Transitory expression of *Dlx5* and *Dlx6* in maxillary arch epithelial precursors is essential for upper jaw morphogenesis

Previously titled: Transitory expression of *Dlx5* and *Dlx6* in maxillary arch precursors is essential for upper jaw morphogenesis

Yorick Gitton, Nicolas Narboux-Nême, Giovanni Levi
Evolution des Régulations Endocriniennes, CNRS, UMR7221, Muséum National d'Histoire Naturelle, Paris, France

**Abstract**

Asymmetric, articulated jaws are characteristic of most vertebrate species; they derive from the first pharyngeal arch (PA1) which generates both maxillary and mandibular components. PA1 is colonized by cranial neural crest cells (CNCCs) which give rise to most bones and tendons of the jaws. The elements formed by different CNCCs contingents are specified by the combinatorial expression of *Dlx* genes. *Dlx5* and *Dlx6* are predominantly expressed by mandibular CNCCs. Analysis of the phenotype of *Dlx5* and *Dlx6* double mutant mice has suggested that they are necessary and sufficient to specify mandibular identity. Here, using 3D reconstruction, we show that inactivation of *Dlx5* and *Dlx6* does not only affect the mandibular arch, but results in the simultaneous transformation of mandibular and maxillary skeletal elements which assume a similar morphology with gain of symmetry. As *Dlx5* and *Dlx6*-expressing cells are not found in the maxillary bud, we have examined the lineage of *Dlx5*-expressing progenitors using an *in vivo* genetic approach. We find that a contingent of cells deriving from epithelial precursors transiently expressing *Dlx5* participate in the formation of the maxillary arch. These cells are mostly located in the distal part of the maxillary arch and might derive from its lambdoidal junction with the olfactory pit. Our observations provide the first genetic demonstration of the ‘Hinge and Caps’ model[1]. We support the notion that ‘cap’ signals could originate from epithelial derivatives of *Dlx5*-expressing progenitors which migrate and colonize the maxillary arch epithelium. Our results imply that *Dlx5* and *Dlx6* control upper and lower jaw morphogenesis through different coordinated mechanisms to generate functional, articulated jaws.
How to cite this article: Gitton Y, Narboux-Nême N and Levi G. Transitory expression of Dlx5 and Dlx6 in maxillary arch epithelial precursors is essential for upper jaw morphogenesis [v3; ref status: indexed, http://f1000r.es/4dd] F1000Research 2014, 2:261 (doi: 10.12688/f1000research.2-261.v3)

Copyright: © 2014 Gitton Y et al. This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Grant information: This research was partially supported by the EU Consortium IDEAL (HEALTH-F2-2011-259679) to GL.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 28 Nov 2013, 2:261 (doi: 10.12688/f1000research.2-261.v1)
First indexed: 03 Oct 2014, 2:261 (doi: 10.12688/f1000research.2-261.v3)
Introduction

The skull of most vertebrates is characterized by the presence of articulated, asymmetric jaws which support the function of a muscularized oral cavity. During embryonic development, the upper and lower jaws derive from the maxillary and mandibular processes of the first pharyngeal arch (PA1). Most cartilaginous and dermatoctral derivatives of PA1 are formed by Cranial Neural Crest Cells (CNCCs). The presence of shared signals remains elusive. Here we revisit the effects of Dlx5/6-dependent signalling centres in proximodorsal patterning of PA1 derivatives. While Dlx1 and Dlx2 are expressed by CNCCs of the maxillary and mandibular components of PA1, Dlx5 and Dlx6 transcripts are present only in mandibular CNCCs. Targeted simultaneous inactivation of Dlx5 and Dlx6 results in the transformation of lower jaw into upper jaw-like structures, underlining the importance of these genes for lower jaw identity. This phenotype is more severe than those observed in both single mutants (for instance, see 21–23,24). The activation of Dlx5 and Dlx6 by endothelin-1 signalling is necessary and sufficient to define lower jaw identity. Interestingly it has been observed that, after inactivation of Dlx5 and Dlx6, maxillary components are also affected despite the fact that these genes are not expressed by maxillary CNCCs. This observation could be accounted for by the presence of shared Dlx5/6-dependent signalling centres in proximodorsal patterning of both the mandibular and maxillary arches; this notion gave rise to the so-called “Hinge and Caps” model of jaw organization. In its original formulation this model predicts the presence of two opposing morphogen gradients, one emanating from the region of the upper/lower jaw articulation (hinge) and one from the distal extremities of PA1 (caps); the origin and nature of these signals remain elusive. Here we revisit the effects of Dlx5 and Dlx6 double inactivation on jaw development and, using a transgenetic lineage tracing approach, we reveal that the maxillary arch epithelium harbours a cellular contingent derived from frontonasal Dlx5/6-expressing progenitors. Our findings suggest that transient Dlx5/6 expression could program these epithelial cells to provide the cues needed for maxillary arch morphogenesis.

