Ciliopathic micrognathia is caused by aberrant skeletal differentiation and remodeling

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AUTHORS: Christian Louis Louis Bonatto Paese, Evan C Brooks, Megan Aarnio-Peterson, and Samantha A Brugmann

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

Reviewer 1

Advance summary and potential significance to field

Paese et al use a chicken genetic model of a ciliopathy to build on Chang et al 2014 and Schock et al 2015 and examine the cellular processes required for mandibular development. The strengths include use of a chicken genetic system and beautiful classical skeletal analysis. The authors concentrate their focus on HH36-39. Both endochondral and intramembranous ossification are affected in ta2 mutants. The authors demonstrate that C2cd3 is required for controlling gene expression and limiting cell proliferation and pre-osteoblast deposition as well as promoting bone mineralization. The quality of the data is high (with minor exceptions noted below). The advance reported here is somewhat modest, but is balanced by the clinical significance and the strengths noted above.

Comments for the author

Major points
Previous work from this group has indicated that ta2 mutants display Hh signaling defects. Here, the authors speculate the mandibular phenotypes described here may be secondary to Hh signaling defects. The authors state at the outset of their work that they are interested in extending the molecular understanding of ta2 mutants. This work, while including beautiful phenotypic analyses, only modestly extends our molecular understanding of ta2 mutant developmental defects. Some few additional experiments, such as correlating the origin of the gene expression defects with altered Hh signal transduction, may significantly extend the insight provided by this work.

The absence of error bars for the control gene expression analyses in Fig 2B (and throughout) suggests that each control replicate was set to one, artificially diminishing the variance among the controls. Statistically setting each control replicate to one is a mistake, as it thereby overestimates significance. Instead, the mean should be set to one.

Minor points
This author has some misgivings about the authors asserting that, “Ciliopathic mutants are particularly vulnerable to cell cycle disruptions as the centrioles required for ciliogenesis are the same organelles required for formation of the mitotic spindle. Thus, ciliary extension and cell cycle progression are inextricably linked processes.” The authors cite Tucker et al 1979, a classic paper. However, Tucker et al demonstrates a correlation between ciliogenesis and cell cycle progression, but does not indicate causation. Indeed, there is precious little evidence that ciliogenesis is necessary for cell cycle progression generally. Moreover, the authors evidence that cell cycle progression is affected in the mandibular bones of ta2 mutants makes the tissue specificity of the role of C2cd3 all the more intriguing.

The text states that differences in Survivin expression are significant whereas the figure indicates that they are not.

Are the authors sure that these cells do not possess cilia in S and G2 phases, as suggested by Figure 2A and older work that has recently been revised (see Ford et al 2018)?

Why does the Sox9+ domain appear to be a solid volume in Fig 2C, but a shell in Fig 2D?

What do the white boxes indicate in Fig 3?

Reviewer 2

Advance summary and potential significance to field

The article by Paese et al. entitled “Ciliopathic micrognathia is caused by aberrant skeletal differentiation and remodeling’” addresses an interesting question that is important to advance the knowledge on how micrognathia occurs in patients with ciliopathies. These discoveries could leave

to the development of new treatments for these patients, which are currently very limited. The authors find changes in cell cycle progression and proliferation, an impairment in the differentiation of neural crest osteochondroprogenitors and in the maturation of osteoblasts, as well as increased bone resorption. My main impression of the article is that is novel and provides interesting advancements to the understanding of how the jaw develops and a role for primary cilia in intramembranous bone development. Overall, I believe that the etiology of ciliopathic micrognathia described in this paper has the potential to influence future research in bone pathways and diseases, as well as craniofacial development.

Comments for the author

I would encourage a revision of the submitted manuscript. My specific comments are below.

Essential revisions:
Line 143: The authors state that the HH39 ta2 mandibles "possessed reduced Alizarin Red staining, indicative of reduced bone deposition (Fig. S1E)." This statement is not necessarily true, since it could also be due to poor mineralization since Alizarin Red binds calcium. The images in Fig. S1E appear to have the same intensity of Alizarin Red, so I do not draw the same conclusion from the data shown.

Line 214-6: Early overexpression of Runx2 in the mandible has been looked at and shown to produce micrognathia in chick. This was done by Hall et al. 2014 using RCAS to overexpress Runx2 at stage HH9.5 and looking at micrognathia with Alizarin Red at HH37. They also looked at cell cycle. Comparing this previous data to the author's new data would further support the author's conclusions.

Ectopic Splenial bone: You mention in the legend of Fig. 1 that you think there is a duplication of the splenial bone, but this in not noted or described in the text. Could you please clarify if you think it is a duplication or ectopic bone and why?

Figures 2, 3, 4 & 5: It is unclear if the standard deviation of the controls was used to calculate the significance. They are not present on any of the qRT-PCR graphs. The standard deviation needs to be added to the graphs and used for the student's t-test analysis. It would also be important for the authors to note if they think these significant differences in qRT-PCR values represent a meaningful biological difference.

Figures 5A&B and 5D&E: When evaluating bone resorption, TRAP is regularly shown in the bone field to note changed levels of bone resorption. Adjacent sections to those used in Fig. 5A&B/D&E stained with TRAP would strength this data.
Figures 5A&B and 5D&E would be strengthened by high magnification images to show the MMP13 coming from the osteocytes and SPP1 coming from the osteoclasts. This might be of interest to the reader, since in Fig. 5B it looks like the MMP13 is coming from large cells on the surface of the bone, maybe osteoclasts? Also for this reason it would be interesting to show adjacent sections stained with MMP13 and SPP1, these do not appear to be adjacent sections. Additionally, the scale bars are not legible and need to be made bigger.

