The transcription factors Foxf1 and Foxf2 integrate the SHH, HGF and TGFβ signaling pathways to drive tongue organogenesis

Jingyue Xu, Han Liu, Yu Lan and Rulang Jiang
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MS TITLE: FOXF Transcription Factors Integrate the SHH, HGF, and TGFβ Signaling Pathways to Drive Tongue Organogenesis

AUTHORS: Jingyue Xu, Han Liu, Yu Lan, and Rulang Jiang

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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

Reviewer 1

Advance summary and potential significance to field

The research article “FOXF transcription factors integrate the SHH, HGF, and TGFb signaling pathways to drive tongue organogenesis” by Xu et al. focuses on the signaling pathway network involved in the tissue-tissue interactions during tongue development. Specifically, this study brings molecular evidence indicating that Foxf1/2 transcription factors directly control the expression of Hgf and Tgfb2/3 downstream of Hedgehog signaling to regulate the development of the tongue. This study advances the previous Foxf1/2 study from the same lab and provides important information related to the regulatory mechanism of tongue development.

Comments for the author

1. Do Foxf1c/c;Wnt1-Cre and Foxf2c/c;Wnt1-Cre mice show any phenotypes? Why do the authors have to delete an extra allele of Foxf1 or Foxf2 in the mutant model? Study rationale and discussion need to be added to this manuscript.

2. Expression of Foxf2 overlaps with muscle actin at E13.5 (Figure 1L), but the muscle phenotype looks milder in Foxf1c/+Foxf2c/cWnt1-Cre than in Foxf1c/cFoxf2c/+Wnt1-Cre (Figure 2E-F). Can the authors provide discussion of this result? Also, does the size of the mandible change in the mutant models?

3. Far fewer MyoD+ cells can be observed in Figure 2H. The contribution of MyoD+ cells is very different in Figure 2H. Are migration and differentiation of muscle progenitor cells affected more in Foxf1c/cFoxf2c/+Wnt1-Cre or this is just sample variation?

4. For ChIP-seq analysis, do the authors have a particular reason for using Foxf2 instead of Foxf1? Based on previous data, Foxf1 mutants show more severe and tendon-related phenotypes. Furthermore, can the authors co-stain Tgfb3/Tgfb2 with Foxf1/Foxf2? The expression patterns of these genes seem quite different. Since ChIP-seq analysis shows Tgfb3 and Tgfb2 are directly bound by Foxf2, one might expect their expression patterns to overlap more in the tongue area (Figure 5A-F).

5. In Figure 5H-I and 5Q-R, expression of pSmad2 seems very different. If 5Q-R represent accurate expression which is indicated by western blot, the authors need to provide more representative figures for 5H-I.

Reviewer 2

Advance summary and potential significance to field

This manuscript from Xu et al., investigates the role of Foxf1 and Foxf2 transcription factors in the development of the tongue. The authors generate NCC-specific compound Foxf1;Foxf2 mutants and discover defects in lingual septum formation and in the development of intrinsic tongue muscles. Through novel ChIP-seq experiments, this manuscript demonstrates that Foxf transcription factors bind at the Hgf, Tgfb2 and Tgfb3 loci, and the expression of these genes is reduced in compound Foxf mutant embryos. These data, together with previous work demonstrating the role for Hgf in myoblast migration, and for TgfB signaling in the development of tendon and ligaments, strongly suggest that the reported phenotypes relate to Foxf regulation of HGF and TGFB signaling, though the authors do not functionally test that Foxf phenotypes are caused by these changes. Overall, the work is clear, of high quality, and contributes significantly to the delineation of regulatory networks regulating tongue development, about which relatively little is currently known.

Comments for the author

Generally, the authors might consider making a more explicit comment on the extent to which early mandibular patterning defects upon loss of Foxf relate to later tongue malformations (see below). A few minor issues might be addressed as well.

- There are clear differences in tongue morphology at the midline in Foxf1c/c;Foxf2c/+Wnt1-Cre embryos, even as early as E12.5. Is it possible that greater phenotypic severity in this genotype might relate to defects in oral/aboral mandible patterning?