Material and methods

Mouse strains and breeding

All animal experimentation was performed in accordance to French national regulations and approved by the MNHN ethical committee (approval n° 68–028/1). For this study we used about 35 dams (including 10 WT, 5 Dlx5+/−, 3 Dlx5/6−/−; 12 B6.129S4-Gt(ROSA)26Sor<tm1Sor/J, 5 B6(CrI)-Dlx5<tm1Sor>ERT2<tm1Zjh/J) and analyzed about 120 embryos, the exact record of animals used, litters obtained, embryos genotyped and number of embryos per litter is on record in our animal house. WT animals were from Charles River France and were maintained in the MNHN mouse facility which is officially certified by the French National Animal well being committee.

Dlx5<tm1Sor>/Dlx6<tm1Sor> knock-in mice were maintained on a mixed B6/D2 genetic background. Double Dlx5 and Dlx6 (Dlx5/6) mutant mice were maintained and genotyped as reported. The inducible Cre driver strain B6(CrI)-Dlx5<tm1Sor>ERT2<tm1Zjh/J (designed by Z. J. Huang) and the Cre reporter strain B6.129S4-Gt(ROSA)26Sor<tm1Sor/J were purchased from Jackson Laboratory (#10705 and #003309 respectively; Maine, USA) through Charles River Laboratories (L’Arbresle, France) and maintained on a C57BL/6J genetic background through heterozygous mating. Double heterozygous embryos were obtained through bi-directional crosses. Induction of Cre recombinase activity was obtained upon single intraperitoneal injection of 5mg of tamoxifen (Sigma-Aldrich), in corn oil. Tamoxifen preparation and administration in pregnant dams followed the Jackson Laboratory’s Guidelines and CNRS/MNHN Animal Handling Guidelines. Dams were anesthetized in a chamber containing 2.5% isoflurane in oxygen, euthanized by cervical dislocation at indicated stages and embryos were collected in phosphate-buffered saline (PBS), then fixed and staged by immersion in ice-cold fixative (2% paraformaldehyde/0.2% glutaraldehyde) for 5 to 15 minutes (depending upon their developmental stage).

β-galactosidase detection

For lacZ expression, embryos were fixed for 15–30 min in 4% paraformaldehyde; X-gal staining was performed as described previously. Vehicle (corn oil) injection in double heterozygous mice did not yield leaking β-galactosidase activity.

Histology and 3D reconstruction

Heads from 18.5dpc (days post coitum) Dlx5/6−/− and wild type mouse embryos were fixed in Bouin’s solution (Sigma, France), embedded in paraffin and complete sets of frontal or parasagittal serial sections (12µm) were prepared. All sections were stained by Mallory’s trichrome as in and photographed (Nikon Digital Site DS-FI1). Pictures were aligned, piled and registered using the Fiji plugin of NIH ImageJ “Register Virtual Stack Slices” (http://fiji.sc/wiki/index.php/Register_Virtual_Stack_Slices). 3D segmentation was performed with Mimics (Materialise, Belgium: http://biomedical.materialise.com/mimics) and visualized using Adobe Acrobat 9 pro.

