Lamb1a regulates atrial growth by limiting second heart field addition during zebrafish heart development

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MS TITLE: Lamb1a regulates atrial growth by limiting excessive, contractility-dependent second heart field addition during zebrafish heart development

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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

In this manuscript, Dr. Emily Noel and colleagues characterize the function of the extracellular matrix Laminin proteins in zebrafish heart development. Human genetic and animal studies (mouse and Drosophila) have indicated a role for Laminin function in heart development and congenital heart disease however the specific function of Laminins in these processes has not been described.

Lamc1, which encodes the single zebrafish gamma subunit of the Laminins (and therefore with its loss should disrupt all laminin function), was knocked down in F0 “crispants”, which resemble previously published lamc1 mutants. Loss of lamc1 was found to result in perturbed cardiac looping at 55hpf and an apparent increase in cardiac size (area measurement of RNA ISH) at 72hpf. A similar, yet milder phenotype was observed in stable lamb1a (encoding a beta subunit) mutants, with arguably a larger effect on chamber size than looping. It is found that lamb1a mutants have an increased atrial (but not ventricular) CM number from 55-72hpf, with a “birthdating” approach used to show that there is an increased number of newly added CMs in the atrium of lamb1a mutant hearts. This alteration of atrial CM number is dependent on cardiac contractility, and appears to reflect alterations in RA and Fgf signalling downstream of a Laminin-based tension sensing mechanism.

Overall, this work suggests a novel and intriguing model where Laminins may bridge a response of mechanical forces from the onset of cardiac contractility to influence the extent of SHF addition of CMs to the venous pole of the heart. The manuscript is clearly written and has a compelling narrative. However, as outlined below, I feel some critical points need to be addressed.

Comments for the author

Major comments:
1. It is stated (lines 188/189) that roles for looping morphogenesis and restriction of cardiac size are uncoupled in lamb1a mutants. However, quantification in Figures 2M and O would seem to suggest that is not the case, particularly at 55hpf. This is an important point which should be addressed. This is especially important as comparing the allele made for this study and the grumpy allele (Figure S3) seem to yield similar but qualitatively different results.

2. It is not entirely clear why lamc1 mutants were not pursued for most of this study. The data presented suggest they have a more severe looping phenotype yet a milder phenotype with respect cardiac chamber size (CM counts are not shown). In the text possible redundant function of other beta subunits is mentioned, but does this also suggest that Laminins may have positive and negative roles in some of these processes?

3. it is not clear why ventricular chamber size is larger in lamb1a mutants, if CM size/spacing and cell number are indistinguishable from WT (Figure 3). Is it possible that the process of RNA ISH fixation reflects differences in cardiac function or some other parameter that is being read out as size of the heart? Inter-CM distances are being measured in hearts presumable fixed/teated in a different manner prior to quantification.

4. The SHF progenitor population is examined via isl1a RNA ISH at 30hpf. However, as SHF addition to the atrium is thought to occur relatively early in development as compared to arterial pole addition, this time point may be too late to capture the initial SHF population that is added to the heart. This should be considered in discussing the results. Is it possible that instead there is a change in proliferation of these cells following addition to the heart?

5. In Figure 6, it is concerning that both p53 MO alone and p53 MO + tnnt2a MO seem to rescue heart area (6E) and atrial cell number (6J/K).

6. The analysis of Fgf and RA signalling is somewhat confusing. Based on expression of aldh1a2 and spry4 they would appear to be more active in the ventricular chamber, yet here atrial phenotypes are being studied. The RA-mediated effects on lamb1a atrial chamber size are show, the CM number is not.

Reviewer 2

Advance summary and potential significance to field

The manuscript "Lamb1a regulates atrial growth by limiting excessive contractility-dependent second heart field addition during zebrafish heart development" is an important work that gives us insight into the contribution of ECM components to the regulation of size and shape of the developing heart and the interaction with other important factors such as contractility and various signalling pathways such as FGF and RA.
The importance of laminins during for example muscle or eye development have been studied extensively (including in zebrafish) but very little has been shown for heart development. This is important in light of various publications showing changes to ECM levels including laminin sub units in diseased and failing hearts and their importance for regenerating cardiomyocytes. This work shows that laminins alongside other ECM molecules studied previously (Fn Versican and others) have an important role in migration of SHF cells to the heart and influencing the morphology and function of the heart.

Comments for the author

Generally, the manuscript is written well and is very thorough but there are a few issues in the manuscript that need to be addressed.
1). The authors show a clear difference in the lamc1 and lamb1a phenotype. As these contribute mainly to the Laminin short arm and the connections to other laminin/ECM molecules one would expect a similar phenotype, which is likely to be more subtle than the known disease-causing alpha chain that is responsible for MDC1A affecting skeletal and cardiac muscle. However, as the authors show while the lamc1 is the only gamma chain contribute in the heart, lamb1b and lamb2 also express. The authors dealt nicely with the lamb1b by creating the 1b mutants and doubles but there is little data shown for lamb2 , which restrict our understanding of the phenotype. The image shown for lamb2 shows some up-regulation in my opinion, contrary to the authors claim, perhaps because the myocardium is less compact than the endocardium and so less obvious. RT-PCR or a later ISH at 2-3 dpf when the measurements of the phenotype are done may be clearer. However, whether lamb2 is up-regulated or not it is still there at least when contraction of the heart is normal. This means that the beta chain expression is not abolished completley and some laminin molecules can form as opposed to the gamma chain deletion by lamc1. In the double lamb1a/1b mutants there is no beta chain in endocardium whilst there is some lamb2 and therefore beta chain in the myocardium. Could the phenotype be primarily influenced by the myocardium availability of Laminin. Also, related to that, when tnnt2a MO is used it may also affect lamb2 expression in the myocardium. I strongly suggest the authors solve this issue and discuss.