- Relating to above, early changes in Hgf expression are also consistent with the possibility that the tongue phenotype characterized here is essentially a hypomorphic readout/extension of early oral/aboral patterning defects previously reported in Foxf1c/c; Foxf2c/c; wnt1-cre mutants. Is HGF expression changed in the Foxf1c/c; Foxf2c/+; wnt1-cre mutants at E10.5? Is Bmp4 deregulated in Foxf1c/c; Foxf2c/+; wnt1-cre mutants? On the other hand, do MyoD expressing cells fail to migrate into Foxf1c/c;Foxf2c/+; wnt1cre E10.5/E11.5 tongue primordia? Some further characterization or discussion of the genotypes used here to determine the extent to which they impact early oral/aboral patterning would be informative to thinking about whether/how later tongue phenotypes might relate to early patterning defects.

Minor

- As compound Foxf1;Foxf2 appear to have palatal shelves that have not elevated at E14.5, and the tongue remains positioned between them. The authors might comment on the extent to which they think overt tongue shape differences are attributable to this fact versus a consequence of malformed tongue musculature

- In Figure 1, higher magnification views of Foxf1/2 and Scx-GFP/muscle actin would be helpful to discern the degree of overlapping expression. The authors might also consider including some additional annotation, to e.g. point out expression overlap, location of transverse muscles, etc. to be more accessible to a broader audience.

- The presence of MyoD+ myocytes, and absence of organized muscle fibers is clear in Figure2G-I. However, it looks as though MyoD+ myocytes are distributed very differently, and possibly differ in abundance. Showing the MyoD channel alone and possibly quantifying MyoD+ cells in different positions in the tongue (e.g. midline vs. lateral) might be informative.

- Although I can see that Foxf1c/+; Foxf2c/c; Wnt1-cre mutants still exhibit a more normal organization of Scx-GFP cells at E13.5, it appears that neither these mutants nor Foxf1c/c; Foxf2c/+; Wnt1cre mutants exhibit any formation of the lingual septum at E16.5. Therefore, I am not sure I understand the basis for claiming that Foxc1 “plays a predominant role in lingual septum tendon formation”. Does a difference between these genotypes exist at a later stage when the septum is more formed in control?

- In Figure 5, arrows would be helpful for the reader to see the described differences. E.g. differentiate between Tgfb2 expression in CNCC-mesenchyme versus myogenic cells. Specifically, I do not see the differences in Tgfb2 expression that are described in the results text. Also, what is red signal in panels 5A,B,D,E?

- Are extrinsic muscles of the tongue affected in Foxf1c/c; Foxf2c/+; wnt1-cre or Foxf1c/+; Foxf2c/c; wnt1-cre mutant embryos?

- Care should be taken in the language used in making the claim that genes are direct targets based only on ChIP-seq + differential expression analysis. These data are consistent with the possibility that Foxf regulates Tgfb, Hgf directly, but does not prove that this is the case, which would require a more detailed interrogation of cis regulatory elements.

Reviewer 3

Advance summary and potential significance to field

This is a straight forward study in which the authors study tongue development. They show that Foxf1/Foxf2 transcription factors in CNCC mandibular mesenchyme regulate myoblast migration into the tongue primordium during tongue initiation. In addition Foxf continue to regulate intrinsic tongue muscle assembly and lingual tendon formation. The authors identified Hgf, Tgfb2 and Tgfb3 as the Foxf1/2direct target genes in embryonic tongue. Through careful and detailed genetic analyses the authors show that Foxf1 and Foxf2 mediate Hedgehog signaling in the regulation of myoblast migration during tongue initiation and intrinsic tongue muscle formation by directly activating HGF and TGFß signaling pathways.

Comments for the author

This is a straight forward study in which the authors study tongue development. They show that Foxf1/Foxf2 transcription factors in CNCC mandibular mesenchyme regulate myoblast migration into the tongue primordium during tongue initiation. In addition Foxf continue to regulate intrinsic tongue muscle assembly and lingual tendon formation. The authors identified Hgf, Tgfb2 and Tgfb3 as the Foxf1/2direct target genes in embryonic tongue. Through careful and detailed genetic analyses the authors show that Foxf1 and Foxf2 mediate Hedgehog signaling in the regulation of myoblast migration during tongue initiation and intrinsic tongue muscle formation by directly activating HGF and TGFß signaling pathways.

