusion of cardiac primordia. This defect together with the presence of Elnb+ cells outside the heart suggest defects in migration.

While the idea that Pbx4 mutation results in fate alteration of SHF cells is extremely interesting, the data in this manuscript are not entirely supportive of this idea. Since Pbx4 is mutated globally, these data do not rule out defect in migration.

We do not disagree that cardiomyocyte progenitor migration may be affected in lzr mutants, at least at early stages, and felt we had acknowledged and explicitly expressed this idea in the text. We have modified the sentence on lines 116-117 of revised manuscript to specifically state that migration is affected. As we show, we do not see obvious defects in the endoderm itself that could explain this, suggesting signals from the endoderm or the ability to receive signals from the endoderm contribute to the defects. We feel that understanding these signals and mechanisms are beyond the scope of this study and do not impinge on the interpretations about the specification and proliferation defects in lzr mutants.

It may be interesting to quantify numbers of endocardial cells to assay a possibility that Nkx2.5 cells with EC potential may be midirected to form endocardial cells.

We have performed this requested experiment and counted the number of endocardial cells within hearts of WT and lzr mutant embryos. We observe a slight decrease in the number of total endocardial cells within lzr heart, but not a statistically significant decrease in either of the individual chambers. Thus, these results support that posterior Nkx2.5+ endothelial cells are not becoming endocardial cells either. We have included this data on lines 219-225 and in a new Fig. S13 for the revised manuscript. It is possible the decrease in endocardial cells reflects improper migration of endocardial cells within the abnormally shaped heart tube of lzr mutants. It is not clear this would reflect a fate transformation with SHF-derivatives as we do not observe endocardial cells within the nkx2.5 scRNA-seq data.

Minor comments:
Kao et al 2015 used similar quantification methods and fish lines to quantify CM numbers and atria and ventricles in Pbx4 MO and came up with a different conclusion about their numbers. Could the authors comment on the differences between their and the current study, and why they think their quantification is right and the one in the previous manuscript is wrong?

We are also curious as to why we observe these different trends. Without knowing exactly what Kao et al did beyond what is in their paper, it is difficult to comment on why we observe these differences. Thus, we are hesitant to speculate in the manuscript and do not want cast aspersions upon the Maves lab, despite the differences we observe. Technical differences include that we used the myl7:DsRed2-NLS transgene with IHC for cardiomyocyte quantification, while they used the myl7:H2A-mcherry and myh6:EGPF transgenes. They also counted far fewer embryos per condition than we did. Given what they reported, to test our hypothesis and observations, we performed the experiments multiple times with lzr mutants and depleted embryos (morphants), the cardiomyocytes counts were performed blinded and by multiple different people, and we employed additional complementary assays. It is not clear why the technical differences would lead to different results.

Despite the claim that heart cells appear to fuse anteriorly at 20s in Pbx4 mutants, the heart morphology does not appear to be consistent among mutants at this stage. For example, while Fig. S2 panel E indeed show that the Mef2cb+ cells are coming together anteriorly prematurely, the fusion of cardiac primordia appears to be delayed in Fig. S3B compared with the control in S3A. Similarly, the fusion of VMHC+ cardiac primordia appears to be delayed in the lzr mutants in Fig. 2B compared with Fig. 2A.

We acknowledge there is some variability to the timing and location of fusion of the bilateral cardiomyocyte fields and tried to indicate this observation with a statement in the text of the original manuscript (lines 112-113 of revised the manuscript). Despite this variability, we found the aberrant morphology of these fields with more anteriorly localized aggregates of cardiomyocytes within the lzr mutants to be very consistent and feel this is evident in all the mentioned figures.

Foxa2 ISH in FIG S4 does not contribute to the argument that endoderm formation is not affected in the mutants; Foxa2 staining in the mutant is more extensive than in control. Is this a consistent difference?

We have not found an overt difference between foxa2 staining of WT and lzr mutant embryos. The lzr embryo in the image in Fig. S4 of the original manuscript had more background, which was not specific to the mutants. This background did not affect the observation and interpretation. We have provided different images in a revised Fig. S4G and H that do not have background staining in the yolk to prevent this confusion.