Figure 5G: OPG and RANKL are both secreted by osteoblasts. The figure needs to be edited to show this is true.

Figure 6: Overall, I found this figure confusing. I believe you mean that the increased number of cells at day 5.5 are osteochondroprogenitors and not pre-osteoblasts. Also the preposteoblasts are the same color as the notochord.
Additionally, there would be pre-osteoblasts and osteoblasts at day 9, as well as pre-osteoblasts, osteoblasts, and osteocytes at day 13. Furthermore, I found the coloring of Meckel's cartilage with chondroprogenitors and chondrocytes to be distracting. I would just keep the Meckel's cartilage one color since this paper does not specifically focus on the chondroprogenitor or chondrocyte. I would also add the HH staging. The legend adds further confusion, at day 5.5 do you mean to show pre-osteoblasts or osteochondroprogenitors? No description is present for day 13 is present in the legend.

Supplementary Fig. 1: In A, did you count the ectopic/duplication of splenial bone in the ta2 embryos when you calculated the bone volume? This should be addressed more clearly in the text and/or figure. You may see a significant difference in the splenial bone if you remove the ectopic/duplicated bone.

Potential extensions of the study: (not needed)

Additional minor comments:
Line 83: I think you mean bone remodeling and not bone resorption.

Line 146-7: The authors look at the skeletal elements of tongue and relate it to the human condition. In the text it needs to be clarified that humans do not have a skeletal element within their tongue.

Line 256: The authors incorrectly referred to their data in Fig 5H in their text as the OPG/RANKL ratio. This need to be corrected. They show RANKL/OPG and not OPG/RANKL. For clarity, I would suggest update the figure legend for Fig 5 G & H and make this clearer on the figure itself.

Fig. 1F & 1K: It is not clear how the mandibular length was taken. Was it from the uCTs or from the Alizarin red staining? This needs to be described further in the text.

Fig. 1I & 1J: It would be helpful to convert the voxels into mm3 or mm2 as it was done for Fig 1C. This should also be done for Supplemental Fig. 1B &C.

Fig. 2, 3, 4, S2 & S3: Scale bars need to be enlarged, they are not currently legible.
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Fig. 2, 3, 4, S2 & S3: Scale bars need to be enlarged, they are not currently legible.

Reviewer 3

Advance summary and potential significance to field

In this article Paese and colleagues report on the mechanisms underlying the micrognathic phenotype of the ciliopathic Talpid2 mutant chicken. They show expansion of the early Runx2+ osteoprogenitors in the mandible in mutant mandibular prominences leading to more pre-osteoblasts. However, a proportion of these pre-osteoblasts fail to mature and form mineralised bone, resulting in the micrognathic phenotype observed. In addition, they see an (indirect?) upregulation of bone resorption markers, which are proposed to cause the decreased mineralised bone volume seen in the mutants. The text is written clearly, and the conclusions are supported by the results, which are generally well-presented. The story follows on nicely from previous work in this group examining earlier cellular and molecular consequences of talpid2 mutation. In general we are enthusiastic about this story.

The authors wish to draw two major conclusions in this paper: 1) that progression of the osteogenic program is disrupted or delayed at crucial transitions, and 2) that there is excess remodelling of the bone (via osteoclast activity). While the data shown are intriguing, we feel that the evidence does not yet show this definitively. We make some specific points and suggestions below.

Comments for the author

Major points:

1) The assessment of key cellular populations during osteogenic progression (Fig 4) is crucial to the conclusions of this paper. The authors could improve these data by addressing the following:
a. Fig 4 – are these sections all at the same rostral-caudal location (assuming these are frontal sections) and if so, where? If not, where are the relative sections - suggest a callout in one of the previous wholemount figures.
b. Col1a1 expression – which is the real staining, the puncta (very specific?) or the lawn of staining across the section? How sure are the authors that this is real staining? Perhaps a positive control (e.g. col1a1 in wt chicken long bone?) could be used. It would also help to see the comparable histology (e.g. via trichrome or pentachrome staining or even a simple H&E) so that we could orient ourselves well.
c. ALPL expression is shown at HH39 but the later more mature marker (OCN) is shown earlier at HH35. It would be helpful to see the extent of ALPL at the earlier stage as well, as authors propose that initial NC-derived mesenchyme do enter the osteogenic lineage and if OCN is already on at HH35 in the wt animals we should also see ALPL at this stage and prior to this stage.
d. OCN expression looks very non-specific here – how confident are the authors that this expression is real? And if it is entirely gone in the mutants how do you explain the osteogenic progression seen in the earlier figures? That seems unlikely, so it important to show some controls and/or immune staining in other regions so we are confident of the specificity of antibody.
e. qRTPCR analyses/methods need more detail, especially as we are not quite convinced by the immunos yet. Can you include variance on the controls? How were the normalisations done? Assume these ΔΔCT? If so, please state this. Best practice for ΔΔCT is to use two loading controls and for each target gene normalise to the control with a similar amplification efficiency… http://blog.mcbryan.co.uk/2013/06/qpcr-normalisation.html

2) The second key point is the idea that there is increased bone resorption/osteoclast activity in the mutants (Figure 5).
a. This again is intriguing, but the same comments hold as for Figure 4 (orientation/location of sections), some accompanying histology so that we can see structures.
b. To show “remodelling” or resorptive activity, the authors should do TRAP staining on these sections to confirm this.

