Molecular profiling of the vestibular lamina highlights a key role for Hedgehog signalling

Tengyang Qiu, Barbora Hutečková, Maisa Seppala, Martyn T. Cobourne, Zhi Chen, Mária Hovořáková, Marcela Buchtová and Abigail S. Tucker
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Original submission

First decision letter

MS ID#: DEVELOP/2022/201464

MS TITLE: Hedgehog signalling from the developing tooth directs the development of the adjacent vestibular lamina, coordinating tooth and vestibule formation

AUTHORS: Tengyang qiu, Bara Huteckova, Maisa H-M Seppala, Martyn Timothy Cobourne, Zhi Chen, Maria Hovorakova, Marcela Buchtova, and Abigail S Tucker

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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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

This research aims to identify the molecular regulatory mechanism of vestibular lamina for significant. structure for tooth development and renewal. These approaches have been available by lower vertebrates or reptiles. Indeed, critical study, but we need to make significant progress due to severe mistakes in preparing the current manuscript to be accepted

Comments for the author

Full Title: Hedgehog signalling from the developing tooth directs the development of the adjacent vestibular lamina, coordinating tooth and vestibule formation

This research aims to identify the molecular regulatory mechanism of VL. They tried to find the critical signalling interactions between VL and tooth germs at the E14 stage. For these analyses, they analyzed the bulk RNA sequencing between VL and TB from the incisor region of the mandible at E14. And they showed the roles of the Hedgehog pathway in VL during tooth development. However, there are many complicated concerns with the figures and manuscript.

Major points:
From the first part of this study, the sampling for transcriptomic analysis is questionable. Is the comparison between VL and TB appropriate? While the dental lamina forms the teeth, the VL forms the gap between the lips and cheeks. They wanted to know the difference between the two laminas. For this reason, it seems appropriate to compare VL and DL without tooth germs at the early developmental stage. Many things are mixed in this study, not focusing on one subject. Furthermore, the sample number is sufficient for the KO study, and the mouse number of KO is only 1 for the analysis, which needs to be seriously improved.
Most of all, the rationale of this study is unclear. The authors should clarify the relationship between the unsolved problems in the previous studies and this study's research objectives (expected outcomes). This paper should be written more on what they want to investigate on. This study's introduction, result, and discussion need to be clarified, and the authors should write more neatly about what they want to address. Especially in the result part, the contents of the discussion and result need to be clarified and it is tough to read. Furthermore, all figures are faint and shallow and I could not see any RNA sequencing data at all.

Minor points
All figures for RNA seq in Figure 1 are very faint and of low quality. The PCA is too fuzzy to see any data on the heatmap.

SM Figure 1 is also very faint. Labels on all figures are not visible. What do the sample names mean in the heatmap, such as 19TB, 21TB, etc.?

In SM figure2, all of the quantification data are fuzzy. And the order of figures should be arranged according to the order of description of the manuscript. In the manuscript, the authors explained that lingual-buccal differences in Otx expression were observed, but the expression did not seem significantly different.
In figure2, the data of RNA seq. are fuzzy. Any data are not seen. Furthermore the expression pattern also is very faint. High magnification figures for each figure should be needed.

Why the authors used the different stages of mice? They should address why they use the stage. Authors need to make their figures coherent. For example, if they want to show the shape of the epithelium, they should decide whether to show it as E-cadherin expression with IHC or HE staining. Please show them consistently.
The figures are inconsistent.

Arrowheads, dotted lines, and arrows are not visible in all figures.

In figure4, why did they show the E13 stage? They should describe. The graph is very faint. Where are Whisker follicles? Please label. They address Gli1 was expressed higher on the buccal side than on the lingual side. But in Figure 4 they showed only the lingual side. The buccal side is not visible.

In figure5, why did they use the E12.5 stage for the BrdU assay? Please explain.
High magnification for co-localization should be needed—any difference between WT and KO.

The authors should label the lingual buccal direction with an arrow at every figure. And they show lingual buccal direction with consistency.

The authors showed only Wnt1-cre. How about K14-Cre (loss of Gas1 in the epithelium)?

In figure 7, with cyclopamine, VL lengths were not changed after two days of in vitro culture. However, they showed almost two times a difference between the control and Cyclopamine groups. Green and black dotted lines were not visible.