Fig. 3
It is not clear whether or not the hearts are shown in the same orientation in Fig. 3A and 3B. It would be helpful to understand the data if embryonic axis were marked in each figure.

We have added arrows to indicate the direction of the arterial pole of the hearts in the images and amended the figure legend (lines 897 and 898 of revised manuscript).

Surplus of Elnb+ cells appear to reside outside of the heart, suggesting defective migration or ectopic differentiation into smooth muscle lineage

We agree, the ectopic Elnb could be from defect migration and/or ectopic differentiation from the expanded heart field. However, we are unclear of what specifically we are being asked to test from this statement and think that presently it would be difficult to experimentally tease these mechanisms apart given our data support that minimally the SHF is expanded in the lzr mutants. If the defect were just from improper migration of progenitor cells, we do not think we would have observed an increase in the number of Elnb-surrounded cells.

It is not clear how Elnb+ nuclei were quantified, the yellow dots do not mark Elnb+ cells in Fig. 3G' - H', as stated in the legend

We have quantified Elnb-surrounded cells similar to a method that was previously reported in Paffett-Lugassy et al, 2017. However, for our analysis we used Imaris. Because Elnb is secreted, we systematically went through the confocal stacks and labeled the DAPI+ nuclei that were surrounded by Elnb. We described this in the original Methods. We have modified the statement to try to make this description clear (lines 498 and 499 of the revised manuscript).

Add a side bar to Fig. 3G – H' to indicate which panels are WT and which are lzr;

We have added side bars to these panels in Fig. 3.

If the stars on bar graphs are meant to indicate statistical significance, please add the p value and the test(s) used.

That asterisks indicate statistical significance in all the graphs of figures and a p-value <0.05 was stated in the figure legend for Figure 1 of the original manuscript (lines 877 and 878 of the revised manuscript). A $p<0.05$ being considered statistically significant and the statistical tests used were also stated in the "Statistical analysis" section of the Methods of the original manuscript (line 598 of the revised manuscript). We have added a sentence reiterating that the asterisks in graphs indicate a $p<0.05$ to the Methods (line 601 of the revised manuscript). In some specific cases, additional information about the tests used and statistical significance was also given in the figure legend. For instance, for comparisons of the cells clusters in the legend for Fig. 9 (lines 946 and 947 of the revised manuscript).

Please add scale bars to all figures

We have added scale bars to all figures. Scales are now stated in all the corresponding figure legends.

Reviewer 3 Advance summary and potential significance to field
The work by Holowiecki et al., investigates the role of Pbx4 in the development of the heart and pharyngeal arches in the zebrafish model. Using a pbx4 mutant and, at times, morphants, the authors describe that pbx4 is required to restrict and segregate the heart field (specifically the ventricle and outflow tract region/OFT) and permit posterior pharyngeal arch artery (pPAA)

development. The scRNAseq data is interesting, exciting, and supports the phenotypes. Unfortunately, I find several flaws with other parts of the work that make it unsuitable for publication. These include:

1.A large proportion of 4 figures in this manuscript have been reported elsewhere (specifically figures 1, 3, 5 and 7). Whilst there are some discrepancies between the two studies, these are relatively minor and the major phenotypes (the expansion of the OFT, increased ventricular cell number, failure to segregate heart field) has already been described.

We respectfully disagree with the sentiment that information in Figures 1, 3, 5, and 7 are unnecessary and the discrepancies between the studies are relatively minor. Our data support that Pbx4 specifically limits the size of the SHF and OFT, which is a very different mechanism than the previously proposed hypothesis that Pbx4 has biphasic roles promotes differentiation and limiting overall cardiomyocyte number. While we agree some similar assays between the studies are presented in Figures 1, 3, and 5, which is expressly acknowledged in the manuscript, these were necessary to show because of the different data and conclusions. Furthermore, the statement from Reviewer 3 overlooks the additional assays performed in these figures to test our hypotheses. We are also not aware of any previous studies that examined temporal expression of nkx2.5 as it was done in Figure 7. Therefore, the previous studies did not provide a foundation for us to build upon and necessitated us presenting some similar assays bolstered by additional experiments so a reader can understand our observations and the rationale for experiments and conclusions presented within this manuscript.