Comments 1. The ChIP seq analysis is relatively superficial and more can be done with the data. They should combine the ChIP seq data with available ATAC and Chromatin mark data which would make the data more impactful. Also if there is any expression data from the tongue this would also help to provide more insight in the complete set of Foxf2 target genes.

2. It's unclear what is shown in figure 5 X. Please clarify 3. Can the authors add a lineage tracing experiment to the data in figure6. This would provide more solid support for the conclusions they are making.

First revision

Author response to reviewers' comments

We thank all three reviewers for the thorough review and constructive comments on our manuscript. We have performed extensive new experiments and data analyses to address the reviewers' suggestions and comments. We incorporated the new data and our response to the reviewers' comments into the revised manuscript. Major changes to the manuscript data include: (1) addition of new Supplementary Figures S1 and S2 showing results of *Foxf1*$^{c/c}$*Wnt1-Cre* and *Foxf2*$^{c/c}$*Wnt1-Cre* single conditional mutants, respectively, related to the rationale for studying tongue development in the compound mutants as requested by Reviewer 1; (2) addition of new Supplementary Figure S3 showing effects on mandible development and oral-aboral patterning in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* and *Foxf1*$^{c/+}$*Foxf2*$^{c/c}$*Wnt1-Cre* embryos in comparison with control littermates, in response to comments from Reviewer 1 and Reviewer 2; (3) addition of new Supplementary Figure S4 comparing HGF expression and the *Hand2-Cre* lineage tracing in the developing mandible and tongue primordium in response to Reviewer 3's comment; (4) addition of Supplementary Figure S6 to address one of Reviewer 2's comments regarding changes in HGF expression and myoblast migration at earlier developmental stages. (5) addition of higher magnification views to Figure 1 to better document overlapping patterns of immunofluorescent staining of Foxf1 and Foxf2, respectively, with Scx-GFP and muscle actin in the developing tongue; (6) changing the original Figure 2 to the revised Figure 2 and Figure 3 by incorporating new data and quantitative analyses to more clearly document the tongue myogenesis defects; (7) replacement of several panels in the original Figure 5 (now Figure 6 in revised manuscript) and added labels to show the data more clearly; (8) Moved original Figure 4F to be part of the revised Figure 7A, which incorporated additional data on the ATAC-seq peaks around the *Hgf* gene and goes better with the flow of the revised manuscript text; (9) the revised manuscript text has incorporated descriptions of new data and additional discussion paragraphs. These new data and text revisions have significantly improved our manuscript and enhanced our conclusions stated in the original manuscript. All new texts in the manuscript are highlighted in yellow for easier re-review. Listed below are point-by-point responses to the reviewers' comments (the reviewers' comments are numbered and shown in italicized font).

Response to Reviewer 1's comments:

1. Do Foxf1c/c;Wnt1-Cre and Foxf2c/c;Wnt1-Cre mice show any phenotypes? Why do the authors have to delete an extra allele of Foxf1 or Foxf2 in the mutant model? Study rationale and discussion need to be added to this manuscript.

We have added new Supplementary Figures S1 and S2 showing the cleft palate and tongue phenotypes of *Foxf1*$^{c/c}$*Wnt1-Cre* and *Foxf2*$^{c/c}$*Wnt1-Cre* mutants, respectively. The *Foxf1*$^{c/c}$*;Wnt1-Cre* mice displayed variable and incompletely penetrant defects in tongue size and shape whereas the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* and *Foxf1*$^{c/+}$*Foxf2*$^{c/c}$*Wnt1-Cre* mutants exhibited consistent and fully penetrant tongue muscle and tendon defects. These results and the rationale for the compound mutant mouse studies have been added to the text on Page 8 of the revised manuscript.

2. Expression of Foxf2 overlaps with muscle actin at E13.5 (Figure 1L), but the muscle phenotype looks milder in Foxf1c/+Foxf2c/cWnt1-Cre than in Foxf1c/cFoxf2c/+Wnt1-Cre (Figure 2E-F). Can the authors provide discussion of this result? Also, does the size of the mandible change in the mutant models?

