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 Endocrinienes, 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.
Corresponding author: Giovanni Levi (glevi@mnhn.fr)

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 [v2; ref status: approved 1, approved with reservations 1, http://f1000r.es/3ek] F1000Research 2014, 2:261 (doi: 10.12688/f1000research.2-261.v2)

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)
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 dermocranial derivatives of PA1 are formed by Cranial Neural Crest Cells (CNCCs). During migration, signals emanating from the endoderm and possibly other PA1 components instruct the CNCCs to unfold the morphogenetic process of the jaws. The nested expression of Dlx homeobox genes, vertebrate homologues of Drosophila Distal-less, has a fundamental role in the specification of the dorsoventral 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. 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 proximity to the extremities 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 transgenic lineage tracing approach, we reveal that the maxillary arch epithelium harbours a cellular contingent derived from frontonasal Dlx5-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° 68-028r1). For this study we used about 35 dams (including 10 WT, 5 Dlx5+/−; 3 Dlx5/6−/−; 12 B6.129S4-Gt(Rosa)26Sortm3(cre/ERT2)Zjh; 5 B6(Cg)-Dlx5tm1(cre/ERT2)Zjh/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.

Dlx5/6−/− 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(Cg)-Dlx5tm1(cre/ERT2)Zjh/J (designed by Z. J. Huang) and the Cre reporter strain B6.129S4-Gt(Rosa)26Sortm3(cre/ERT2)Zjh/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 and euthanized by cervical dislocation at indicated stages and embryos were collected in phosphate-buffered saline (PBS), then staged and fixed 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-F1i). Pictures were aligned, piled and registered using the Fiji plug-in 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 18.5 dpc (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. Exam-
ing 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 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 Dlx5 expression in maxillary arch progenitors**

In 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 (Figure 2A,A’). To
understand the origin of the Dlx5/6-dependent defect of the upper
jaw we used a genetic approach to follow the lineage of Dlx5-
precursors in the head. To this end we brought the R26R-lacZ
reporter into the Dlx5-creERT2 driver background and we activated
cre-recombinase activity by tamoxifen treatment of the pregnant
dam at ts9 (7 dpc). We monitored β-Gal reporter activity from
ts15 (10 dpc) to ts20 (12.5 dpc). At ts15 we observed a stream of
β-Gal-positive cells extending from the lambdoidal junction, which
joins the olfactory pit with the distal maxillary arch1-3, 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 epi-
thelial 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 Dlx5-positive cells, we performed serial paraffin sections of Dlx5-creERT2; R26R-lacZ stained mouse embryos (12.5 dpc) after tamoxifen treatment of pregnant dams at 7dpc/Theiler stage 9 (ts9) (Figure 3). While in the mandibular arch ß-Gal staining is limited, as expected, to CNCCs derivatives, 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 in 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 Dlx5/6.

