The hedgehog co-receptor BOC differentially regulates SHH signaling during craniofacial development

Martha L. Echevarria-Andino and Benjamin L. Allen
DOI: 10.1242/dev.189076

Editor: James Briscoe

Review timeline
Original submission: 4 February 2020
Editorial decision: 2 March 2020
First revision received: 6 June 2020
Editorial decision: 29 June 2020
Second revision received: 21 September 2020
Editorial decision: 23 September 2020
Third revision received: 2 October 2020
Accepted: 6 October 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/189076

MS TITLE: The Hedgehog Co-Receptor BOC Differentially Regulates SHH Signaling During Craniofacial Development

AUTHORS: Martha Echevarria-Andino and Benjamin Allen

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. All three referees request additional analysis of the proliferation experiments that led to the conclusion that BOC decreases neural crest-derived mesenchymal proliferation. In addition, each of the referees has several constructive comments that should help improve the clarify of the manuscript. I would particularly draw your attention to the comments from Referee 2 regarding the presentation of the statistical analyses.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

Reviewer 1

Advance summary and potential significance to field

- clear characterization of phenotypes present in Gas1-/-;Boc-/- double mutants, including three Hh-responsive tissues of NT, face, and limb
- Beautiful and clear images
- well-written manuscript
- Clear connection to human conditions
- Clarifies previously conflicting results from Hh co-receptor mouse phenotypes

Comments for the author

- The study is mostly descriptive, with only new descriptions being of the Gas1-/-;Boc-/- double mutants. All single mutants, the triple Gas1-/-;Boc-/-;Cdon-/- have been described before, but this study focused on Gas1-/-;Boc-/- on an all C57B/6 background.

- The lack of phenotype present in Boc-/- does not create a compelling argument for a substantial and required role during development, it is still unclear how phenotypes only arise when it is knocked out in conjunction with a partner, such as GAS1

- The Cdo data itself is not integrated into the story, it is not included in the double mutants, so single Cdo mutants do not add support for these data

- No mechanism. The authors hint at increased proliferation in mesenchyme in Gas1-/-;Boc-/- being the cause for partial rescue of the nasal bone, and suggest that decreased proliferation is responsible for the decreased nasal bone size in Gas1-/-, but there is not adequate support of this being the predominant mechanism of action.

- Proliferation studies do not explain the 'rescue' seen in telencephalic vesicle division of Gas1-/-;Boc-/- mutants, since these embryos do not display increased proliferation in the forebrain neuroepithelium

- No molecular quantification provided. No qPCR, no RNAscope, no RNA-seq.

- Model in Figure 7 proposes cytoplasmic domain contributions and Hh-independent activity, but neither of these were tested in any of the tissues proposed in this study.

Reviewer 2

Advance summary and potential significance to field

This manuscript investigates the roles of three Hh co-receptors, Gas1, Cdon, and Boc, in craniofacial and neural development, primarily in mouse. Interesting observations are made that these co-receptors have differing roles in the face, brain, and limbs, thus demonstrating the complexity of Hh signaling regulation in development. Strikingly, loss of Boc appears to partially rescue the holoprosencephaly and a subset of the facial skeletal defects of Gas1 mutants, while combined loss of Boc and Gas1 has a synergistic effect of creating ectopic lower jawbone. The expression and genetic analyses are carefully performed, though for some quantitation and statistical analyses are lacking. The paper also nicely shows how genetic background can influence the interpretation of genetic analysis. I think this paper would be of interest toward understanding Hh complexity in developmental patterning, though I have several issues that would first need to be addressed before I would recommend it for publication.

Comments for the author

1. Several times throughout the manuscript, including in the Intro and Discussion, reference is made to a zebrafish study claiming that Boc antagonizes Hh in the lower jaw (Bergeron 2011).

These data however, are quite weak and show at low resolution in supplementary data an apparent thickening of Meckel's cartilage that is not quantified. Further, there is no direct analysis of Hh signaling in the lower jaw of zebrafish boc mutants. This is in contrast to the more convincing data of the present study. I would recommend placing less emphasis on this previous study, in particular in how it may contrast with mouse data. I would recommend either to discuss in more detail the limitations of this zebrafish study or to delete all mention of it. Otherwise, it gives the potentially erroneous impression that Boc may function differently in fish and mouse.

2. In statistical analyses throughout the manuscript, it is not clear if Bonferroni correction was applied to account for multiple comparisons. Two-tailed student's t-test is not sufficient when multiple groups are being compared. Exact p values should also be stated for each experiment, even when >0.05. There are also several instances where quantitation/statistics were not performed. Rescue of nasal bone in Gas1/Boc v. Gas1 needs statistical analysis in Fig. 5 and Fig. S7. Fig. 3E,F rescue needs statistical analysis. Cranial vault and forelimb defects in Fig. S7 require quantification and statistical analysis. In Fig. 6, need to show if Gas1/Boc proliferation is significantly different from Gas1 single mutants to show if proliferation is rescued. It is not sufficient to show lack of significant differences between Gas1/Boc and wt, as Gas1/Boc and Gas1 mutants may also not be statistically different. This could be due to low sample number.

3. In Fig. 6, it is not clear if pH3+ cells were normalized to number of cells in tissue. In other words are there less pH3+ cells in Gas1 and Cdon mutants simply due to less cells overall, or is the proportion of cells staining for pH3 affected?

4. Line 109: It should be stated where exactly the SHH source is in both tissues. Line 359: please explain in more detail source of Shh and how loss of ligand sequestration in Boc mutants could account for the different craniofacial defects in single and compound mutants. For example, do differences in the lower and upper jaw skeleton vis-a vis Boc regulation correlate with their location relative to a Shh source? Outlining in more detail how ligand sequestration could account for the opposite affects might be useful.

5. Fig. 7 should also summarize effects of Boc on neural crest mesenchyme and craniofacial epithelia relevant to craniofacial defects described.

6. Fig. 1C, Cdon expression in PCP is difficult to appreciate.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Echevarria-Andino and Allen describes the individual and combined contributions of the HH co-receptors Gas1, Cdon and Boc during brain, face, and to a lesser extent, limb development. Through a combination of genetic experiments conducted in mice and in ovo experiments conducted in chicks, the authors explore the functions of Boc both independent and dependent of Gas1 and Cdon function. In a surprising discovery, Boc by itself does not alter the expression domains of Gli1, suggesting that it does not achieve its phenotypic effects by reducing Hh pathway activity. This is distinct from the effects of Gas1 and Cdon which are also considered mediators of Hh signaling. In an interesting twist, the authors provide data indicating that loss of Boc results in wider faces, which suggests an increase in Hh signaling in the mutants. These specific results provide a cautionary note to those who propose that all signaling pathways function equivalently, regardless of tissue.
It was also interesting to note the enormous range of holoprosencephalic phenotypes related to disruptions in Hh signaling, from normal TV division and MNP separation, to incomplete TV division seen in Gas1-/- and Cdon-/- embryos (Fig. 2). Boc-/- embryos appear to be resistant to these malformations (Fig. 2), and it is not entirely clear why this is the case. This is despite the fact that the genes are co-expressed during TV and MNP development (Fig. 1). Precisely why there is such enormous variation is not completely answered here, but that should not be viewed as a criticism; rather, the authors are careful to analyze the mice on the same genetic background and thus they are pointing out the extreme variation seen amongst mice carrying mutations in these Hh

mediators. Similar extreme variations exist in patients, so it will be worthwhile to continue to explore the basis for these variations.
The greatest strength of this work rests in the near-exhaustive, carefully executed analyses on size/shape differences in Gas1, Boc, and Cdon mutants. I also found it very informative that in addition to analyzing craniofacial structures, the authors considered that the same Hh pathway mediators might function differently in other tissues. I have only minor concerns about the suitability of this work for the readers of Development; these are listed below.