The *Wnt1-Cre* transgenic mice express Cre recombinase specifically in the premigratory neural crest cells and has no Cre activity in the muscle lineages. Thus, our study of the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* and *Foxf1*$^{c/+}$*Foxf2*$^{c/c}$*Wnt1-Cre* mice only addresses the function of Foxf1 and Foxf2 expression in the neural crest-derived mesenchyme in tongue development. We state this experimental design clearly in the revised manuscript text and emphasize that the muscle defects in these mutant mice indicate that "Foxf1 and Foxf2 in the CNCC-derived tongue mesenchyme play an important cell non-autonomous role in regulating intrinsic muscle formation" (Pages 8-10). We have added data in Supplementary Figure S3 to show the effects on mandible size and patterning in the mutant mouse models. We have added new text in the Results section on Pages 8-11 and in the Discussion section on Pages 20-21 to describe and discuss these new data and the underlying mechanisms.

3. Far fewer MyoD+ cells can be observed in Figure 2H. The contribution of MyoD+ cells is very different in Figure 2H. Are migration and differentiation of muscle progenitor cells affected more in Foxf1c/cFoxf2c/+Wnt1-Cre or this is just sample variation?

We have revised the original Figure 2 by incorporating new data and quantitative analyses. The original Figure 2G-I panels showing analysis of MyoD and muscle actin positive cells in the E12.5 embryos have been revised and shown in Figure 3D-F, with the corresponding MyoD-only staining patterns shown in Figure 3A-C. A schematic showing the regional areas for quantification of MyoD+ cells is included in Figure 3G and the quantification and statistical analysis results shown in Figure 3H and Figure 3I. These results show clear differences in the regional distribution of the MyoD+ myoblasts in the E12.5 tongue in the control and mutant embryos. In addition, we added new data in Supplementary Figure S6 showing a defect in myoblast migration to the tongue primordium at E11.5 in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* embryo in comparison with the control and *Foxf1*$^{c/+}$*Foxf2*$^{c/c}$*Wnt1-Cre* littermates. We have incorporated these results into the manuscript text on Pages 9-10 and Page 17. In addition, we have added a paragraph in the Discussion section on Pages 20-21 addressing the differences in early myoblast migration during tongue formation in the two mutant models.

4. For ChIP-seq analysis, do the authors have a particular reason for using Foxf2 instead of Foxf1? Based on previous data, Foxf1 mutants show more severe and tendon-related phenotypes. Furthermore, can the authors co-stain Tgfb3/Tgfb2 with Foxf1/Foxf2? The expression patterns of these genes seem quite different. Since ChIP-seq analysis shows Tgfb3 and Tgfb2 are directly bound by Foxf2, one might expect their expression patterns to overlap more in the tongue area (Figure 5A-F).

Success in ChIP-seq analysis of endogenous transcription factor binding sites depends on high quality ChIP-grade specific antibodies. Most antibodies do not work well for ChIP-seq analysis. The anti-FLAG antibody has been reported for successful ChIP-seq identification of genome-wide binding sites of multiple transcription factors using transgenic or knockin animals or cell lines. We took advantage of the *Foxf2*^{Flag} mice, in which the DNA sequence encoding the 3xFLAG tag was inserted in-frame immediately preceding the translational STOP codon of the *Foxf2* gene (Xu et al., 2020), to perform ChIP-seq analysis of the endogenous Foxf2-binding sites in the developing tongue. We revised the text on Page 12 to briefly explain this experimental approach. Since Foxf1 and Foxf2 share 100% amino acid sequence identity in the DNA-binding Forkhead domain and our analyses of the compound mutant mice indicated that Foxf2 partly complemented Foxf1 function in mandible and tongue development, many of the Foxf2-binding sites discovered from our ChIP-seq data likely represent shared binding sites for Foxf1 and Foxf2 in the cells that expressed both transcription factors.