They concluded the cilia relationship in VL with only SAG experiment. The authors exaggerated their conclusions and put them in a diagram. The conclusion is overstated, and more experiments with cilia are needed.

Diagram also should be changed. Too exaggerated.

Keywords should be changed. Cilia is too exaggerated. Even VL has no mention in the keyword.

The title should be changed. Too long.

The authors should provide more information about the figures in the figure legend. Too lacking.

For the materials and methods, the authors should give the information in detail about the company, country, city, and catalogue numbers…..And detailed primer information for the probe should be needed.

The discussion part should be vigorously changed. The authors should discuss the current study and compare it with the previous research. What is the significance of their findings? It is necessary to compare and discuss the differences from previous studies and what novelty is. How much improvement here compared to the earlier results or conclusions?

Reviewer 2

Advance summary and potential significance to field

In the manuscript entitled, “Hedgehog signalling from the developing tooth directs the development of the adjacent vestibular lamina, coordinating tooth and vestibule formation”, Qiu et al. present evidence that HH signaling plays a role in the development of the vestibular lamina in mice. Overall, the work will be of interest to the readership of Development, and the data are generally of high quality. However, as detailed below, there are a number of issues ranging from readability of figures to statistical considerations and a lack of key controls for some experiments that need to be addressed prior to further consideration of this manuscript.

Comments for the author

Major Comments:

1) For the bulk RNA-sequencing presented in Figure 1, it is unclear how the authors controlled for potentially different relative amounts of epithelium and mesenchyme between the two dissected structures (i.e., the VL and TB). As an example, if the dissected VL simply contains relatively more epithelial cells than the TB, then epithelial-expressed genes would appear as enriched (and vice versa if there were greater mesenchyme). Have the authors examined this possibility? If so, it would be useful to include some analysis to assure the reader that these samples have similar numbers of epithelial and mesenchymal cells, which would increase

confidence that the differentially expressed genes are due to fundamental differences in the molecular signatures, as proposed by the authors.

2) The observation that the VL is similarly truncated in Gas1+/- and Gas1-/- embryos is quite interesting (Figure 3A-C). The authors should label the VL in the images and provide quantitation of the degree of truncation across WT, Gas1+/-, and Gas1-/- embryos.

3) In Figure 3D-G, the authors need to include a Gas1-/- E15.5 control for comparison with the Gas1-/-;Boc+/- and Gas1-/-;Boc-/- embryos. Similar to the above comment, the authors should quantify the degree of truncation across the different genotypes.

4) In Figure 4, the authors should examine HH target gene expression in Gas1+/- embryos, especially since loss of one copy of Gas1 is sufficient to yield a truncated VL.

5) The data in Figure 6 are quite exciting. However, a key control, Wnt1Cre is missing. This is of particular importance since Wnt1Cre animals are known to display developmental phenotypes (PMID: 23648512). It would be of further interest to assess Wnt1Cre;Gas1fl/+ animals. Presumably these animals also result in a truncated VL? And, again, quantitation of the VL truncation phenotype is absent.

6) The concentration of cyclopamine used in Figure 7 is extremely high and known to cause off-target effects (PMID: 18754008). It is also odd that the authors use Gli1 as a readout in Figure S5 and Ptch1 as a readout in Figure S6.
While it is not possible to directly compare, it seems that Ptch1 expression is much lower in the control in Figure S6 compared to Gli1 expression in Figure S5. I would recommend using the same target in both experiments to increase confidence that SAG and cyclopamine are working as expected.

7) I would recommend that the authors address the severity of the VL truncation phenotype observed in Gas1+/- mutants in the discussion (perhaps replacing the Evc section with this discussion). Especially, if the levels of HH pathway activity are not reduced in the Gas1+/- embryos. Perhaps this suggest a HH-independent role for Gas1 in VL development?

Minor Comments:

1) In the abstract, I believe it should be multiple frenula, not frenulum.

2) The text boxes in the volcano plot in Figure 1C are not readable.

3) In Figure 1D, the authors should be explicit whether the heat maps represent four distinct E14 embryos. Also, it is unclear why the labeling is different (e.g., 17 TB vs. 18 VL). Did the TB and VL samples come from the same embryos?

4) Again, the labels in Supplemental Figure 1A-C and Supplemental Figure 2 are not readable.