2.Several of the images are unclear and difficult to see. This includes the lineage tracing (which appears to contribute to the atrium as well as the ventricle) and the Elnb and pHH3 staining.

We strive to present our data in as clear a manner possible for the reader and would like to make requested changes. However, without specifically stating what the reviewer thinks should be changed to make then more clear (as was done by the other reviewers), it is difficult to directly address this issue. We feel our lineage tracing is on par with what has been presented in other manuscripts, including Paffett-Lugassy et al, 2013 and Paffett-Lugassy et al, 2017. Furthermore, these experiments was praised by the other Reviewers. While we could break up the channels for the lineage tracing, we thought that it did not provide additional information and made the figures excessively large. For the Elnb figure, it is not clear what should be changed. We have made changes regarding the orientation of the hearts requested by Reviewer 2. For the pHH3, we provided an image of all the staining and a higher magnification with the 3 channels and merged image. It is not clear that including a representative single co-labeled cell from the analysis would provide additional information. However, we could do that if requested.

3.Line 169 “We found that at 28 hpf lzr mutants have an increased percentage of pHH3+/Nkx2.5+ progenitors (Fig. 4G).” compared to Line 172 “nor an increased rate of proliferation in differentiated CMs of lzr mutants at 28 hpf (Fig. S10)”. This directly contradicts

We apologize for the confusion. The point is that we observe an increase in proliferation of Nkx2.5+ progenitors and not in differentiated Nkx2.5+ cardiomyocytes. We have modified the sentences (lines 168-172 of the revised manuscript) and changed the designation to “pHH3+/Nkx2.5+/Mhc-“ (line 168) to help clarify the statement and point.

4.There are some experiments that appear underpowered. A “not significant” result is described but this may be due to insufficient numbers

Without knowing what specific experiments that are being referred to, it is difficult for us to directly address this issue. Power analysis shows that to be appropriately powered to 80% confidence with $p<0.05$ for the slight differences observed in the “not significant” samples would require at least 300 samples per condition, which is not experimentally feasible. While we agree testing for significance and being appropriately powered is desirable, this is often not achievable with these types of biological studies and even frowned upon for post-hoc analysis. Therefore, we have repeated experiments and performed complementary analyses whenever possible. Overall, we feel the numbers/quantification reported are consistent with standards set in the field.

5.There are several claims that are too strong

We appreciate this comment. However, as with the comments 2 and 4, without stating specific examples Reviewer 3 feels are too strong it is difficult for us to change or directly address the claims.

Reviewer 3 Comments for the author
1. The orientation of the heart is unclear in many images and is not always consistent

We have added arrows to Figs 3,4,8,S3,S4,S8,S9,S10,S17, and S19 to help make the orientations of hearts within images clear. While we sought to make the orientation of the hearts consistent, at least within figures, this was not always possible due how they are oriented in the original images.

2.Several things are missing from figure legends, including descriptions of arrows and arrowheads, experimental details needed to interpret the data (sorted cells versus whole embryos), numbers of embryos in panels, nomenclature of transgenics is incorrect and italics of mutant, gene and mRNA names is absent

a. We have added descriptions to the legends of Figures 1 and 5 and included descriptions of all the arrows and arrowheads in the figure legends for the Figures indicated in the response to the preceding comment.

b. All the n's for embryos examined and used for the quantification in the graphs were reported in the figure legends and with individual data points within the graphs.

c. We did catch a typo for one of the transgene names where we had used a previous designation for the gene name. Otherwise, we have followed all the nomenclature rules for genes/mRNA/protein and transgene names/allele designations as they are currently indicated in ZFIN. While we did not give the whole designation of the transgenes in the text to conserve space and for clarity, the specific transgene and mutant alleles used are indicated in the Methods (lines 448-456 of the revised manuscript).

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Second decision letter

MS ID#: DEVELOP/2019/185652

MS TITLE: Pbx4 limits heart size and fosters arch artery formation through partitioning second heart field progenitors and restricting proliferation

AUTHORS: Andrew Holowiecki, Kelsey Linstrum, Padmapriyadarshini Ravisankar, Kashish Chetal, Nathan Salomonis, and Joshua Waxman

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 BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. In particular Reviewer 3's comments 2-5 on the reproduction of previous work should be more clearly acknowledged beyond the initial reference to previous work.