2). Another issue is that the assays the authors use extensively such as for looping ratio and myl7 area show that the lamb1a-/- mutants as well (as some of the older existing alleles) have highly significant differences between sibs and mutants but then when the same mutants are compared in the double het incross there is no significance at all for many comparisons, which weakens the authors' claims. For example, Fig. 2M, N compared with S4 M,N. The statistics methods should be re-visited and the authors should come up with an explanation to this and which is actually a true reflection of the phenotype.

3) One possibility for the growth of the heart in the mutant is that it affects proliferation. The authors seem to ignore that possibility. Is the amount of extra cell migration to the poles enough to count for the expanding of the chambers? They should address this, especially as during day 3 of development and onwards that becomes an important growth contributor.

4) Minor comment: In the methods, lines 543-4 -make distinction between Derrick 2021 references.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, Derrick et al. illustrate novel roles for laminins in limiting heart size through inhibition of cellular expansion at the inflow tract. The authors also dissect a contractility-dependent function for lamb1a mutants in restricting the posterior second heart field (SHF) addition to the venous pole. Blocking cardiac contractility rescues the cardiac morphogenetic abnormality and the contribution of excess SHF cells to the lamb1a mutant atrium. Finally, lamb1a mutants exhibit defects in the FGF-RA signaling axis and treatment with RA partially rescues the expanded cardiac size.

This study holds interest for investigators studying cardiac development and the signaling and biomechanical cues that regulate accumulation of the late-differentiating populations. Moreover, these data offer intriguing insights into the importance of extracellular matrix proteins in establishing proper cardiac chamber proportions. The experiments are performed in a technically sound and elegant manner and sufficient detail is included. Thus, this manuscript would appeal to the authorship of Development. However, a few essential issues remain that need to be addressed in order to strengthen the paper.

Comments for the author

Major Points:
1. The images in Figure S1 are not clear which may be a function of the quality of the PDF file. If these images could be improved, it might be helpful to have the tissue-specific expression of laminins in the body of the manuscript. Also, why are the tissue-specific expression patterns only represented at 30 hpf?

2. In Fig. 1, lamc1 expression at 55 hpf appears to be endocardial. Furthermore, in Fig. S1, the myocardial expression of lamc1 at 30 hpf is not convincing. The discrepancy between these data and the conclusions drawn in the text need to be rectified.

3. There are several contexts where the data is overstated. For example, in lines 125-126, the dynamic spatiotemporal expression of laminins may prompt further investigation of their role in early cardiac morphogenesis. However, it is too extreme to indicate that these findings implicate a function in “driving” heart tube development. It would be advisable to review these types of statements throughout the manuscript to ensure that the conclusions adequately represent the findings.

4. The placement of the 72 hpf morphometrics for the lamc1 crispants in Fig. S2E-F is disjointed.

5. Although cell counting is employed in Fig. 3L, it should be used more pervasively throughout the manuscript to quantify the size of the cardiac chambers. Given the ease with which this technique can be applied and the potential for inaccuracy through assessment of myl7 expression domains, it would strengthen the data to document ventricular and atrial cardiomyocyte cell counts for the initial representation of each mutant phenotype.

6. Although the images in Fig. 2B and 2J demonstrate different degrees of cardiac looping in comparison to wild-type, the looping ratios calculated in Fig. 2E and 2M both show a statistically significant difference.
Given the images, the quantitative findings are confusing.

7. In lines 181-184, the authors conclude that lamc1 and lamb1a play “distinct roles”, yet, again, this is an overstatement. While lamb1b does not compensate for the loss of lamb1a, there could be other factors at play. Furthermore, the differences in the phenotypes between lamc1 and lamb1a are relatively subtle.

8. In Fig. 3, the data would be more compelling if cell size was measured directly with a membrane-bound marker rather than assessing internuclear distance, an indirect measure of cell size.

9. In Fig. 4D and 4E, the y-axis label is confusing as the cell counts appear to be represented as a ratio.

10. Given that ltbp3 has been identified as a marker of anterior SHF progenitors, it is unclear why the authors include this population in their evaluation of those late-differentiating cells added to the venous pole in Fig. S5.

11. In Fig. 6K, how do the authors explain the lack of a statistically significant difference between the sibling and lamb1a mutant embryos injected with tp53 MO?

12. In Fig. 7, the expression of spry4 in the lamb1a mutant does not appear to be significantly different than the wild-type sibling embryos. The conclusions drawn in the text are not justified by these data.

13. Fig. S7P is difficult to interpret and appears to contradict prior data. Why is there not a statistically significant difference between sibling and lamb1a mutant embryos in the myh6 expression area?

Minor Points:
1. In line 24, it is unclear what the authors mean by “to compact around the developing atrioventricular canal.”
2. The statements in lines 81-82 and 87-86 about the roles of laminins in heart development appear to be contradictory. Furthermore, the statement in line 96 is repetitive with those mentioned above.
3. Line 370 has an odd citation.

First revision

Author response to reviewers' comments

We thank the reviewers for their positive appraisal of our manuscript, in particular highlighting the interest and importance of a role for laminin in biomechanical regulation of cardiac growth, as well as their positive view of the quality of our manuscript and data. We also thank the reviewers for their detailed and helpful feedback and suggestions on how to improve the manuscript, and we provide responses to their specific comments and concerns below.

Reviewer 1
1. It is stated (lines 188/189) that roles for looping morphogenesis and restriction of cardiac size are uncoupled in lamb1a mutants. However, quantification in Figures 2M and O would seem to suggest that is not the case, particularly at 55hpf. This is an important point which should be addressed. This is especially important as comparing the allele made for this study and the grumpy allele (Figure S3) seem to yield similar but qualitatively different results.
We have tried to clarify what we mean in this sentence, not only to improve our description of the phenotypes, but also to help improve the justification for why we have pursued the *lamb1a* mutant (related to point 2 below). It is specifically the relatively mild impact of morphological defects in relation to the severely enlarged hearts in *lamb1a* mutants which makes this mutant a more useful model in which we can study regulation of cardiac size by laminin in relative isolation from other cardiac defects. We have slightly reorganised this part of the manuscript to concisely summarise what the two models represent, and why we have chosen to pursue the *lamb1a* mutant - this can now be found at 204-213.
Regarding the *grumpy* allele - we do agree that there appears to be slight differences in the morphological phenotypes between these alleles. We tried to acquire further embryos to allow us to dig into the phenotype in this allele a little more, but were unable to do so from the

(currently non-)breeding pairs we have. We have instead inserted a small addition in the text highlighting that there may be slight differences in the morphology of the heart at 55hpf between our allele and the *grumpy* allele.