Results

Dlx5/6 inactivation results in upper and lower jaw transformation with gain of symmetry

Previous reports suggest that double inactivation of Dlx5 and Dlx6 results in lower-to-upper jaw transformation; these reports also indicated that the upper jaw of these mice is not normal. To better visualize the jaw phenotype of Dlx5/6 mutants, we performed 3D reconstructions of craniofacial elements of 18.5dpce (days post coitum) embryos. Frontal view of the mutant jaws (Figure 1, upper panel) shows an obvious gain of symmetry compared to a WT animal. Examining the defects of the lower and upper jaws separately (Figure 1, middle and lower panels), it is evident that both are transformed. In the absence of Dlx5 and Dlx6 the dentary and the upper...
Figure 1. Three-dimensional reconstruction of the dentary and maxillary bones of 18.5dpc wild type and \textit{Dlx5/6}\(^{-/-}\) mouse embryos. Upper row: Frontal view of WT and \textit{Dlx5/6}\(^{-/-}\) oral apparatus. Skeletal elements are grey, the tongue is red and incisors are violet. Middle row: Dorsal view of the dentary bone of WT and \textit{Dlx5/6}\(^{-/-}\) 18.5dpc mice. Lower row: Ventral view of the maxillary components of WT and \textit{Dlx5/6}\(^{-/-}\) 18.5dpc mice. Note that the inactivation of \textit{Dlx5/6} results in the transformation of both lower and upper jaw skeletal elements into new structures which appear more similar to each other than to their WT counterpart. cp, coronoid processes; dt, dentary bone; li, lower incisor; t, tongue; ui, upper incisor; za, zygomatic arch; za\(^{*}\), zygomatic arch-like structure deriving from lower jaw transformation; za\(^{'}\), zygomatic arch-like structure deriving from upper jaw transformation.

Jaw bones do not form correctly and are replaced by remarkably similar skeletal structures. In the mutant embryos, both the upper and lower jaw skeletal elements are reduced in size, are not fused in the midline, and display a lateral process positionally homologous to the wild type zygomatic arch. Thus the upper and lower jaw mutant bones resemble each other more closely than usually found in their normal counterparts.

**Transient \textit{Dlx5} expression in maxillary arch progenitors**

In \textit{Dlx5-lacZ} heterozygous Theiler stage (ts) 19 (12 dpc) embryos the reporter is active in the olfactory pit and mandibular arch, but not in the maxillary arch; this pattern of expression does not change upon tamoxifen treatment of the pregnant dam at ts9 (7 dpc). We monitored \(\beta\)-Gal reporter activity from ts15 (10 dpc) to ts20 (12.5 dpc) (n=10 embryos per stage). At ts15 we observed a stream of \(\beta\)-Gal-positive cells extending from the lambdoidal junction, which joins the olfactory pit with the distal maxillary arch\(^{26}\), towards the body of the maxillary arch (Figure 2B, B’). At ts19 and ts20 (Figure 2C, C’; D, D’) reporter-expressing cells are found in the upper epithelial lining of the maxillary arch (arrowheads in Figure 2C’, 2D’) and in two distinct proximal and distal territories of the arch body (red asterisk in Figure 2C’).

To determine more precisely the tissue distribution of craniofacial derivatives of \textit{Dlx5}-positive cells, we set apart two illustrative among the ten embryos per stage, and performed serial paraffin sections of \textit{Dlx5-creERT2; R26R-lacZ} \(\beta\)-Gal-stained mouse embryos.
Figure 2. Lineage of Dlx5-expressing cells in the maxillary arch. β-Galactosidase activity in the cephalic region of Dlx5-lacZ (A, A’) and Dlx5-creERT2; R26R-lacZ mouse embryos (B–D’). In all cases pregnant dams were treated with tamoxifen at 7dpc/Theiler stage 9 (ts9) and embryos were collected at the indicated Theiler stage. A, A’) As expected, even after tamoxifen treatment, Dlx5 is expressed in the mandibular arch (md), in the olfactory pit (olf), in the otic vesicle (ov), in the striatum (st) and in the hind limb (hl), but not in the maxillary arch. B, B’) Permanent activation of lacZ reporter expression in derivatives of Dlx5-expressing early progenitors (ts9) reveals the presence of a positive cellular contingent in the ts15 lambdoidal junction (λ) between the olfactory pit and the maxillary process. C, C’, D, D’) At later developmental stages (ts19, ts20) a contingent of lacZ positive cells populates the distal domain of the maxillary arch. hl, hind limb; md, mandibular arch; mx, maxillary arch; olf, olfactory pit; ov, otic vesicle; bt, basal telencephalon; λ, lambdoidal junction; red asterisk/black arrowheads, territories of the maxillary arch colonized by derivatives of Dlx5-expressing progenitors. Bar: A–D 1mm; A’–D’ 250µm.