Reviewer 1

*Advance summary and potential significance to field*

In this manuscript, Waxman and colleagues examine the role of Pbx4 (zebrafish homologue of mammalian Pbx1) in second heart field (SHF) development. Zebrafish lazarus (pbx4) mutants have previously demonstrated cardiac defects, whereas in mice deletion of the related Pbx1 causes defects in outflow tract (OFT) development, supporting an association between PBX1 mutations and congenital heart disease in humans. Despite these observations, the precise function of Pbx1/4 in cardiac development remain to be elucidated.

The authors show two apparently distinct phenotypes in lazarus mutants: an absence of partitioning of posterior pharyngeal arch progenitors (and their subsequent differentiation), and a later increased number of ventricular CMs and OFT smooth muscle cells. The increased chamber size evident in lazarus mutants is due to increased SHF-mediated contribution of ventricular cardiomyocytes (CMs) and smooth muscle, supporting a role in OFT development. Via elegant lineage tracing and single cell RNAseq analysis, the authors demonstrate that the expanded SHF progenitor pool in lazarus mutants is due to a loss in posterior pharyngeal arch artery (pPAA) fate.

This work provides a detailed analysis of cardiac defects arising from Pbx4, in particular based on the lineage tracing and scRNAseq methodologies used. The scRNAseq analysis of the Nkx2.5 population at 28hpf provides a novel and detailed initial examination of heterogeneity in the SHF progenitor population, which will be of interest to the cardiac development field in general. Overall, the quality and rigour of this work is of a high level.

*Comments for the author*

The authors have addressed all my concerns.

Reviewer 2

*Advance summary and potential significance to field*

The manuscript “Pbx4 limits heart size and fosters arch artery formation through partitioning second heart field progenitors and restricting proliferation” by Holowiecki et al demonstrates intricate stratification of progenitors within the second heart field (SHF) in zebrafish. The authors uncover a novel regulation of SHF fates and show that the transcription factor Pbx4 regulates the allocation of SHF progenitor cells into endothelial lineage of the pharyngeal arch arteries and the late adding cells of zebrafish outflow tract. This study is novel, elegant, and of high quality. The authors have addressed all of my previous comments, and I have only two minor comments to add.

*Comments for the author*

Minor comments:
1. In the abstract, it is not clear what the portion of the sentence “ and assimilate characteristics of normally discrete proliferative progenitor and anterior, differentiated cardiomyocyte populations” means. It's not clear what the authors mean by the word "assimilate" in the context of the sentence.

2. To get an impression of whether the observed phenotypes were a direct or indirect consequence of the loss of Pbx4, could the authors comment on cell types which express Pbx4 at various stage examined in this study? For example, is Pbx4 expressed in the SHF cells fated to be added to the heart and in the SHF cells fated to become PAA ECs?

Reviewer 3

*Advance summary and potential significance to field*

A revisitation of the role Pbx4 in cardiac arterial pole development is presented. The addition of lineage tracing and scSeq data provides new developmental and molecular information into the phenotype of the mutants.

*Comments for the author*

Despite the responses of the authors to the comments raised, I am not satisfied they have been addressed. I raise specifically two issues that I think need resolving before the manuscript is suitable for publication.

First: “While we agree some similar assays between the studies are presented in Figures 1, 3 and 5, which is expressly acknowledged in the manuscript these were necessary to show because of the different data and conclusions”

I disagree with this comment. There are some minor discrepancies, however the majority of the work (detailed below) has been performed and published with similar conclusions drawn. I think there is sufficient novelty in this current work for publication in Development (this is a credit to the lineage tracing and scSeq data) however, it does not include the repetitious data. To be clear where the repetition is, I detail it below. At the very least, acknowledgement and reference to the prior work is needed.