Some minor comments:
3) Fig1 a. Posterior portion of dentary bone appears much thicker in the mutant as assessed by alizarin red staining (Figure 1B). This goes unmentioned in the text and is contrary to the main argument of the paper. Is this a tooth like structure or bone?
b. Scale bars in fig 1 do not seem in agreement with mandible length measurements from the microCT analysis.
c. Ectopic skeletal element (medially located) could be marked in figure (unclear if this is on the dentary bone or the duplicated 4) FigS1 medial cartilage spurs on meckel’s should be noted as well 5) Fig2 a. C+E (and figS2 A+B) box slightly unclear with opacity (reduce opacity or outline box)
b. Significant decrease in proliferation of sox9-ve cells, unmentioned in text (fig2J). This should be discussed in text.
c. SOX9+ve area could be expressed as a percentage of total mnp area d. Also generally a description of quantification of this data automatic/manual?
6) Fig3 a. DE, IJ and O are hard to see, increase brightness?
b. Hand2 staining spanning midline in control not mentioned
7) Supplemental 3D movies (if possible) for figures 2/3 would help to clarify the spatial arrangement of staining
8) Figs 4 and 5 – include scheme for section orientation or be clearer in text
9) Figure 6 – HH stages as well as hours? Perhaps clearer to be consistent with other figures?
10) N numbers are for biological replicates over one experiment or multiple days?
11) Discussion
a. 299-303 – reasonable speculation?
b. 320-325 – something about this is weird – timings of signals and expression of markers? They say differentiation but do they mean maturation? Was this unclear for you?

12) 507 and 509 – first author umlaut missing (line 507)
13) 603 – need author names again?

First revision

Author response to reviewers' comments

We thank the Reviewers for their helpful and insightful comments. Overall the Reviewers were positive about the paper, noting that the work builds off previous findings and has 'clinical significance' and "addresses an interesting question that is important to advance the knowledge on how micrognathia occurs in patients with ciliopathies". As per Reviewers comments we have added molecular analyses, validation of several probes and antibodies, reimaged results and reanalyzed qRT-PCR data. These new data resulted in **new Figure 2**, **new Supplemental Figure 2** and **new Supplemental Figure 5**. Please see individual Reviewer comments below and our responses in italics. Edited and new sections in the manuscript are indicated in blue text.

Reviewer 1 Advance Summary and Potential Significance to Field...

Paese et al use a chicken genetic model of a ciliopathy to build on Chang et al 2014 and Schock et al 2015 and examine the cellular processes required for mandibular development. The strengths include use of a chicken genetic system and beautiful classical skeletal analysis. The authors concentrate their focus on HH36-39. Both endochondral and intramembranous ossification are affected in ta2 mutants. The authors demonstrate that C2cd3 is required for controlling gene expression and limiting cell proliferation and pre-osteoblast deposition as well as promoting bone mineralization. The quality of the data is high (with minor exceptions noted below). The advance reported here is somewhat modest but is balanced by the clinical significance and the strengths noted above.

Reviewer 1 Comments for the Author...

Major points
Previous work from this group has indicated that ta2 mutants display Hh signaling defects. Here, the authors speculate the mandibular phenotypes described here may be secondary to Hh signaling defects. The authors state at the outset of their work that they are interested in extending the molecular understanding of ta2 mutants. This work, while including beautiful phenotypic analyses, only modestly extends our molecular understanding of ta2 mutant developmental defects. Some few additional experiments, such as correlating the origin of the gene expression defects with altered Hh signal transduction, may significantly extend the insight provided by this work.

We appreciate the Reviewer's comment and agree that adding molecular analyses to the work will significantly improve the depth of the project. In response, we have added ***new Figure 2*** *and* ***new Supplemental Figure 2,*** *in which we cross-reference previously published Gli ChIP-seq (Elliott et al., 2020; Lorberbaum et al., 2016) and bulk RNA-Seq from the talpid^2 MNP (Chang et al., 2014) to determine potential Gli targets that are differentially expressed in the talpid^2 MNP. By cross-referencing these datasets and performing gene ontology (GO) analysis, we identified Gli-dependent cell processes and gene networks that were significantly impacted in ciliopathic micrognathia. We validated these findings by performing qRT-PCR for three genes from the 'positive regulation of cell cycle' that are associated with the G1-S phase transition of the cell cycle. In addition to providing molecular insight into the basis for ciliopathic micrognathia, these newly provided data also provide strong rationale for examining the cell cycle and osteogenesis in later figures. Description of this new data is detailed in the text in blue (pages 7-8, lines 165-184).*

The absence of error bars for the control gene expression analyses in Fig 2B (and throughout) suggests that each control replicate was set to one, artificially diminishing the variance among the controls. Statistically, setting each control replicate to one is a mistake, as it thereby overestimates significance. Instead, the mean should be set to one.

We understand and appreciate this concern that was brought up by more than one Reviewer. Our previous analysis was based on the relative standard curve, in which deviation smaller than 0.5 was normalized to 1. We appreciate Reviewer's concern with this methodology, and as such we have re-analyzed the qRT-PCR data with the control replicates as separate entities. All the graphs and text throughout the manuscript are updated to reflect this change. Importantly, this re-analysis has not changed the overall conclusions of any experiments.