(12.5 dpc) after tamoxifen treatment of pregnant dams at 7dpc/ Theiler stage 9 (ts9) (Figure 3). While in the mandibular arch β-Gal staining is limited, as expected, to CNCCs derivatives, the analysis of the complete set of serial sections shows that only epithelial cells lining the maxillary arch are positive. As no Dlx5-positive epithelial cells are present in the maxillary arch of normal embryos, we conclude that a population of epithelial cells derived from the Dlx5-positive frontonasal process participates to the formation of the maxillary arch.

Discussion

In this study we have re-examined the skeletal jaw phenotype of Dlx5/6 mutant mice. We confirm that both the mandibular and maxillary arches are transformed. The profound change in the shape of the maxillary arch is difficult to explain, as this region does not derive from a Dlx5/6-expressing territory. Indeed, in normal embryos maxillary CNCCs and the overlying epithelium do not express Dlx5 and Dlx6. Lineage analysis to identify derivatives of Dlx5-positive progenitors reveals a new population of cells extending from the olfactory pit through the lambdoidal junction towards the maxillary arch12,22. These derivatives of Dlx5-positive cells have lost Dlx5 expression as seen by Dlx5 in situ hybridization (see for example14,15,27,28) and by lacZ-Dlx5 knock-in (15, and Figure 2A’). We have previously shown that early Dlx5 and Dlx6 expression in the anterior neural fold is essential for nasal capsule patterning29; our present findings suggest that the same population of cells could also contribute to maxillary patterning. The epithelial cell contingent might well exert a patterning role upon the maxillary arch providing spatial cues to the underlying mesenchyme. A further argument supporting the notion that Dlx5/6 patterning of the upper jaw does not require their expression in CNCCs derives from our recent observation that selective ablation of these genes in CNCCs does not affect upper jaw morphology (Gitton et al., in preparation). This observation fits with the prediction of the ‘Hinge and Caps’ model1,3,22, and suggests that ‘cap’ signals could originate from derivatives of Dlx5-expressing frontonasal progenitors. Even if, after migration in the maxillary arch, these cells lose Dlx5 expression, it appears that the early expression of Dlx5 confers them the capacity to pattern maxillary arch CNCCs, which do not themselves express Dlx5 and Dlx6. As the compound Dlx5/6 mutant displays an upper jaw malformation which is not observed in either single mutant16, it appears that Dlx5 and Dlx6 exert redundant functions not only on lower,
but also on upper jaw morphogenesis. Whether there is a specific and transiently-expressing Dlx6 cell population during upper jaw morphogenesis remains, ideally, to be formally determined using an inducible Cre-targeted Dlx6 strain. Our lineage strategy is based upon a strain with an inducible Cre recombinase inserted into the Dlx5 open reading frame. Based on previous work it is likely that the resultant reporter expression reflects the activity of jaw-specific regulatory elements known to control both Dlx5 and Dlx6 transcription, as observed for instance through similar transgenic analysis32.

It appears, therefore, that Dlx5 and Dlx6 pattern the upper and lower jaw through very different mechanisms, which must be coordinated to generate asymmetric, articulated, muscularized jaws.

Author contributions
GL and YG conceived the study and designed the experiments. YG and NN-N carried out the research. GL and YG prepared the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests
No competing interests were disclosed.

Grant information
This research was partially supported by the EU Consortium IDEAL (HEALTH-F2-2011-259679) to GL.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
This work was made possible thanks to the excellent technical assistance of Mss. Anastasia Fontaine, Aurélie Hagneau, Ocilia Fernandes and Gladys Alfama.