Comments for the author

Minor concerns
The authors move one step closer to understanding if variations in craniofacial morphology correlate with changes in Hh pathway activity, by examining Gli1 expression. Here, the analyses are limited to in situ hybridization; it would be helpful to see if changes in gene expression levels are then translated into differences in protein expression.
The same question, whether changes in Gli1 expression seen in Gas1;Boc mutants translates to changes in protein expression, applies to Fig. 3.
The proliferation changes were only assessed using phospho-histone H3, which is a general marker of cells undergoing mitosis. It would be helpful if there were information about the phases of the cell cycle that might be impacted, as could be revealed by BrdU/EdU dual labeling. This, however, would require the generation of additional embryos and in keeping with the 3R's concerning the use of animals for research, this should be considered a suggestion and not a requirement for the current manuscript.

First revision

Author response to reviewers' comments

Thank you for the thoughtful reviews of our recent submission to *Development*. We were excited to read that the reviewers expressed considerable interest in our work. We have carefully considered the reviewers' comments and appreciate their constructive feedback. To address the reviewer's comments we pursued a number of additional experiments, revised the presentation of the statistical analyses and made changes to the text to greatly improve the quality of our manuscript and clarify our conclusions.

Specifically, major changes to the paper include:

1) Revisions to all the statistical analyses performed in the manuscript to account for multiple comparisons throughout the different datasets;

2) A revised Figure 1 that more clearly demonstrates *Cdon* expression in the prechordal plate at E8.5;

3) A revised Figure 2 that directly addresses a reviewer suggestion to quantify the effects of the individual deletion of *Gas1* and *Boc* on *Gli1* expression and GLI1 protein levels, which further validates a tissue- specific antagonist role for *Boc* during HH-dependent craniofacial development;

4) A new Supplemental Figure 6 that that quantifies the effects of the individual deletion of *Gas1* and *Boc* on *Gli1* expression and GLI1 protein levels in the developing forelimb bud, which further supports that *Boc* functions in a tissue-specific manner;

5) A revised Supplemental Figure 8 that includes additional quantitation of the nasal bone width, directly addressing a reviewer suggestion;

6) A revised Figure 6 in which we quantitate phospho-histone H3+ cells in the surface

ectoderm, forebrain neuroepithelium, and neural crest-derived mesenchyme of E10.5 forebrains normalized to the total number of DAPI+ cells in each tissue compartment;

7) A revised Figure 7, in which we include the effects BOC on the surface ectoderm of the craniofacial structures.

Together with the data from our original submission, our results demonstrate that BOC differentially regulates HH signaling during craniofacial development. The individual deletion of *Boc* at E10.5 results in facial widening and a significant increase in *Gli1* expression at E11.5 in the nasal processes. This is the first evidence that demonstrates that BOC works in opposition to GAS1 and CDON specifically during craniofacial development. Additionally, the deletion of *Boc* in a *Gas1* null background partially rescues the craniofacial defects observed in *Gas1* single mutants. The rescue of the craniofacial defects observed in *Gas1;Boc* mutants is restricted to certain craniofacial structures, while other HH responsive tissues are more severely affected in these double mutants. These findings indicate that BOC regulates HH signaling in a tissue-specific manner during mouse embryogenesis. These tissue-specific effects could be mediated by the coupled selective reduction of proliferation and the restriction of HH pathway activity mediated by *Boc*. Given, the reviewers' overall positive comments, our responses to their suggestions and the additional experimental data that we now provide, we are hopeful that you will find that the revised manuscript is sufficient to warrant publication in *Development*. We continue to believe that this work will be of wide interest to the readership of your journal and we look forward to your assessment of our revised manuscript. Please find below a point-by-point response to the reviewers' comments (*italicized*) highlighted in blue. Please also find enclosed a revised manuscript (following the *Development* manuscript preparation guidelines) with all changes to text highlighted in yellow.

Specific Responses to Reviewer #1 (reviewer comments *italicized*)

1) "Clear characterization of phenotypes present in Gas1$^{-/-}$;Boc$^{-/-}$ double mutants, including three Hh-responsive tissues of NT, face, and limb. Beautiful and clear images. Well-written manuscript. Clear connection to human conditions. Clarifies previously conflicting results from Hh co-receptor mouse phenotypes."

We thank the reviewer for these positive comments.

2) "The study is mostly descriptive, with only new descriptions being of the Gas1$^{-/-}$;Boc$^{-/-}$ double mutants. All single mutants, the triple Gas1$^{-/-}$;Boc$^{-/-}$;Cdon$^{-/-}$ have been described before, but this study focused on Gas1$^{-/-}$;Boc$^{-/-}$ on an all C57B/6 background."

The reviewer is correct that the HH co-receptor single and compound mutants have been previously published. However, these reports (Allen et al., 2007; Cole and Krauss, 2003; Seppala et al., 2007; Seppala et al., 2014; Zhang et al., 2011; Zhang et al., 2006) have examined these mutants in different genetic backgrounds. Part of what we demonstrate in this study is that the severity of the craniofacial defects observed in the HH co- receptor mutants is dependent on the genetic background of the mouse model. Among other findings, our data revealed that, even within the same genetic background, at E10.5 *Boc* mutant embryos display facial widening, while *Gas1* and *Cdon* display significantly variable HPE phenotypes. Further, and as the reviewer noted, we also describe a novel craniofacial phenotype in *Gas1;Boc* double mutant embryos that are consistent with *Boc* functioning as a tissue-specific HH pathway antagonist.

We agree with the reviewer that additional mechanistic insight would be helpful. To address this concern, we now include two new pieces of data quantifying changes in the level of the general HH pathway target, *Gli1*, across multiple tissues. For more details, please see our response below to comment #7 from Reviewer 1.

3) "The lack of phenotype present in Boc$^{-/-}$ does not create a compelling argument for a substantial and required role during development, it is still unclear how phenotypes only arise when it is knocked out in conjunction with a partner, such as GAS1."