Minor points

This author has some misgivings about the authors asserting that, "Ciliopathic mutants are particularly vulnerable to cell cycle disruptions as the centrioles required for ciliogenesis are the same organelles required for formation of the mitotic spindle. Thus, ciliary extension and cell cycle progression are inextricably linked processes." The authors cite Tucker et al 1979, a classic paper. However, Tucker et al demonstrates a correlation between ciliogenesis and cell cycle progression, but does not indicate causation. Indeed, there is precious little evidence that ciliogenesis is necessary for cell cycle progression generally. Moreover, the authors evidence that cell cycle progression is affected in the mandibular bones of ta2 mutants makes the tissue specificity of the role of C2cd3 all the more intriguing.

We appreciate the Reviewer giving us the opportunity to better explain our rationale and provide additional data and citations of support. We have responded in two ways.

*First, we have edited the text to better explain our rationale (blue text p. 8, lines 188-198). Indeed, the Tucker reference does not imply causation, and by citing this reference we were not implying causation. Rather, we were attempting to use the most original reference for the fact that there is a correlation between ciliogenesis and entry/exit into the cell cycle. This correlation has been well documented in cell culture (Doxsey et al., 2005). In brief, formation of primary cilia begins at the G1/G0 phase of the cell cycle and begins to disassemble as cells re-enter the cell cycle (Tucker et al., 1979; Kim and Tsiokas, 2011). The mature cilium is fully extended in G0. Upon cell cycle re-entry, ciliary resorption begins and the balance of cilium assembly and disassembly, via microtubule stability, is shifted toward disassembly (Kim et al., 2015; Yeh et al., 2013). As cells enter the cell-cycle, ciliary disassembly occurs 1-2 h after serum stimulation (Izawa et al., 2015; Pugacheva et al., 2007; Tucker et al., 1979). After disassembly, the basal body is released from cilia, thereby freeing up centrioles (centrosome) to function as microtubule organizing center (MTOC) or spindle poles during mitosis (Nigg et al., 2011; Kobayashi et al., 2011). We have updated our schematic to better represent these details (see **Fig. 3A**).*

*Second, as stated above, we have added **new Fig. 2** which includes GO analyses. These analyses revealed that cell-cycle regulation was one of the processes impacted in ta^2 embryos. We now use this data, along with literature cited above, to validate our rationale for examining if and how the cell-cycle is impaired in ta^2 embryos.*

The text states that differences in Survivin expression are significant whereas the figure indicates that they are not.

*We have removed this data from the manuscript and have replaced it with new qRT-PCR data analyses for two new markers of positive cell cycle regulation as demonstrated from our unbiased GO-term analyses (**see Fig. 3B**).*

Are the authors sure that these cells do not possess cilia in S and G2 phases, as suggested by Figure 2A and older work that has recently been revised (see Ford et al 2018)?

*As per numerous reviews (Pan, 2007; Izawa et al., 2015; Keeling, 2016) we propose that cilia are undergoing axonemal shortening and in the processes of disassembly during S/G2; however, we do concede that cilia may not be completely absent. As such we have edited **Fig. 3A** to reflect that the cilium is significantly shortened in G2/S and preparing to release the basal body as the cell moves into M phase.*

Why does the Sox9+ domain appear to be a solid volume in Fig 2C, but a shell in Fig 2D?

*Both the Sox9+ domain in old Figure 2C and 2D (now **new Fig. 3C and 3D**) were imaged with a confocal microscope and the surface was reconstructed with Imaris software. The reason for the difference pointed out by the Reviewer is that in Fig. 3C (solid) the MNPs were imaged at 40x and reconstructed with a 25 degrees angle for the whole visualization, whereas the MNPs in Fig. 3D were imaged at 100x and the surface was reconstructed at a 0 degree angle, for the visualization*

of the Sox9+ domain with the PHH3 puncta surrounding it. The solid versus shell rendering does not impact overall conclusions.

What do the white boxes indicate in Fig 3?
*The white-dotted line boxes in former Figure 3 (now **new Figure 4 D,E,I,J,N,O**) indicate the region imaged in higher magnification (now **new Figure 4D',E',I',J',N',O'**) to show the Meckel's cartilage. We have updated the figure legend to indicate this.*

Reviewer 2 Advance Summary and Potential Significance to Field...

The article by Paese et al. entitled "Ciliopathic micrognathia is caused by aberrant skeletal differentiation and remodeling'" addresses an interesting question that is important to advance the knowledge on how micrognathia occurs in patients with ciliopathies. These discoveries could leave to the development of new treatments for these patients, which are currently very limited. The authors find changes in cell cycle progression and proliferation, an impairment in the differentiation of neural crest osteochondroprogenitors and in the maturation of osteoblasts, as well as increased bone resorption. My main impression of the article is that is novel and provides interesting advancements to the understanding of how the jaw develops and a role for primary cilia in intramembranous bone development. Overall, I believe that the etiology of ciliopathic micrognathia described in this paper has the potential to influence future research in bone pathways and diseases, as well as craniofacial development.

Reviewer 2 Comments for the Author...

I would encourage a revision of the submitted manuscript. My specific comments are below.

