Canonical Wnt signaling regulates soft palate development through mediating ciliary homeostasis

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MS TITLE: Canonical Wnt signaling regulates soft palate development through mediating ciliary homeostasis

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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. 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 manuscript reports studies of the function of beta-catenin mediated Wnt signaling in soft palate development. Disruption of soft palate development is a common birth defect but the molecular mechanisms controlling soft palate development are not well understood. In this study, the authors analyzed mouse embryos with tissue-specific inactivation of beta-catinin in the cranial neural crest derived palate mesenchyme using the Osr2-Cre driver. They show that the mutant embryos had significant reduction in palatal mesenchyme cell proliferation at E13.5, both by percentage of pH3-positive mitotic cells and by percentage of pH3-positive cells in a 2-hour BrdU chased cells, which suggested a defect in cell cycle progression from S- to M-phase. Using RNA-seq analysis, the authors found that the beta-catenin cko soft palate had increased expression of ciliogenesis associated genes including Ttll3, Ttll6, and Ift88. Bioinformatic analysis of the Ttll3 promoter DNA sequence identified a Tcf7/Tcf7l2 binding site, and CUT&RUN-qPCR analysis revealed enrichment of Tcf7l2 binding at the Ttll3 promoter. They performed primary soft palatal mesenchyme cell culture and showed that siRNA knockdown of Tcf7l2 caused significant increase in Ttll3 mRNA expression. They further showed that siRNA knockdown of Ttll3 increased proliferation index of both control and mutant soft palatal mesenchyme cells. They conclude the beta-catenin/Tcf7l2 normally regulates palatal mesenchyme cell proliferation by repressing Ttll3 expression and that increased Ttll3 in the mutant palatal mesenchyme caused stabilization of primary cilia and prevented cell cycle progression. The authors hypothesized that primary cilia in palatal mesenchyme cells regulate soft palate myogenesis by regulating expression of one of the Notch ligands Dlk1, one of the top enriched molecules in the perimysial cells in the scRNA-seq dataset. They showed that ttll3 siRNA knockdown in the cultured palatal mesenchyme cells caused increased expressionof Dlk1. They conclude that Wnt/beta-catenin signaling regulates soft palate development, including palatal mesenchyme cell proliferation and perimysial cell differentiation, through directly regulating ciliary homeostasis. Overall, the hypothesis that Wnt signaling regulates soft palate development through mediating ciliary homeostasis is interesting, but the experimental evidence supporting this hypothesis were mostly indirect and the manuscript left out important detailed information in both the Results and Methods sections.

Comments for the author

1. A major weakness of the manuscript is lack of direct experimental evidence that the increased expression of Ttll3 is a major cause of the cell proliferation and myogenesis defects in the beta-catenin cko palate.Wnt signaling is well known to regulate cell proliferation and the cell cycle through several other target genes. The increase in the percentage of ciliated cells in the beta-catenin cko mutant embryos could be a downstream effect, rather than the cause, of cell cycle disruption. Although the manuscript presented data that Ttll3 siRNA knockdown increased cell proliferation in the palatal mesenchyme cultures, the beta-catenin cko cells still showed significantly reduced proliferation than the control cells when comparing both siRNA knockdown samples (Fig. 5K) and these results only demonstrate an in vitro effect of Ttll3 siRNA knockdown. It has been shown that complete lack of tubulin glycylation in Ttll3-/-Ttll8-/- double mutant mice did not affect ciliogenesis and most ciliary function in vivo, with the double mutant mice developing and surviving normally (Gadadhar et al., Science 371:eabd4914, 2021). Since Ttll3-/- mice survive and can breed, a demonstration that Ttll3 loss of function could rescue palatal mesenchyme proliferation and/or other defects in the beta-catenin cko embryos would provide convincing evidence in support of the conclusions in this manuscript.

2. In the beginning of the Results section (Fig. 1), the authors stated that “the palatal shelf development and the number of MyoD positive cells at the level of LVP were comparable between control and mutants at E13.5 (Fig. 1I-J and K-L) and “the difference in the number of Myod1 positive cells started to be apparent in Osr2-Cre;b-catenin-fl/fl mice at E14.0 in comparison to the control (Fig. 1M and O). The frozen sections shown in these Fig. 1 panels are not sufficient to orient the reader regarding the palatal defects in the mutant embryos. It is necessary to show whole mount views of the upper jaw/palatal shelves, with appropriate markers to show the anterior and posterior regions of the palatal shelves, to support the statement. The position where the sections correspond to at the LVP level should be indicated on the whole mount pictures. In addition,

quantitative data for the numbers of MyoD positive LVP precursor cells in the palatal shelves need to be provided.

3. Fig. 2 shows data from BrdU and pH3 detection to indicate defect in palatal mesenchyme cell proliferation in the mutant embryos. Fig. 2I shows that about 15% of total cells were pH3 positive in the control, Fig. 2L shows ~80% of the BrdU-labeled cells were also pH3 positive. How were the cells counted and what areas of the palatal mesenchyme cells were counted? The percentage for pH3 positivity seems unreasonably high. Since the authors use pH3 as a marker of the M phase and 80% of the 2-hour BrdU labeled cells were pH3 positive, the data would suggest that these cells have very short S- and G2- phases and relatively long M-phase. How about also showing the percentage of total cells that were BrdU positive?

4. The CUT&RUN-qPCR method needs to be described in more detail. Fig. 4E shows enrichment of Tcf7l2 at the Ttll3 promoter, from which the authors concluded that "the b-catenin/Tcf7l2 complex functions as a repressor of the ciliary gene Ttll3" (Page 10). Was b-catenin also enriched at the Ttll3 promoter in the palatal mesenchyme? It is well known that Tcfl2 acts as a repressor in the absence of Wnt signaling but upon canonical Wnt signaling activation beta-catenin interacts with Tcf7l2 to activate target gene expression. However, the authors concluded that the beta-catenin/Tcf7l2 complex directly repressed Ttll3 expression. Can the authors provide a reference showing beta-catenin/Tcf7l2 complex directly repressed target gene expression upon Wnt activation? Does Wnt treatment of soft palate mesenchyme cells significantly repress Ttll3 expression or Ttll3 promoter activity?

5. The lack of LVP myogenesis in the beta-catenin cko mice is likely a secondary defect following earlier disruption and arrest of the posterior palatal shelves. The manuscript shows decreased expression of Dlk1 in the mutant palatal mesenchyme and increase of Dlk1 expression in Ttll3 siRNA-treated palatal mesenchyme cells in culture, but there is no evidence that Dlk1 is required for LVP myogenesis. These data are insufficient to reach the conclusion that "impaired ciliary disassembly leads to differentiation defects of mesenchymal cells and indirectly disrupts myogenesis through decreased expression of Dlk1" (abstract). Without experimental evidence demonstrating direct and specific function of some downstream target genes in LVP myogenesis, the defect in soft palate myogenesis in the beta-catenin cko mutants is better regarded as a secondary consequence of earlier disruption of development of the palatal mesenchyme.

6. Fig. 3Q shows a large number of concentrated Ttll3 signals in the lower center area that does not correlate with areas of reduced Tcf7l2 signals in Fig. 4H. Are the signals in Fig. 3Q real and representative or there was too much background noise in this panel?

Minor points:
- The first paragraph in the Results section (Page 6) does not belong in this section.
- The methods section mentioned qPCR validation of Ttll3, Tcf7l2, and Dlk1 mRNA expression, but the data were not included in the manuscript.

Reviewer 2

Advance summary and potential significance to field

The authors use a variety of multi-omic approaches to show that b-catenin disruption leads to a failure of cilia to disassemble, resulting in cell cycle changes with myogenic cells that will form the LVP in anterior pharynx (soft palate). These changes appear to result from aberrant changes to Tcf activity. Thus, Wnt signaling drives soft palate morphogenesis through the regulation of ciliogenesis; it remains to be seen if this is an event behind other ciliopathies, though it is intriguing to think that this mechanisms may be responsible for multiple developmental disease. It is equally exciting to ponder the possibility that posterior palate fusion changes may be addressable through modulating b-catenin signaling.

Comments for the author

In this manuscript, the authors illustrate that conditional loss of b-catenin in the NCC-derived facial mesenchyme in the area of soft palate development disrupts muscle proliferation and likely

subsequent differentiation. The authors use a variety of multi-omic approaches to show that b-catenin disruption leads to a failure of cilia to disassemble, resulting in cell cycle changes. These changes appear to result from Tcf activity. The authors show that the proliferation defect can be rescued in vitro by knocking down Tcf expression. Taken together, the findings illustrate how Wnt signaling can drive soft palate morphogenesis through the regulation of ciliogenesis and that some posterior palate defects may be addressable through treatments that reduce Tcf activity or boost Tcf-repressed genes. Overall, the manuscript is quite concise (sometimes too much so) and well-written.

1. In Figure S1, the authors state that antibody staining illustrated that the active form of b-catenin was absent from the CNC-derived mesenchymal cells while persisting in the epithelial cells. Based on tdTomato fluorescence, the authors should offer a more conservative estimate for the reduction in b-catenin activity in the CNC-derived cells. Figure S1H illustrates that there is still active b-catenin in the CNC derived mesenchyme, though it is substantially reduced in the area in which Osr2-Cre is active. It is unlikely that any Cre can reduce a targeted gene to zero. If the authors want to stick with their statement, they should perform a more nuanced experiment in which the fluorescence is quantified. I do not think this is necessary if the authors more cautiously interpret their findings.

2. I am not clear why Figs. S1 and S2 are not made into a single figure and added as the new Figure 1. The authors only have 6 figures and this addition would strengthen the manuscript. I also think that Fig. S3A should be in the manuscript as part of a final cartoon figure.

3. In Figure 1, the authors state that the change in number of Myod1 positive cells became apparent at E14.0, which looks correct. Is there also a failure of myogenic cells to migrate medially due to the proliferation failure (or perhaps better put, can myogenic cells with reduced proliferation still migrate?). The presented data suggest that the cells do not differentiate in myofibrils that extend medially. Would disruption of b-catenin in this area at a later time point disrupt migration post-MyoD positive cell proliferation? These are hard questions to address, though are important in understanding why the LVP does not properly form.