2. It is not entirely clear why lamc1 mutants were not pursued for most of this study. The data presented suggest they have a more severe looping phenotype yet a milder phenotype with respect cardiac chamber size (CM counts are not shown). In the text possible redundant function of other beta subunits is mentioned, but does this also suggest that Laminins may have positive and negative roles in some of these Processes?
In a similar vein to our previous comment, it is specifically the relatively mild impact of morphological defects in *lamb1a* mutants when compared to *lamc1* mutants/crispants which permits a more focused mechanistic analysis of the role of laminin in regulating heart growth. We have expanded slightly our analysis of the *lamc1* crispant phenotype, which supports this approach. This includes: comparative analysis of heart size at 30hpf in *lamb1a* and *lamc1* crispants; analysis of FHF and SHF cell number in *lamb1a* mutants at 30hpf, and analysis of atrial number and venous pole SHF addition in *lamc1* crispants at 55hpf. From this data we can see that 1) *lamb1a* mutants do not have defects in size of the heart or number of FHF CMs at 30hpf (Fig S6); 2) lamb1a mutants do not have a significantly increased number of SHF cells at the venous pole at 30hpf (Fig S6), together supporting an ongoing defect in SHF addition; 3) *lamc1* crispants have an apparent reduction in heart size at 30hpf, (Fig S6, S7), suggesting an early defect in size of the heart tube which could be coupled with the morphogenetic defects at 55hpf; 4) *lamc1* crispants do not have an increased number of DsRed + CMs at 55hpf, for which we have included the atrial data (Fig S6), but do appear to have an increase in SHF cells at the venous pole. The phenotypes between the two mutants in these analyses do not appear comparable, and this may be as the reviewer suggests due to an earlier opposing role for laminin prior to heart tube assembly and onset of looping morphogenesis that is captured in the *lamc1* crispant and not the *lamb1a* mutant. Together this suggests that laminins may indeed play positive and negative roles in this process, first promoting the right number of cells in the heart, and then restricting it. This extra comparative analysis of progressive phenotype in terms of cell number has been added as a supplemental figure (Fig S6). We refer to this data in the results to help support the ongoing analysis of the *lamb1a* mutant, and discuss the implications of this data in the discussion.

3. it is not clear why ventricular chamber size is larger in lamb1a mutants, if CM size/spacing and cell number are indistinguishable from WT (Figure 3). Is it possible that the process of RNA ISH fixation reflects differences in cardiac function or some other parameter that is being read out as size of the heart? Inter-CM distances are being measured in hearts presumable fixed/teated in a different manner prior to quantification.
We do not believe that fixation or processing contributes to the fact that we do not observe defects in ventricular CM spacing or number in the *lamb1a* mutants (and initial fixation method is the same for samples which will be processed either by in situ or immunohistochemistry). However we did want to understand why the ventricles in *lamb1a* mutants are enlarged. We attempted to quantify GFP+;DsRed- SHF cell number at the arterial pole using DAPI to visualise cell nuclei, but cells at the OFT are very tightly packed making it challenging to perform this analysis with confidence (as compared to the well- spaced nuclei of the venous pole). We instead decided to quantify the amount of GFP+;DsRed- tissue (which represents SHF-derived tissue) at the arterial pole, distal to the last dsRed+ nucleus in the ventricle/outflow tract. To do this we reoriented each sample to allow transverse reslicing into the arterial pole. Once the first dsRed+ cardiomyocyte was observed, all subsequent slices were discarded, and the GFP channel selected, creating a small stack representing only the GFP+ SHF-derived component of the arterial pole. The 3D Object Counter Fiji plugin was used to threshold, identify, and quantify the arterial pole SHF myocardium. This analysis revealed a significant increase in SHF contribution to the arterial pole in *lamb1a* mutants, and is now included in Figure 4. This further supports the mild increase in *spry4* expression we observed, which we have also now moved to Fig S7.

4. The SHF progenitor population is examined via isl1a RNA ISH at 30hpf. However, as SHF addition to the atrium is thought to occur relatively early in development as compared to arterial pole addition, this time point may be too late to capture the initial SHF population that is added to the heart. This should be considered in discussing the results. Is it possible that instead there is a change in proliferation of these cells following addition to the heart?
To address the first comment, we have analysed *isl1* expression at a slightly earlier stage of heart

development, 24hpf, to help capture this initial SHF population added to the venous pole of the heart. We do not see any difference in the *isl1* expression domain at 24hpf in *lamb1a* mutants compared to sibling, and we have replaced the 30hpf *isl1* expression data in (now) Supplemental Figure S7 with this earlier analysis to better support our conclusions.
In regards to the second comment, we believe the elevated number of SHF cells in *lamb1a* mutants at 55hpf demonstrates that it is ongoing SHF addition which underlies the cardiomegaly in *lamb1a* mutants. However, we have analysed cell proliferation using pH3 immunostaining at 55hpf to determine whether there is an increase in proliferation in these CMs once they have been incorporated into the heart. We do not find any increase in proliferation in *lamb1a* mutant cardiomyocytes or endocardial cells at 55hpf compared to controls, and we have included this data in Fis S7. However, we do acknowledge that this data consists of only a snapshot of proliferation over this time, and we include a short sentence in the discussion stating that we cannot explicitly rule out small increases in CM proliferation in SHF cells added early to the venous pole that we cannot detect using our methods.