Our data indicate that *Boc*$^{-/-}$ embryos do exhibit a phenotype, namely a significant widening of the medial nasal process phenotype at E10.5. Importantly (and as described in more detail below), we

now also demonstrate increased *Gli1* levels in *Boc* mutant embryos (see Figure 2N). *Boc* mutants also display axon guidance defects in SHH-dependent commissural axon guidance (Okada et al., 2006). Further, we have previously demonstrated functional redundancy between *Gas1*, *Cdon* and *Boc*, which together are required to transduce HH signals (Allen et al., 2011). These data demonstrates that, while BOC alone is not required during mouse embryogenesis, it does function to regulate HH signaling in multiple tissues. Finally, there is a substantial body of evidence demonstrating overlapping functions for other cell surface HH pathway components, (e.g., *Hhip* and *Ptch2*) which also function redundantly, and whose roles are only revealed when these genes are deleted in combination with the loss of the canonical HH receptor and pathway antagonist, *Ptch1* (Holtz et al., 2015; Holtz et al., 2013; Jeong and McMahon, 2005).

4) "The Cdo data itself is not integrated into the story, it is not included in the double mutants, so single Cdo mutants do not add support for these data."

While we agree with the reviewer that the *Cdon* data are not integrated into the double mutant analyses, we respectfully disagree that the data are not integrated into the story. For example, in Figure 1, the differences in *Gas1*, *Cdon* and *Boc* expression, in particular the much broader ventral expression of *Boc* compared to *Cdon*, set the stage for investigating potential differences in the contribution of these co-receptors to HH-dependent craniofacial development. In particular, we now include new data in Figure 1 clarifying the unique expression of *Cdon* in the prechordal plate (For more details, please see our response below to comment #12 from Reviewer 2). Further, in Figure 2, our data demonstrate significant phenotypic differences between *Cdon* and *Boc* mutants, despite their structural similarities, and similar capabilities to bind to SHH ligand and promote HH signaling. Finally, in Figure 6, we demonstrate differential contributions of CDON and BOC to cranial neural crest-derived mesenchyme proliferation. These direct comparisons of *Cdon* and *Boc* mutant phenotypes on congenic backgrounds provide significant support for the distinct and tissue-specific roles of these co-receptors that comprise a major finding of our paper.

5) "No mechanism. The authors hint at increased proliferation in mesenchyme in $Gas1^{-/-}$;$Boc^{-/-}$ being the cause for partial rescue of the nasal bone, and suggest that decreased proliferation is responsible for the decreased nasal bone size in $Gas1^{-/-}$, but there is not adequate support of this being the predominant mechanism of action.

We agree with the reviewer that the increased mesenchymal proliferation following *Boc* deletion, while one possible mechanism, is not necessarily the primary cause of the phenotypic rescue in *Gas1;Boc* double mutants. Therefore, we have revised the text and tempered our conclusions appropriately to clarify this issue. We propose multiple potential mechanisms (Figure 7) that could explain this phenotype; however, a full exploration of these possibilities will require significant further investigation, that will likely constitute multiple additional papers.

6) "Proliferation studies do not explain the 'rescue' seen in telencephalic vesicle division of $Gas1^{-/-}$;$Boc^{-/-}$ mutants, since these embryos do not display increased proliferation in the forebrain neuroepithelium"

As indicated in the our response above, we have tempered our conclusions to indicate that increased proliferation is not the only mechanism responsible for the rescue observed in *Gas1;Boc* mutants. We only examined the telencephalic vesicle division morphologically at E10.5; at later stages of development we observed that rescue is limited to the nasal bone and nasal capsule of these embryos. We did not formally examine the division of the brain at later stages. We have clarified our discussion to emphasize the multifunctional role of BOC in these different tissues (Lines #334-339).

7) "No molecular quantification provided. No qPCR, no RNAscope, no RNA-seq."

We agree with the reviewer that additional molecular quantitation is important. To address this concern, we examined *Gli1* expression by qRT-PCR and GLI1 protein levels by western blot analysis. Specifically, we micro-dissected nasal processes (removing the forebrain neuroepithelium, and keeping the olfactory epithelium) (Fig. 2M-N) and forelimb buds (Fig. S6) from E11.5 wildtype, $Gas1^{-/-}$ and $Boc^{-/-}$ mutant embryos. Our qRT-PCR results are consistent with the whole mount *in situ* hybridization data (Fig. 3, Fig. S4), demonstrating that *Gas1* mutants have

a significant reduction in *Gli1* expression in nasal processes and forelimb buds (Fig. 2N and Fig. S6A). To our surprise the qRT-PCR also revealed that *Boc* mutants have a significant, increase in *Gli1* expression consistent with the widening of the medial nasal process at E10.5 (Fig. 2N). Notably, this increase in *Gli1* expression is restricted to the nasal processes, as forelimb buds display normal levels of *Gli1* (Fig. S6A). Additionally, our protein expression analysis has revealed that *Gas1*$^{-/-}$ embryos exhibit decreased GLI1 protein expression in the nasal processes, while *Boc*$^{-/-}$ embryos maintain normal GLI1 protein expression (Fig. 2O, Fig. S3G). Notably, this difference is less apparent in the forelimb buds where the levels of GLI1 protein are slightly reduced in *Gas1* mutants and seem to remain unchanged in *Boc* mutants (Fig. S6B-C). Unfortunately, we were not able to collect *Gas1*$^{-/-}$;*Boc*$^{-/-}$ double mutants. Further experiments will be required to determine how the simultaneous deletion of GAS1 and BOC alter the levels of GLI1 protein. Regardless, these new findings further confirm that BOC selectively antagonizes HH signaling during craniofacial development. We thank the reviewer for this important and helpful suggestion.

8) "Model in Figure 7 proposes cytoplasmic domain contributions and Hh-independent activity, but neither of these were tested in any of the tissues proposed in this study."

The reviewer is correct that our model (Figure 7) proposes potential contributions of BOC that we do not experimentally address in our current study. We included this model to place our work in the context of other studies, and to fully consider potential alternative mechanisms (as the Reviewer appropriately noted in comment # 2 above) to explain the tissue-specific contributions of BOC to HH-dependent patterning. We have modified our discussion to clarify this point (Lines #385-387).

Specific Responses to Reviewer #2 (reviewer comments *italicized*)
1) "This manuscript investigates the roles of three Hh co-receptors, Gas1, Cdon, and Boc, in craniofacial and neural development, primarily in mouse. Interesting observations are made that these co-receptors have differing roles in the face, brain, and limbs, thus demonstrating the complexity of Hh signaling regulation in development. Strikingly, loss of Boc appears to partially rescue the holoprosencephaly and a subset of the facial skeletal defects of Gas1 mutants, while combined loss of Boc and Gas1 has a synergistic effect of creating ectopic lower jawbone. The expression and genetic analyses are carefully performed, though for some quantitation and statistical analyses are lacking. The paper also nicely shows how genetic background can influence the interpretation of genetic analysis. I think this paper would be of interest toward understanding Hh complexity in developmental patterning, though I have several issues that would first need to be addressed before I would recommend it for publication."