4. In Figure 2, the authors state that there is decreased proliferation of cells in conditional knockout mice. However, it appears that the overall number of pH3 cells is unchanged, though there are fewer in the Osr2 domain. In contrast, there appear to be more medial pH3 positive cells in cko mice as compared to control embryos. The authors should denote in which zone they are counting proliferating cells. As an aside, why are there more MyoD cells in the cko mice? Are they mislocalized cells? Does loss of b-catenin cause the cells to aberrantly locate in the tissue?

5. Please include 1-2 sentences in the Results that states what the SCENIC plot is illustrating.

Reviewer 3

Advance summary and potential significance to field

1. Wnt/β-catenin signaling in the embryonic palatal mesenchymal cells regulates myogenesis cell non-autnomously.
2. Wnt/β-catenin signaling in the embryonic palatal mesenchymal cells may directly inhibit ciliogenesis.

Comments for the author

The study identify the role of beta-catenin in regulating proliferation of soft palatal mesenchymal cells via regulating ciliogenesis. The manuscript is well written and the data are of high quality. The conclusions are potentially interesting if the authors can further clarify some issues.

Major comments:

1. While the data about increased number of ciliated cells and reduced proliferation of soft palatal mesenchymal cells in Osr2-Cre;β-catenin fl/fl embryo at E13.5 were solid (Fig. 2), stating Wnt/β-

catenin signaling acts through Tcf7l2 to directly repress Ttll3 expression lacks convincing support of appropriate experiments. The authors stated without any citation that “Binding of β-catenin to these transcription factors in the nucleus further enables activation or repression of Wnt responsive genes”. It is known that Tcf and Lef1 are transcription repressors without β-catenin binding, rising β-catenin levels switch inhibitory TCF7/Tcfl or Lef1 complexes to activating complexes to activate gene expression. I did not see any demonstration/indication of the inhibitory role of β-catenin in Ttll3 expression. The cultured palatal mesenchymal cells are likely in a state of low β-catenin or no β-catenin stimulation, in which Tcfl2 can play an inhibitory role.

2. The authors showed clearly that the proliferation defect was in the palatal mesenchyme. They hypothesized with limited data that myogenic reduction was due to the problems in the palatal mesenchyme. The conclusion of Dlk1 mediates Wnt/β-catenin signaling in the palatal mesenchyme in myogenesis can be further potentiated.

First revision

Author response to reviewers' comments

Response to Reviewers’ comments

We appreciate the reviewers’ suggested changes and recommendations for our manuscript. We have added additional experiments and explanations to address all of reviewers’ comments. Please see below our point-by-point responses to all the comments.

Reviewer 1 Advance Summary and Potential Significance to Field:

This manuscript reports studies of the function of beta-catenin mediated Wnt signaling in soft palate development. Disruption of soft palate development is a common birth defect but the molecular mechanisms controlling soft palate development are not well understood. In this study, the authors analyzed mouse embryos with tissue-specific inactivation of beta-catinin in the cranial neural crest derived palate mesenchyme using the Osr2-Cre driver. They show that the mutant embryos had significant reduction in palatal mesenchyme cell proliferation at E13.5, both by percentage of pH3-positive mitotic cells and by percentage of pH3-positive cells in a 2-hour BrdU chased cells, which suggested a defect in cell cycle progression from S- to M-phase. Using RNA-seq analysis, the authors found that the beta-catenin cko soft palate had increased expression of ciliogenesis associated genes including Ttll3, Ttll6, and Ift88. Bioinformatic analysis of the Ttll3 promoter DNA sequence identified a Tcf7/Tcf7l2 binding site, and CUT&RUN-qPCR analysis revealed enrichment of Tcf7l2 binding at the Ttll3 promoter. They performed primary soft palatal mesenchyme cell culture and showed that siRNA knockdown of Tcf7l2 caused significant increase in Ttll3 mRNA expression. They further showed that siRNA knockdown of Ttll3 increased proliferation index of both control and mutant soft palatal mesenchyme cells. They conclude the beta-catenin/Tcf7l2 normally regulates palatal mesenchyme cell proliferation by repressing Ttll3 expression and that increased Ttll3 in the mutant palatal mesenchyme caused stabilization of primary cilia and prevented cell cycle progression. The authors hypothesized that primary cilia in palatal mesenchyme cells regulate soft palate myogenesis by regulating expression of one of the Notch ligands Dlk1, one of the top enriched molecules in the perimysial cells in the scRNA-seq dataset. They showed that ttll3 siRNA knockdown in the cultured palatal mesenchyme cells caused increased expression of Dlk1. They conclude that Wnt/beta-catenin signaling regulates soft palate development, including palatal mesenchyme cell proliferation and perimysial cell differentiation, through directly regulating ciliary homeostasis. Overall, the hypothesis that Wnt signaling regulates soft palate development through mediating ciliary homeostasis is interesting, but the experimental evidence supporting this hypothesis were mostly indirect and the manuscript left out important detailed information in both the Results and Methods sections.

Reviewer 1 Comments for the Author:

1.A major weakness of the manuscript is lack of direct experimental evidence that the increased expression of Ttll3 is a major cause of the cell proliferation and myogenesis defects in the beta-catenin cko palate. Wnt signaling is well known to regulate cell proliferation and the cell cycle through several other target genes. The increase in the percentage of ciliated cells in the beta-catenin cko mutant embryos could be a downstream effect, rather than the cause, of cell cycle disruption. Although the manuscript presented data that Ttll3 siRNA knockdown increased cell proliferation in the palatal mesenchyme cultures, the beta-catenin cko cells still showed significantly reduced proliferation than the control cells when comparing both siRNA knockdown samples (Fig. 5K) and these results only demonstrate an in vitro effect of Ttll3 siRNA knockdown. It has been shown that complete lack of tubulin glycylation in Ttll3-/-Ttll8-/- double mutant mice did not affect ciliogenesis and most ciliary function in vivo, with the double mutant mice developing and surviving normally (Gadadhar et al., Science 371:eabd4914, 2021). Since Ttll3-/- mice survive and can breed, a demonstration that Ttll3 loss of function could rescue palatal mesenchyme proliferation and/or other defects in the beta-catenin cko embryos would provide convincing evidence in support of the conclusions in this manuscript.

We appreciate the suggestion to test whether restoring *Ttll3* expression can rescue soft palatal defects in the *Osr2-Cre;β-catenin$^{fl/fl}$* embryos. Since we do not have access to the *Ttll3$^{-/-}$* mouse model, alternatively, we used an explant culture system which we further optimized in our lab (Feng et al., 2022; Ruigrok et al, 2017; Ruigrok et al, 2018) to culture 300 µm thick embryonic slices of the soft palatal region. This method enabled local siRNA injection while the three-dimensional structure of the culture region was maintained. More specifically, we injected control or *Ttll3* siRNA locally into the soft palatal mesenchyme surrounding muscle progenitors in the LVP region from *Osr2-Cre;β-catenin$^{fl/fl}$* embryonic heads. We found that *Ttll3* siRNA treatment restored the level of *Ttll3* and increased the number of MyoD+ cells in *Osr2-Cre;β-catenin$^{fl/fl}$* samples, compared to slices treated with the control siRNA (Fig. 6L-P).

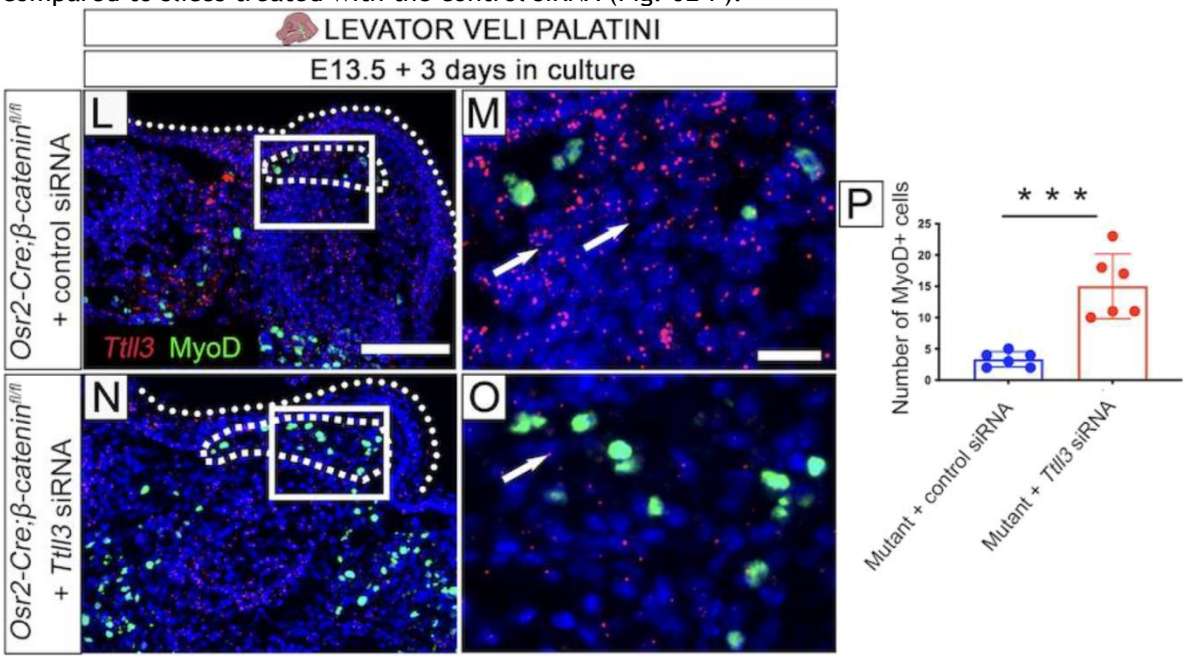


Fig. 6. Dysfunctional ciliary disassembly restrains cell cycle progression of soft palatal cells in *Osr2-Cre;β-catenin$^{fl/fl}$* mice. (L-O) *Ttll3* (red) *in situ* RNAScope hybridization and MyoD (green) immunofluorescence staining of *Osr2-Cre;β-catenin$^{fl/fl}$* samples injected with control (L-M) or *Ttll3* siRNA (N-O). (P) Quantification of the numbers of MyoD+ cells in *Osr2-Cre;β-catenin$^{fl/fl}$* (mutant) samples injected with control or *Ttll3* siRNA. ***p-value = 0.0003. N = 6. For P statistical significance was evaluated by unpaired t-test with two-tailed calculations. Data is presented as mean ± s.e.m. Schematic drawing of the mouse head in the top panel above the figures L-O depicts the position and angle of sectioning. White boxes in L and N show approximate locations of higher magnification images in M and O, respectively. White dotted lines in L and N indicate palatal

shelves. White dashed lines outline the LVP muscle region stained with MyoD in L and N. Scale bar in L indicates 100 μm in L and N; scale bar in M indicates 20 μm in M and O.