5. In Figure 6, it is concerning that both p53 MO alone and p53 MO + tnnt2a MO seem to rescue heart area (6E) and atrial cell number (6J/K).
We would like to highlight that in the referred Figure 6, there is not a significant rescue of *lamb1a* phenotype in the p53-injected *lamb1a* mutants when compared to control uninjected *lamb1a* mutants, for either heart size or cell area. Rather the changes in *lamb1a* mutants compared to controls were not significant in those samples.
Since the change in number of cells being added to the SHF is small, and the number of tp53 MO-injected siblings was fewer compared to other experimental groups, we hypothesised this experiment was underpowered. We performed a further repeat of this experiment, injecting both *tp53* MO alone and *tnnt2a* MO with tp53MO into sibling and *lamb1a* mutant embryos to better align sample number between groups, quantifying SHF addition at the venous pole. Analysis of this more complete data set demonstrates that the *tp53* MO is not rescuing SHF addition, and Figure 6 has been updated with this extra data. We have not repeated the ISH analysis of the rescue (data in Fig 6E). The lack of statistical significance in that data could be due to a) similarly smaller sample number in the *tp53* MO- injected group, or b) a mild effect of *tp53* MO injection on morphology of the tissue.
However, as quantification of cell number better reflects the specific process we wish to investigate in this experiment (i.e. SHF addition), we hope that expanding this analysis has addressed the concerns of the reviewer.

6. The analysis of Fgf and RA signalling is somewhat confusing. Based on expression of aldh1a2 and spry4, they would appear to be more active in the ventricular chamber, yet here atrial phenotypes are being studied. The RA-mediated effects on lamb1a atrial chamber size are show, the CM number is not.
We agree that the analysis of the FGF/RA data in our original version of the manuscript could be difficult to interpret in the context of the phenotypes we described in the original manuscript. In line with our response to point 3, analysis of recently added SHF tissue at the arterial pole of the heart revealed a significant increase in SHF contribution to the arterial pole in *lamb1a* mutants. We have included this data in Figure 4, and have moved the *spry4* expression data to Fig S7, which we hope helps understand the relevance of the altered *spry4* expression. This in turn allows us to better highlight the expression data demonstrating that *aldh1a2* is first upregulated at 30hpf throughout the heart tube - including the atrium, at the stage we believe is the onset of the increased SHF addition to the venous pole in *lamb1a* mutants. We hope that the separation of these data improve the presentation. We have not been able to perform cell counts in the RA treated sibling and *lamb1a* mutants due to a vast number of experimental samples required for the correct number of replicates within treatment groups and subsequent technical repeats, particularly in light of a phenotype we believe to be only a partial rescue. It has not been feasible to perform an experiment of this magnitude within the revision in the current environment, however we have tried to emphasise better the caveats in interpreting the RA-treatment data in the discussion.

Reviewer 2
1). The authors show a clear difference in the lamc1 and lamb1a phenotype. As these contribute mainly to the Laminin short arm and the connections to other laminin/ECM molecules one would expect a similar phenotype, which is likely to be more subtle than the known disease-causing

alpha chain that is responsible for MDC1A affecting skeletal and cardiac muscle. However, as the authors show while the lamc1 is the only gamma chain contribute in the heart, lamb1b and lamb2 also express. The authors dealt nicely with the lamb1b by creating the 1b mutants and doubles but there is little data shown for lamb2 , which restrict our understanding of the phenotype. The image shown for lamb2 shows some up- regulation in my opinion, contrary to the authors claim, perhaps because the myocardium is less compact than the endocardium and so less obvious. RT-PCR or a later ISH at 2-3 dpf when the measurements of the phenotype are done may be clearer. However, whether lamb2 is up-regulated or not it is still there at least when contraction of the heart is normal. This means that the beta chain expression is not abolished completley and some laminin molecules can form as opposed to the gamma chain deletion by lamc1. In the double lamb1a/1b mutants there is no beta chain in endocardium whilst there is some lamb2 and therefore beta chain in the myocardium. Could the phenotype be primarily influenced by the myocardium availability of Laminin. Also, related to that, when tnnt2a MO is used it may also affect lamb2 expression in the myocardium. I strongly suggest the authors solve this issue and discuss.
We agree with the reviewer that one would expect the same phenotype upon loss of either *lamc1* or *lamb1a* - as long as *lamb1a* is the only beta subunit forming laminin trimers in the context of the developing heart up until 72hpf of development.

The question of upregulation of *lamb2* in *lamb1a* mutants is difficult to directly address. *lamb2* is expressed at very high levels in the somites (Jacoby et al, 2009), and thus qPCR analysis on wild type vs *lamb1a* mutant embryos would not be particularly informative, since any changes in cardiac signal would be lost amongst somite signal. We have extended our analysis of *lamb2* expression in *lamb1a* mutants to 55hpf, to try and ascertain whether there is indeed a persistent clear upregulation (this is now included in Supplemental Figure S5).

As at 30hpf, there is not a clear and strong increase in *lamb2* signal, and levels are relatively comparable, although we acknowledge this is not quantitative. We do notice for other cardiomyocyte genes, such as *myl7*, expression particularly in the atrium of *lamb1a* mutants can appear stronger, but this may be due to for example more compacted cells/tissue (internuclear distance in the atrium of *lamb1a* mutants is reduced for example, see Fig 3).

Nevertheless, while the question of whether *lamb2* is upregulated or not is unclear, we agree with the reviewer that it is of course expressed in the heart, thus still present in *lamb1a* mutants, and therefore there is a possibility that laminin trimers could form in which *lamb2* subunits are incorporated.

Based on these data we therefore used CRISPR-Cas9 mediated mutagenesis to generate *lamb2* F0 crispants - a similar approach to that we took to analyse the role of *lamc1*. We analysed heart size and morphology in *lamb2* crispants as well as in *lamb1a* mutant/*lamb2* crispant doubles, and found that loss of *lamb2* had no impact on heart development in either context. Most importantly, we did not observe a recapitulation of the severe *lamc1* heart looping defects in embryos lacking both *lamb1a* and *lamb2*, and this suggests that phenotype is not affected by general myocardial availability of laminin subunits. This data has the caveat that *lamb2* crispants are morphologically normal, in line with published *lamb2* mutants (Jacoby et al, 2009), and we lack a straightforward functional readout for loss of *lamb2*. However, our methodology is consistent with published approaches to F0 mutagenesis to disrupt the gene, in line with the same methodology we used to successfully mutagenise *lamc1*, and we used PCR analysis to confirm mutagenesis at target sites. The *lamb2* expression and functional data is now included in a new Supplemental Figure S5, with relevant discussion in the text.