We thank the reviewer for these positive comments.

2) "Several times throughout the manuscript, including in the Intro and Discussion, reference is made to a zebrafish study claiming that Boc antagonizes Hh in the lower jaw (Bergeron 2011). These data, however, are quite weak and show at low resolution in supplementary data an apparent thickening of Meckel's cartilage that is not quantified. Further, there is no direct analysis of Hh signaling in the lower jaw of zebrafish boc mutants. This is in contrast to the more convincing data of the present study. I would recommend placing less emphasis on this previous study, in particular in how it may contrast with mouse data. I would recommend either to discuss in more detail the limitations of this zebrafish study or to delete all mention of it. Otherwise, it gives the potentially erroneous impression that Boc may function differently in fish and mouse."

We thank the reviewer for this suggestion. We have revised our text as suggested removing it from the introduction. We did feel it was important to keep our reference to this work in the discussion; however, we now include a discussion of the limitations of this study, as the reviewer suggested (Lines # 316-320)

3) "In statistical analyses throughout the manuscript, it is not clear if Bonferroni correction was applied to account for multiple comparisons. Two-tailed student's t-test is not sufficient when multiple groups are being compared. Exact p values should also be stated for each experiment, even when >0.05. There are also several instances where quantitation/statistics were not performed.

We thank the reviewer for this important suggestion. To perform the appropriate statistical analyses we met with staff of the University of Michigan Consulting for Statistics, Computing and Analytics Research (CSCAR). Following the suggestions of the Reviewer and CSCAR, we decided to determine the p-values using two-tailed *Student's t - tests*, with the Bonferroni correction to account for multiple comparisons in each dataset. The adjusted p-values for each dataset are described in the figure legends of their respective figure. We also evaluated the possibility to perform a one-way ANOVA with the Bonferroni correction post hoc. However, some of our datasets do not meet the criteria of having normal distribution to perform this test. To be consistent thought the manuscript, we employed the Bonferroni correction similarly in all of our datasets. With this method we should obtain similar results as performing a one-way ANOVA. We have also included the exact p- value for each comparison, even when they are not statistically significant. We also respond individually to each instance in which we do not perform quantitation/statistics. Please see all the comments and their respective responses below.

4) "Rescue of nasal bone in Gas1/Boc v. Gas1 needs statistical analysis in Fig. 5 and Fig. S7."

To address this suggestion, we measured the nasal bone width in E18.5 wildtype, *Gas1*$^{-/-}$, *Boc*$^{-/-}$ and *Gas1*$^{-/-}$*;Boc*$^{-/-}$ embryos and included the quantitation in Fig. S8E-F. Even though the nasal bone width in *Gas*$^{-/-}$*;Boc*$^{-/-}$ fails to reach statistical significance, we consistently observe that the patterning of this bone is ameliorated in *Gas1;Boc* double mutants. We now include this information in the Results (Lines #254-256).

5) "Fig. 3E,F rescue needs statistical analysis."

To perform statistical analyses in the these type of data sets, which display the frequencies of the telencephalic division and medial nasal processes separation in our mutants, we need to utilize Chi-Square or Fisher's exact tests. These types of tests are designed to analyze contingency tables that usually have a large number of samples. The Chi-Square is extremely sensitive to sample number and is not recommended to use it when the sample number is less than 5. Thus, we have to disregard this test since we have some categories with less than 5 embryos. On the other hand, the Fisher's exact test can be used when the sample sizes are small and it only evaluates the differences of just two variables. Ideally we would use the Fisher's exact test to compare our data, however this test will not allow us to compare the multiple phenotypes observed in our mutants. Additionally, in our consultation with CSCAR, we were advised that our sample numbers are too to establish statistical significant differences. These types of tests when the sample numbers are too small provide inaccurate results.
Even though we have collected a considerable number of mutants, these are not enough to establish statistically significant differences. Due to the high variability in our phenotypes we would have to collect many additional embryos to establish statistical differences. This would fall well outside the time period allotted for revisions, and so we propose to retain our original presentation our data as frequencies, which shows the entire phenotypic spectrum that we observe in our mutants without drawing conclusions regarding statistical significance.

6) "Cranial vault and forelimb defects in Fig. S7 require quantification and statistical analysis."

The purpose of including these data in the manuscript is to strengthen our conclusions about the tissue- specific roles of BOC during vertebrate embryogenesis. Our data demonstrates that the rescue of the craniofacial defects in *Gas1;Boc* mutants is restricted specifically to the nasal bone and nasal capsule, consistent with the increased levels of *Gli1* in the nasal processes of *Boc*$^{-/-}$. By showing how severely affected are other craniofacial structures and other HH responsive tissues such as the limb we demonstrate the specificity of the rescue and the tissue-specific roles of BOC. While this is an important point, the characterization and quantitation of the defects in the cranial vault and forelimb defects fall outside the scope of this manuscript.

7) "In Fig. 6, need to show if Gas1/Boc proliferation is significantly different from Gas1 single mutants to show if proliferation is rescued. It is not sufficient to show lack of significant differences between Gas1/Boc and wt, as Gas1/Boc and Gas1 mutants may also not be statistically different. This could be due to low sample number."

We agree with the reviewer, and have revised the statistical analyses performed in Fig. 6 to

include *Gas1* vs. *Gas1;Boc* comparisons (Fig. 6F-H) across all the datasets. Our results show that *Gas1* and *Gas1;Boc* mutants do no display any statistically significant differences in the proliferation of the surface ectoderm, forebrain neuroepeithelium and/or mesenchyme. We agree with the reviewer, this could be due to low sample number. However, we were not able to collect more *Gas1;Boc* embryos to add to our quantifications. Based on our results of the proliferation quantitation in the mesenchyme of the craniofacial structures, *Gas1;Boc* embryos exhibit a higher mean (17.4) vs. *Gas1* (14.5) of phospho-histone H3+ cells. We predict that with more samples this trend will be consistent. Even, though this is not statistically significant we consider that this increase in proliferation contributes to the rescue of the craniofacial defects observed in *Gas1;Boc* mutants (Lines #270-274) .

8) "In Fig. 6, it is not clear if pH3+ cells were normalized to number of cells in tissue. In other words, are there less pH3+ cells in Gas1 and Cdon mutants simply due to less cells overall, or is the proportion of cells staining for pH3 affected?"