1. Fig 1 – if you remove the nppa images, the figure is near identical to Kao et al., Fig 4a-g. In both manuscripts, the number of ventricular cells is increased and only in Kao et al, is the atrium increased. This discrepancy is acknowledged with a reference. Given that the remainder of this work describes the ventricular defect, I don't see why this discrepancy is very important (the two poles of the heart have previously been described to be regulated differently). However, for the sake of forming a frame-work for the remainder of the manuscript, I can see why the authors wish to include it and I would not argue this point.
2. Fig 2a-d – vmhc staining at 20s + 24 hpf versus Fig2K-R (Kao et al) – vmhc staining at 21s and 27s. The conclusion is slightly different. Here the authors convincingly describe the abnormal fusion and morphology of the tube whereas Kao et al describe a delay in fusion and differentiation. This is not referenced or compared.
3. Fig 3d-e – Elnb antibody staining at 72 hpf versus Fig4 H-J (Kao et al) - Elnb antibody staining at 72 hpf. Same observation (expanded Elnb staining with “extensions”). This is not referenced or acknowledged in the text and is repetitious of previously published work.
4. Fig 5c+d – ltbp3 staining at 28 hpf versus Fig4M+N (Kao et al) – ltbp3 staining at 24 hpf. Both interpret it as broader staining. This is not referenced or acknowledged in the text and is repetitious of previously published work.
5. Fig 7E-H – nkx2.5 expression showing discontinuous staining in the wildtype versus continuous in the mutants versus Fig3E+F (Kao et al) – hand2 staining showing discontinuous anterior versus posterior staining in wildtype versus continuous in mutants. Whilst this is not exactly the same stain or stage, it is the same conceptual data. That is, an expansion of the cardiac progenitor population into the posterior of the embryo. It's inclusion is therefore valid however reference or acknowledgement in the text to the similarity with previously pubished work is appropriate.

Second point: “We feel our lineage tracing is on par with what has been presented in other manuscripts, including Paffett-Lugassy et al, 2013 and Paffett-Lugassy et al, 2017.”

Making a side-by-side comparison, this is a reasonable comment. However, using the same example, Paffett-Lugassy et al, 2017 in Fig 3, have a cartoon depicting the uncaged region. Fig 8 would benefit from something similar and would make orienting the experiment easier for the reader.

**Second revision**

Author response to reviewers' comments

Response to Reviews - DEVELOP/2019/185652_R1

We are grateful for the overall positive evaluation of the revised manuscript. Although we adequately addressed Reviewer 1's comments with our previous revision, Reviewer 2 had some remaining minor issues. Reviewer 3, however, did not feel we adequately addressed two of their previous concerns. In this revised manuscript, we have tried to directly address each of these concerns. Our responses to the specific comments are indicated below in blue. We have highlighted the changes made in response to their comments within the manuscript in gray.

Reviewer 1 Advance summary and potential significance to field
In this manuscript, Waxman and colleagues examine the role of Pbx4 (zebrafish homologue of mammalian Pbx1) in second heart field (SHF) development. Zebrafish lazarus (pbx4) mutants have previously demonstrated cardiac defects, whereas in mice deletion of the related Pbx1 causes defects in outflow tract (OFT) development, supporting an association between PBX1 mutations and congenital heart disease in humans. Despite these observations, the precise function of Pbx1/4 in cardiac development remain to be elucidated.

The authors show two apparently distinct phenotypes in lazarus mutants: an absence of partitioning of posterior pharyngeal arch progenitors (and their subsequent differentiation), and a later increased number of ventricular CMs and OFT smooth muscle cells. The increased chamber size evident in lazarus mutants is due to increased SHF-mediated contribution of ventricular cardiomyocytes (CMs) and smooth muscle, supporting a role in OFT development. Via elegant lineage tracing and single cell RNAseq analysis, the authors demonstrate that the expanded SHF progenitor pool in lazarus mutants is due to a loss in posterior pharyngeal arch artery (pPAA) fate.

This work provides a detailed analysis of cardiac defects arising from Pbx4, in particular based on the lineage tracing and scRNAseq methodologies used. The scRNAseq analysis of the Nkx2.5 population at 28hpf provides a novel and detailed initial examination of heterogeneity in the SHF progenitor population, which will be of interest to the cardiac development field in general. Overall, the quality and rigour of this work is of a high level.

Reviewer 1 Comments for the author
The authors have addressed all my concerns.
Thank you.