Interestingly, we also analysed *lamb2* expression in *tnnt2a* morphants, in which contractility is abrogated, and there we did observe an increase in intensity of *lamb2* expression, included below as a figure for the reviewer. Again in this context we cannot be certain that this increase in intensity is due to increased levels of expression, or altered morphology and organisation of the tissue. However, given that knocking down *lamb2* does not appear to have an effect on heart development, either in a wild type or *lamb1a* mutant context, we have not pursued this further.

We have removed unpublished data provided for the referees in confidence.

2). Another issue is that the assays the authors use extensively such as for looping ratio and myl7 area show that the lamb1a-/- mutants as well (as some of the older existing alleles) have highly significant differences between sibs and mutants but then when the same mutants are compared in the double het incross there is no significance at all for many comparisons, which weakens the authors' claims. For example, Fig. 2M, N compared with S4 M,N. The statistics methods should be re-visited and the authors should come up with an explanation to this, and which is actually a true reflection of the phenotype.
Our original analysis of *lamb1a/lamb1b* double homozygous mutants, generated through incross of double heterozygous carriers (Fig S4), was carried out on reduced numbers of mutant embryos compared to the original characterisation of the *lamb1a* mutants (Fig 2) due to the low frequency of double mutants within these crosses (1/16 embryos), as well as low frequency of double homozygous wild type siblings used as the appropriate wild type controls Reduction in heart size at 55hpf in *lamb1a* mutants is relatively subtle, yet we believe this is the most pertinent stage to analyse since it is at this stage we see profound differences between the morphology of the *lamb1a* and *lamc1* mutants - and in the context of this experiment we are investigating whether loss of both *lamb1* paralogs recapitulates the *lamc1* looping morphogenesis phenotype. We have therefore carried out further crosses to bring the number of double mutants analysed more in line with the number analysed in Figure 2, where the *lamb1a* phenotype is first introduced. This expanded analysis recapitulates the phenotypes analysed in *lamb1a* mutants in Figure 2, and further demonstrates no additional significant changes to heart size or morphology in *lamb1a/lamb1b* doubles, strengthening our conclusion that *lamb1b* does not compensate for loss of *lamb1a* in heart development. This expanded data is included in Figure S4. The statistical tests used are appropriate for the data types.

Our analysis of the older '*grumpy*' allele of *lamb1a* reveals very similar defects in heart size as compared to our *lamb1a* alleles (compare Fig 2N,P and with Fig S3, N,P). However we do acknowledge that there appears to be a more severe effect on looping morphology in our new *lamb1a* alleles. We were unable to generate any new *grumpy* allele mutants (due to husbandry issues) to investigate this further, and have slightly amended and expanded the text to reflect that the phenotypes are similar between our *lamb1a* alleles, but there may be small differences in the morphology at 55hpf.

3. One possibility for the growth of the heart in the mutant is that it affects proliferation. The authors seem to ignore that possibility. Is the amount of extra cell migration to the poles enough to count for the expanding of the chambers? They should address this, especially as during day 3 of development and onwards that becomes an important growth contributor. Determining whether the number of extra SHF cells added to the atrium in *lamb1a* mutants is sufficient to drive the increase in atrial size is a challenging undertaking, since it would require a tight correlative analysis of FHF and SHF cell number, CM proliferation, CM size, and atrial size over time in wild type embryos alone. However, while we believe the elevated number of SHF cells in *lamb1a* mutants at 55hpf demonstrates that it is ongoing SHF addition which underlies the cardiomegaly in *lamb1a* mutants, we acknowledge that we did not analyse proliferation in our mutants. We have analysed cell proliferation using pH3 immunostaining at 55hpf to determine whether there is an increase in proliferation in these CMs once they have been incorporated into the heart. This is a time point when excess SHF addition in the atrium is already quantifiable, but also a stage when we should be able to capture proliferative increase which would lead to the significant cardiomegaly at 72hpf. We do not find any increase in proliferation in *lamb1a* mutant cardiomyocytes or endocardial cells at 55hpf compared to controls, and we have included this data in Supplemental Figure 7 However, we do acknowledge that this data consists of only a snapshot of proliferation over this time, and we include a short sentence in the discussion stating that we cannot explicitly rule out small increases in CM proliferation in SHF cells added early to the venous pole that we cannot detect using this method.

4) Minor comment: In the methods, lines 543-4 -make distinction between Derrick 2021 references.
We have amended this in the text

Reviewer 3:
1. The images in Figure S1 are not clear which may be a function of the quality of the PDF file. If these images could be improved, it might be helpful to have the tissue-specific expression of

laminins in the body of the manuscript. Also, why are the tissue-specific expression patterns only represented at 30 hpf?
We have included a less compressed version of Figure S1 in the revised submission. We have only analysed tissue specificity of laminin expression at 30hpf since we predominantly investigate the requirement for laminins prior to 55hpf (much of our analysis is performed at 55hpf) - therefore expression at 30hpf is the most relevant to the processes we analyse.
Since we do not focus on investigating tissue-specific requirements for laminins in heart development, we do not consider the tissue-specific expression requires sufficient highlighting as a main figure, and believe it is better placed as a supplemental file to not interrupt the main figure flow.