Essential revisions:
Line 143: The authors state that the HH39 ta2 mandibles "possessed reduced Alizarin Red staining, indicative of reduced bone deposition (Fig. S1E)." This statement is not necessarily true, since it could also be due to poor mineralization since Alizarin Red binds calcium. The images in Fig. S1E appear to have the same intensity of Alizarin Red, so I do not draw the same conclusion from the data shown.
We have now updated the text to reflect the reviewer's comment (see blue text p.6, line 144).

Line 214-6: Early overexpression of Runx2 in the mandible has been looked at and shown to produce micrognathia in chick. This was done by Hall et al. 2014 using RCAS to overexpress Runx2 at stage HH9.5 and looking at micrognathia with Alizarin Red at HH37. They also looked at cell cycle. Comparing this previous data to the author's new data would further support the author's conclusions.
We appreciate the reviewer's comments and have now updated the text with findings from this paper, which supports our results (see blue text p. 9, lines 219-223 and p. 10, lines 233-235). We have also added key points from this manuscript to our discussion (see blue text p. 14, lines 333-335 and p.14, lines 337-340).

Ectopic Splenial bone: You mention in the legend of Fig. 1 that you think there is a duplication of the splenial bone, but this in not noted or described in the text. Could you please clarify if you think it is a duplication or ectopic bone and why?
On p. 6, lines 129-133, we note that there is an ectopic bone medially adjacent to the splenial. We have now updated the figure legend match this description. As there is no evidence to suggest it is a duplication and as this feature is not the main focus of the manuscript, we have simply noted the ectopic structure.

Figures 2, 3, 4 & 5: It is unclear if the standard deviation of the controls was used to calculate the significance. They are not present on any of the qRT-PCR graphs. The standard deviation needs to be added to the graphs and used for the student's t-test analysis. It would also be important for the authors to note if they think these significant differences in qRT-PCR values represent a meaningful biological difference.
Our previous analysis was performed by normalizing the Control triplicates that had a deviance smaller than 0.5 to 1. We have now updated the analysis in the manuscript based in the Livak's paper from 2011, in which the $2^{-\Delta\Delta CT}$ method was standardized (see blue text p. 23, lines 550-552).

Figures 5A&B and 5D&E: When evaluating bone resorption, TRAP is regularly shown in the bone field to note changed levels of bone resorption. Adjacent sections to those used in Fig. 5A&B/D&E stained with TRAP would strength this data. Figures 5A&B and 5D&E would be strengthened by high magnification images to show the MMP13 coming from the osteocytes and SPP1 coming from the osteoclasts. This might be of interest to the reader, since in Fig. 5B it looks like the MMP13 is coming from large cells on the surface of the bone, maybe osteoclasts? Also for this reason it would be interesting to show adjacent sections stained with MMP13 and SPP1, these do not appear to be adjacent sections. Additionally, the scale bars are not legible and need to be made bigger.
We appreciate the Reviewer's suggestion and have now performed TRAP staining on sections of the HH39 mandible. These data in ***new Fig. 6*** *support our previous hypothesis of increased of bone resorption in the ta^2 mandible. We have also updated the figures for MMP13 and SPP1 RNAscopes in higher magnification, as suggested by the Reviewer, to more clearly show expression in the osteocyte and osteoclast, respectively.*

Figure 5G: OPG and RANKL are both secreted by osteoblasts. The figure needs to be edited to show this is true.
This figure has been significantly edited. See new ***Fig. 6I****.*

Figure 6: Overall, I found this figure confusing. I believe you mean that the increased number of cells at day 5.5 are osteochondroprogenitors and not pre-osteoblasts. Also the preposteoblasts are the same color as the notochord. Additionally, there would be pre-osteoblasts and osteoblasts at day 9, as well as pre-osteoblasts, osteoblasts, and osteocytes at day 13. Furthermore, I found the coloring of Meckel's cartilage with chondroprogenitors and chondrocytes to be distracting. I would just keep the Meckel's cartilage one color since this paper does not specifically focus on the chondroprogenitor or chondrocyte. I would also add the HH staging. The legend adds further confusion, at day 5.5 do you mean to show pre-osteoblasts or osteochondroprogenitors? No description is present for day 13 is present in the legend.
*We have updated the figure (****new Figure 7****) to reflect the reviewer's comments.*

Supplementary Fig. 1: In A, did you count the ectopic/duplication of splenial bone in the ta2 embryos when you calculated the bone volume? This should be addressed more clearly in the text and/or figure. You may see a significant difference in the splenial bone if you remove the ectopic/duplicated bone.
We have updated the text and analysis with both the ectopic bone excluded (Fig 1C) and included (Fig S1A), which matches the reviewer's suggestion (see blue text p. 6, lines 129-133)

Potential extensions of the study: (not needed)

Additional minor comments:
Line 83: I think you mean bone remodeling and not bone resorption.
We have updated the text to reflect the reviewer's comments (see blue text p. 4, lines 83 and 84)

Line 146-7: The authors look at the skeletal elements of tongue and relate it to the human condition. In the text it needs to be clarified that humans do not have a skeletal element within their tongue.
We have significantly updated the text in response to Reviewer's comment (see blue text p.6-7, lines 150-163).

Line 256: The authors incorrectly referred to their data in Fig 5H in their text as the OPG/RANKL ratio. This need to be corrected. They show RANKL/OPG and not OPG/RANKL. For clarity, I would suggest update the figure legend for Fig 5 G & H and make this clearer on the figure itself.
We appreciate the comment. Both the text and figure legend have been updated (see blue text p. 13 line 300, and pg. 18, line 417).