Reviewer 2 Advance summary and potential significance to field
The manuscript "Pbx4 limits heart size and fosters arch artery formation through partitioning second heart field progenitors and restricting proliferation" by Holowiecki et al demonstrates intricate stratification of progenitors within the second heart field (SHF) in zebrafish. The authors uncover a novel regulation of SHF fates and show that the transcription factor Pbx4 regulates the allocation of SHF progenitor cells into endothelial lineage of the pharyngeal arch arteries and the late adding cells of zebrafish outflow tract. This study is novel, elegant, and of high quality. The authors have addressed all of my previous comments, and I have only two minor comments to add.

Reviewer 2 Comments for the author
Minor comments:
1. In the abstract, it is not clear what the portion of the sentence " and assimilate characteristics of normally discrete proliferative progenitor and anterior, differentiated cardiomyocyte populations" means. It's not clear what the authors mean by the word "assimilate" in the context of the sentence.

Thank you for pointing out that this sentence is not clear. We have changed the sentence in the abstract (lines 40-42 in the Abstract of the revised manuscript).

2. To get an impression of whether the observed phenotypes were a direct or indirect consequence of the loss of Pbx4, could the authors comment on cell types which express Pbx4 at various stage

examined in this study? For example, is Pbx4 expressed in the SHF cells fated to be added to the heart and in the SHF cells fated to become PAA ECs?

From previous studies, Popperl et al, 2000 and Kao et al, 2015, it appears that Pbx4 is ubiquitous. Our work with the published Pbx4 antibody in the paper agrees with this, which is why it wasn't included. Additionally, we found the pbx4 was expressed in all cells from the scRNA-seq, supporting it is in both SHF progenitors and differentiated cardiomyocytes. We did mention this result in the Discussion of the previous manuscripts and referenced that it is presented in Fig. S15. We apologize greater emphasis was not put on this statement previously. We have modified the sentences on lines 385-387 of the revised manuscript to better emphasize the ubiquitous expression of pbx4 in the cardiac lineages.

Reviewer 3 Advance summary and potential significance to field
A revisitation of the role Pbx4 in cardiac arterial pole development is presented. The addition of lineage tracing and scSeq data provides new developmental and molecular information into the phenotype of the mutants.

Reviewer 3 Comments for the author
Despite the responses of the authors to the comments raised, I am not satisfied they have been addressed. I raise specifically two issues that I think need resolving before the manuscript is suitable for publication.

First: "While we agree some similar assays between the studies are presented in Figures 1, 3, and 5, which is expressly acknowledged in the manuscript, these were necessary to show because of the different data and conclusions"

I disagree with this comment. There are some minor discrepancies, however the majority of the work (detailed below) has been performed and published, with similar conclusions drawn. I think there is sufficient novelty in this current work for publication in Development (this is a credit to the lineage tracing and scSeq data) however, it does not include the repetitious data. To be clear where the repetition is, I detail it below. At the very least, acknowledgement and reference to the prior work is needed.

1) We thank Reviewer 3 for now providing detailed comments. We also very much appreciate that Reviewer 3 still thinks there is sufficient novelty to our study, despite our differences in opinion. Direct responses to each of their specific comments is below.

2) We still respectfully disagree with Reviewer 3's comment that the differences in observations and conclusions between our study and Kao et al are so minimal that they are repetitive and unnecessary. The details of each of the experiments and how we each came to conclusions are important. Our previous comments for Figure 1 really apply to all the figures that included the same assays. We feel our results using the same markers needed to be shown because our observations and conclusions are different than Kao et al. Thus, their results did not provide rationale for our experiments and a foundation to build upon. If we were to provide data only showing the new markers and assays without also showing the results using the same markers, it would be confusing to the reader and would not be sufficient to provide a necessary basis for our experiments. We would have undoubtedly been asked by reviewers to provide this data. Additionally, our data are necessary for interested readers to be able to compare the papers, if they choose. With Reviewer 3's comments, we feel they are largely overlooking key differences in the observations, the critical new information provided from the additional assays we performed, and selectively choosing statements made in Kao et al. that support oversimplified and unsupported conclusions.