2. In Fig. 1, lamc1 expression at 55 hpf appears to be endocardial. Furthermore, in Fig. S1, the myocardial expression of lamc1 at 30 hpf is not convincing. The discrepancy between these data and the conclusions drawn in the text need to be rectified.
After reviewing the in situ expression data for *lamc1* at 55hpf, we do not agree with the reviewer that *lamc1* expression at 55hpf is endocardial. Within Figure 1, comparing *lamc1* expression (panel 1N) with *lama4* expression (1D) at 55hpf the domain of expression of *lamc1* is broader than that of *lama4* (an endocardial gene), and more comparable to *lamb2* (1L, a myocardial gene). We also disagree that the myocardial expression of *lamc1* in Figure S1 is not convincing - in the myocardial merge shown in panel F there is clearly expression of *lamc1* overlapping with the *myl7:GFP* expression domain, which can also be better appreciated in the magnified insert. In line with our previous point that the focus of this manuscript is not to interrogate tissue specific contribution of laminin isoforms further, we do not believe we need to amend the manuscript here, but hope that a better resolution of figure for the supplemental data will aid readers in appraising the data.

3. There are several contexts where the data is overstated. For example, in lines 125-126, the dynamic spatiotemporal expression of laminins may prompt further investigation of their role in early cardiac morphogenesis. However, it is too extreme to indicate that these findings implicate a function in “driving” heart tube development. It would be advisable to review these types of statements throughout the manuscript to ensure that the conclusions adequately represent the findings.
We have revisited this statement as well as assessed the strength of other conclusions throughout the manuscript to make sure conclusions or assertions we make are appropriate to the data presented. In the example given above we have removed the word ‘driven’ and changed the wording to ‘...may play a role in early heart tube morphogenesis’.

4. The placement of the 72 hpf morphometrics for the lamc1 crispants in Fig. S2E-F is disjointed.
We have amended the figure accordingly

5. Although cell counting is employed in Fig. 3L, it should be used more pervasively throughout the manuscript to quantify the size of the cardiac chambers. Given the ease with which this technique can be applied and the potential for inaccuracy through assessment of myl7 expression domains, it would strengthen the data to document ventricular and atrial cardiomyocyte cell counts for the initial representation of each mutant phenotype.
We do agree with the reviewer that quantification of cell number provides a more detailed understanding of the nature of the cardiac phenotypes in our mutants than just *myl7* domain alone. While we did not have the relevant transgenic line in the background of the *lamc1 sleepy* mutants or the *lamb1a grumpy* allele, and not sufficient time within the revision period to generate them, we have instead used lamc1 CRISPR F0 mutagenesis in the *myl7GFP;DsRed* transgenic background to analyse cell number. We believe this approach is appropriate since we have demonstrated that *lamc1* crispants recapitulate the *lamc1* mutant phenotype. This analysis has revealed that *lamc1* mutants do not recapitulate the increase in dsRed positive cell number observed in the atrium of *lamb1a* mutants at 55hpf, but do show an increase in second heart field number, supporting our finding that laminin restricts this process. Furthermore, in combination with an expanded analysis of heart phenotypes at 30hpf (see response to reviewer 1), this discrepancy between mutants suggests that *lamc1* mutants have defects in size or morphology of the heart tube at the onset of looping morphogenesis, which may confound the analysis of the role of laminins in cell addition independent of tube and looping morphogenesis during this 24-55hpf time window. This data has now been included in Supplemental Figure S6. We did not

observe any defects in ventricular cell number in the *lamc1* crispants, but have not included this data in the figure since at this point of the manuscript we are focussing on atrial growth and cell addition to the venous pole, and so we maintained this focus in the figure for the *lamc1* crispant analysis.

6. Although the images in Fig. 2B and 2J demonstrate different degrees of cardiac looping in comparison to wild-type, the looping ratios calculated in Fig. 2E and 2M both show a statistically significant difference. Given the images, the quantitative findings are confusing. While the quantifications of cardiac looping in Figure 2 do both show a reduction in heart looping ratio in comparison to their respective siblings, at 55hpf *lamc1* crispants do have a lower average looping ratio when compared to *lamb1a* mutants (mean of 1.14 in *lamc1* crispants vs 1.36 in *lamb1a* mutants). An unpaired t-test comparing the looping ratio of these two mutants at 55hpf demonstrates a significant difference between them ($p < 0.0001$), supporting our conclusion that the looping phenotypes are a)both different from their respective siblings, but also b)different from each other, which is reflected in the graphs. We have included the info about this comparative analysis of looping ratio between mutants in the text.

7. In lines 181-184, the authors conclude that lamc1 and lamb1a play “distinct roles”, yet, again, this is an overstatement. While lamb1b does not compensate for the loss of lamb1a, there could be other factors at play. Furthermore, the differences in the phenotypes between lamc1 and lamb1a are relatively subtle.
We agree that while our data suggests that the impact of loss of *lamb1a* and *lamc1* on looping morphology and heart size are different, our data does not fully support completely distinct roles for these two subunits in these processes, and this may be due to a compounded effect of other factors (temporal requirements, differential maternal contribution etc). We have amended this sentence to simply state that our data identify two previously- uncharacterised requirements laminin (in general) in both these processes, without claiming distinct roles for each subunit, and include a simple statement outlining these possibilities, including functionally or temporally different requirements, or different levels of maternal contribution.

8. In Fig. 3, the data would be more compelling if cell size was measured directly with a membrane-bound marker rather than assessing internuclear distance, an indirect measure of cell size.
We do agree that internuclear distance is an indirect measure of cell size, however currently no published transgenic line or antibody marks clearly the membrane of atrial cells (the use of transgenic lines such as myl7:RasGFP, antibodies such as DMGRASP, or injection of fluorescent membrane markers such as Lyn:TdTomato has been restricted to analysis of ventricular tissue, where cells are tighter packed, signal is stronger, and chamber structure is better preserved in fixed samples). Furthermore the use of internuclear distance as a proxy for cell size in the atrium has been previously published in Bornhorst et al, Nature Communications 2019 (PMID 31511517), and we think this represents the best approach given the lack of more appropriate tools, the generation of which is beyond the scope of these revisions.