In our initial analysis we did not normalized the phospo-histone H3 + cells to the total number of cells in the tissue. We thank the reviewer for this suggestion and have revised our quantitation accordingly. We isolated the forebrain neuroepithelum and the nasal processes mesenchyme and surface ectoderm in our images to perform the unbiased quantification of phospho-histone H3+ cells using the analyze particles tool of Image J, and also quantified the number of DAPI+ cells in each region. After revising our quantitation, our data indicate that the proportion of phospho-histone H3+ cells is not affected in our different mutants. Notably, our conclusion about *Boc* and *Gas1;Boc* mutants remains the same, these embryos exhibit increased proliferation specifically in the neural crest-derived mesenchyme in comparison to wildtype embryos. Our data also have revealed that *Gas1* mutants exhibit a slight increase in proliferation, however it fails to be statistically significant.

9) "Line 109: It should be stated where exactly the SHH source is in both tissues.

We have revised the text to indicate that the expression of BOC extends closer to the ventral neuroepithelium in the forebrain and closer to the notochord and floor plate in the neural tube (Lines #106-109).

10) Line 359: please explain in more detail source of Shh and how loss of ligand sequestration in Boc mutants could account for the different craniofacial defects in single and compound mutants. For example, do differences in the lower and upper jaw skeleton vis-a vis Boc regulation correlate with their location relative to a Shh source? Outlining in more detail how ligand sequestration could account for the opposite affects might be useful."

We have revised the text to discuss how the loss of *Boc* could modulate the distribution of SHH protein in the surface ectoderm of the medial nasal process, contributing to the phenotypic differences observed in single and compound mutants. (Lines #376-380). We thank the reviewer for this suggestion to improve the explanation of our model in the discussion.

11) "Fig. 7 should also summarize effects of Boc on neural crest mesenchyme and craniofacial epithelia relevant to craniofacial defects described."

We thank the reviewer for their suggestion, and have modified Figure 7 as requested to include effects on craniofacial epithelia (Fig 7, lines #353-354). Regarding the neural crest-derived mesenchyme, since we did not formally explore the effects of *Boc* deletion on neural crest-derived mesenchyme compared to non-neural crest mensenchyme, we decided to keep our general reference to forebrain mesenchyme.

12) "Fig. 1C, Cdon expression in PCP is difficult to appreciate."

We agree with Reviewer #2, and now include a Revised Figure 1, that includes insets in panels A-D showing a dorsal view of E8.5 embryos that highlights *Cdon* expression in the prechordal plate. We thank the reviewer for this helpful suggestion.

Specific Responses to Reviewer #3 (reviewer comments *italicized*)

1) "The manuscript by Echevarria-Andino and Allen describes the individual and combined contributions of the HH co-receptors Gas1, Cdon and Boc during brain, face, and to a lesser extent, limb development. Through a combination of genetic experiments conducted in mice, and in ovo experiments conducted in chicks, the authors explore the functions of Boc both independent and dependent of Gas1 and Cdon function. In a surprising discovery, Boc by itself does not alter the expression domains of Gli1, suggesting that it does not achieve its phenotypic effects by reducing Hh pathway activity. This is distinct from the effects of Gas1 and Cdon which are also considered mediators of Hh signaling. In an interesting twist, the authors provide data indicating that loss of Boc results in wider faces, which suggests an increase in Hh signaling in the mutants. These specific results provide a cautionary note to those who propose that all signaling pathways function equivalently, regardless of tissue. It was also interesting to note the enormous range of holoprosencephalic phenotypes related to disruptions in Hh signaling, from normal TV division and MNP separation, to incomplete TV division seen in Gas1$^{-/-}$ and Cdon$^{-/-}$ embryos (Fig. 2). Boc$^{-/-}$ embryos appear to be resistant to these malformations (Fig. 2), and it is not entirely clear why this is the case. This is despite the fact that the genes are co-expressed during TV and MNP development (Fig. 1). Precisely why there is such enormous variation is not completely answered here, but that should not be viewed as a criticism; rather, the authors are careful to analyze the mice on the same genetic background and thus they are pointing out the extreme variation seen amongst mice carrying mutations in these Hh mediators. Similar extreme variations exist in patients, so it will be worthwhile to continue to explore the basis for these variations. The greatest strength of this work rests in the near-exhaustive, carefully executed analyses on size/shape differences in Gas1, Boc, and Cdon mutants. I also found it very informative that in addition to analyzing craniofacial structures, the authors considered that the same Hh pathway mediators might function differently in other tissues. I have only minor concerns about the suitability of this work for the readers of Development; these are listed below."

We thank the reviewer for these positive comments.

"Minor concerns. The authors move one step closer to understanding if variations in craniofacial morphology correlate with changes in Hh pathway activity, by examining Gli1 expression. Here, the analyses are limited to in situ hybridization; it would be helpful to see if changes in gene expression levels are then translated into differences in protein expression. The same question, whether changes in Gli1 expression seen in Gas1;Boc mutants translates to changes in protein expression, applies to Fig. 3.

We agree with the reviewer and, as noted above (see comment #7 from Reviewer 1), we now include qPCR and western blot data, which further supports our comparison of *Gas1* and *Boc* single mutants.
Unfortunately, we were not able to collect enough *Gas1;Boc* double mutants to complete this analysis. We thank the reviewer for their helpful suggestion.

2) The proliferation changes were only assessed using phospho-histone H3, which is a general marker of cells undergoing mitosis. It would be helpful if there were information about the phases of the cell cycle that might be impacted, as could be revealed by BrdU/EdU dual labeling. This, however, would require the generation of additional embryos and in keeping with the 3R's concerning the use of animals for research, this should be considered a suggestion and not a requirement for the current manuscript."

We thank the reviewer for this suggestion, and agree that additional EdU analysis would be helpful. However, due to experimental restrictions on animal work at the University of Michigan related to COVID-19, we were not allowed to perform these additional experiments. Given the reviewers acknowledgement of limiting the use of animals for research, we hope this will not diminish their enthusiasm for our work.

References

Allen, B. L., Song, J. Y., Izzi, L., Althaus, I. W., Kang, J. S., Charron, F., Krauss, R. S. and McMahon, A. P. (2011). Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. *Dev Cell* 20, 775-787.
Allen, B. L., Tenzen, T. and McMahon, A. P. (2007). The Hedgehog-binding proteins Gas1 and Cdo