2. In the beginning of the Results section (Fig. 1), the authors stated that "the palatal shelf development and the number of MyoD positive cells at the level of LVP were comparable between control and mutants at E13.5 (Fig. 1I-J and K-L) and "the difference in the number of Myod1 positive cells started to be apparent in Osr2-Cre;b-catenin-fl/fl mice at E14.0 in comparison to the control (Fig. 1M and O). The frozen sections shown in these Fig. 1 panels are not sufficient to orient the reader regarding the palatal defects in the mutant embryos. It is necessary to show whole mount views of the upper jaw/palatal shelves, with appropriate markers to show the anterior and posterior regions of the palatal shelves, to support the statement. The position where the sections correspond to at the LVP level should be indicated on the whole mount pictures. In addition, quantitative data for the numbers of MyoD positive LVP precursor cells in the palatal shelves need to be provided.

We appreciate this suggestion to clarify how the sections correspond to the region of LVP and provide quantitative data for the numbers of MyoD+ LVP precursor cells to highlight the muscle phenotype in *Osr2-Cre;β-catenin*$^{fl/fl}$ mice. We have added the below data:

1. To clarify the position and orientation of the provided sections, we added a whole mount view of the upper jaws of control and *Osr2-Cre;β-catenin*$^{fl/fl}$ mice at E18.5 as a representative stage, as suggested (Fig. 2A and C). Moreover, we are providing sagittal views of soft tissue CT scans for both control and *Osr2-Cre;β-catenin*$^{fl/fl}$ mice (Fig. 2B and D). We also use orange lines in these figures to mark the position of the frontal views visualized in the corresponding frozen sections, indicating the region of LVP.

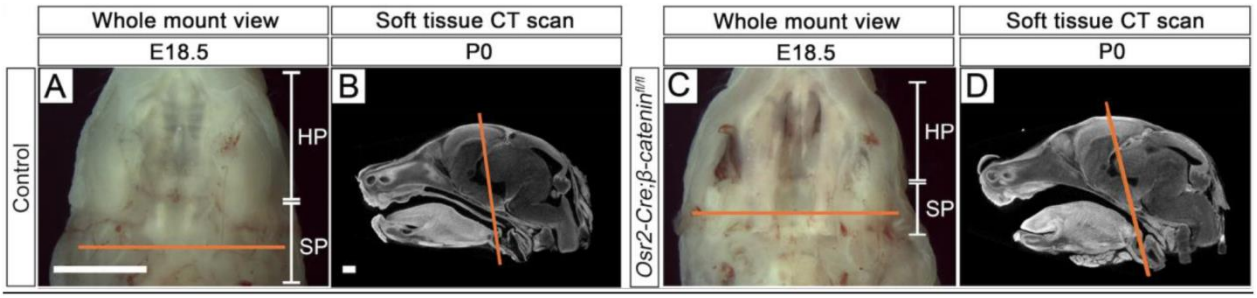


Fig. 2. Wnt signaling from CNC-derived mesenchymal cells is essential for guiding myogenesis through tissue-tissue interactions. (A, C) Intraoral whole mount view of the maxilla from control (A) and*Osr2-Cre;β-catenin*$^{fl/fl}$ mice (C) at E18.5. (B, D) Soft tissue CT scans at P0 of control (B) and *Osr2-Cre;β-catenin*$^{fl/fl}$ mice (D). Orange lines in A-D indicate positions of frontal views in E-T.

2. We added quantification analysis of MyoD+ LVP precursors at E13.5, E14.0 and E14.5 in control and *Osr2-Cre;β-catenin*$^{fl/fl}$ samples in Fig. 2U-W. We have confirmed there was no difference in the numbers of MyoD+ cells between control and *Osr2-Cre;β-catenin*$^{fl/fl}$ mice at E13.5 (Fig. 2U); however, the numbers of MyoD+ cells in *Osr2-Cre;β-catenin*$^{fl/fl}$ mice at E14.0 and E14.5 were significantly reduced in comparison to the control littermates (Fig. 2V and W).

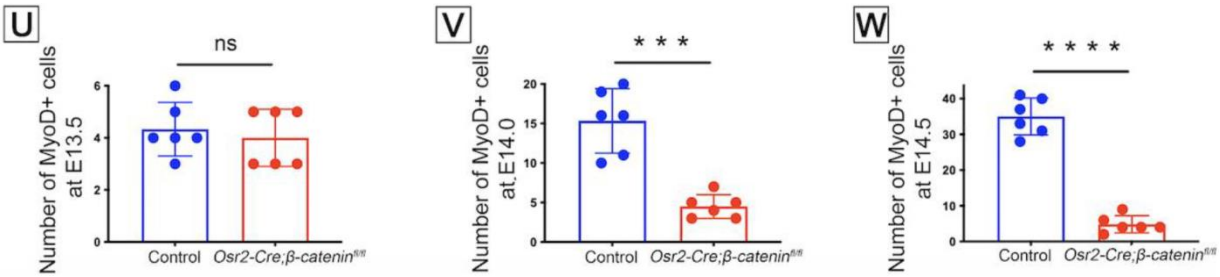


Fig. 2. Wnt signaling from CNC-derived mesenchymal cells is essential for guiding myogenesis through tissue-tissue interactions. (U-W) Quantification of the number of MyoD+ cells at E13.5 (U) ns = not significant, E14.0 (V) ***p-value = 0.0001, and E14.5 (W) ****p-value <0.0001. N = 6 for U-W.

Statistical significance was evaluated by unpaired t-test with two-tailed calculations. Data is presented as mean ± s.e.m.

3. Fig. 2 shows data from BrdU and pH3 detection to indicate defect in palatal mesenchyme cell proliferation in the mutant embryos. Fig. 2I shows that about 15% of total cells were pH3 positive in the control, Fig. 2L shows ~80% of the BrdU-labeled cells were also pH3 positive. How were the cells counted and what areas of the palatal mesenchyme cells were counted? The percentage for pH3 positivity seems unreasonably high. Since the authors use pH3 as a marker of the M phase and 80% of the 2-hour BrdU labeled cells were pH3 positive, the data would suggest that these cells have very short S- and G2- phases and relatively long M-phase. How about also showing the percentage of total cells that were BrdU positive?

We thank the reviewer for these comments. We added a more detailed description to clarify the quantification methods for these ratios below:

The graph provided in original Fig. 2I (now numbered Fig. 3I in the revised manuscript) shows the percentage of pH3+ cells out of all the soft palatal mesenchymal cells. Original Fig. 2L (now numbered Fig. 3L) shows the percentage of BrdU+/pH3+ cells out of the BrdU+ cells.
The soft palatal mesenchymal region was used for this quantification (excluding epithelium). This region is outlined in original Figure 2 (now numbered Figure 3).

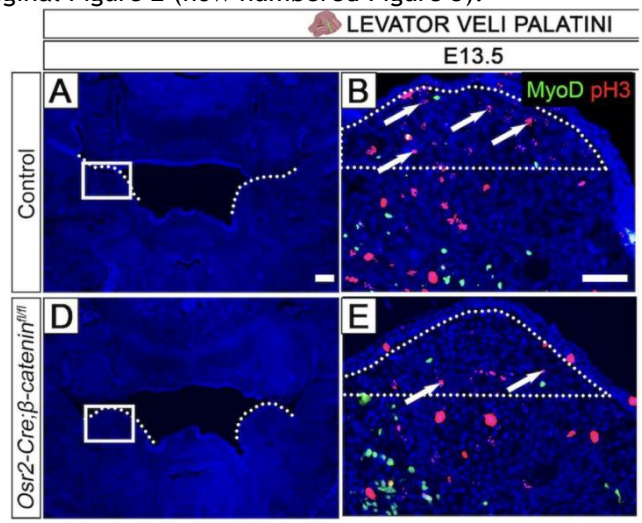
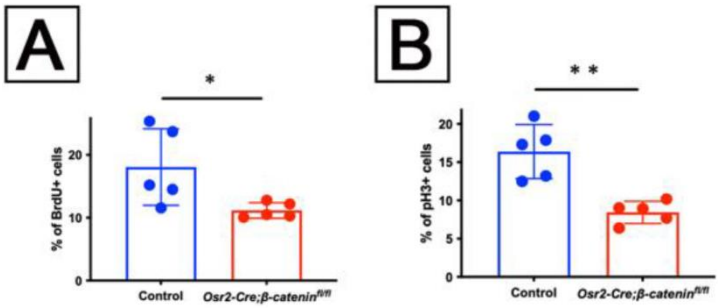


Fig. 3. *Osr2-Cre;β-catenin*$^{fl/fl}$ mice display decreased proliferation of the mesenchymal cells. (A, D) DAPI staining of control (A) and *Osr2-Cre;β-catenin*$^{fl/fl}$ (D) sections at the level of the LVP at E13.5. (B, E) MyoD (green) and pH3 (red) in control (B) and *Osr2-Cre;β-catenin*$^{fl/fl}$ mice (E). White dotted lines in B and E indicate area of quantification.

As suggested, we also quantified the percentage of BrdU+ cells out of the total palatal mesenchymal cells (Response letter figure 1A). We have also used the same samples to confirm the percentage of pH3+ cells out of the total number of cells in the soft palatal mesenchyme (Response letter figure 1B). These results are consistent with the presented data in revised Fig. 3.



Response letter figure 1. *Osr2-Cre;β-catenin*$^{fl/fl}$ mice display decreased proliferation of mesenchymal cells. (A) Quantification of the percentage of BrdU positive cells out of total palatal

mesenchymal cells. *p-value = 0.0378. N = 5. (B) Quantification of the percentage of pH3 positive cells out of total mesenchymal cells. **p-value = 0.0016. N = 5. Statistical significance was evaluated by unpaired t-test with two-tailed calculations. Data is presented as mean ± s.e.m.