Fig. 1F & 1K: It is not clear how the mandibular length was taken. Was it from the uCTs or from the Alizarin red staining? This needs to be described further in the text.
Fig. 1I & 1J: It would be helpful to convert the voxels into mm3 or mm2 as it was done for Fig 1C. This should also be done for Supplemental Fig. 1B &C.

We have updated the methodology section explaining how these measurements were taken. The length measurements were done by manually measuring the total length of the whole mount Alizarin Red-stained mandibles in Leica LAS X (see blue text p. 20, lines 477-479). The measurements in voxels were automatically performed by the Imaris software in the microCT samples (as described in p. 20-21, lines 483-487). Although we understand your comment regarding converting voxels to millimeters, converting cubic pixels (that are extremely accurate) to cubic metric measurements is not as accurate, so we opted to maintain the original measurements in these graphs.

Fig. 2, 3, 4, S2 & S3: Scale bars need to be enlarged, they are not currently legible.
We updated the scale bars in all the figures.

Reviewer 3 Advance Summary and Potential Significance to Field...

In this article Paese and colleagues report on the mechanisms underlying the micrognathic phenotype of the ciliopathic Talpid2 mutant chicken. They show expansion of the early Runx2+ osteoprogenitors in the mandible in mutant mandibular prominences leading to more pre-osteoblasts. However, a proportion of these pre-osteoblasts fail to mature and form mineralised bone, resulting in the micrognathic phenotype observed. In addition, they see an (indirect?) upregulation of bone resorption markers, which are proposed to cause the decreased mineralised bone volume seen in the mutants. The text is written clearly, and the conclusions are supported by the results, which are generally well-presented. The story follows on nicely from previous work in this group examining earlier cellular and molecular consequences of talpid2 mutation. In general we are enthusiastic about this story.

The authors wish to draw two major conclusions in this paper: 1) that progression of the osteogenic program is disrupted or delayed at crucial transitions, and 2) that there is excess remodelling of the bone (via osteoclast activity). While the data shown are intriguing, we feel that the evidence does not yet show this definitively. We make some specific points and suggestions below.

Reviewer 3 Comments for the Author...

Major points:

1) The assessment of key cellular populations during osteogenic progression (Fig 4) is crucial to the conclusions of this paper. The authors could improve these data by addressing the following:
a. Fig 4 – are these sections all at the same rostral-caudal location (assuming these are frontal sections) and if so, where? If not, where are the relative sections - suggest a callout in one of the previous wholemount figures.
*Originally, we had performed COL1A1 and OCN immunostaining on whole mandibles. We have now repeated these experiments on frontal sections. We have included a pentachrome stain of the frontal section at HH35 (**new Figure 5B**) to indicate plane of section through the angular and surangular bones.*

b. Col1a1 expression – which is the real staining, the puncta (very specific?) or the lawn of staining across the section? How sure are the authors that this is real staining? Perhaps a positive control (e.g. col1a1 in wt chicken long bone?) could be used. It would also help to see the comparable histology (e.g. via trichrome or pentachrome staining or even a simple H&E) so that we could orient ourselves well.
*We appreciate the reviewer comment and have added positive and negative controls from a control HH35 femur (long bone) in **new Supplemental Figure 5** to assuage Reviewer's concerns. Furthermore, we have repeated the COL1A1 staining in section (**new Fig. 5C-D**), rather than wholemount, to eliminate background staining.*

c. ALPL expression is shown at HH39 but the later more mature marker (OCN) is shown earlier at HH35. It would be helpful to see the extent of ALPL at the earlier stage as well, as authors propose that initial NC-derived mesenchyme do enter the osteogenic lineage and if OCN is already on at HH35 in the wt animals, we should also see ALPL at this stage and prior to this stage.
*We appreciate the Reviewer's comment and have added ALPL RNAscope in situ hybridization in frontal sections of HH35 mandibles for the control and talpid^2 (**new Figure 5F-G**), together with the quantitative analysis via qRT-PCR.*

d. OCN expression looks very non-specific here – how confident are the authors that this expression is real? And if it is entirely gone in the mutants, how do you explain the osteogenic progression seen in the earlier figures? That seems unlikely, so it important to show some controls and/or immune staining in other regions so we are confident of the specificity of antibody.
We appreciate the Reviewer's comment regarding the efficiency of the antibody. We have now performed OCN on a control HH35 femur (long bone) in ***new Supplemental Figure 5*** *to assuage Reviewer's concerns. We also repeated OCN staining in the frontal sections through the surangular and angular bones of the HH35 control and talpid^2 mandible (as indicated from the pentachrome stain in* ***new Fig. 5B****). We do not conclude that expression is entirely gone, as our qPCR data (****new Fig. 5K****) does show some low-level expression.*

e. qRTPCR analyses/methods need more detail, especially as we are not quite convinced by the immunos yet. Can you include variance on the controls? How were the normalisations done? Assume these ΔΔCT? If so, please state this. Best practice for ΔΔCT is to use two loading controls and for each target gene normalise to the control with a similar amplification efficiency… http://blog.mcbryan.co.uk/2013/06/qpcr-normalisation.html
As with the other reviewers that pointed this out, we appreciate the comments. Our previous analyses were not utilizing the Livak's method, only the relative standard curve for each analysis, assuming that a standard deviation smaller than 0.5 was considered good to normalize the controls to 1. We understand the implications of this in the analysis, and have now updated all the analysis for the qRT-PCR assuming that each triplicate control is an entity by itself. All the normalizations were done accordingly to Livak's paper, and an updated methodology is described in the Materials and Methods section. Note that this does not change the conclusions of the analysis in the manuscript (see blue text p. 23 lines 550-552).