cooperate to positively regulate Shh signaling during mouse development. *Genes Dev* **21**, 1244-1257.
Cole, F. and Krauss, R. S. (2003). Microform holoprosencephaly in mice that lack the Ig superfamily member Cdon. *Curr Biol* **13**, 411-415.
Holtz, A. M., Griffiths, S. C., Davis, S. J., Bishop, B., Siebold, C. and Allen, B. L. (2015). Secreted HHIP1 interacts with heparan sulfate and regulates Hedgehog ligand localization and function. *J Cell Biol* **209**, 739-757.
Holtz, A. M., Peterson, K. A., Nishi, Y., Morin, S., Song, J. Y., Charron, F., McMahon, A. P. and Allen, B. L. (2013). Essential role for ligand-dependent feedback antagonism of vertebrate hedgehog signaling by PTCH1, PTCH2 and HHIP1 during neural patterning. *Development* 140, 3423-3434.
Jeong, J. and McMahon, A. P. (2005). Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. *Development* **132**, 143-154.
Okada, A., Charron, F., Morin, S., Shin, D. S., Wong, K., Fabre, P. J., Tessier-Lavigne, M. and McConnell, S. K. (2006). Boc is a receptor for sonic hedgehog in the guidance of commissural axons. *Nature* **444**, 369-373.
Seppala, M., Depew, M. J., Martinelli, D. C., Fan, C. M., Sharpe, P. T. and Cobourne, M. T. (2007). Gas1 is a modifier for holoprosencephaly and genetically interacts with sonic hedgehog. *J Clin Invest* **117**, 1575-1584.
Seppala, M., Xavier, G. M., Fan, C. M. and Cobourne, M. T. (2014). Boc modifies the spectrum of holoprosencephaly in the absence of Gas1 function. *Biol Open* **3**, 728-740.
Zhang, W., Hong, M., Bae, G. U., Kang, J. S. and Krauss, R. S. (2011). Boc modifies the holoprosencephaly spectrum of Cdo mutant mice. *Dis Model Mech* **4**, 368-380.
Zhang, W., Kang, J. S., Cole, F., Yi, M. J. and Krauss, R. S. (2006). Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly. *Dev Cell* **10**, 657-665.

Second decision letter

MS ID#: DEVELOP/2020/189076

MS TITLE: The Hedgehog Co-Receptor BOC Differentially Regulates SHH Signaling During Craniofacial Development

AUTHORS: Martha Echevarria-Andino and Benjamin Allen

I have now received 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 continue to express interest in your work, but Referee 2 has significant criticisms that need to be addressed before we can consider publication. All the referees recognise the valuable and careful mutant analyses you are reporting, however, Referee 2 is concerned that the conclusion that craniofacial defects are rescued in the double mutant and that this is the result of increased cell proliferation are not adequately supported by the data. Further data or analysis would be needed to support these conclusions, or the claims would need to be removed. As these revisions will alter the conclusions of the study, or involve substantial new data, I am afraid I have no choice other than to indicate that a major revision is required .

If you are able to address the issues raised by Referee 2, 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.

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

This study examines the role of GAS1, CDON and BOC during craniofacial development.Boc deletion in a Gas1 null background generates a tissue-specific partial rescue of the craniofacial defects observed in Gas1 single mutants. This contrasts with HH-dependent phenotypes in other tissues that significantly worsen following combined deletion of Gas1 and Boc. Mechanistically, BOC selectively restricts neural crest-derived mesenchymal proliferation. Together, these data indicate that BOC acts as a multi-functional regulator of HH signaling during craniofacial development, alternately promoting or restraining HH pathway activity in a tissue-specific fashion.

Comments for the author

Authors comprehensively addressed all reviewers comments.

Reviewer 2

Advance summary and potential significance to field

The authors show a subtle increase in internasal width in single Boc mutants suggesting a different effect of Boc mutations in this part of the face compared to the rest of the body. However, the extent to which Boc loss rescues Gas1 craniofacial defects is less clear.

Comments for the author

This revision decreases my enthusiasm for the study as the new statistical analysis does not clearly support loss of Boc rescuing the craniofacial or brain defects of Gas1 mutants. In particular, the statistical analysis in Fig. S8F shows a wide range of nasal bone width in Gas1 mutants, with Gas1/Boc double mutants falling in the middle of the range of Gas1 mutants with no statistical difference. Other phenotypes that are claimed to be rescued are still not supported by statistics. In Fig. 3E, Gas1/Boc double mutants have 31% normal division compared to 12% for Gas1, but on the other hand Gas1/Boc have 38% no division compared to 12% for Gas1. Similar concerns exist for the MNP separation phenotypes in Fig. 3F.
As the authors point out in the response, the n numbers are low which preclude statistical tests, but this does not mean that rescue can still be claimed. For mesenchymal proliferation, Gas1/Boc double mutants are no different than Gas1 mutants, again not supporting rescue or increased proliferation being the cause of rescue. What we are left with is a very subtle increase in Gli1 RNA (but not protein) levels in Boc mutants on a C57BL/6J background, which corresponds to an equally subtle increase in internasal distance. However, the data supporting a role for Boc rescuing craniofacial defects of Gas1 are not strong enough for publication, and the subtle increase in internasal width in the single Boc mutants seems a marginal advance for Development given the extensive previous analysis of combinatorial Gas1/Boc/Cdon mutants.

Second revision

Author response to reviewers' comments

Specific Responses to Reviewer #1 (reviewer comments *italicized*; author responses highlighted in blue):
"This study examines the role of GAS1, CDON and BOC during craniofacial development. Boc deletion in a Gas1 null background generates a tissue-specific partial rescue of the craniofacial defects observed in Gas1 single mutants. This contrasts with HH-dependent phenotypes in other tissues that significantly worsen following combined deletion of Gas1 and Boc. Mechanistically, BOC selectively restricts neural crest-derived mesenchymal proliferation. Together, these data indicate that BOC acts as a multi-functional regulator of HH signaling during craniofacial development, alternately promoting or restraining HH pathway activity in a tissue- specific fashion.

Authors comprehensively addressed all reviewers comments."

We thank the reviewer for these positive comments.

Specific Responses to Reviewer #2 (reviewer comments *italicized*):
"The authors show a subtle increase in internasal width in single Boc mutants, suggesting a different effect of Boc mutations in this part of the face compared to the rest of the body. However, the extent to which Boc loss rescues Gas1 craniofacial defects is less clear.

This revision decreases my enthusiasm for the study as the new statistical analysis does not clearly support loss of Boc rescuing the craniofacial or brain defects of Gas1 mutants. In particular, the statistical analysis in Fig. S8F shows a wide range of nasal bone width in Gas1 mutants, with Gas1/Boc double mutants falling in the middle of the range of Gas1 mutants with no statistical difference. Other phenotypes that are claimed to be rescued are still not supported by statistics. In Fig. 3E, Gas1/Boc double mutants have 31% normal division compared to 12% for Gas1, but on the other hand Gas1/Boc have 38% no division compared to 12% for Gas1. Similar concerns exist for the MNP separation phenotypes in Fig. 3F. As the authors point out in the response, the n numbers are low which preclude statistical tests, but this does not mean that rescue can still be claimed.