Although our ChIP-seq identified Foxf2-binding sites in both the *Tgfb2* and *Tgfb3* genes, these two genes exhibited very different patterns of expression in the developing tongue mesenchyme. These results indicate that these two genes are differentially regulated and likely involve other transcription factors in addition to Foxf1 and Foxf2. We have added labels to the panels showing expression of *Tgfb2* and *Tgfb3* mRNAs in the original Figure 5 (now Figure 6 in the revised manuscript) to clearly point out the differences in the mutant and control samples. We have added a brief discussion of the differential regulation of *Tgfb2* and *Tgfb3* in the developing tongue and their role in Foxf1/Foxf2-mediated regulation of lingual tendon and tongue muscle development on Page 23.

5. In Figure 5H-I and 5Q-R, expression of pSmad2 seems very different. If 5Q-R represent accurate expression which is indicated by western blot, the authors need to provide more representative figures for 5H-I.

We have re-examined multiple serial sections of each sample from three different biological replicates. We have replaced the data in the original Figure 5G, I, J, L, M, and O with better matched sections in the corresponding panels in the revised Figure 6.

Response to Reviewer 2's comments:

1. There are clear differences in tongue morphology at the midline in Foxf1c/c;Foxf2c/+Wnt1- Cre embryos, even as early as E12.5. Is it possible that greater phenotypic severity in this genotype might relate to defects in oral/aboral mandible patterning?

We have added new experimental data in Fig. 2 and Supplementary Fig. S3, and Fig. S6 to specifically address this question and related mechanism. The *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* mice exhibited significant reduction in the length of the mandible and the size of the tongue. We found that expression of the BMP target genes *Msx1* and *Msx2* was expanded to the oral side of the most distal region of the mandibular arch mesenchyme at E10.5 in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* embryos (Fig. S3). However, no overt disruption of the oral-aboral patterning of the mandible, including the mandibular bone and incisor teeth, was observed at later stages, in contrast to the duplication of mandibular bone at the oral side at the expense of tongue formation in the *Foxf1*$^{c/c}$*Foxf2*$^{c/c}$*Wnt1-Cre* embryos reported previously (Xu et al., 2019). We agree with the reviewer that the early patterning defect likely contributed to the more severe tongue developmental defect in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* embryos than in the *Foxf1*$^{c/+}$*Foxf2*$^{c/c}$*Wnt1-Cre* embryos. Indeed, we found that the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* embryos exhibited more scattered myoblasts in the caudal region of the mandible at E11.5, compared with the concentrated myoblast migration towards the tongue primordium (Supplementary Fig. S6). We include all these results in the Results section (Pages 10 – 11 and Page 17). In addition, we added a new paragraph in the Discussion section specifically discussing the effect and contribution of the early mandibular patterning defect to tongue development in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* mice (Pages 20 – 21).

2. Relating to above, early changes in Hgf expression are also consistent with the possibility that the tongue phenotype characterized here is essentially a hypomorphic readout/extension of early oral/aboral patterning defects previously reported in Foxf1c/c; Foxf2c/c; wnt1-cre mutants. Is HGF expression changed in the Foxf1c/c; Foxf2c/+; wnt1-cre mutants at E10.5? Is Bmp4 deregulated in Foxf1c/c; Foxf2c/+; wnt1-cre mutants? On the other hand, do MyoD expressing cells fail to migrate into Foxf1c/c;Foxf2c/+; wnt1cre E10.5/E11.5 tongue primordia? Some further characterization or discussion of the genotypes used here to determine the extent to which they impact early oral/aboral patterning would be informative to thinking about whether/how later tongue phenotypes might relate to early patterning defects.

We performed new experiments and added new data on the expression of BMP target genes in the E10.5 mandibular arch (Supplementary Fig. S3) as well as expression of HGF and MyoD in the developing tongue primordium/mandible at E11.5 (Supplementary Fig. S6). In addition, we quantified the regional distribution of MyoD+ cells in the anterior versus posterior halves and in the medial versus lateral domains of the developing tongue at E12.5 (Fig. 3). Together, these data support our conclusion that Foxf1/Foxf2-mediated activation of HGF expression is crucial for directing myoblast migration into the tongue primordium. Since a previous study (Bonafede et al., 2006 – new reference added in revised manuscript) showed that BMP signaling restricts the position of premuscle masses of myoblasts in the developing limb buds by indirectly antagonizing HGF-directed myoblast migration, we postulated that the earlier expanded BMP signaling activity to the

oral side of the distal mandibular arch in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* embryos likely contributed to the more severe disruption of tongue myogenesis and anteriorward tongue growth in these mutants compared with the *Foxf1*$^{c/+}$*Foxf2*$^{c/c}$*Wnt1-Cre* embryos. The added results (Pages 10 -11 and Page 17) and discussion (Pages 20 – 21) made these points clear.