9. In Fig. 4D and 4E, the y-axis label is confusing as the cell counts appear to be represented as a ratio.
We have amended the axis label in the figure to improve clarity

10. Given that ltbp3 has been identified as a marker of anterior SHF progenitors, it is unclear why the authors include this population in their evaluation of those late-differentiating cells added to the venous pole in Fig. S5.
We agree that this data is distracting and doesn’t provide a tangible contribution to the manuscript, so we have removed it.

11. In Fig. 6K, how do the authors explain the lack of a statistically significant difference between the sibling and lamb1a mutant embryos injected with tp53 MO?
Since the change in number of cells being added to the SHF is small, and the number of *tp53* MO-injected siblings was fewer compared to other experimental groups, we hypothesised this experiment was underpowered. We performed a further repeat of this experiment, injecting both *tp53* MO alone and *tnnt2a* MO with *tp53* MO into sibling and *lamb1a* mutant embryos to better align sample number between groups, quantifying SHF addition at the venous pole.

Analysis of this more complete data set demonstrates that *tp53* MO is not rescuing SHF addition, and Figure 6 has been updated with this extra data.

12. In Fig. 7, the expression of spry4 in the lamb1a mutant does not appear to be significantly different than the wild-type sibling embryos. The conclusions drawn in the text are not justified by these data.
To understand the increase in ventricle size in *lamb1a* mutants at 55hpf, we performed a quantification of the GFP+ (but dsRed -) SHF myocardium at the arterial pole of the heart. This reveals an increase in SHF myocardium, suggesting that, similar to the venous pole, laminin may be regulating SHF addition at the arterial pole too. This increase in volume is mild, but we do believe this is linked to a mild increase in *spry4* expression at 55hpf in *lamb1a* mutants, and while we focus predominantly on the role of laminin in growth of the atrium/venous pole, we do think it is relevant to keep this data in Supplemental format. We have therefore removed all *spry4* data from Figure 7 and have moved the initial analysis of *spry4* expression at 55hpf in *lamb1a* mutants (originally panels 7E/F) to new Supplemental Figure S7. We have completely removed the *spry4* expression analysis in *tnnt2a* morphants as this was not so relevant to the main conclusions from that experiment.

13. Fig. S7P is difficult to interpret and appears to contradict prior data. Why is there not a statistically significant difference between sibling and lamb1a mutant embryos in the myh6 expression area?
We believe there is not a statistically significant difference between sibling and *lamb1a* mutants in *myh6* expression area (this is now Fig S9P) due to a combination of a) comparatively low number of 'experimental 'units' analysed (4 technical RA treatment repeats, which each represents an average measurement derived from 10+ embryos) when compared to the original characterisation of atrial size in the *lamb1a* mutants (a dataset comprising 64 sibling embryos and 29 mutant embryos, Fig 3F); and b) partial rescue, which we note in the results section and return to in the discussion - global application of RA is unlikely to completely recover normal wild type patterned/restricted activity of RA, and thus is unlikely to rescue fully. We acknowledge the difficulty in interpreting these data, and have expanded the relevant sentence to include this caveat in our discussion of the data: 'Supporting this hypothesis, timed RA treatments during early SHF addition partially rescued heart size in *lamb1a* mutants at 3dpf (Fig. S9). However, global upregulation of RA is likely too broad to restore the careful balance of RA-FGF levels, and it is therefore difficult to interpret the specific contribution of disrupted RA signalling to the increased SHF addition and cardiomegaly in *lamb1a* mutants given the complex antagonistic interactions.'

Minor Points:
1. In line 24, it is unclear what the authors mean by "to compact around the developing atrioventricular Canal."
We have simplified this description

2. The statements in lines 81-82 and 87-86 about the roles of laminins in heart development appear to be contradictory. Furthermore, the statement in line 96 is repetitive with those mentioned above.
We have amended the first referenced section to better highlight that while requirements for laminins in vertebrate heart development and function are suggested by previous studies, mechanistically there is poor understanding of the roles that laminins play in these contexts. We have removed the end of the last sentence to prevent repetition.

3. Line 370 has an odd citation.
Formatting error - removed

Second decision letter

MS ID#: DEVELOP/2021/199691

MS TITLE: Lamb1a regulates atrial growth by limiting second heart field addition during zebrafish heart development

AUTHORS: Christopher J Derrick, Eric J G Pollitt, Ashley Sanchez Sevilla Uruchurtu, Farah Hussein, Andrew J Grierson, and Emily S Noel

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.

The overall evaluation is positive and we would like to publish your work in Development, provided that the referees' very minor comments can be satisfactorily addressed in the text.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Dr. Emily Noel and colleagues characterize the function of the extracellular matrix Laminin proteins in zebrafish heart development. Human genetic and animal studies (mouse and Drosophila) have indicated a role for Laminin function in heart development and congenital heart disease however the specific function of Laminins in these processes has not been described.

Lamc1, which encodes the single zebrafish gamma subunit of the Laminins (and therefore with its loss should disrupt all laminin function), was knocked down in F0 “crispants”, which resemble previously published lamc1 mutants. Loss of lamc1 was found to result in perturbed cardiac looping at 55hpf and an apparent increase in cardiac size (area measurement of RNA ISH) at 72hpf. A similar, yet milder phenotype was observed in stable lamb1a (encoding a beta subunit) mutants, with arguably a larger effect on chamber size than looping. It is found that lamb1a mutants have an increased atrial (but not ventricular) CM number from 55-72hpf, with a “birthdating” approach used to show that there is an increased number of newly added CMs in the atrium of lamb1a mutant hearts. This alteration of atrial CM number is dependent on cardiac contractility, and appears to reflect alterations in RA and Fgf signalling downstream of a Laminin-based tension sensing mechanism.

Overall, this work suggests a novel and intriguing model where Laminins may bridge a response of mechanical forces from the onset of cardiac contractility to influence the extent of SHF addition of CMs to the venous pole of the heart. The manuscript is clearly written and has a compelling narrative.

Comments for the author

The authors have largely addressed my previous comments, and included a large amount of supplemental data to do so.