4.The CUT&RUN-qPCR method needs to be described in more detail. Fig. 4E shows enrichment of Tcf7l2 at the Ttll3 promoter, from which the authors concluded that "the b-catenin/Tcf7l2 complex functions as a repressor of the ciliary gene Ttll3" (Page 10). Was b-catenin also enriched at the Ttll3 promoter in the palatal mesenchyme? It is well known that Tcfl2 acts as a repressor in the absence of Wnt signaling but upon canonical Wnt signaling activation beta-catenin interacts with Tcf7l2 to activate target gene expression. However, the authors concluded that the beta-catenin/Tcf7l2 complex directly repressed Ttll3 expression. Can the authors provide a reference showing beta-catenin/Tcf7l2 complex directly repressed target gene expression upon Wnt activation? Does Wnt treatment of soft palate mesenchyme cells significantly repress Ttll3 expression or Ttll3 promoter activity?

We thank the reviewer for the suggestion to perform more experiments supporting the statement that canonical Wnt signaling is influencing the expression of *Ttll3*. We have edited the Results section, added references concerning the repressive role of ß-catenin, and performed additional experiments:

1. As suggested, we added more details regarding the Cut and Run Assay method in the Results section as follows on pages 9-10, lines 215-219:

We identified a motif (chr6113388069-113388082) that enables binding of Tcf7l2 to the *Ttll3* promoter region (Fig. 5D). We utilized a Cut and Run (Cleavage Under Targets and Release Using Nuclease) assay (Skene & Henikoff, 2017) and confirmed direct binding of Tcf7l2 to the predicted region at the *Ttll3* promoter site (Fig. 5E). This data suggested that *Ttll3* is a direct target of canonical Wnt signaling in the soft palate mediated by Tcf7l2.

2. We have added additional references supporting the statement that ß-catenin and or ß-catenin/Tcf complex can directly repress targeted genes (page 9, lines 198-199 and page 14, line 351):
    a. Expression of *Ifit1* and *Ifit2* is negatively regulated by Wnt signaling, specifically by ß-catenin/Tcf7l2 complex (Ohsugi et al, 2017).
    b. ß-catenin can act as a suppressor through the negative regulatory elements in *Xenopus*, mouse, and human systems (Kim et al, 2017).
    c. Knockdown of *Tcf7l2*, Wnt signaling pathway effector, using siRNA results in a significant elevation of *PEPCK* and *G-6-Pase* mRNA levels (Ip et al, 2012)
    d. ß-catenin is associated with both activation and repression of cell-specific gene expression programs, which expands on how Wnt/ß-catenin drives complex cell behaviors (Pagella et al., 2022, bioRxiv).

3. In order to further support our conclusions, and to mimic the *in vivo* conditions in *Osr2-Cre;ß-catenin*$^{fl/fl}$ mice, we used siRNA targeting *ß-catenin* and analyzed the expression of *Ttll3*. Comparable to the *Tcf7l2* siRNA results, we observed an increase of *Ttll3* expression after efficient knockdown of *ß-catenin* (Fig. 5L and M). These results suggest a repressive function of ß-catenin with respect to *Ttll3*. Furthermore, when the cells were treated with a combined *Tcf7l2* and *ß-catenin* siRNA (using a half dose of each), we still observed an increase in *Ttll3* expression (Fig. 5N) comparable to that of full dose of *ß-catenin* siRNA or *Tcf7l2* siRNA alone. This additional set of experiments suggests that ß-catenin and Tcf7l2 repress *Ttll3* expression to regulate soft palate development.

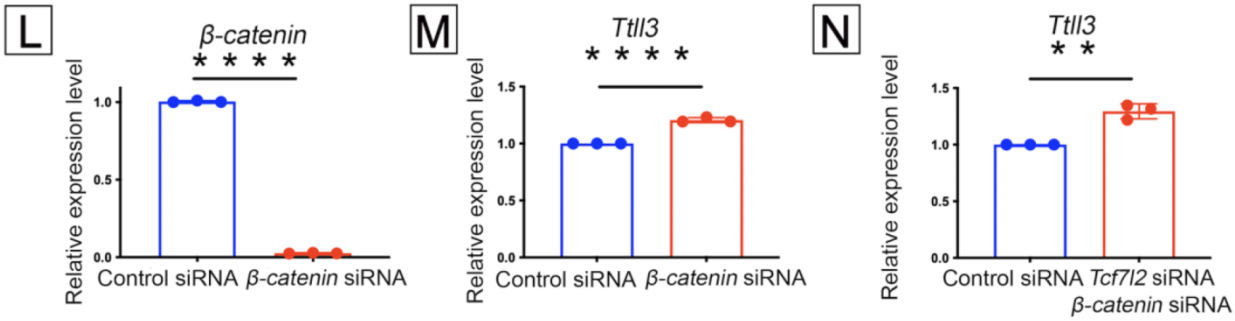


Fig. 5. Wnt signaling directly regulates ciliary genes. (L-N) qPCR analysis. Efficiency of *β-catenin* siRNA. ****p-value <0.0001 (L). Increased expression of *Ttll3* after *β-catenin* siRNA treatment. ****p-value <0.0001 (M). Increased expression of *Ttll3* after *β-catenin* and *Tcf7l2* siRNA treatment. **p-value = 0.0015 (N). For L, M, and N statistical significance was evaluated by unpaired t-test with two-tailed calculations. Data is presented as mean ± s.e.m.

4. Considering that ß-catenin does not bind directly to the DNA, we attempted to use two different ß-catenin antibodies and the same specific primers designed for the Tcf7l2-targeted region of binding to *Ttll3* promoter region in a classical Cut and Run protocol. However, we did not see enrichment on the *Ttll3* promoter using either ß-catenin antibody.While searching the literature, we found that several other scientific groups have attempted to use a classical Cut and Run method for profiling the Wnt/ß-catenin transcriptional complex and gained reliable results for transcription factors such as Lef/Tcf, however they failed to produce consistent binding patterns for ß-catenin, which is a non-DNA-binding factor using the classical Cut and Run protocol (Zambanini et al., 2022). Although Zambanini have very recently introduced a novel biochemical modification of the Cut and Run protocol (published December 1, 2022) which could potentially better identify ß-catenin binding to the promoter of targeted genes, due to the revision time frame, we were unable to test whether this newly published protocol would work for our tissue or not. Thus, due to the technical limitation of the current classical Cut and Run method, we are unable to draw a definitive conclusion as to whether ß-catenin can directly bind to the promoter of *Ttll3*.

5. The lack of LVP myogenesis in the beta-catenin cko mice is likely a secondary defect following earlier disruption and arrest of the posterior palatal shelves. The manuscript shows decreased expression of Dlk1 in the mutant palatal mesenchyme and increase of Dlk1 expression in Ttll3 siRNA-treated palatal mesenchyme cells in culture, but there is no evidence that Dlk1 is required for LVP myogenesis. These data are insufficient to reach the conclusion that "impaired ciliary disassembly leads to differentiation defects of mesenchymal cells and indirectly disrupts myogenesis through decreased expression of Dlk1" (abstract). Without experimental evidence demonstrating direct and specific function of some downstream target genes in LVP myogenesis, the defect in soft palate myogenesis in the beta-catenin cko mutants is better regarded as a secondary consequence of earlier disruption of development of the palatal mesenchyme.

We appreciate this comment focusing on the requirement of *Dlk1* in LVP myogenesis. In order to generate this data, we optimized an *in vitro* embryonic head slice culture system (Feng et al., 2022; Ruigrok et al, 2017; Ruigrok et al, 2018) in combination with local siRNA injection. More specifically, we injected *Dlk1* or control siRNA into the soft palatal mesenchyme of 300 µm thick slices containing the LVP region from control embryos.

We confirmed efficient decrease of *Dlk1* following the injection of *Dlk1* siRNA into the cultured tissue slices (Fig. 7I-L). The number of MyoD+ LVP precursors was lower in the palatal shelves injected with *Dlk1* siRNA (Fig. 7K-M); while the MyoD+ LVP precursors continued to proliferate and differentiate in the soft palatal shelves injected with control siRNA (Fig. 7I-J). This experiment showed that palatal mesenchymal-derived *Dlk1* is functionally required for soft palatal myogenesis of LVP.

To further support our conclusion that "impaired ciliary disassembly leads to differentiation defects of mesenchymal cells and indirectly disrupts myogenesis through decreased expression of *Dlk1*", we have also treated *Osr2-Cre;β-catenin*$^{fl/fl}$ slice cultures with *Ttll3* siRNA injection to restore its expression in *Osr2-Cre;β-catenin*$^{fl/fl}$ samples. Following the *Ttll3* siRNA treatment, we observed restored expression of *Dlk1* (Fig. 7N-Q). This demonstrates that *Ttll3* expression regulates the expression of *Dlk1* in the soft palatal mesenchymal cells and ultimately myogenesis through cell-cell interaction.