3 (minor). As compound Foxf1;Foxf2 appear to have palatal shelves that have not elevated at E14.5, and the tongue remains positioned between them. The authors might comment on the extent to which they think overt tongue shape differences are attributable to this fact versus a consequence of malformed tongue musculature

We have added data on *Foxf1*$^{c/c}$*Wnt1-Cre* and *Foxf2*$^{c/c}$*Wnt1-Cre* mice in Fig. S1 and Fig. S2. Although both *Foxf1*$^{c/c}$*Wnt1-Cre* and *Foxf2*$^{c/c}$*Wnt1-Cre* mice exhibited cleft palate with the tongue partly wedged in between the palatal shelves, the former showed disruption of tongue musculature but the latter did not. Thus, the cleft palate defect may impact the tongue shape due to the limited space in the oral cavity but does not change the muscle structures. These new results are described on Page 8. On the other hand, our analysis of tongue myogenesis defects in the mutant embryos focus on early stages prior to palate shelf elevation, which represent primary effects on tongue development separately from palate development.

4 (minor). In Figure 1, higher magnification views of Foxf1/2 and Scx-GFP/muscle actin would be helpful to discern the degree of overlapping expression. The authors might also consider including some additional annotation, to e.g. point out expression overlap, location of transverse muscles, etc. to be more accessible to a broader audience.

We have added higher magnification panels in Fig. 1 to show the overlapping expression of Foxf1 and Foxf2 with Scx-GFP in the developing lingual septum (Panels H' and K') as well as the lack of significant overlap of Foxf1 and Foxf2 expression with muscle actin in the developing tongue muscles (Panels I' and L').

5 (minor). The presence of MyoD+ myocytes, and absence of organized muscle fibers is clear in Figure2G-I. However, it looks as though MyoD+ myocytes are distributed very differently, and possibly differ in abundance. Showing the MyoD channel alone and possibly quantifying MyoD+ cells in different positions in the tongue (e.g. midline vs. lateral) might be informative.

We have added MyoD-only panels and quantified MyoD+ cell distribution in the new Fig. 3 and described in the Results section (Pages 9 – 10).

6 (minor). Although I can see that Foxf1c/+; Foxf2c/c; Wnt1-cre mutants still exhibit a more normal organization of Scx-GFP cells at E13.5, it appears that neither these mutants nor Foxf1c/c; Foxf2c/+; Wnt1cre mutants exhibit any formation of the lingual septum at E16.5. Therefore, I am not sure I understand the basis for claiming that Foxc1 "plays a predominant role in lingual septum tendon formation". Does a difference between these genotypes exist at a later stage when the septum is more formed in control?

We have added whole mount views of E16.5 tongue with Scx-GFP marking the midline lingual septum tendon (Fig. 2A-C). The lingual septum defect is obviously more severe in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* mutants than in the *Foxf1*$^{c/+}$*Foxf2*$^{c/c}$*Wnt1-Cre* mutants. However, in light of the new data identifying a defect in early tongue myoblast migration in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* embryos, we have replaced the original statement "Foxf1 plays a more predominant role in lingual septum tendon formation" to "these results suggest that Foxf1 and Foxf2 act partly redundantly to regulate lingual tendon formation" (Page 14).

7 (minor). In Figure 5, arrows would be helpful for the reader to see the described differences. E.g. differentiate between Tgfb2 expression in CNCC-mesenchyme versus myogenic cells. Specifically, I do not see the differences in Tgfb2 expression that are described in the results text. Also, what is red signal in panels 5A,B,D,E?