5) The authors should indicate the genetic backgrounds of the animals used in this study.

6) The authors should explicitly indicate when they are using paired and un-paired t-tests.

7) In Figure 4 G,H, Figure 5K, and Figure 7 C, F, the authors need to include individual data points in their histograms.

8) In Figure 4 G,H, the authors should indicate what units are represented on the Y axes.

9) I would recommend adding labels to Figure S4. It is also not clear why there is a Figure S4(1) and S4(2). Just make a single Figure S4.

First revision

Author response to reviewers' comments

We thank the reviewers for their helpful comments which have greatly enhanced the paper. In addition to the changes below we have also changed the abstract length etc to fit with the format of Development.

Reviewer 1 Advance Summary and Potential Significance to Field:
This research aims to identify the molecular regulatory mechanism of vestibular lamina for significant. structure for tooth development and renewal. These approaches have been available by lower vertebrates or reptiles. Indeed, critical study, but we need to make significant progress due to severe mistakes in preparing the current manuscript to be accepted

Reviewer 1 Comments for the Author:
Full Title: Hedgehog signalling from the developing tooth directs the development of the adjacent vestibular lamina, coordinating tooth and vestibule formation

This research aims to identify the molecular regulatory mechanism of VL. They tried to find the critical signalling interactions between VL and tooth germs at the E14 stage. For these analyses, they analyzed the bulk RNA sequencing between VL and TB from the incisor region of the mandible at E14. And they showed the roles of the Hedgehog pathway in VL during tooth development. However, there are many complicated concerns with the figures and manuscript.

Major points:
From the first part of this study, the sampling for transcriptomic analysis is questionable. Is the comparison between VL and TB appropriate? While the dental lamina forms the teeth, the VL forms the gap between the lips and cheeks. They wanted to know the difference between the two laminas. For this reason, it seems appropriate to compare VL and DL without tooth germs at the early developmental stage.

We are sorry for the lack of clarity. We have made the aims and choice of stage for transcriptomics more evident by revising the text in the title, abstract and introduction.

The main aim was to produce a molecular signature that was specific to the VL.
We have added a new supplementary montage showing expression of the genes found by the screen to be upregulated in the VL.

We picked E14 as this allowed us to compare odontogenic and non-odontogenic tissues. The VL can form tooth buds after stabilisation of Wnt signalling from E13.5 to E15.5, so still has the potential to form teeth at E14 but normally doesn't.

We additionally, wanted to investigate whether the teeth and VL interact during development, by comparing signalling pathway expression in the two.

We agree that an earlier time point for comparing the two laminas before they diverge would be an interesting project in order to understand early fate decisions, but this is not within the remit of this paper.

Many things are mixed in this study, not focusing on one subject.

We hope the flow of the paper is now clearer, with linking sentences to show how we moved from the transcriptomic data to focusing on hedgehog signalling.

Furthermore, the sample number is sufficient for the KO study, and the mouse number of KO is only 1 for the analysis, which needs to be seriously improved.

Regarding the mouse mutants we have included more numbers in our analysis (see methods section on mice and results text). In a few instances when we have only one KO for a specific stage (for example BOC -/-) but have at least four stages of this mutant, with mutants all showing the same

phenotype. This is because the mutants came from our collaborator and we do not have the line in house. For our main line, *Gas1*, however, we have a large number of mutants analysed, N = 18. So we feel the data is very robust. We have made it clearer throughout the text the numbers of mice analysed.

Most of all, the rationale of this study is unclear. The authors should clarify the relationship between the unsolved problems in the previous studies and this study's research objectives (expected outcomes). This paper should be written more on what they want to investigate on. This study's introduction, result, and discussion need to be clarified, and the authors should write more neatly about what they want to address. Especially in the result part, the contents of the discussion and result need to be clarified, and it is tough to read.

As mentioned above, we completely rewritten the last part of the introduction with changes to the title and abstract. To make the discussion clearer we have added in extra sections and put sub-headings to direct the rationale for the text. We stress the novelty, in that this is the first comprehensive molecular analysis of the vestibular lamina.

Furthermore, all figures are faint and shallow, and I could not see any RNA sequencing data at all.

We have gone through and improved all figures, increasing font size for the figures. Some of the issues are probably also due to low resolution of the original images in the PDF for review.