References
1. Depew MJ, Compagnucci C: Tweaking the hinge and caps: testing a model of the organization of jaws. J Exp Zool B Mol Dev Evol. 2008; 310(4): 315–35. [PubMed Abstract] [Publisher Full Text]
2. Depew MJ, Simpson CA, Morasso M, et al.: Reassessing the Dlx code: the genetic regulation of branchial arch skeletal pattern and development. J Anat. 2005; 207(5): 501–61. [PubMed Abstract] [Publisher Full Text | Free Full Text]
3. Compagnucci C, Debais-Thibaud M, Coisson M, et al.: Pattern and polarity in the development and evolution of the gnathostome jaw: both conservation and heterotopy in the branchial arches of the shark, Scyliorhinus canicula. Dev Biol. 2013; 377(2): 428–48. [PubMed Abstract] [Publisher Full Text]
4. Tan SS, Morris-Kay GM: Analysis of cranial neural crest cell migration and early fates in postimplantation rat chimaeras. J Embryol Exp Morphol. 1986; 98: 21–56. [PubMed Abstract]
5. Couly GF, Cotet PM, Le Douarin NM: The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. Development. 1993; 117(2): 409–29. [PubMed Abstract]
Open Peer Review

Current Referee Status:  

Version 3

Referee Report 03 October 2014

doi:10.5256/f1000research.5665.r5031

Robert Knight
Craniofacial Development and Stem Cell Biology, King's College London, Guy's Hospital, London, UK

All of my questions and requests have been satisfied and I recommend indexation with no further modifications.

This is an interesting story and it is intriguing that Dlx genes function in different cellular compartments to pattern the upper and lower jaws, especially considering our assumptions about the homology of upper and lower skeletal elements in the pharyngeal arches. I look forward to reading about the conditional mouse alleles.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 2

Referee Report 04 August 2014

doi:10.5256/f1000research.4412.r5678

Robert Knight
Craniofacial Development and Stem Cell Biology, King's College London, Guy's Hospital, London, UK

This paper by Gitton et al. re-examines the 'Hinge and Caps' model posited by Michael Depew and adds a new piece of evidence supporting this theory. This model argues that changes to mandibular and maxillary skeletal elements in mice mutant for various Dlx gene mutations, reflect a transformation of the upper and lower jaws due to a loss of signals from the 'caps' at the ends of the jaws to the 'hinges'. Detailed descriptions of the phenotypes of the various Dlx mutants have previously been published by the Depew lab and others and in this study the authors show a similar phenotype as has previously been shown for the Dlx5/6 mutant mice. The authors display 3D reconstructions emphasising the symmetry of the transformed skeletal elements, but do not elaborate beyond the characterisations of these mutants that haven previously been performed.

The authors then describe the expression of an inducible Dlx5 transgene in the maxillary arch and show
that \textit{Dlx5} expressing cells are restricted to ectoderm, although I could not determine how many animals were examined to determine this. The authors further state that these cells no longer express \textit{Dlx5} when they arrive at this point, but again this is not shown, but rather referenced by other work. The authors argue that this ectodermal positioning of the \textit{Dlx5}-lineage cells in the maxillary arch, in conjunction with the phenotype of the \textit{Dlx5/6} double mutant, argues that the 'caps' signal is an ectoderm signal. The authors then cite unpublished data for a CNCC-specific knockout of \textit{Dlx5/6} and state this has no phenotype in the maxillary arch, but fail to show this.

This paper is interesting and posits an attractive idea – that a discrete population of cells located at a point in the ectoderm can act as a positional signal to pattern forming skeletal elements. The lineage tracing experiments are elegant and could be elaborated on to show if the restriction of the \textit{Dlx5} lineage cells are restricted to the ectoderm throughout the maxillary arch, as only one plane is shown in Figure 3.

One thing that I was not able to glean from this work, is whether there is any \textit{Dlx6} expression or \textit{Dlx6} -derived cells in the maxillary arch and if so, is this in ectoderm-derived or CNCC-derived cells? This is critical to ascertain as the premise of the work is that a \textit{Dlx5} expressing cell lineage in the ectoderm is controlling patterning of the maxillary arch, yet the mutant being examined is a compound \textit{Dlx5/6} mutant. Is there redundancy between these genes in patterning the maxillary arch? Can the authors reference work describing the phenotype of the maxillary arch elements in \textit{Dlx6} and \textit{Dlx5} single mutant animals? \citet{Beverdam2002}, highlighted the combinatorial consequences of \textit{Dlx5} and \textit{Dlx6} loss of function, leading to a graded alteration in morphology, indicative of skeletal transformations. This point needs to be clarified in the text.