We have added arrows and arrowheads to point to the corresponding domains in the different samples. We have added description of the differences in *Tgfb3* and *Tgfb2* expression (Page 13) and provided additional discussion of the differential regulation of these genes (Page 23).

The red signal in the original panels 5A, B, D, E (now Fig. 6A, B, D, E) resulted from the eosin counterstaining of the blood cells. We have added this explanation to the figure legend.

8 (minor). Are extrinsic muscles of the tongue affected in Foxf1c/c; Foxf2c/+; wnt1-cre or Foxf1c/+; Foxf2c/c; wnt1-cre mutant embryos?

We have added identification of extrinsic muscles in the revised Fig. 2 and description in the results section on Page 9. These extrinsic muscles were all identified in both mutant genotypes but the palatoglossus and styloglossus muscles were disorganized in the posterior region of the tongue in the *Foxf1*$^{c/c}$*Foxf2*$^{c/+}$*Wnt1-Cre* embryos (Page 9).

9 (minor). Care should be taken in the language used in making the claim that genes are direct targets based only on ChIP-seq + differential expression analysis. These data are consistent with the possibility that Foxf regulates Tgfb, Hgf directly, but does not prove that this is the case, which would require a more detailed interrogation of cis regulatory elements.

We have deleted the word "direct" or rephrased when describing the target genes.

Response to Reviewer 3's comments:

1. The ChIP seq analysis is relatively superficial and more can be done with the data. They should combine the ChIP seq data with available ATAC and Chromatin mark data which would make the data more impactful. Also if there is any expression data from the tongue this would also help to provide more insight in the complete set of Foxf2 target genes.

We have not found any available ATAC-seq or Chromatin marker data for mouse embryonic tongue. We did find ATAC-seq data from dissected E10.5 mandibular arch tissues, which is related to the stage of myoblast migration into the mandible and tongue primordium. Thus, we have incorporated the E10.5 ATAC-seq data into our analysis and show that multiple Foxf2 binding peaks overlapped with ATAC-seq peaks in/around the *Hgf* gene, including the promoter region (Figure 7A). No bulk or single cell RNA-seq data on early embryonic tongue is available yet. We plan to perform those experiments in the future.

2. It's unclear what is shown in figure 5 X. Please clarify.

The original Fig. 5X (now Fig. 6X) shows quantitative comparison of pSmad2/3 in the tongue tissues of E13.5 *Foxf1*$^{c/+}$*Foxf2*$^{c/c}$*Wnt1-Cre* embryos and their control littermates. We added the description into the Results section (Page 14).

3. Can the authors add a lineage tracing experiment to the data in figure6. This would provide more solid support for the conclusions they are making.

We have added Supplementary Fig. S4 showing lineage tracing of *Hand2-Cre* in the developing mandibular arch and tongue primordium from E10.5 to E11.5. In addition, we added data comparing the domains of expression of *Hgf* mRNAs and HGF protein with the lacZ lineage reporter in the E11.5 embryos to show that HGF expression in the tongue primordium is in the *Hand2-Cre* lineage cells. These results are described on Page 15.

Second decision letter

MS ID#: DEVELOP/2022/200667

MS TITLE: FOXF Transcription Factors Integrate the SHH, HGF, and TGFβ Signaling Pathways to Drive Tongue Organogenesis

AUTHORS: Jingyue Xu, Han Liu, Yu Lan, and Rulang Jiang
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.

Reviewer 1

Advance summary and potential significance to field

The research article “FOXF transcription factors integrate the SHH, HGF, and TGFb signaling pathways to drive tongue organogenesis” by Xu et al. focuses on the signaling pathway network involved in the tissue-tissue interactions during tongue development. Specifically, this study brings molecular evidence indicating that Foxf1/2 transcription factors directly control the expression of Hgf and Tgfb2/3 downstream of Hedgehog signaling to regulate the development of the tongue. This study advances the previous Foxf1/2 study from the same lab, but several points need to be addressed regarding the molecular mechanism.

Comments for the author

All concerns have been addressed.

Reviewer 3

Advance summary and potential significance to field

Concerns addressed

Comments for the author

Concerns adequately addressed