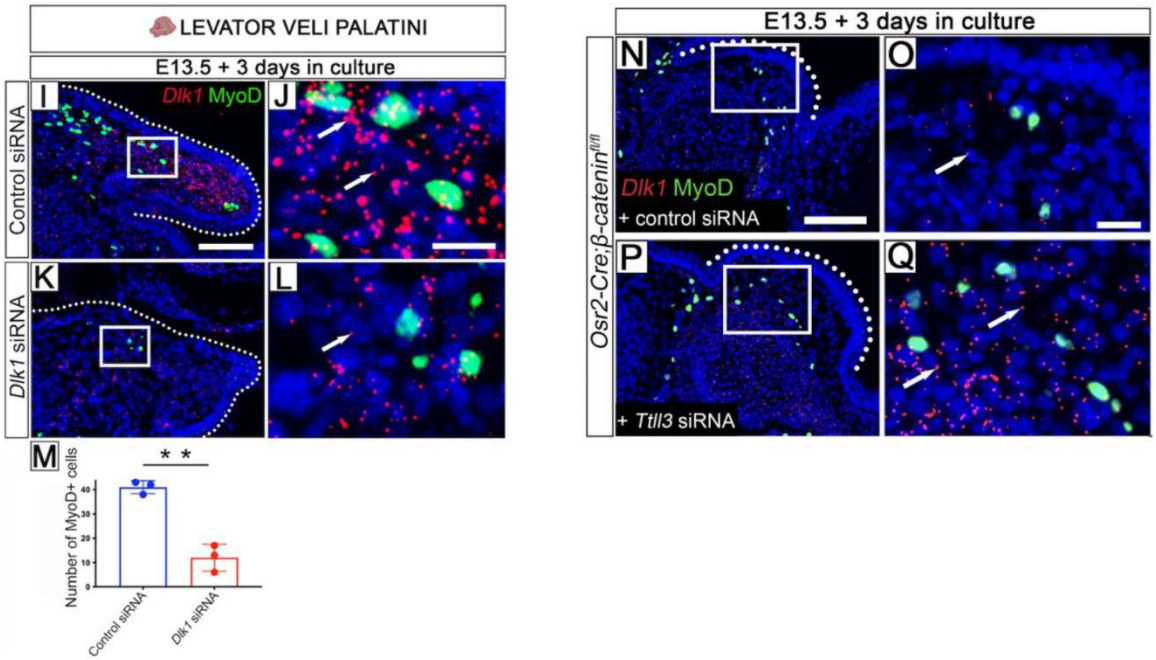


Fig. 7. Primary cilia influence the soft palate muscle development via regulating the perimysial fate of CNC-derived palatal mesenchyme cells, which further disrupts soft palate myogenesis. (I-L) *Dlk1* (red) *in situ* RNAScope hybridization and MyoD (green) immunofluorescence staining of control samples injected with control (I-J) or *Dlk1* siRNA (K-L). (M) Quantification of MyoD+ cells in control samples injected with control or *Dlk1* siRNA. **p-value = 0.0012. For M statistical significance was evaluated by unpaired t-test with two-tailed calculations. Data is presented as mean ± s.e.m. (N-Q) *Dlk1* (red) *in situ* RNAScope hybridization and MyoD (green) immunofluorescence staining of *Osr2-Cre;β-catenin*$^{fl/fl}$ samples injected with control (N-O) or *Ttll3* siRNA (P-Q). Schematic drawing of the mouse head in the top panel above the figures I-L depicts the position and angle of sectioning. White boxes in I, K, N, and P show approximate location of higher magnification images in J, L, O, and Q, respectively. White dotted lines indicate palatal shelves in I, K, N, and P. White arrows in J, L, O, and Q point to positive signal. Scale bar in I indicates 100 μm in I and K; scale bar in J indicates 20 μm in J and L; scale bar in N indicates 100 μm in N and P; scale bar in O indicates 20 μm in O and Q.

6. Fig. 3Q shows a large number of concentrated Ttll3 signals in the lower center area that does not correlate with areas of reduced Tcf7l2 signals in Fig. 4H. Are the signals in Fig. 3Q real and representative or there was too much background noise in this panel?

We thank the reviewer for this comment. We repeated the experiment and consistently saw this concentrated *Ttll3* signal in *Osr2-Cre;β-catenin*$^{fl/fl}$ sections. We have replaced the above-mentioned figures with better quality ones, in which the increased *Ttll3* signal in the primordium of the hyoid bone is still visible. This data might suggest that *Ttll3* has a broader expression pattern in comparison to the *Tcf7l2*-expressing region.

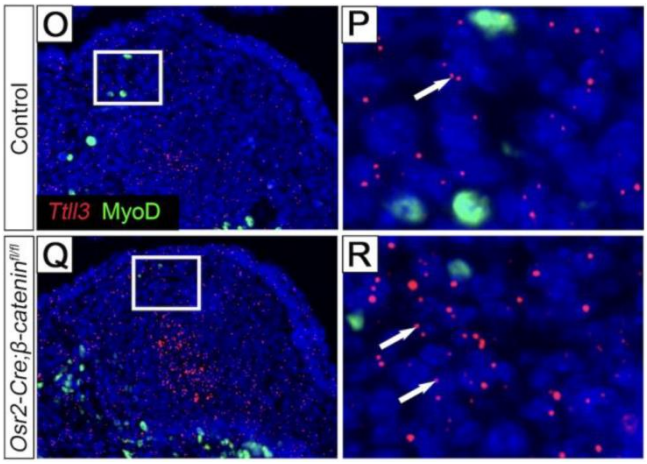


Fig. 4. Loss of Wnt signaling in mesenchymal cells leads to an increase in the number of cilia. (O-R) *Ttll3* (red) *in situ* RNAScope hybridization and MyoD (green) immunofluorescence staining in control (O-P) and *Osr2-Cre;ß-catenin*$^{fl/fl}$ mice (Q-R). White boxes in O and Q are showing a higher magnification in P and, respectively.

Minor points:
-The first paragraph in the Results section (Page 6) does not belong in this section.

We thank the reviewer for pointing this out. The first paragraph of the Results section has been adjusted (page 6, lines 99-104):

To identify how cell signaling regulates soft palate development, we screened several signaling pathways including Wnt, Fgf, and Hh (Janeckova et al, 2019). Wnt signaling emerged as a strong candidate since it was primarily activated in the CNC-derived mesenchymal cells surrounding the mesoderm-derived myogenic cells during the early development of the soft palate. This corroborated a previously published study, in which it was shown that mesenchymal Wnt signaling mediates TGF-ß epithelial signaling to guide myogenesis in the soft palate (Iwata et al, 2014).

-The methods section mentioned qPCR validation of Ttll3, Tcf7l2, and Dlk1 mRNA expression, but the data were not included in the manuscript.

We thank the reviewer for this comment. Validation of the changed expression of *Ttll3*, *Tcf7l2*, and *Dlk1* was done through RNAScope. We have added the details in Materials and Methods section accordingly. The original sentence was edited for clarification as follows: "The soft palatal tissue was also used for primary cell culture followed by qPCR analysis." (page 19, line 488).

Reviewer 2 Advance Summary and Potential Significance to Field:

The authors use a variety of multi-omic approaches to show that b-catenin disruption leads to a failure of cilia to disassemble, resulting in cell cycle changes with myogenic cells that will form the LVP in anterior pharynx (soft palate). These changes appear to result from aberrant changes to Tcf activity. Thus, Wnt signaling drives soft palate morphogenesis through the regulation of ciliogenesis; it remains to be seen if this is an event behind other ciliopathies, though it is intriguing to think that this mechanisms may be responsible for multiple developmental disease. It is equally exciting to ponder the possibility that posterior palate fusion changes may be addressable through modulating b-catenin signaling.

Reviewer 2 Comments for the Author:
In this manuscript, the authors illustrate that conditional loss of b-catenin in the NCC-derived facial mesenchyme in the area of soft palate development disrupts muscle proliferation and likely subsequent differentiation. The authors use a variety of multi-omic approaches to show that b-catenin disruption leads to a failure of cilia to disassemble, resulting in cell cycle changes. These changes appear to result from Tcf activity. The authors show that the proliferation defect can be rescued in vitro by knocking down Tcf expression. Taken together,

the findings illustrate how Wnt signaling can drive soft palate morphogenesis through the regulation of ciliogenesis and that some posterior palate defects may be addressable through treatments that reduce Tcf activity or boost Tcf-repressed genes. Overall, the manuscript is quite concise (sometimes too much so) and well-written.

1. In Figure S1, the authors state that antibody staining illustrated that the active form of b-catenin was absent from the CNC-derived mesenchymal cells while persisting in the epithelial cells. Based on tdTomato fluorescence, the authors should offer a more conservative estimate for the reduction in b-catenin activity in the CNC-derived cells. Figure S1H illustrates that there is still active b-catenin in the CNC derived mesenchyme, though it is substantially reduced in the area in which Osr2-Cre is active. It is unlikely that any Cre can reduce a targeted gene to zero. If the authors want to stick with their statement, they should perform a more nuanced experiment in which the fluorescence is quantified. I do not think this is necessary if the authors more cautiously interpret their findings.

We are appreciative of this comment. We have modified the original Figure S1, which now appears in the main text as Figure 1. The Results section describing the reduction in β-catenin activity in the CNC-derived cells has been changed after re-evaluating the figure as below (page 6, lines 106-112):

To test whether canonical Wnt signaling is functionally required for regulating tissue-tissue interactions in soft palate development, we deleted *β-catenin*, the key mediator of Wnt signaling, in the CNC-derived mesenchymal cells, without affecting Wnt signaling in epithelial or myogenic cells in *Osr2-Cre;β-catenin*$^{fl/fl}$ mice (Chen et al, 2009) (Fig. 1A-B and E-F). We confirmed efficient reduction of β-catenin expression in the CNC-derived mesenchymal cells (Fig. 1C-D and G-H). In contrast, β-catenin expression was not affected in the epithelial cells, which are not targeted by *Osr2-Cre* (Fig. 1C-D and G-H).

2. I am not clear why Figs. S1 and S2 are not made into a single figure and added as the new Figure 1. The authors only have 6 figures and this addition would strengthen the manuscript. I also think that Fig. S3A should be in the manuscript as part of a final cartoon figure.

We thank the reviewer for these recommendations. We have combined Figs. S1 and S2 into a new Figure 1 as suggested. Moreover, we added a final cartoon figure to summarize the entire study (Fig. 8).