Major points:

1. How many animals were examined for \textit{lacZ} reporter gene expression in the maxillary arch by paraffin wax sectioning? Another example and an indication of the number of animals examined is required.

2. The authors should show that the expression of \textit{Dlx5} is absent from the maxillary arch at comparable stages to the \textit{lacZ} staining (this is referenced, but not shown at comparable stages to the \textit{lacZ} stained animals in).

3. I would like to see what expression of \textit{Dlx6} is in the maxillary arch at comparable stages to those examined by the \textit{Dlx5-lacZ} transgene.

4. I would like to see the data for the mice in which \textit{Dlx5} is knocked out in CNCC (unpublished data cited in the Discussion). The central premise of the paper is that the \textit{Dlx5} lineage cells in the epithelial of the maxillary arch are acting as the caps, in which case it is crucial to show that a knockout of \textit{Dlx5/6} in the CNCC does not affect the morphology of the maxillary skeleton.

5. Please reference descriptions of maxillary arches in \textit{Dlx5} and \textit{Dlx6} single mutants, describe the expression of both genes in the maxilla and highlight the redundancy between these genes in arch patterning. These should be incorporated into the Discussion.

\textbf{I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.}
**Competing Interests:** No competing interests were disclosed.

Giovanni Levi, CNRS/MNHN, France

“The authors display 3D reconstructions emphasising the symmetry of the transformed skeletal elements, but do not elaborate beyond the characterisations of these mutants that haven previously been performed.”

As discussed in our first revision in response to Referee 1, in this manuscript we have focused our attention on the simultaneous alteration of both upper and lower jaws of Dlx5/6 double mutant embryos. In previous reports, the skeletal analysis was interpreted suggesting that the lower jaw was transformed into an upper jaw with gain of symmetry. Now we stress the fact that the acquisition of new symmetry derives from morphological changes occurring both in upper and lower jaws.

“The lineage tracing experiments are elegant and could be elaborated on to show if the restriction of the Dlx5 lineage cells are restricted to the ectoderm throughout the maxillary arch, as only one plane is shown in Figure 3.”

As stated in the revised version of the paper we have analysed complete sets of serially sectioned embryos and we conclude that Dlx5 lineage derivatives are restricted to the ectoderm in each section analysed. We have now added the sentence: “the analysis of the complete set of serial sections” to stress this point. The specific section shown in Fig. 3 was chosen as it shows simultaneously the maxillary and the mandibular arches.

“One thing that I was not able to glean from this work, is whether there is any Dlx6 expression or Dlx6-derived cells in the maxillary arch and if so, is this in ectoderm-derived or CNCC-derived cells?” and … “The authors further state that these cells no longer express Dlx5 when they arrive at this point, but again this is not shown, but rather referenced by other work.”

In situ hybridization and transcriptomic analyses by our group as well as others has, to our opinion, settled the issue (eg: Ozeki et al., 2004; Charité et al., 2001; Jeong et al., 2008). Maxillary cells, at any time analysed, do not express Dlx6, this does not rule out a transient and early Dlx6 expression in maxillary-fated cells in the nasal prominence. We have added a paragraph at the end of the discussion to emphasize this issue, including the sentence: ’As the compound Dlx5/6 mutant displays an upper jaw malformation which is not observed in either single mutant (Jeong et al., 2008), it appears that Dlx5 and Dlx6 exert redundant functions not only on lower, but also on upper jaw morphogenesis. Whether there is a specific and transiently-expressing Dlx6 cell population during upper jaw morphogenesis remains, ideally, to be formally determined using an inducible Cre-targeted Dlx6 strain…”

“The authors then describe the expression of an inducible Dlx5 transgene in the maxillary arch and show that Dlx5 expressing cells are restricted to ectoderm, although I could not determine how many animals were examined to determine this.”

We have now modified the text to include this data (n=10 embryos per stage).

“The authors then cite unpublished data for a CNCC-specific knockout of Dlx5/6 and state this has
The extensive analysis of this new series of conditional Dlx5/6 mutant is the subject of another paper in preparation in which we include also the use of other tissue-specific cre-recombinase mice. Deletion of Dlx5 and Dlx6 in CNCCs, from E7 on, results in a mandibular phenotype without any detectable alteration of maxillary skeletal elements.