Reviewer 2

Advance summary and potential significance to field

This paper focuses on a relatively less studied aspect of the role of the various laminins during heart development. It makes some order in the expression and function of the various laminin genes, which is important in view of the increasing understanding of ECM role in heart development.

Comments for the author

I am generally happy with the changes made to the manuscript following my comments and of the other reviewers, and therefore would recommend the manuscript for publication. However there are still a few minor issues that needs attention before publication:

1. The authors write: “Blocking cardiac contractility in lamb1aΔ25 mutant embryos significantly reduced heart size at 55hpf and 72hpf compared to control lamb1aΔ25 mutants, suggesting that excess SHF addition is mediated by contractility upon loss of lamb1a (Fig. 6E, Fig. S8A).”
The figure sent for the reviewer eyes shows lamb2 upregulation when tnnt2a MO is used. If that is the case, one can also imagine another indirect possibility in which lamb2 is upregulated upon lack of contractility, compensating on the lack of lamb1a and preventing the excess SHF addition. Should refer to it in discussion.

2. The authors added crispant studies for lamb2. It is stated that 4 targets were chosen and in ‘Materials and Methods’ they say: "…Efficacy of mutagenesis was confirmed through PCR amplification of the targeted region of genomic DNA…" -
please elaborate more in this section so the method would be clearer-do the authors mean that the regions amplified were sequenced following PCR and the reads assessed or any other method?. How many embryos were checked? Was each embryo checked for all 4 regions? what was the percentage of embryos, which had at least one truncation? Since this is not a stable mutant it is important to understand that the results represent embryos in which both alleles were truncated.

3. There is a mistake in Supplemental figure S1 legend- it should say "…while lama5 and lamb2 are expressed in the myocardium (C,D)...". (and not the current
lama5 and lama1b).

Reviewer 3

Advance summary and potential significance to field

The authors have thoroughly responded to all reviewers' comments. While there were limitations in their ability to complete some of the indicated experiments, adequate additional data clearly address the most important concerns. Thus, I believe that this manuscript is appropriate and ready for publication in Development.

Comments for the author

The authors have thoroughly responded to all reviewers' comments. While there were limitations in their ability to complete some of the indicated experiments, adequate additional data clearly address the most important concerns. Thus, I believe that this manuscript is appropriate and ready for publication in Development.

Second revision

Author response to reviewers' comments

We thank all reviewers for appraising a revised version of our manuscript. All reviewers were satisfied with our experimental revisions, and Reviewer 2 has suggested some further minor amendments to the manuscript text. Our response to Reviewer 2 follows:

1. The authors write: “Blocking cardiac contractility in lamb1aΔ25 mutant embryos significantly reduced heart size at 55hpf and 72hpf compared to control lamb1aΔ25 mutants, suggesting that excess SHF addition is mediated by contractility upon loss of lamb1a (Fig. 6E, Fig. S8A).”
The figure sent for the reviewer eyes shows lamb2 upregulation when tnnt2a MO is used. If that is the case, one can also imagine another indirect possibility in which lamb2 is upregulated upon lack of contractility, compensating on the lack of lamb1a and preventing the excess SHF addition. Should refer to it in discussion.
While we agree that there appears to be an upregulation of *lamb2* transcript upon loss of contractility, we have not performed this analysis in *lamb1a* mutants where contractility is blocked, and our analysis of *lamb2* crispant knockdown in *lamb1a* mutants suggests that *lamb2*

itself would not be sufficient to compensate, or rescue, the SHF defect in *lamb1a* mutants where contractility is blocked. It is also unclear whether an increase in *lamb2* expression would result in extra laminin trimers being deposited from the myocardium, since this still requires assembly with the relevant alpha and gamma subunits. Direct speculation that a potential upregulation of *lamb2* in *lamb1a* mutants in which contractility is blocked could rescue the SHF defects is thus rather a specific supposition not entirely supported by the data we currently have, and which would require complex genetic experiments to resolve. However, there is evidence from literature that mechanical loading does impact ECM content (for a review see Humprey et al., Nat. Rev. Mol. Cell. Biol. 2014), and it is possible that contractility alters expression of multiple cardiac ECM components – of which *lamb2* may be only one. An altered ECM environment in *lamb1a* mutants where contractility is abrogated could change SHF dynamics, and could be sufficient to recover the SHF defects in *lamb1a* mutants. This broader role for contractility in potential regulation of ECM content is an important consideration, and we have included the following passage in the discussion:
'Mechanical loading has been implicated as a moderator of ECM content in other contexts, for example bone (Humphrey et al., 2014). It is possible therefore that loss of heart contractility could affect composition of the cardiac ECM, for example through upregulation of ECM components, which could restore a suitable environment for SHF addition in *lamb1a* mutants.'
(Lines 432-435)

2. The authors added crispant studies for lamb2. It is stated that 4 targets were chosen and in 'Materials and Methods' they say: "…Efficacy of mutagenesis was confirmed through PCR amplification of the targeted region of genomic DNA…" - please elaborate more in this section so the method would be clearer-do the authors mean that the regions amplified were sequenced following PCR and the reads assessed or any other method?. How many embryos were checked? Was each embryo checked for all 4 regions? what was the percentage of embryos, which had at least one truncation? Since this is not a stable mutant it is important to understand that the results represent embryos in which both alleles were truncated.
We have expanded the relevant methods section to include extra details on *lamb2* CRISPR genotyping analyses.

3. There is a mistake in Supplemental figure S1 legend- it should say "…while lama5 and lamb2 are expressed in the myocardium (C,D)...". (and not the current lama5 and lama1b).
We thank the reviewer for drawing our attention to this mistake and have rectified it.

Third decision letter

MS ID#: DEVELOP/2021/199691

MS TITLE: Lamb1a regulates atrial growth by limiting second heart field addition during zebrafish heart development

AUTHORS: Christopher J Derrick, Eric J G Pollitt, Ashley Sanchez Sevilla Uruchurtu, Farah Hussein, Andrew J Grierson, and Emily S Noel
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.