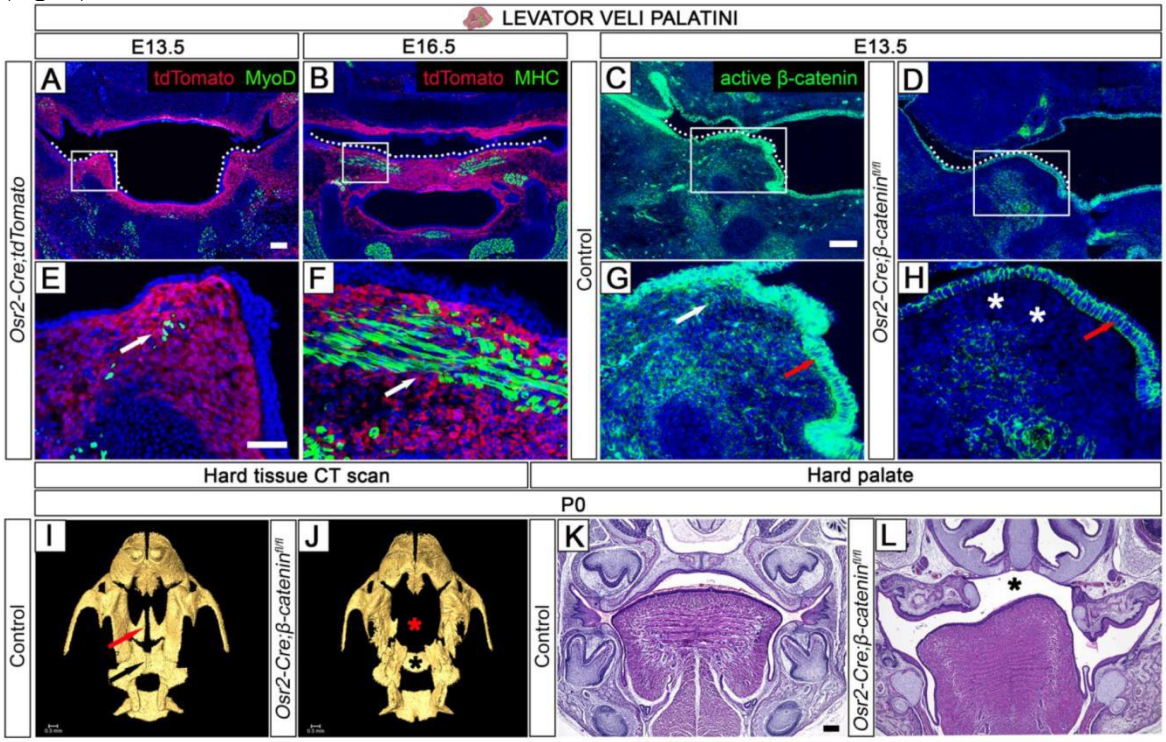


Fig. 1. Wnt signaling from CNC-derived mesenchymal cells is essential for palatogenesis. (A-B, E-F) *Osr2-Cre;tdTomato* mice at E13.5 (A, E) and E16.5 (B, F). (C-D, G-H) Active ß-catenin (green) immunofluorescence staining at E13.5 in control (C, G) and *Osr2-Cre;ß-catenin*$^{fl/fl}$ mice (D, H). (I-J) Hard tissue CT scans at P0 showing defects in the palatine process of the maxilla (red asterisk in J) and palatine bone (black asterisk in J) of *Osr2-Cre;ß-catenin*$^{fl/fl}$ mice in comparison to the normal palatine process of the maxilla (red arrow in I) and palatine bone (black arrow in I) in controls. (K-L) Hematoxylin and eosin staining at the level of the hard palate in control (K) and *Osr2-Cre;ß-catenin*$^{fl/fl}$ mice (L) at P0. Black asterisk in L identifies the cleft palate in *Osr2-Cre;ß-catenin*$^{fl/fl}$. Boxes in A-D show locations of higher magnification images in E-H, respectively. Schematic drawing of the mouse head in the top panel depicts the position and angle of sectioning. White dotted lines indicate palatal shelves in A-D. White arrows in E-G point to positive areas in the mesenchyme. Asterisks in H show reduced signal in the mesenchyme. Red arrows in G and H point to positive areas in the epithelium. Scale bar in A indicates 100 µm in A and B; scale bar in C indicates 100 µm in C and D; scale bar in E indicates 50 µm in E-H; scale bar in I indicates 0.3 mm in I and J; scale bar in K indicates 200 µm in K and L, respectively.

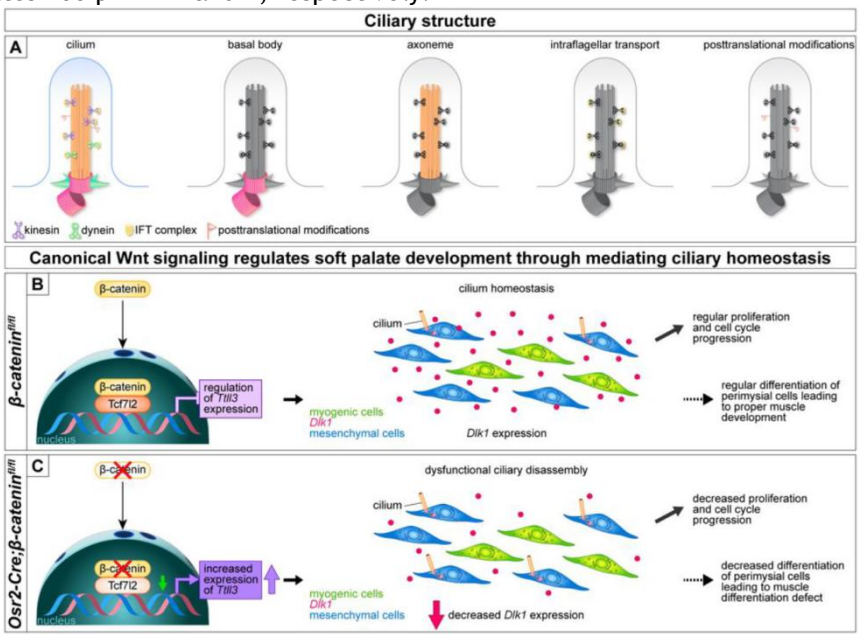


Fig. 8. **Loss of Wnt signaling in mesenchymal cells leads to disruption of ciliary homeostasis and defective muscle development.** (A) Schematic drawings of primary cilia focusing on structure (basal body and ciliary axoneme), intraflagellar transport and posttranslational modifications. (B) In control mice, stabilized ß-catenin translocates to the nucleus and regulates *Ttll3* expression through *Tcf7l2*, which maintains ciliary homeostasis, regular proliferation, cell cycle progression, and differentiation of perimysial cells. This ultimately results in proper development of the soft palatal muscles. (C) In *Osr2-Cre;ß-catenin*$^{fl/fl}$ mice, deletion of ß-catenin from CNC-derived mesenchymal cells causes decreased expression of *Tcf7l2* and increased expression of *Ttll3*, which leads to dysfunctional ciliary disassembly, decreased proliferation and cell cycle progression of perimysial cells. As a result, we observe decreased *Dlk1* expression and defective soft palatal muscle development.

3. In Figure 1, the authors state that the change in number of Myod1 positive cells became apparent at E14.0, which looks correct. Is there also a failure of myogenic cells to migrate medially due to the proliferation failure (or perhaps better put, can myogenic cells with reduced proliferation still migrate?). The presented data suggest that the cells do not differentiate in myofibrils that extend medially. Would disruption of b-catenin in this area at a later time point disrupt migration post-MyoD positive cell proliferation? These are hard questions to address, though are important in understanding why the LVP does not properly form.

We appreciate this comment to further investigate the fate of cells in the defective LVP muscle in *Osr2-Cre;β-catenin*$^{fl/fl}$ mice.

We have optimized a system for slice culture (Feng et al., 2022; Ruigrok et al, 2017; Ruigrok et al, 2018) of *Osr2-Cre;β-catenin*$^{fl/fl}$ embryos at E13.5 and cultured slices of their soft palatal tissues for three days. We noticed that the scarce MyoD+ cells that are present in the soft palate in *Osr2-Cre;β-catenin*$^{fl/fl}$ embryos at E13.5 and they do not migrate or proliferate even after 3 days in culture. This is consistent with the *in vivo* data, where at E13.5 we can see comparable numbers of MyoD+ cells in control and *Osr2-Cre;β-catenin*$^{fl/fl}$ embryos. In control samples at E14.0, the number of MyoD+ cells increases, whereas the number of MyoD+ cells in *Osr2-Cre;β-catenin*$^{fl/fl}$ embryos does not (Fig. 2U-W) .

4. In Figure 2, the authors state that there is decreased proliferation of cells in conditional knockout mice. However, it appears that the overall number of pH3 cells is unchanged, though there are fewer in the Osr2 domain. In contrast, there appear to be more medial pH3 positive cells in cko mice as compared to control embryos. The authors should denote in which zone they are counting proliferating cells. As an aside, why are there more MyoD cells in the cko mice? Are they mislocalized cells? Does loss of b-catenin cause the cells to aberrantly locate in the tissue?

We thank the reviewer for this comment. The proliferating cells were counted in the soft palatal mesenchyme only, not including the epithelium. We have outlined this region in revised Fig. 3B and E for clarification. After thorough evaluation, we confirmed that there were not more MyoD+ cells in *Osr2-Cre;β-catenin*$^{fl/fl}$ embryos and replaced the images with more representative ones.

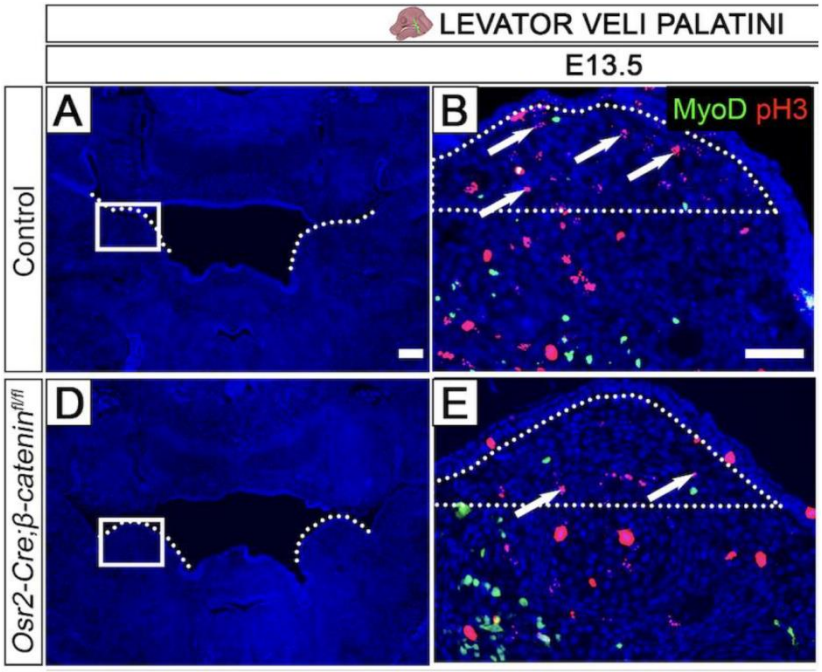


Fig. 3. *Osr2-Cre;β-catenin*$^{fl/fl}$ mice display decreased proliferation of the mesenchymal cells. (A, D) DAPI staining of control (A) and *Osr2-Cre;β-catenin*$^{fl/fl}$ (D) sections at the level of the LVP at E13.5. (B, E) MyoD (green) and pH3 (red) in control (B) and *Osr2-Cre;β-catenin*$^{fl/fl}$ mice (E). White dotted lines in B and E indicate area of quantification.