"How many animals were examined for lacZ reporter gene expression in the maxillary arch by paraffin wax sectioning? Another example and an indication of the number of animals examined is required."

As stated above (and now included in the text) we have analysed 10 embryos per stage. Illustrative results are provided in Figures 2 and 3. The results section has now been modified to precise this point.

"The authors should show that the expression of Dlx5 is absent from the maxillary arch at comparable stages to the lacZ staining (this is referenced, but not shown at comparable stages to the lacZ stained animals in"

"I would like to see what expression of Dlx6 is in the maxillary arch at comparable stages to those examined by the Dlx5-lacZ transgene."

Dlx 5/6 expression profiles have been already largely described previously (eg: Ozeki et al., 2004; Charité et al., 2001) through in situ hybridization analysis and transcriptomic profiling (Jeong et al., 2008) of the first pharyngeal arch throughout craniofacial embryogenesis. Neither transcripts have been detected in significant amounts in the maxillary compartment of all analysed species (eg Debiais-Thibaud et al., 2013) at these stages.

"I would like to see the data for the mice in which Dlx5 is knocked out in CNCC (unpublished data cited in the Discussion). The central premise of the paper is that the Dlx5 lineage cells in the epithelial of the maxillary arch are acting as the caps, in which case it is crucial to show that a knockout of Dlx5/6 in the CNCC does not affect the morphology of the maxillary skeleton."

As stated above, the analysis of this new series of conditional Dlx5/6 mutant is the subject of another paper in preparation in which we include also the use of other tissue-specific cre-recombinase mice. A remarkable morphological feature of these mutant mice is that after Dlx5/6 deletion only in CNCCs the lower jaw is transformed, but the nasal capsule and the upper jaw are normal reinforcing the notion that Dlx5/6 act on different cellular populations. If the reviewer is interested in seeing some of these images we are glad to provide them personally to him, however, in order to maintain the novelty of our results we cannot attach these pictures to this reply as on F1000Research this will be published online.

"Please reference descriptions of maxillary arches in Dlx5 and Dlx6 single mutants, describe the expression of both genes in the maxilla and highlight the redundancy between these genes in arch patterning. These should be incorporated into the Discussion."

We added a sentence stating that the morphological defects observed in the jaws of single mutants were previously described to be less severe than in the double mutant (Jeong et al., 2008), and we pointed to relevant references.

**Competing Interests:** No competing interest.
The changes made by Gitton and colleagues in this revised manuscript have improved and clarified their new finding regarding Dlx5/6 contributions to upper jaw morphogenesis. In particular, the addition of Figure 3 and the changes to the text more clearly describe the role of epithelial expression of Dlx5 to upper jaw patterning. However, I do not necessarily agree with the statement that these "observations provide the first genetic demonstration of the ‘Hinge and Caps' model" (p.1 Abstract). Previous genetic evidence supporting the Hinge and Caps model comes from references 3, 14, 20 cited in this article, as well as Ferretti et al. 2011 and Inman et al., 2013. Nonetheless, the data shown here add to these previous examples supporting the Hinge and Caps model and further our understanding of the role of Dlx5/6 in jaw morphogenesis.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.
The question that Gitton and colleagues proposed is an important one, as the role of Dlx5/6 in jaw morphogenesis is clearly not limited to lower jaw identity. The 3D reconstructions provide improved morphological detail of the Dlx5/6 mutants, and clearly show the abnormal upper jaw morphology in these mutants.