3) We agree it is critically important to properly cite and acknowledge previous work. This is a practice that is far too often not done. We have no desire to be repetitive or repeat data in the literature that could stand as a foundation and provide rationale for experiments. However, we do feel it is important for groups to present conflicting data that shows different results within the literature. As requested, we have added references throughout the text to the prior work to better acknowledge when similar assays were performed and highlight the differences. We have also

revised the first paragraph of the Discussion (lines 373-382) to specifically acknowledge and compare the differences seen in the assays and interpretations.

1. Fig 1 – if you remove the nppa images, the figure is near identical to Kao et al., Fig 4a-g. In both manuscripts, the number of ventricular cells is increased and only in Kao et al, is the atrium increased. This discrepancy is acknowledged with a reference. Given that the remainder of this work describes the ventricular defect, I don't see why this discrepancy is very important (the two poles of the heart have previously been described to be regulated differently). However, for the sake of forming a frame-work for the remainder of the manuscript, I can see why the authors wish to include it and I would not argue this point.

We appreciate that Reviewer 3 can see our point with respect to Figure 1. However, as stated above, we still disagree with their interpretation that the discrepancies are minimal and not important. While there are mechanisms that differentially regulate development at the poles of the heart, there are also mechanisms that can similarly affect both populations. A specific effect on ventricular cardiomyocytes vs a broad effect on atrial and ventricular cardiomyocytes minimally implies different temporal mechanisms and effects on progenitor fields. One cannot assume that because we both show ventricular cardiomyocytes are increased, this means the data support similar mechanisms within the heart field underlie these defects. They do not. Thus, we feel the inference from Reviewer 3 is an oversimplification and interpretation that is not supported by data.

2. Fig 2a-d – vmhc staining at 20s + 24 hpf versus Fig2K-R (Kao et al) – vmhc staining at 21s and 27s. The conclusion is slightly different. Here the authors convincingly describe the abnormal fusion and morphology of the tube whereas Kao et al describe a delay in fusion and differentiation. This is not referenced or compared.

We have added a statement and reference to lines 105. We also specifically state this difference now in the Discussion on lines 373-382 of the revised manuscript.

3. Fig 3d-e – Elnb antibody staining at 72 hpf versus Fig4 H-J (Kao et al) - Elnb antibody staining at 72 hpf. Same observation (expanded Elnb staining with “extensions”). This is not referenced or acknowledged in the text and is repetitious of previously published work.

We feel this is a misrepresentation and cherry-picking of their data and interpretations. Kao et al’s conclusions are not that overall there are enlarged or increased OFTs in lzr mutants but that lzr mutants have “abnormal” outflow tracts. Their abstract states, “we find that pbx4 mutant larvae have aberrant outflow tracts. . . .” Furthermore, within the text they state, “The outflow tract defects show variability: the outflow tract smooth muscle marker elastinb (elnb; [29]) is reduced in some pbx4b557-/- embryos (Figure 4I) and appears expanded in some pbx4b557-/- embryos (Figure 4J).” While some of the lzr hearts were “expanded and bifurcated” this was less than half (4/9). Additionally, Kao et al. performed in situ hybridization not immunohistochemistry and did not quantify the number of elnb+ cells/surrounded cells, as we did. They only reported the number of embryos examined with the respective phenotypes, of which the “expanded” were a minority. Why Kao et al observe the variability is a question that we cannot answer. Nothing in their paper supports a mechanism that would underlie these variable defects. Overall, we feel it is not correct to simply conclude from the Kao et al’s data and statements that we make the “same observation.”

We have specifically stated these different observations in the Discussion on lines 373-382 of the revised manuscript.

4. Fig 5c+d – ltbp3 staining at 28 hpf versus Fig4M+N (Kao et al) – ltbp3 staining at 24 hpf. Both interpret it as broader staining. This is not referenced or acknowledged in the text and is repetitious of previously published work.