5. Please include 1-2 sentences in the Results that states what the SCENIC plot is illustrating.

We are thankful for this comment and have taken the opportunity to add more explanation of the SCENIC plot. The Results section has been adjusted accordingly as below (page 9, lines 205-209):

To determine whether Tcf7 or Tcf7l2 presented a better candidate for further analysis, we analyzed our scRNAseq data from E13.5 soft palatal shelves (Fig. 5B) (Han et al, 2021) using the R package SCENIC (Aibar et al, 2017). This allowed us to identify the main genetic regulatory

networks (regulons) in early soft palate development to guide identification of the transcription factors that play biological roles in particular clusters identified by scRNAseq (Aibar et al, 2017).

Reviewer 3 Advance Summary and Potential Significance to Field:
1. Wnt/ß-catenin signaling in the embryonic palatal mesenchymal cells regulates myogenesis cell non-autnomously.
2. Wnt/ß-catenin signaling in the embryonic palatal mesenchymal cells may directly inhibit ciliogenesis.

Reviewer 3 Comments for the Author:

The study identify the role of beta-catenin in regulating proliferation of soft palatal mesenchymal cells via regulating ciliogenesis. The manuscript is well written and the data are of high quality. The conclusions are potentially interesting if the authors can further clarify some issues.
Major comments:

1. While the data about increased number of ciliated cells and reduced proliferation of soft palatal mesenchymal cells in Osr2-Cre;ß-catenin fl/fl embryo at E13.5 were solid (Fig. 2), stating Wnt/ß-catenin signaling acts through Tcf7l2 to directly repress Ttll3 expression lacks convincing support of appropriate experiments. The authors stated without any citation that "Binding of ß-catenin to these transcription factors in the nucleus further enables activation or repression of Wnt responsive genes". It is known that Tcf and Lef1 are transcription repressors without ß-catenin binding, rising ß-catenin levels switch inhibitory TCF7/Tcfl or Lef1 complexes to activating complexes to activate gene expression. I did not see any demonstration/indication of the inhibitory role of ß-catenin in Ttll3 expression. The cultured palatal mesenchymal cells are likely in a state of low ß-catenin or no ß-catenin stimulation, in which Tcfl2 can play an inhibitory role.

We appreciate this suggestion to further explore the repressive role of canonical Wnt signaling on *Ttll3* expression. To demonstrate the inhibitory role of ß-catenin on *Ttll3* expression, we used *ß-catenin* siRNA and observed an increase in *Ttll3* expression after efficient knockdown of *ß-catenin* (Fig. 5L and M). To support our statement that Wnt/ß-catenin signaling acts through Tcf7l2 to repress *Ttll3* expression, we performed an experiment in which we used half doses of both *ß-catenin* and *Tcf7l2* siRNA together. After this treatment, we observed an increase of *Ttll3* expression (Fig. 5N), further supporting the conclusion that both ß-catenin and Tcf7l2 regulate the expression of *Ttll3*.

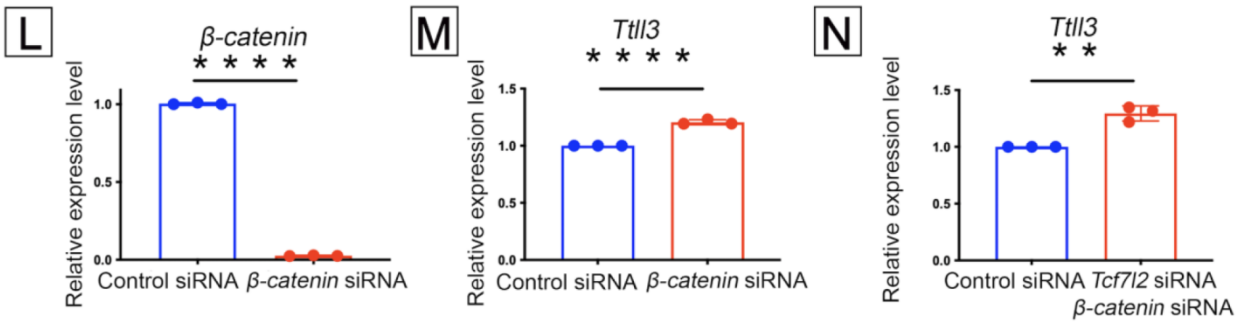


Fig. 5. Wnt signaling directly regulates ciliary genes. (L-N) Efficiency of *ß-catenin* siRNA. ****p-value <0.0001 (L). Increased expression of *Ttll3* after *ß-catenin* siRNA treatment. ****p-value <0.0001 (M). Increased expression of *Ttll3* after *ß-catenin* and *Tcf7l2* siRNA treatment. **p-value = 0.0015 (N). For L, M, and N statistical significance was evaluated by unpaired t-test with two-tailed calculations. Data is presented as mean ± s.e.m.

Furthermore, in our revised manuscript, we provide examples of references supporting the statement that ß-catenin and/or ß-catenin/Tcf complex can directly repress targeted genes (page 9, lines 198-199 and page 14, line 351):

a) Expression of *Ifit1* and *Ifit2* is negatively regulated by Wnt signaling, specifically by ß-catenin/Tcf7l2 complex (Ohsugi et al, 2017).
b) ß-catenin can act as a suppressor through the negative regulatory elements in *Xenopus*, mouse, and human systems (Kim et al, 2017).
c) Knockdown of *Tcf7l2*, Wnt signaling pathway effector, using siRNA results in a significant elevation of *PEPCK* and *G-6-Pase* mRNA levels (Ip et al, 2012)
d) ß-catenin is associated with both activation and repression of cell-specific gene expression programs, which expands on how Wnt/ß-catenin drives complex cell behaviors (Pagella et al., 2022, bioRxiv).

2. The authors showed clearly that the proliferation defect was in the palatal mesenchyme. They hypothesized with limited data that myogenic reduction was due to the problems in the palatal mesenchyme. The conclusion of Dlk1 mediates Wnt/ ß-catenin signaling in the palatal mesenchyme in myogenesis can be further potentiated.
We appreciate this comment focusing on our conclusion that *Dlk1* mediates canonical Wnt signaling in palatal mesenchyme myogenesis. For this purpose, we optimized an *in vitro* slice culture system (Ruigrok et al, 2017; Ruigrok et al, 2018) in combination with local siRNA injection. Slices containing LVP region from control embryos were injected with control or *Dlk1* siRNA into the soft palatal mesenchyme.

We confirmed efficient decrease of *Dlk1* following the injection of *Dlk1* siRNA into the cultured tissue slices (Fig. 7I-L). The number of MyoD+ LVP precursors was lower in the palatal shelves injected with *Dlk1* siRNA (Fig. 7K-M); while the MyoD+ LVP precursors continued to proliferate and differentiate in the soft palatal shelf injected with control siRNA (Fig. 7I-J). This experiment showed that palatal mesenchymal-derived *Dlk1* is functionally required for soft palatal myogenesis of the LVP.

Moreover, we used *Osr2-Cre;β-catenin*$^{fl/fl}$ slice cultures containing the LVP muscle and injected them with *Ttll3* siRNA in order to restore its expression in *Osr2-Cre;β-catenin*$^{fl/fl}$ samples. After the injection of *Ttll3* siRNA, we observed restored expression of *Dlk1* in *Osr2-Cre;β-catenin*$^{fl/fl}$ samples in comparison to the *Osr2-Cre;β-catenin*$^{fl/fl}$ samples injected with control siRNA where the *Dlk1* expression stayed very low (Fig. 7N-Q).

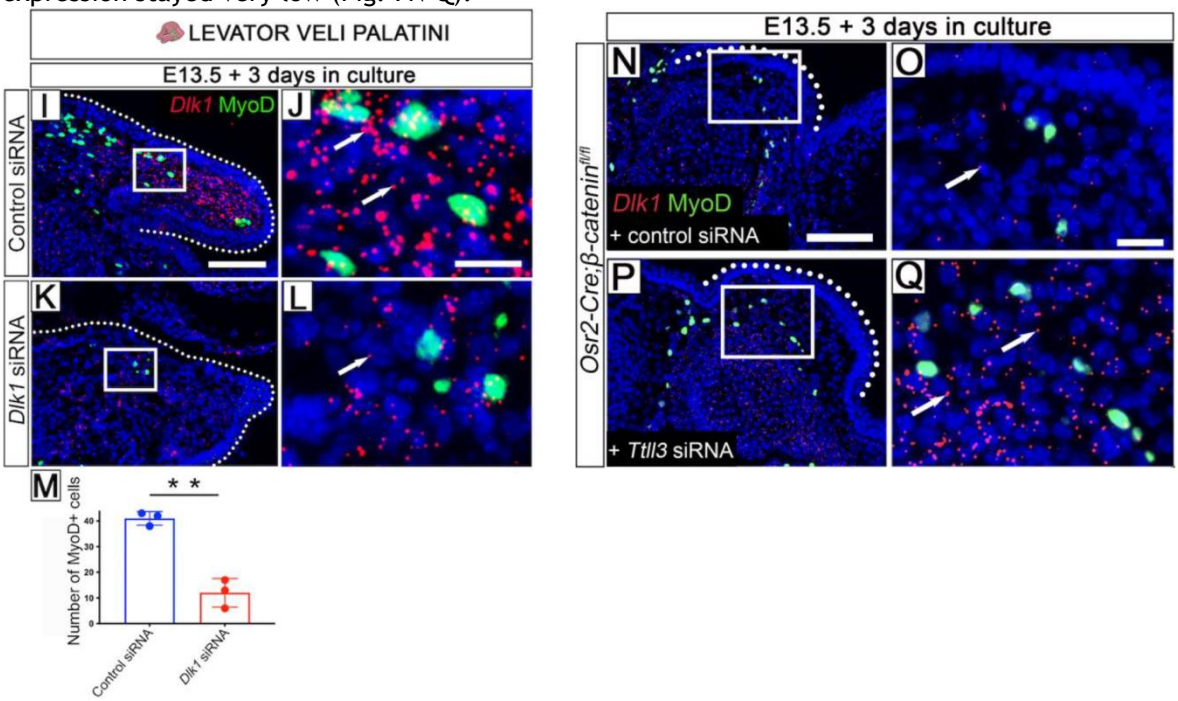


Fig. 7. Primary cilia influence the soft palate muscle development via regulating the perimysial fate of CNC-derived palatal mesenchyme cells, which further disrupts soft palate myogenesis. (I-L) *Dlk1* (red) *in situ* RNAScope hybridization and MyoD (green) immunofluorescence staining of control samples injected with control (I-J) or *Dlk1* siRNA (K-L). (M) Quantification of MyoD+ cells in control samples injected with control or *Dlk1* siRNA. **p-value = 0.0012. For M statistical