The main concern I have with this manuscript as it stands is the way the two hypotheses are described, as well as their interpretation. The first hypothesis refers to Dlx5 expression in the epithelium. It is well known that Dlx5 is expressed in the surface cephalic ectoderm and in the epithelia of the nasal pits, where it is important in regulating the competence of the epithelia to signal to the underlying mesenchyme that gives rise to the nasal capsule and upper jaw. It is this role of Dlx5 in the epithelium that is predicted by, and consistent with, the Hinge and Caps hypothesis. The second hypothesis, as it is phrased, suggests that Dlx5 may be expressed in the mesenchyme of the distal upper jaw. The authors do not say mesenchyme, but this is implied by the phrase "cells populating the maxillary arch." This point needs clarification. If the authors simply mean the epithelium overlying the maxillary arch, this is not really different from hypothesis #1, except to suggest that proliferation of cells near the olfactory pit later contribute to the maxillary epithelium. It does not really provide an alternate biological explanation for the mutant phenotype. Additionally, to clarify this point, it would be nice to see sections of the embryos shown in Figure 2 that would clearly show where Lac-Z is expressed- in the epithelia or the mesenchyme. If it is absent from the mesenchyme, then it is incorrect to say that Dlx5/6 expression (transitory or not) in maxillary arch precursors is essential for upper jaw morphogenesis, as the title suggests.

Other minor points:

The authors state that CNCCs populating PA1 come from the prosencephalic and anterior mesencephalic neural folds. In fact, neural crest populating PA1 derives from the posterior mesencephalon and the first and second rhombomeres of the hindbrain.

The authors point out the importance of asymmetric, articulated jaws for predation. It would be more appropriate to say that the evolution of asymmetric jaws has been important for the diversification of vertebrates, as the symmetric jaws of sharks are quite sufficient for predation. This point is also relevant for the evolution of Dlx5/6 expression in the mesenchyme. Although still nested, Dlx gene expression in sharks is distinct from that of mouse and chick, and in fact, Dlx5 expression in shark embryos occurs in the mesenchyme of the upper jaw. This difference in expression may be related to the degree of symmetry in upper and lower jaw morphology (see Compagnucci C et al., 2013).

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

**Competing Interests:** No competing interests were disclosed.
Dlx5/6 mutants, we think that what she calls the “two hypotheses” of this paper needs further consideration.

This paper is based on experimental evidence. We are not formulating any hypothesis, but we provide experimental evidence supporting an existing hypothesis: the “Hinge and Caps hypothesis” (for instance Fish JL et al., 2011). We show that indeed cells derived from the frontonasal epithelium after losing the expression of Dlx5/6 migrate to the epithelium overlaying the maxillary arch. This is what we meant saying “cells populating the maxillary arch.”; in no way did we hint to the possibility that mesenchymal cells populating the maxillary arch did express at any time Dlx5/6. The whole text of the manuscript has been reformulated to clarify this point. We have now added a new figure (Figure 3) demonstrating experimentally that derivatives of Dlx5/6 positive cells in the upper jaw are epithelial and not mesenchymal. To make this point even clearer we have changed the title and several sentences of the paper referring now to “Dlx5/6 epithelial precursors”.

Regarding the first hypothesis that the reviewer claims that we have formulated: “That loss of Dlx5 in the epithelia overlying the developing upper jaw primordia disrupts signaling to the underlying CNC (as previously hypothesized by the Hinge and Caps model of jaw development)” it is important to note that Dlx5 is NEVER expressed by the epithelia overlying the developing upper jaw primordia. What we show is that derivatives of cells from the frontonasal primordial (FNP) migrate, after having downregulated Dlx5/6, to the upper jaw and then play an important role in defining upper jaw identity. These cells carry therefore a “memory” of having expressed Dlx5/6 before migrating to the epithelia overlying the upper jaw primordia.

As the reviewer asks: “to clarify this point, it would be nice to see sections of the embryos shown in Figure 2 that would clearly show where Lac-Z is expressed- in the epithelia or the mesenchyme.” we have added Figure 3.

Other minor points:

The authors state that CNCCs populating PA1 come from the prosencephalic and anterior mesencephalic neural folds. In fact, neural crest populating PA1 derives from the posterior mesencephalon and the first and second rhombomeres of the hindbrain.

We removed the sentence as the origin of CNCCs is not particularly relevant to the paper.

It would be more appropriate to say that the evolution of asymmetric jaws has been important for the diversification of vertebrates, as the symmetric jaws of sharks are quite sufficient for predation.

We agree with the reviewer and the discussion has been modified accordingly including the cited reference.

Thanking you again for the time and energy you give to the reviewing process,

Sincerely yours,

YG, NNN, GL

Competing Interests: No competing interests were disclosed.