Similar to above, we feel this is also a misrepresentation and cherry-picking of their data and interpretations. Within the text Kao et al. state, “We find that expression of ltbp3 is disrupted in pbx4b557-/- embryos (Figure 4M,N)” and that “we see a consistent, diffuse, defective ltbp3 expression pattern that appears broader but also weaker than that in controls (Figure 4M,N).” Within the figure legend they state, “ltbp3 expression in second heart field domain (arrows) appears broader yet weaker in pbx4b557-/- embryos (n = 10, all weaker expression).” Although

they state “broader,” the emphasis of their observation is on the “weakness” of the expression, which implies there is decreased expression. However, they perform no quantitative assessment of ltbp3 expression in Pbx4-deficient embryos to support this. In contrast, we did not observe the weakness with ltbp3 staining in lzr mutants. Instead, we find there is expanded expression at the arterial pole of the hearts and that there is actually quantitatively increased ltpb3 expression within nkx2.5+ cells of Pbx4-deficient embryos. Thus, these observations and interpretations are not the same.

We have specifically stated these different observations in the Discussion on lines 373-382 of the revised manuscript.

5. Fig 7E-H – nkx2.5 expression showing discontinuous staining in the wildtype versus continuous in the mutants versus Fig3E+F (Kao et al) – hand2 staining showing discontinuous anterior versus posterior staining in wildtype versus continuous in mutants. Whilst this is not exactly the same stain or stage, it is the same conceptual data. That is, an expansion of the cardiac progenitor population into the posterior of the embryo. It's inclusion is therefore valid however reference or acknowledgement in the text to the similarity with previously pubished work is appropriate.

We feel here too this is a misrepresentation of the data. Hand2 and nkx2.5 have overlapping expression, but do not mark the exact same cell populations at the 8-10 somite stages (Schoenebeck et al, 2007). While Kao et al postulate about what their observations with respect to hand2 mean in their Discussion, there is no data actually provided in their manuscript that shows there is really a posterior expansion or how what they report leads to a defect on the SHF. If Reviewer 3 is not familiar with the dynamics of hand2 expression, please also recognize that hand2 expression at earlier stages of somitogenesis starts out continuous throughout the lateral plate mesoderm and then separates into anterior and posterior fields by the 10 somite stage (Yelon et al, 2000). For contrast, the gata4 and gata5 expression, whose posterior boundaries are the same as hand2 within the ALPM at the 10 somite stage (Schoenebeck et al, 2007), are not extended posteriorly and do not support their interpretations. Although hand2 expression could theoretically be specifically affected in lzr mutants, since hand2 is expressed throughout the ALPM at earlier stages, they cannot properly conclude hand2 is expanded posteriorly or anteriorly without proper additional assays.

Additionally, where we agree with Kao et al. is that early cardiomyocyte specification is not overtly affected. We did not observe a defect in specification markers during early somitogenesis. In this case, it would be redundant to show, which is why we did not include these data in the manuscript. However, when we performed in situ for hand2, we could not corroborate a posterior expansion in lzr mutants. While it is difficult to speculate about the differences, one possibility is that the embryo that was picked for the panel is in Figure 3 of Kao et al is generally delayed relative to the control, which is suggested from the broader krox20 staining and shorter embryo.

With respect to our data, we do not show nkx2.5 at the 10 somite stage as we did not find a difference. Our data also do not show a posterior expansion in that the nkx2.5 field is bigger at the 14 somite stage. We show nkx2.5 expression starts out the same and as development proceeds is partitioned differently, with a posterior expansion of cardiac fates within the nkx2.5+ field. Thus, these details should not be dismissed as essentially the “same conceptual idea” as what was proposed before.

Second point: “We feel our lineage tracing is on par with what has been presented in other manuscripts, including Paffett-Lugassy et al, 2013 and Paffett-Lugassy et al, 2017.”

Making a side-by-side comparison, this is a reasonable comment. However, using the same example, Paffett-Lugassy et al, 2017 in Fig 3, have a cartoon depicting the uncaged region. Fig 8 would benefit from something similar and would make orienting the experiment easier for the reader.

We have modified Fig 8 to include schematics of the experimental approaches. These are referenced in the paragraph on lines 240-263 and in the figure legend.

Third decision letter

MS ID#: DEVELOP/2019/185652

MS TITLE: Pbx4 limits heart size and fosters arch artery formation through partitioning second heart field progenitors and restricting proliferation

AUTHORS: Andrew Holowiecki, Kelsey Linstrum, Padmapriyadarshini Ravisankar, Kashish Chetal, Nathan Salomonis, and Joshua Waxman
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.