significance was evaluated by unpaired t-test with two-tailed calculations. Data is presented as mean ± s.e.m. (N-Q) *Dlk1* (red) *in situ* RNAScope hybridization and MyoD (green) immunofluorescence staining of *Osr2-Cre;β-catenin*$^{fl/fl}$ samples injected with control (N-O) or *Ttll3* siRNA (P-Q). Schematic drawing of the mouse head in the top panel above the figures I-L depicts the position and angle of sectioning. White boxes in I, K, N, and P show approximate location of higher magnification images in J, L, O, and Q, respectively. White dotted lines indicate palatal shelves in I, K, N, and P. White arrows in J, L, O, and Q point to positive signal. Scale bar in I indicates 100 μm in I and K;scale bar in J indicates 20 μm in J and L; scale bar in N indicates 100 μm in N and P; scale bar in O indicates 20 μm in O and Q.

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Second decision letter

MS ID#: DEVELOP/2022/201189

MS TITLE: Canonical Wnt signaling regulates soft palate development through mediating ciliary homeostasis

AUTHORS: Eva Janeckova, Jifan Feng, Tingwei Guo, Xia Han, Aileen Ghobadi, Angelita Araujo-Villalba, Md Shaifur Rahman, Heliya Ziaei, Thach-Vu Ho, Siddhika Pareek, Jasmine Alvarez, and Yang Chai

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, as you will see, Reviewer 1 has raised some very minor points that should be very easily addressed by some simple text changes. Please attend to these minor comments in your revised manuscript. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. Your manuscript will not require any further review, rather once uploaded I will accept the final version.

Reviewer 1

Advance summary and potential significance to field

This paper identifies a novel crucial role of beta-catenin signaling in regulating primary cilia homeostasis in the developing palatal mesenchyme.

Comments for the author

The authors did an excellent job in addressing the review comments on the original submission through new experimentation and the new results help support the main conclusions of the manuscript. The revised manuscript is much improved. However, clarification in several places of the manuscript text is needed to avoid misgiving and misunderstanding of the results presented:

1. The Summary Statement (Lines 28 - 29) needs to be clearer regarding the role of canonical Wnt signaling in controlling ciliary hoemostasis in the CNCC-derived palatal mesenchyme and indirectly affects muscle development in the soft palate.

2. Lines 43 - 44 in the Abstract section, the sentence "we found that restoring Ttll3 expression rescues mesenchymal cell proliferation and myogenesis in Osr2-Cre;b-cateninf//fl samples" needs to be revised to stating siRNA-mediated reduction of Ttll3 expression partly rescues mesenchymal cell proliferation and myogenesis in the palatal explant cultures from Osr2-Cre;b-cateninfl/fl embryos. The phrase "restoring Ttll3 expression" is an incorrect description of the experimental result presented.

3. Lines 188 - 190, the entire sentence "Increased expression of cilium-related genes, especially that of Tll3, enhances the stability ...." should be deleted as no experimental data is presented and this is not a result in this paper.

4. Lines 266 - 268, the sentence "This indicated that restoring the Ttll3 expression level..." should be deleted or revised. Again, the experimental data showed reduction of Ttll3 expression after siRNA-mediated knockdown in the palatal explant, which is not the same as "restoring the Ttll3 expression level" and "alleviate the myogenic defect observed in Osr2-Cre;b-cateninfl/fl mice" is a speculation (not a result).

Reviewer 2

Advance summary and potential significance to field

The authors illustrate that b-catenin signaling is essential for soft palate development though the regulation of ciliogenesis. Loss of b-catenin in the soft palate leads to clefting, due apparently to decreased mesenchyme proliferation and myogenic differentiation. This is accompanied by an increase in ciliogenesis. The authors also show that expression of the cilium modifier gene Ttl3 was increased. The authors now show using slice culture and siRNA that up regulation of Ttl3 (due to a failure of Wnt-mediated repression) represses Dlk1 expression. Loss of Dlk1 subsequently results in mesenchymal and myogenic differentiation defects. This is the first example of Wnt signaling regulating soft palate development.

Comments for the author

The authors addressed my concerns. The additional experimentation to address concerns on the direct role of Ttl3 in repressing Dlk1 is quite convincing and the reorganization of figures helps the story. It is noted that examples of negative regulation by b-catenin is not novel, though it is in this context. The additional slice culture/siRNA treatment is convincing and the results have been carefully interpreted. This study represents a novel finding in how b-catenin can differentially regulate development of different craniofacial features through differential gene induction and repression.

Minor comment:
1. The tiny little heads at the top of each figure are almost comical. I realize that the authors were asked to add this, but they can only be seen above 400x. The figures are square or rectangular, but if that is as good as it gets, the authors should just remove it. Why not just refer to Figure 2B, D.

Second revision

Author response to reviewers' comments

Response to Reviewers’ comments

We appreciate all of the reviewers’ comments and suggested changes. We have addressed all of them in the manuscript and we provide point-by-point responses below in this response letter.

Reviewer 1 Advance summary and potential significance to field
This paper identifies a novel crucial role of beta-catenin signaling in regulating primary cilia homeostasis in the developing palatal mesenchyme.

Reviewer 1 Comments for the author

The authors did an excellent job in addressing the review comments on the original submission through new experimentation and the new results help support the main conclusions of the manuscript. The revised manuscript is much improved. However, clarification in several places of the manuscript text is needed to avoid misgiving and misunderstanding of the results presented:

1. The Summary Statement (Lines 28 - 29) needs to be clearer regarding the role of canonical Wnt signaling in controlling ciliary hoemostasis in the CNCC-derived palatal mesenchyme and indirectly affects muscle development in the soft palate.

We thank the reviewer for this comment, and we adjusted our Summary Statement (page 2, lines 28-29) as follows:

This study reveals a crucial role of canonical Wnt signaling in controlling ciliary homeostasis in CNC-derived palatal mesenchyme to indirectly regulate muscle development in the soft palate.

2. Lines 43 - 44 in the Abstract section, the sentence "we found that restoring Ttll3 expression rescues mesenchymal cell proliferation and myogenesis in Osr2-Cre;b-cateninf//fl samples" needs to be revised to stating siRNA-mediated reduction of Ttll3 expression partly rescues mesenchymal cell proliferation and myogenesis in the palatal explant cultures from Osr2-Cre;b-cateninfl/fl embryos. The phrase "restoring Ttll3 expression" is an incorrect description of the experimental result presented.

We thank the reviewer for this comment. Please see changed Abstract (page 3, lines 42-44) to read as follows:

Moreover, we found that siRNA-mediated reduction of Ttll3 expression partly rescues mesenchymal cell proliferation and myogenesis in the palatal explant cultures from Osr2-Cre;β-cateninfl/fl embryos.

3. Lines 188 - 190, the entire sentence "Increased expression of cilium-related genes, especially that of Tll3, enhances the stability ...." should be deleted as no experimental data is presented and this is not a result in this paper.

We deleted this entire sentence from the manuscript as suggested by the reviewer.

4. Lines 266 - 268, the sentence "This indicated that restoring the Ttll3 expression level..." should be deleted or revised. Again, the experimental data showed reduction of Ttll3 expression after siRNA-mediated knockdown in the palatal explant, which is not the same as "restoring the Ttll3 expression level" and "alleviate the myogenic defect observed in Osr2-Cre;b-cateninfl/fl mice" is a speculation (not a result).

This sentence was deleted from the manuscript as recommended.

Reviewer 2 Advance summary and potential significance to field
The authors illustrate that b-catenin signaling is essential for soft palate development though the regulation of ciliogenesis. Loss of b-catenin in the soft palate leads to clefting, due apparently to decreased mesenchyme proliferation and myogenic differentiation. This is accompanied by an increase in ciliogenesis. The authors also show that expression of the cilium modifier gene Ttl3 was increased. The authors now show using slice culture and siRNA that up regulation of Ttl3 (due to a failure of Wnt-mediated repression) represses Dlk1 expression. Loss of Dlk1 subsequently results in mesenchymal and myogenic differentiation defects. This is the first example of Wnt signaling regulating soft palate development.

Reviewer 2 Comments for the author

The authors addressed my concerns. The additional experimentation to address concerns on the direct role of Ttl3 in repressing Dlk1 is quite convincing and the reorganization of figures helps the story. It is noted that examples of negative regulation by b-catenin is not novel, though it is in this context. The additional slice culture/siRNA treatment is convincing and the results have been carefully interpreted. This study represents a novel finding in how b-catenin can differentially regulate development of different craniofacial features through differential gene induction and repression.

Minor comment:

1. The tiny little heads at the top of each figure are almost comical. I realize that the authors were asked to add this, but they can only be seen above 400x. The figures are square or rectangular, but if that is as good as it gets, the authors should just remove it. Why not just refer to Figure 2B, D.

We thank the reviewer for this comment. The schematic drawings of the mouse heads were deleted from all figures that contained them (Figs. 1, 3, 4, 5, 6, 7, and Fig. S1) and the references to them were deleted from the corresponding figure legends.

Third decision letter

MS ID#: DEVELOP/2022/201189

MS TITLE: Canonical Wnt signaling regulates soft palate development through mediating ciliary homeostasis

AUTHORS: Eva Janeckova, Jifan Feng, Tingwei Guo, Xia Han, Aileen Ghobadi, Angelita Araujo-Villalba, Md Shaifur Rahman, Heliya Ziaei, Thach-Vu Ho, Siddhika Pareek, Jasmine Alvarez, and Yang Chai

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.