We thank the reviewer for their critical assessment of our data, as it is both correct, and highlights a flaw in the communication of our results. Specifically, in our previous manuscript we did not adequately highlight the significant phenotypic differences that we observe between *Gas1* mutants and *Gas1;Boc* double mutants. To address this flaw, we have generated a new supplemental figure (Supplemental Figure 9) that quantifies five different phenotypic differences between these embryos at E18.5. Specifically, we demonstrate the following:

1) *Gas1;Boc* mutants have a significantly (p= 0.0102) reduced head width when compared with *Gas1* mutants;

2) When normalized to head width, *Gas1;Boc* mutants display a significantly (p= 0.0228) increased interocular distance compared to *Gas1* mutants;

3) *Gas1;Boc* mutants also display a significantly increased snout width (p= 0.0451) compared to *Gas1* mutants;

4) Examination of the frequency of a single nostril versus two nostrils reveals a qualitative phenotypic difference, where all *Gas1;Boc* mutants have two partially fused nostrils (8/8). In comparison, a subset of *Gas1* mutants (5/12) present with a single nostril;

5) Finally, analysis of the medial lip notch distance reveals a statistically significant increase (p

= 0.013) in *Gas1;Boc* mutants compared to *Gas1* mutants.

Notably, these data are all consistent with the notion that *Boc* deletion partially rescues the craniofacial phenotypes observed in *Gas1* mutants, and are consistent with the data that we present throughout the manuscript. We believe that these additional analyses, which demonstrate statistically significant improvements in the craniofacial phenotypes of *Gas1;Boc* double mutants compared to *Gas1* mutants, will adequately address the reviewer's concerns.

For mesenchymal proliferation, Gas1/Boc double mutants are no different than Gas1 mutants, again not supporting rescue or increased proliferation being the cause of rescue.

The reviewer again raises an important point. While we do not detect significant differences in mesenchymal proliferation between *Gas1* single mutants and *Gas1;Boc* double mutants, we do observe a significant increase in mesenchymal proliferation between *Boc* mutants and wildtype embryos ($p = 0.0028$; Figure 6H), which also display increased *Gli1* expression and increased facial widening. Notably, mesenchymal proliferation is also increased in *Gas1* single mutants compared to wildtype embryos, although this does not quite reach statistical significance. However, this does raise the issue of whether increased mesenchymal proliferation is the mechanism to explain the phenotypic rescue that we observe. In fact, we do not think this is the case, and so we spend significant time in the discussion considering alternative possibilities. For example, we discuss the broader domain of *Boc* expression and its potential regulation of SHH ligand distribution. We also consider the unique cytoplasmic domain that BOC possesses, and its potential functional contribution. Notably, we summarize what we believe to be the most likely contributions of BOC to HH signal transduction in Figure 7B.

What we are left with is a very subtle increase in Gli1 RNA (but not protein) levels in Boc mutants on a C57BL/6J background, which corresponds to an equally subtle increase in internasal distance. However, the data supporting a role for Boc rescuing craniofacial defects of Gas1 are not strong enough for publication, and the subtle increase in internasal width in the single Boc mutants seems a marginal advance for Development given the extensive previous analysis of combinatorial Gas1/Boc/Cdon mutants."

We respectfully disagree with the reviewer's assessment of the totality of the work presented in the manuscript. Specifically, our work provides the following novel findings:

1) A comprehensive temporal comparison of *Gas1*, *Cdon* and *Boc* expression during the onset of craniofacial development, where we discovered that, in contrast to *Gas1* and *Cdon*, *Boc* is more broadly expressed and more closely expressed to the source of SHH than either of these two co-receptors in multiple tissues during craniofacial development;

2) A careful phenotypic analysis of *Gas1*, *Cdon* and *Boc* single mutants on a congenic C57BL/6J genetic background, where we made two key findings, namely that *Gas1* and *Cdon* single mutants display variable and significant holoprosencephaly phenotypes, while *Boc* deletion does not result in any overt holoprosencephaly phenotype;

3) Instead, *Boc* mutants display a significant increase in facial widening and a significant *increase* in Hedgehog pathway activity as measured by the direct transcriptional target, *Gli1*; conversely, *Gas1* and *Cdon* mutants display significantly decreased internasal distance, and *Gas1* mutants exhibit significantly *decreased Gli1* expression;

4) Combined deletion of *Gas1* and *Boc* results in an amelioration of the holoprosencephalic phenotype observed in *Gas1* single mutants, and correlates with increased *Gli1* expression levels, consistent with the notion of *Boc* as a tissue-specific Hedgehog pathway antagonist;

5) Notably, in the same *Gas1;Boc* mutant embryos that display increased Hedgehog pathway activity in developing craniofacial structures, we observe reduced Hedgehog pathway activity in other Hedgehog- dependent tissues, including the limb bud and the neural tube;

6) Analysis of Hedgehog-dependent neural patterning in the forebrain and the neural tube demonstrate differential contributions of *Boc* to these two neuroepithelia;

7) Importantly, the rescue of the craniofacial structure phenotypes in *Gas1;Boc* mutant embryos is maintained over developmental time (from E10.5 to E18.5), highlighting both the important contribution of *Boc* to proper craniofacial development, and its differential contribution to distinct structures of the face;

8) Finally, we present evidence that *Boc* selectively restricts neural crest-derived mesenchymal cell proliferation, identifying a potential mechanism for the distinct and tissue-specific effects of *Boc* deletion on craniofacial development.

Third decision letter

MS ID#: DEVELOP/2020/189076

MS TITLE: The Hedgehog Co-Receptor BOC Differentially Regulates SHH Signaling During Craniofacial Development

AUTHORS: Martha Echevarria-Andino and Benjamin Allen

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referee's comments can be satisfactorily addressed. As you will see Reviewer 2 recognises the new data strengthen the evidence for rescue of Gas1 phenotypes after Boc loss. Nevertheless, the reviewer is still concerned that the data do not indicate improved nasal bone morphology and asks for changes to the text to clarify this. The reviewer also has helpful suggestions for improving the presentation and interpretation of the data in Fig S9.Please attend to all of the reviewer's comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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.

Reviewer 2

Advance summary and potential significance to field

I appreciate the explanation provided by the authors for why this study is significant and I admit to perhaps having been too harsh in this regard in my previous review. My main problems in the previous version were the lack of data supporting rescue of Gas1 by Boc loss. The new Fig. S9 now provides key quantitative data supporting partial rescue of a subset of Gas1 craniofacial phenotypes by loss of Boc.

Comments for the author

I have a few remaining issues that should be addressed, but otherwise now support publication.

1. I still do not understand how the authors can claim that the nasal bone is partially restored in Gas1/Boc double mutants. Again, the images in Fig. 5M'-P' do not seem representative as in Fig. S8A-D the Gas1 mutant examples shown are either normal nasal bone or absent nasal bone, and the Gas1/Boc double mutant examples both represent reduced nasal bone. The quantitation of nasal bone width shows Gas1/Boc nasal bones to be intermediate in the spectrum of nasal bone phenotypes in Gas1 single mutants. I do not see anywhere evidence for "improved nasal bone morphology". The following two sections quoted below should be modified to not imply rescue of the nasal bone in Gas1/Boc double mutants. The claims of other craniofacial phenotypic rescue should solely be based on the data in the new Fig. S9.