2) The second key point is the idea that there is increased bone resorption/osteoclast activity in the mutants (Figure 5).
a. This again is intriguing, but the same comments hold as for Figure 4 (orientation/location of sections), some accompanying histology so that we can see structures.
*We have now updated old Figure 5 (****new Figure 6****). While these slides are within the same frontal section plane as indicated in Fig.5B, since these are high magnification images, we did not add a pentachrome section to orient the reader. Rather we described the section as being through the surangular bone.*

b. To show "remodelling" or resorptive activity, the authors should do TRAP staining on these sections to confirm this.
*We have now performed TRAP staining in HH39 sections (****new Fig 6A, B****) to show the increased TRAP staining in talpid^2 samples.*

Some minor comments:
3) Fig1
a. Posterior portion of dentary bone appears much thicker in the mutant as assessed by alizarin red staining (Figure 1B). This goes unmentioned in the text and is contrary to the main argument of the paper. Is this a tooth like structure or bone?
We agree with the Reviewer and included a sentence in results stating that although some elements in the mandible were dysplastic and have a thicker appearance, the overall length of the mandible is reduced. This is commonly seen in ciliopathic mutants" (page 6 lines 146-149).

b. Scale bars in fig 1 do not seem in agreement with mandible length measurements from the microCT analysis.
The scale bars were re-analyzed to make sure that they are all uniform.

c. Ectopic skeletal element (medially located) could be marked in figure (unclear if this is on the dentary bone or the duplicated
The ectopic skeletal element was marked in the inset in the microCT analysis as yellow, to differentiate from the endogenous splenial bone. The literature and our earlier analysis were not conclusive about the origins of this element, but ongoing research in our lab is focused in this question.

4) Fig S1 medial cartilage spurs on meckel's should be noted as well
The Reviewer has a very good eye. The medial spurs on Meckel's cartilage are a very interesting feature and are currently a topic of a separate research project in our lab.

5) Fig2
a. C+E (and figS2 A+B) box slightly unclear with opacity (reduce opacity or outline box)
We have now updated the figures to reflect the Reviewer's comment.

b. Significant decrease in proliferation of sox9-ve cells, unmentioned in text (fig2J). This should be discussed in text.
We appreciate the Reviewer's comment and have now mentioned the significant decrease in PHH3 expression in SOX9 negative cells. (page 9 line 216)

c. SOX9+ve area could be expressed as a percentage of total mnp area
We appreciate the Reviewer's comment in this regard, and we add in the text the percentage of each group, maintaining the graph as voxels to be consistent with the other analysis. (page 9 lines 213-214).

d. Also generally a description of quantification of this data, automatic/manual?
The methodology for the quantification performed in Bitplane Imaris was included in the material and methods, which is an algorithm run by the software. "For the vector rendering, images from the confocal were loaded in Bitplane iMaris and the surface algorithm was run. The selection of the surface was made by subtracting the background and adjusting the brightness until no signal was detected outside of the surface. The spots rendering for PHH3 and CC3 were adjusted for the detection of 2µm signals, and background subtraction was applied." (page 23 line 539-543)

6) Fig3
a. DE, IJ and O are hard to see, increase brightness?
*The brightness and contrast were adjusted to improve the visualization of the figure, which is now **Fig. 4**.*

b. Hand2 staining spanning midline in control not mentioned
The proximal expression of Hand2 in the control is more ectodermal than mesenchymal, thus the presence of the transcripts in the midline is expected and was also previously published (Barron et al., 2011).

7) Supplemental 3D movies (if possible) for figures 2/3 would help to clarify the spatial arrangement of staining
*We have included **Supplementary Movie 1**, that contains a 3D movie of RUNX2 expression together with SOX9 in the MNP, to demonstrate the spatial arrangement of the staining.*

8) Figs 4 and 5 – include scheme for section orientation or be clearer in Text
*We have added a pentachrome staining (**new Fig. 5B)** in order to orient the reader for subsequent molecular analyses.*

9) Figure 6 – HH stages as well as hours? Perhaps clearer to be consistent with other figures?
*We appreciate the reviewer comment and **new Fig. 7** was updated to reflect it.*

10) N numbers are for biological replicates over one experiment or multiple days?
We appreciate the Reviewer's comment regarding our analyses. The number of embryos used for the replicates come from the same batch of eggs, normally 3 for controls and 3 for mutants, unless stated otherwise in the text or the figure descriptions (see in blue p. 19-20, lines 461-462).

12) 507 and 509 – first author umlaut missing (line 507)
We have now corrected the citation.

13) 603 – need author names again?
We have now corrected the citation.