Minor points
All figures for RNA seq in Figure 1 are very faint and of low quality. The PCA is too fuzzy to see any data on the heatmap.

Corrected

SM Figure 1 is also very faint. Labels on all figures are not visible. What do the sample names mean in the heatmap, such as 19TB, 21TB, etc.?

Apologies for the confusion. The labels represent the numbers from the original dissections. So tooth bud dissection from litter 21 is TB21. Slices were generated from embryos and the tooth bud and VL dissected. VL and TBs were pooled from a litter to generate enough tissue. After dissection and extraction, we checked all the pooled samples for quality of RNA and picked the 5 litters with the best scores. These are now referred to as TB 1-5 etc for clarity and the process is written in the methods.

In SM figure2, all of the quantification data are fuzzy.

Corrected

And the order of figures should be arranged according to the order of description of the manuscript.

Figures have been rearranged

In the manuscript, the authors explained that lingual-buccal differences in Otx expression were observed, but the expression did not seem significantly different.

We have now added labels to the figures so that it is clear what is being shown. The lingual-buccal/labial differences are very pronounced for Otx1, which we hope are now obvious.

In figure2, the data of RNA seq. are fuzzy. Any data are not seen. Furthermore, the expression pattern also is very faint. High magnification figures for each figure should be needed.

The figure has been improved

Why the authors used the different stages of mice?
They should address why they use the stage.

We now describe the use of the different stages in the text. For example “E12.5 (prior to obvious morphological differences in the laminas) and at E15.5 (when the two structures were clearly distinct morphologically)”. This has now been made clearer throughout.

Authors need to make their figures coherent. For example, if they want to show the shape of the epithelium, they should decide whether to show it as E-cadherin expression with IHC or HE staining. Please show them consistently.
The figures are inconsistent.

The figures have been amended with new images added for consistency. For early stages we use E-cadherin to outline the epithelium, while for older stages we have used histology to show the overall structure of the lower jaw.

Arrowheads, dotted lines, and arrows are not visible in all figures.

These have been amended to make them clearer.

In figure4, why did they show the E13 stage? They should describe.

We now show the phenotype in the mutants at E13.5 in Figure 3. This makes it more logical to focus on this stage for the molecular analysis in Figure 4.

The graph is very faint. Where are Whisker follicles? Please label.

We have added asterisks to indicate the area of the forming whisker follicles. The graph has been amended.

They address Gli1 was expressed higher on the buccal side than on the lingual side. But in Figure 4, they showed only the lingual side. The buccal side is not visible.

Similar to the point about Otx1, we appreciate that he labelling of the sides of the lamina was unclear. We have therefore, added labels to indicate the buccal side, which is clearly visible. Apologies that this was not clear in the first version.

In figure5, why did they use the E12.5 stage for the BrdU assay? Please explain.

This is now explained. For proliferation changes our rationale was to look before the phenotype was generated. Therefore, proliferation was analysed at E12.5 before extension of the VL. The phenotype is therefore a consequence of the proliferation changes rather than being a subsequent change.

High magnification for co-localization should be needed—any difference between WT and KO.

Higher magnification is now added.

The authors should label the lingual buccal direction with an arrow at every figure. And they show lingual buccal direction with consistency.

We have added labels to show the lingual-buccal/labial direction in each figure. We have additionally added schematics in Figure 2 to show the different planes of section used, to show the labial and buccal parts of the VL. As the VL is a 3D epithelial sheet of cells we feel it is important to show a couple of orientations to indicate gene expression all around the lamina. This also helps analysis using Genepaint, where the images are all in sagittal plane.

The authors showed only Wnt1-cre. How about K14-Cre (loss of Gas1 in the epithelium)?

Unfortunately, we do not have the Gas1 floxed line and Wnt1creGas1fl fixed embryos were provided by our collaborator in the USA. We are thus not able to make the K14cre conditionals but we agree this would be very interesting as an extension of the project.

In figure 7, with cyclopamine, VL lengths were not changed after two days of in vitro culture. However, they showed almost two times a difference between the control and Cyclopamine groups.

Green and black dotted lines were not visible.

Yes, the VL lengths were changed. This is clear in the supplementary figures and Figure 7. We have now included additional graphs, comparing the total length and the increase in length from Day 0. As slices can differ we feel that the increase in length (i.e. length at day 2 minus length at Day 0) is a better measure but we have now also included total length of the VL. Both are significantly different.
The lines have been made clearer.