Figure 2. Lineage of Dlx5-expressing cells in the maxillary arch. ß-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.
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. 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, Debiais-Thibaud M, Coolen 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, Scyllorhinus canicula. Dev Biol. 2013; 377(2): 428–48. PubMed Abstract | Publisher Full Text
4. Tan SS, Morriss-Kay GM: Analysis of cranial neural crest cell migration and early fates in postimplantation rat chimaeras. J Embryol Exp Morphol. 1986; 98: 21–58. PubMed Abstract
5. Couly GF, Coltey 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
6. Creuzet S, Couly G, Vincent C, et al.: Negative effect of Hox gene expression on the development of the neural crest-derived facial skeleton. Development. 2002; 129(18): 4301–13. PubMed Abstract
7. Kontges G, Lumsden A: Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. Development. 1996; 122(10): 3229–42. PubMed Abstract
8. Noden DM: Vertebrate craniofacial development: novel approaches and new dilemmas. Curr Opin Genet Dev. 1992; 2(4): 576–81. PubMed Abstract | Publisher Full Text
9. Trainor PA, Tam PP: Cranial paraxial mesoderm and neural crest cells of the mouse embryo: co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches. Development. 1995; 121(8): 2569–82. PubMed Abstract
10. Ruhin B, Creuzet S, Vincent C, et al.: Patterning of the hyoid cartilage depends upon signals arising from the ventral foregut endoderm. Dev Dyn. 2003; 228(2): 219–46. PubMed Abstract | Publisher Full Text
11. Couly G, Creuzet S, Bennaceur S, et al.: Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. Development. 2002; 129(4): 1061–73. PubMed Abstract
12. Merlo GR, Zerega B, Paleari L, et al.: Multiple functions of Dlx genes. Int J Dev Biol. 2000; 44(6): 619–26. PubMed Abstract
13. Beverdam A, Merlo GR, Paleari L, et al.: Jaw transformation with gain of symmetry after Dlx5/Dlx6 inactivation: mirror of the past? Genesis. 2002; 34(4): 221–7. PubMed Abstract | Publisher Full Text
14. Depew MJ, Lufkin T, Rubenstein JL: Specification of jaw subdivisions by Dlx genes. Science. 2002; 298(5592): 381–5. PubMed Abstract | Publisher Full Text
15. Clouthier DE, Hosoda K, Richardson JA, et al.: Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. Development. 1998; 125(5): 813–24. PubMed Abstract
16. Ozeki H, Kurihara Y, Torami K, et al.: Endothelin-1 regulates the dorsoventral branchial arch patterning in mice. Mech Dev. 2004; 121(4): 887–95. PubMed Abstract | Publisher Full Text
17. Ruest LB, Xiang X, Lim KC, et al.: Endothelin-A receptor-dependent and -independent signaling pathways in establishing mandibular identity. Development. 2004; 131(18): 4413–23. PubMed Abstract | Publisher Full Text | Free Full Text
18. Fukuhara S, Kurihara Y, Arima Y, et al.: Temporal requirement of signaling cascade involving endothelin-1/endothelin receptor type A in branchial arch development. Mech Dev. 2004; 121(10): 1223–33. PubMed Abstract | Publisher Full Text | Free Full Text
19. Sato T, Kurihara Y, Asai R, et al.: An endothelin-1 switch specifies maxillomandibular identity. Proc Natl Acad Sci U S A. 2008. PubMed Abstract | Publisher Full Text | Free Full Text
20. Fish JL, Vilimoare B, Kibernick K, et al.: Satb2, modularity, and the evolvability of the vertebrate jaw. Evol Dev. 2011; 13(6): 549–64. PubMed Abstract | Publisher Full Text | Free Full Text
21. Acampora D, Merlo GR, Paleari L, et al.: Craniofacial, visceral and bone defects in mice lacking the Distal-less-related gene Dlx5. Development. 1999; 126(17): 3795–805. PubMed Abstract
22. Merlo GR, Paleari L, Manters S, et al.: Mouse model of split hand/foot malformation type I. Genesis. 2002; 33(2): 97–101. PubMed Abstract | Publisher Full Text | Free Full Text
23. Taniguchi H, He M, Wu P, et al.: A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. Neuron. 2011; 71(6): 995–1013. PubMed Abstract | Publisher Full Text | Free Full Text
24. Gitton Y, Cohen-Tannoudji M, Wassef M: Specification of somatosensory area identity in cortical explants. J Neurosci. 1999; 19(12): 4889–98. PubMed Abstract
25. Tamarin A, Boyde A: Facial and visceral arch development in the mouse embryo: a study by scanning electron microscopy. J Anat. 1977; 124(Pt 3): 563–80. PubMed Abstract | Free Full Text
26. Depew MJ, Liu JK, Long JE, et al.: Dlx5 regulates regional development of the branchial arches and sensory capsules. Development. 1999; 126(17): 3831–46. PubMed Abstract
27. Gitton Y, Benouaiche L, Vincent C, et al.: Dlx5 and Dlx6 expression in the anterior neural fold is essential for patterning the dorsal nasal capsule. Development. 2011; 138(5): 897–903. PubMed Abstract | Publisher Full Text | Free Full Text
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 Dlx5/6 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 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 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 Dlx5-lineage cells in the maxillary arch, in conjunction with the phenotype of the 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 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 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 Dlx6 expression or 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 Dlx5 expressing cell lineage in the ectoderm is controlling patterning of the maxillary arch, yet the mutant being examined is a compound 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 Dlx6 and Dlx5 single mutant animals? Beverdam et al. (2002), highlighted the combinatorial consequences of Dlx5 and 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 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 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).

3. 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.

4. 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 lineage cells in the Dlx5 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.

5. 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.

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.

Author Response 10 Sep 2014

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 (e.g.: 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 no phenotype in the maxillary arch, but fail to show this”

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 (e.g: 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 (e.g. 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.
**Competing Interests:** No competing interests were disclosed.

---

**Jennifer Fish**  
Orthopaedic Surgery, University of California, San Francisco, San Francisco, CA, USA

In this manuscript, Gitton and colleagues explore the role of Dlx5/6 in upper jaw morphogenesis. Dlx5/6 have largely been recognized for their role in lower jaw identity, based on the fact that loss of these genes in mice results in a loss of lower jaw identity. Previous reports have further suggested that loss of Dlx5/6 in mice causes a transformation of identity from that of lower jaw to upper jaw. In this manuscript, Gitton and colleagues present 3D reconstructions of WT and Dlx5/6 mutant mouse jaws, which allow for a more detailed analysis of the jaw phenotype. They note that the Dlx5/6 jaws not only exhibit dysmorphic lower jaw structures, but the upper jaw elements are also abnormal. They propose two hypotheses that could explain this data: 1) That loss of Dlx5 in the epithelia overlying the developing upper jaw primorida disrupts signaling to the underlying CNC (as previously hypothesized by the Hinge and Caps model of jaw development), or 2) that Dlx5 is transiently expressed in cells that will later populate the maxillary arch, and that this transient expression is essential for subsequent upper jaw morphogenesis. Using lineage tracing experiments, the authors conclude that Dlx5 is indeed transiently expressed in precursors that will populate the maxillary arch, and also provide support for the Hinge and Caps model.

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 epithelia 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 mesenchepalic neural folds. In fact, neural crest populating PA1 derives from the posterior mesencephalon and the first and second rhomobomeres 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.

Author Response 06 May 2014
Giovanni Levi, CNRS/MNHN, France

We want, first of all, to thank Dr. Fish for her rapid review of our report. Her suggestions gave us the possibility to modify and, in our view, to improve our article taking in account her input.

While we thank the reviewer for recognizing the importance of the question addressed in this study and for providing improved morphological analysis showing the abnormal upper jaw morphology of 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 primorida 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.