Second decision letter

MS ID#: DEVELOP/2020/194175

MS TITLE: Ciliopathic micrognathia is caused by aberrant skeletal differentiation and remodeling

AUTHORS: Christian Louis Louis Bonatto Paese, Evan C. C Brooks, Megan Aarnio-Peterson, and Samantha A Brugmann

I have received one reviewer report on the above manuscript, and have reached a decision after considering your response to the other two reviews and the revision made to the manuscript. Reviewer 2 comments and Editor's note are appended below, or you can access the reviewer comments online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the points raised can be satisfactorily addressed. Please attend to the comments in your revised manuscript and outline them in a point-by-point response.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Editor’s note:

Revision and Response to Reviewer 1 on the major concern of the relationship of formation and disassembly of the cilia and cell cycle progression are satisfactory.

Response to Reviewer 3:
Critique 10 a-d: Revision of Figure 5 and supplementary figure 5 are adequate.
Critique 1e:The relevant revised text may be page 24 line 559-560. (No further response is needed.).
Critique 2a: May also indicate in the text that the sections were taken through the surangular bone.
Critique 4: may help to mark the medial spur and add a note (as in the response) to the legend of Suppl Fig 1D, E.
Critique 5d:The relevant text may be page 23 line 547-551. (No further response is needed.)
Critique 6b:May add the response to the figure legend for clarification.
Critique 10:The relevant text may be page 20 line 465-466. (No further response is needed.)

Reviewer 2

Advance summary and potential significance to field

The article by Paese et al. entitled “Ciliopathic micrognathia is caused by aberrant skeletal differentiation and remodeling’” addresses an interesting question that is important to advance the knowledge on how micrognathia occurs in patients with ciliopathies. These discoveries could leave to the development of new treatments for these patients, which are currently very limited. The authors find changes in cell cycle progression and proliferation, an impairment in the differentiation of neural crest osteochondroprogenitors and in the maturation of osteoblasts, as well as increased bone resorption. My main impression of the article is still that is novel and provides interesting advancements to the understanding of how the jaw develops and a role for primary cilia in intramembranous bone development. Overall, I believe that the etiology of ciliopathic micrognathia described in this paper has the potential to influence future research in bone pathways and diseases, as well as craniofacial development.

Comments for the author

Overall, I think that the authors have done a very good job addressing all of the reviewers' suggestions. My specific further minor comments are below.

Minor comments:
Fig 1 and Fig S1: I still had some confusion when reading the text about Fig 1 and Fig S1 and I think I have identified the reason. I am now assuming that all of the mm measurements were taken from the alizarin red stained embryos and all the voxel measurements were taken from the uCT analyzed embryos. I think a sentence should be added for clarity either in the methods, results, or figure legends. I was trying to figure out why there was a significant volume difference between almost all the individual bones of the mandibles between the control and ta2 chicks but not when taken together. It might just be because of a difference in technique since there are no other mineralized bones in the mandible. There was also some confusion about length differences coming from reviewer #3, which might have been from the same lack of clarity. For future studies, the authors should note that they would be able to pull individual bone volumes from the uCT data to keep the data consistent.

Fig 1G: This is mislabeled as HH36, it should be HH39.

Fig 1G & 1H: It would be helpful to label the views in each for readership.

Second revision

Author response to reviewers' comments

We thank the Editor and Reviewer for their additional comments. We have made all the minor edits requested. Please see our specific point by point responses below in blue text.

Response to Editor's note (Reviewer 3):

Critique 2a: May also indicate in the text that the sections were taken through the surangular bone.
We appreciate the Editor's comment and have now included in the text which bones were analyzed (p. 11, line 264).

Critique 4: may help to mark the medial spur and add a note (as in the response) to the legend of Suppl Fig 1D, E.
We have now included black arrows in Fig S1E and a statement in the figure legend referring to the abnormal growth present in the *ta*^{2} mandibles.

Critique 6b: May add the response to the figure legend for clarification.
We have now included the description of *HAND2* expression in the midline in the Fig. 4 legend to clarify Reviewer number 3 comment.

Reviewer 2 Comments for the Author:
Overall, I think that the authors have done a very good job addressing all of the reviewers' suggestions. My specific further minor comments are below.

Minor comments:
Fig 1 and Fig S1: I still had some confusion when reading the text about Fig 1 and Fig S1 and I think I have identified the reason. I am now assuming that all of the mm measurements were taken from the alizarin red stained embryos and all the voxel measurements were taken from the uCT analyzed embryos. I think a sentence should be added for clarity either in the methods, results, or figure legends. I was trying to figure out why there was a significant volume difference between almost all

the individual bones of the mandibles between the control and ta2 chicks but not when taken together. It might just be because of a difference in technique since there are no other mineralized bones in the mandible. There was also some confusion about length differences coming from reviewer #3, which might have been from the same lack of clarity. For future studies, the authors should note that they would be able to pull individual bone volumes from the uCT data to keep the data consistent.
We appreciate the Reviewer's comment and edited the methods section for clarity (p. 20, line 482 and p. 21, line 487).

Fig 1G: This is mislabeled as HH36, it should be HH39.
We appreciate the reviewer's comment and have now fixed Fig. 1G label.

Fig 1G & 1H: It would be helpful to label the views in each for readership.
We have now included orientation (dorsal, ventral or lateral) below each microCT image, to ensure the clarity for the reader.

Third decision letter

MS ID#: DEVELOP/2020/194175

MS TITLE: Ciliopathic micrognathia is caused by aberrant skeletal differentiation and remodeling

AUTHORS: Christian Louis Bonatto Paese, Evan C Brooks, Megan Aarnio-Peterson, and Samantha A Brugmann
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.