They concluded the cilia relationship in VL with only SAG experiment. The authors exaggerated their conclusions and put them in a diagram. The conclusion is overstated, and more experiments with cilia are needed. Diagram also should be changed. Too exaggerated.

The schematic in Figure 8A-C is based purely on published data and shows the published understanding of Hh signalling. We added the primary cilia on the diagram as this is an integral part of the Hh pathway and allowed us to show the link between VL defects after changes to the Hh pathway and in ciliopathies. We are not suggesting that our results tell us anything about cilia, but they do explain why people with ciliopathies have frenulum defects. We feel this is an important finding from the paper.
We have made it clear in the figure legend that 8A-C are based on published understanding of the Hh pathway.

Keywords should be changed. Cilia is too exaggerated. Even VL has no mention in the keyword.

We have changed the keywords and added in VL. We have changed cilia to ciliopathies, as VL defects are very closely associated with ciliopathies.

The title should be changed. Too long.

We agree, the title has been shortened with more of an emphasis on the first aim, to create a molecular signature of the developing VL.

The authors should provide more information about the figures in the figure legend. Too lacking.

The figure legends have been rewritten.

For the materials and methods, the authors should give the information in detail about the company, country, city, and catalogue numbers…..

These have been added where appropriate.

And detailed primer information for the probe should be needed.

Probe information is now added, apologies for the omission.

The discussion part should be vigorously changed. The authors should discuss the current study and compare it with the previous research. What is the significance of their findings? It is necessary to compare and discuss the differences from previous studies and what novelty is. How much improvement here compared to the earlier results or conclusions?

We have added sub-headings to the discussion and added some more points. No previous molecular analysis of the VL has been caried out so there is little to compare to and all the findings presented here are novel.

Reviewer 2 Advance Summary and Potential Significance to Field:
In the manuscript entitled, “Hedgehog signalling from the developing tooth directs the development of the adjacent vestibular lamina, coordinating tooth and vestibule formation”, Qiu et

al. present evidence that HH signaling plays a role in the development of the vestibular lamina in mice. Overall, the work will be of interest to the readership of Development, and the data are generally of high quality. However, as detailed below, there are a number of issues ranging from readability of figures to statistical considerations and a lack of key controls for some experiments that need to be addressed prior to further consideration of this manuscript.

Reviewer 2 Comments for the Author:

Major Comments:

1)For the bulk RNA-sequencing presented in Figure 1, it is unclear how the authors controlled for potentially different relative amounts of epithelium and mesenchyme between the two dissected structures (i.e., the VL and TB).

As an example, if the dissected VL simply contains relatively more epithelial cells than the TB, then epithelial-expressed genes would appear as enriched (and vice versa, if there were greater mesenchyme). Have the authors examined this possibility? If so, it would be useful to include some analysis to assure the reader that these samples have similar numbers of epithelial and mesenchymal cells, which would increase confidence that the differentially expressed genes are due to fundamental differences in the molecular signatures, as proposed by the authors.

Thanks to the reviewer for highlighting this important point. We did indeed compare relative expression levels of epithelial and mesenchymal markers (Ecadherin and vimentin respectively) in the VL and TB samples. The level of vimentin as a readout of mesenchyme was very similar, and although the VL samples appeared to have more epithelium than the TB samples, this was not significant. This data has been added to Supplementary Figure 1E,F, and is now referred to in the text. We are therefore confident that the types of tissue included in our comparison were equivalent.

2)The observation that the VL is similarly truncated in Gas1+/- and Gas1-/- embryos is quite interesting (Figure 3A-C). The authors should label the VL in the images and provide quantitation of the degree of truncation across WT, Gas1+/-, and Gas1-/- embryos.

This was a great idea. We have now quantified the differences at E13.5 and show that the truncation is statistically significant in the mutants (see new Figure 4). The VL is now labelled.

3)In Figure 3D-G, the authors need to include a Gas1-/- E15.5 control for comparison with the Gas1-/-;Boc+/- and Gas1-/-;Boc-/- embryos. Similar to the above comment, the authors should quantify the degree of truncation across the different genotypes.