Lines 250-255: "The 3D reconstructions indicated that the nasal bone in Gas1-/-;Boc-/- mutants is partially restored compared to Gas1-/- mutants where this bone is smaller and fragmented (Fig. 5N', P'). As we observed at E10.5 (Fig. 2), there is a spectrum of HPE phenotypes in Gas1 mutants (Fig. S8A-D); while quantitation does not reveal significant differences in nasal bone width between Gas1 and Gas1;Boc mutants, we do observe improved nasal bone morphology in Gas1;Boc mutants (Fig. S8A'-D', E-F).

Lines 343-344: "Specifically, Gas1;Boc double mutants display increased MNP separation at E10.5, and restoration of the nasal capsule and nasal bone at E18.5."

2. In the new Fig. S9, it is difficult to appreciate whether phenotypes are improved or worsened in the Gas1/Boc double vs. Gas1 single mutants as no quantitation is shown for wild-type controls. It is essential to show quantitation in wild-type controls for each category, and ideally Boc single mutants if the data exist. Without controls, it cannot be stated whether double mutants enhance or rescue the stated phenotypes.

3. Statistics should be performed for rescue of nostrils frequency in new Fig. S9E. I believe a Fisher Exact Test is most appropriate, which in this case would result in p=0.0547. This reviewer does not believe that p<0.05 should be a magic threshold, so it would seem that such a p value could still be discussed as likely partial rescue.

4. I wonder if the title should be modified a bit. "Differentially" compared to what? Gas1 and Cdon? Or to different parts of the face?

"The Hedgehog Co-Receptor BOC Differentially Regulates SHH Signaling During Craniofacial Development"

Third revision

Author response to reviewers' comments

Specific Responses to Reviewer #2 (reviewer comments *italicized*):
I appreciate the explanation provided by the authors for why this study is significant and I admit to perhaps having been too harsh in this regard in my previous review. My main problems in the previous version were the lack of data supporting rescue of Gas1 by Boc loss. The new Fig. S9 now provides key quantitative data supporting partial rescue of a subset of Gas1 craniofacial phenotypes by loss of Boc.

I have a few remaining issues that should be addressed, but otherwise now support publication.

We thank the reviewer for the positive comments and the constructive criticism.

1) *I still do not understand how the authors can claim that the nasal bone is partially restored in Gas1/Boc double mutants. Again, the images in Fig. 5M'-P' do not seem representative as in Fig. S8A-D the Gas1 mutant examples shown are either normal nasal bone or absent nasal bone, and the Gas1/Boc double mutant examples both represent reduced nasal bone. The quantitation of*

nasal bone width shows Gas1/Boc nasal bones to be intermediate in the spectrum of nasal bone phenotypes in Gas1 single mutants. I do not see anywhere evidence for "improved nasal bone morphology". The following two sections quoted below should be modified to not imply rescue of the nasal bone in Gas1/Boc double mutants. The claims of other craniofacial phenotypic rescue should solely be based on the data in the new Fig. S9.
We thank the reviewer for this suggestion. We have revised the text in these sections according to the reviewers' comments and highlighted the results from Supplemental Figure 9. Please see below for specific alterations to each section.

Lines 250-255: "The 3D reconstructions indicated that the nasal bone in Gas1-/-;Boc-/- mutants is partially restored compared to Gas1-/- mutants where this bone is smaller and fragmented (Fig. 5N', P'). As we observed at E10.5 (Fig. 2), there is a spectrum of HPE phenotypes in Gas1 mutants (Fig. S8A-D); while quantitation does not reveal significant differences in nasal bone width between Gas1 and Gas1;Boc mutants, we do observe improved nasal bone morphology in Gas1;Boc mutants (Fig. S8A'-D', E-F).

We have changed the text in lines 250-255 to read, "The 3D reconstructions indicated that the nasal bone in *Gas1*$^{-/-}$;*Boc*$^{-/-}$ embryos is reduced in size and partially fused when compared to wildtype embryos (Fig. 5M', P'). As we observed at E10.5 (Fig. 2), there is a spectrum of HPE phenotypes in *Gas1* mutants, ranging from reduced and fused nasal bone to fragments of nasal bone (Fig. 5N', Fig. S8A-D). *Gas1;Boc* mutants display an intermediate nasal bone phenotype when compared to the spectrum of phenotypes in *Gas1* single mutants (Fig. 5N'P', Fig. S8A'-D')."

Lines 343-344: "Specifically, Gas1;Boc double mutants display increased MNP separation at E10.5, and restoration of the nasal capsule and nasal bone at E18.5."

We have changed the text in lines 342-344 to read, "Specifically, *Gas1;Boc* double mutants display increased MNP separation at E10.5, and increased interocular distance, partially restored nostril frequency, and broader medial lip notch distance at E18.5."

2) *In the new Fig. S9, it is difficult to appreciate whether phenotypes are improved or worsened in the Gas1/Boc double vs. Gas1 single mutants as no quantitation is shown for wild-type controls. It is essential to show quantitation in wild-type controls for each category, and ideally Boc single mutants if the data exist. Without controls, it cannot be stated whether double mutants enhance or rescue the stated phenotypes.*

We have revised Supplemental Figure 9 to include quantitation for E18.5 wildtype and *Boc*$^{-/-}$ embryos. The addition of these data support our previous conclusions that *Gas1;Boc* mutants display a less severe phenotype than *Gas1* mutants in a subset of craniofacial structures. Specifically, *Gas1;Boc* mutants display a reduced head *width, similar snout width and significantly increased interocular distance when compared to* Gas1 *mutants Further,* Gas1;Boc *mutants exhibit a significantly wider medial lip notch than* Gas1 *mutants.*

3) *Statistics should be performed for rescue of nostrils frequency in new Fig. S9E. I believe a Fisher Exact Test is most appropriate, which in this case would result in p=0.0547. This reviewer does not believe that p<0.05 should be a magic threshold, so it would seem that such a p value could still be discussed as likely partial rescue.*

We thank the reviewer for the suggestion. We have revised Supplemental Figure 9E to include the Fisher's Exact Test statistical analysis.

4) *I wonder if the title should be modified a bit. "Differentially" compared to what? Gas1 and Cdon? Or to different parts of the face?*

"*The Hedgehog Co-Receptor BOC Differentially Regulates SHH Signaling During Craniofacial Development*"

We thank the reviewer for the suggestion. In this manuscript, the word "differentially" refers both to the distinct contribution of BOC to SHH-dependent craniofacial development compared to GAS1 and CDON, as well as the tissue-specific contributions of BOC to craniofacial development.

Therefore, we feel that the title remains an accurate description of the results provided in this manuscript. However, we are willing to discuss this further with the editor, if necessary.

Fourth decision letter

MS ID#: DEVELOP/2020/189076

MS TITLE: The Hedgehog Co-Receptor BOC Differentially Regulates SHH Signaling During Craniofacial Development

AUTHORS: Martha Echevarria-Andino and Benjamin Allen
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.