We have replaced and added new images to Figure 3 to make this comparison clearer. We were not able to quantify the truncation across all the genotypes as the BOC embryos were generated by our collaborator and we do not have three samples at a single stage for all combinations. For Boc -/-, the VL is normal at all stages N = 4 but we don't have three embryos at a single stage. For the Gas1-/-;Boc+/- we have enough samples and have added a quantification to show that the phenotype mimics that of the Gas1 -/- (Figure 4). For the Gas1-/-;Boc-/- mutants, we do not have enough samples at individual stages but we show results now from two stages where the phenotype is very clear (Figure 3). Embryo numbers are now more clearly shown in the main results section and in the methods.

4)In Figure 4, the authors should examine HH target gene expression in Gas1+/- embryos, especially since loss of one copy of Gas1 is sufficient to yield a truncated VL.

Thanks for this suggestion. The *Gas1* het phenotype is variable and so we now show that in embryos with no truncation the *Gli1* expression is normal, while in those with a truncation the restriction of *Gli1* to the labial/buccal side of the lamina is lost. This is now part of a revised Figure 4.

5)The data in Figure 6 are quite exciting. However, a key control, Wnt1Cre, is missing. This is of particular importance since Wnt1Cre animals are known to display developmental phenotypes (PMID: 23648512). It would be of further interest to assess Wnt1Cre;Gas1fl/+ animals. Presumably

these animals also result in a truncated VL? And, again, quantitation of the VL truncation phenotype is absent.

Apologies for not including this. We now show that the Wnt1cre control has a normal VL, agreeing with the finding that these mice do not have craniofacial defects (see revised Figure 6). We have now quantified the change to show it is significant. We agree that the Wnt1creGas1f/+ would be an interesting addition but we only had one embryo with this genotype and it did not have a truncated VL. However, as some Gas1 Hets do not have a phenotype we would need to look at many more mutants to be able to provide any insight into whether these mice had a phenotype or not.

6)The concentration of cyclopamine used in Figure 7 is extremely high and known to cause off-target effects (PMID: 18754008). It is also odd that the authors use Gli1 as a readout in Figure S5 and Ptch1 as a readout in Figure S6.
While it is not possible to directly compare, it seems that Ptch1 expression is much lower in the control in Figure S6 compared to Gli1 expression in Figure S5. I would recommend using the same target in both experiments to increase confidence that SAG and cyclopamine are working as expected.

We initially started with 20uM cyclopamine, as concentration previously used in slice cultures (PMID: 26755699), but we did not observe an effect on the VL or TB. This data is now added. We then moved to 50uM. We have added expression of Ecadherin (Cdh1) to show that the cells are still viable at this concentration after 2 days in culture. The study mentioned by the reviewer used cell lines, where the penetrance of small molecules is rather different from explant cultures so concentrations are not comparable.
We now use *Gli1* and *Ptc1* as readouts of both the cylopamine and SAG experiments. Thanks for requesting this.

7)I would recommend that the authors address the severity of the VL truncation phenotype observed in Gas1+/- mutants in the discussion (perhaps replacing the Evc section with this discussion). Especially, if the levels of HH pathway activity are not reduced in the Gas1+/- embryos. Perhaps this suggest a HH-independent role for Gas1 in VL development?
This has now been added to the discussion. We have kept the EvC section as we feel the results from the paper clearly explain why EvC and other ciliopathies have vestibule defects. We think this would be interesting to those who work with ciliopathies.
Minor Comments:

1)In the abstract, I believe it should be multiple frenula, not frenulum.
Good point, this has been changed.

2)The text boxes in the volcano plot in Figure 1C are not readable.

Higher resolution used and larger font.

3)In Figure 1D, the authors should be explicit whether the heat maps represent four distinct E14 embryos. Also, it is unclear why the labeling is different (e.g., 17 TB vs. 18 VL). Did the TB and VL samples come from the same embryos?

Apologies for the confusion. The labels represent the numbers from the original dissections. So tooth bud dissections from litter 21 is TB21. Slices were generated from embryos and the tooth bud and VL dissected. VL and TBs were separately pooled from a litter to generate enough tissue. In total we dissected tissue from 11 litters at E14. After dissection and extraction, we checked all the pooled samples for quality of RNA and picked the 5 litters with the best quality scores. These are now referred to as TB 1-5 etc for clarity and the process is written in the methods. As we picked the best litters the TB and VL are not necessarily from the same embryos.

4)Again, the labels in Supplemental Figure 1A-C and Supplemental Figure 2 are not readable.

Corrected

5)The authors should indicate the genetic backgrounds of the animals used in this study.

Added to methods

6)The authors should explicitly indicate when they are using paired and un-paired t-tests.

Apologies, we used unpaired T-tests throughout. This has been changed in the methods.

7)In Figure 4 G,H, Figure 5K, and Figure 7 C, F, the authors need to include individual data points in their histograms.

Added

8)In Figure 4 G,H, the authors should indicate what units are represented on the Y axes.

Added

9)I would recommend adding labels to Figure S4. It is also not clear why there is a Figure S4(1) and S4(2). Just make a single Figure S4.

The supplementary figures have been rearranged and split into separate figures.

Second decision letter

MS ID#: DEVELOP/2022/201464

MS TITLE: Molecular profiling of the vestibular lamina highlights a key role for Hedgehog signalling

AUTHORS: Tengyang qiu, Bara Huteckova, Maisa H-M Seppala, Martyn Timothy Cobourne, Zhi Chen, Maria Hovorakova, Marcela Buchtova, and Abigail S Tucker

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 very positive and we would like to publish a revised manuscript in Development. However Reviewer1 has a few very minor comments regarding minor corrections, so I have returned the manuscript to enable you to make these very final small changes. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. Your manuscript will not require any further review rather I will accept it as soon as the final version has been uploaded.

Reviewer 1

Advance summary and potential significance to field

The authoirs should modify the main manuscript with major changes for better understanding.

Comments for the author

Manuscript Number: DEVELOP/2022/201464

Full Title: Molecular profiling of the vestibular lamina highlights a key role in Hedgehog signalling

All of what I critiqued in the last review have also been made. However, a few things need to change.

1. In figure 3, VL length quantification is required for better understanding.

2. In figure2, please label the arrowheads or arrows where the author wants to show the expression.
3. In Figure 2K, please label Meckel's cartilage.
4. What is Fig3L'?
5. In figure2, please change the location of figure numbers "A", and "B" to the left-upper side of the figures instead of the right.
6. In figure5, please match the magnification size to Figures 5A, B, and other immunostaining figures, unless there is a specific histological explanation.
7. For SM figure (2, 3, 5) graphs, the font size should be more significant for better readability.
8. In SM Fig2H', please change the better quality.

Reviewer 2

Advance summary and potential significance to field

The authors have made substantive changes to the text and figures, including the addition of new data that support their conclusions. Overall, I find the revised manuscript to be significantly improved and support the publication of this work.

Comments for the author

As noted above, the authors have been very responsive to the initial set of reviewer comments. The subsequent revisions have resulted in a greatly improved manuscript that will be of interest to developmental biologists, oral biologists and investigators studying the HH signaling pathway.

Second revision

Author response to reviewers' comments

1. In figure 3, VL length quantification is required for better understanding.

The length of the VL is quantified in Figure 4, where Gas1 het, hom and Gas1/Boc double mutants are compared in Graph 4G.
It was not possible to quantify the VL in BOC single mutants as we did not have enough specimens at the same stage.

2. In figure2, please label the arrowheads or arrows where the author wants to show the expression.

Additional arrows have been added in Figure 2 to highlight the structures with expression.

3. In Figure 2K, please label Meckel's cartilage.

Label has been added in 2K

4. What is Fig3L'?

Sorry this was a mistake in the text, it should be S3L.

5. In figure2, please change the location of figure numbers "A", and "B" to the left-upper side of the figures instead of the right.

This has been corrected.

6. In figure5, please match the magnification size to Figures 5A, B, and other immunostaining figures, unless there is a specific histological explanation.

In Figure 5A and B we intentionally show a lower power view to orientate the reader, before focusing on the zoomed in VL and DL.

7. For SM figure (2, 3, 5) graphs, the font size should be more significant for better readability.

We have made the font size larger in these figures.

8. In SM Fig2H', please change the better quality.

We have changed the contrast of this image as it was a little overexposed. It is now clearer.

Third decision letter

MS ID#: DEVELOP/2022/201464

MS TITLE: Molecular profiling of the vestibular lamina highlights a key role for Hedgehog signalling

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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.