RESEARCH ARTICLE

Transitory expression of *Dlx5* and *Dlx6* in maxillary arch precursors is essential for upper jaw morphogenesis [v1; ref status: approved with reservations 1, http://f1000r.es/2e8]

Yorick Gitton, Nicolas Narboux-Nême, Giovanni Levi

Evolution des Régulations Endocriniennes, CNRS, UMR7221, Muséum National d'Histoire Naturelle, Paris, France

v1 **First published:** 28 Nov 2013, **2**:261 (doi: 10.12688/f1000research.2-261.v1)
Second version: 16 May 2014, **2**:261 (doi: 10.12688/f1000research.2-261.v2)
Latest published: 16 Sep 2014, **2**:261 (doi: 10.12688/f1000research.2-261.v3)

Abstract

Asymmetric, articulated jaws support active predation in vertebrates; 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 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 findings extend current models of jaw morphogenesis and provide an explanation for the maxillary defects of *Dlx5* and *Dlx6* mutants. Our results imply that Dlx5 and Dlx6 model the upper and the lower PA1 components through different morphogenetic mechanisms which are, however, coordinated as they give rise to functional, articulated jaws.

Open Peer Review

Referee Status: ✓ ✓

	Invited Referees 1	2
REVISED **version 3** published 16 Sep 2014		✓ report
REVISED **version 2** published 16 May 2014	✓ report	? report
version 1 published 28 Nov 2013	? report	

1 **Jennifer Fish**, University of California, San Francisco USA

2 **Robert Knight**, King's College London, Guy's Hospital UK

Discuss this article

Comments (0)

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 precursors is essential for upper jaw morphogenesis [v1; ref status: approved with reservations 1, http://f1000r.es/2e8]** *F1000Research* 2013, **2**:261 (doi: 10.12688/f1000research.2-261.v1)

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 vertebrate skull is characterized by the presence of articulated, asymmetric jaws which support the function of a muscularized oral cavity essential for predation. During embryonic development, the upper and lower jaws derive from the maxillary and mandibular processes of the first pharyngeal arch (PA1). Most cartilaginous and dermatocranial derivatives of PA1 are formed by Cranial Neural Crest Cells (CNCCs) emigrating from the prosencephalic and anterior mesencephalic neural folds[1–6]. During migration, signals emanating from the endoderm and possibly other PA1 components instruct the CNCCs to unfold the morphogenetic process of the jaws[5,7,8]. 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[9,10]. The six *Dlx* genes found in mammals are arranged as closely associated bigenic clusters: *Dlx1, Dlx2; Dlx3, Dlx4; Dlx5, Dlx6*; these pairs of genes are often coregulated and display a partially redundant function. 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[11–14]. Interestingly it has been observed[15,16] that, after inactivation of *Dlx5* and *Dlx6*, the maxillary component is also affected despite the fact that these genes are not expressed in 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[17]. 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). While the origin and nature of these signals remain elusive, the possibility that transient *Dlx* expression in a contingent of cells populating the maxillary arch could play a role in its morphogenesis has not been yet analyzed. 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 harbours a cellular contingent derived from *Dlx5* progenitors.

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$^{lacZ/+}$*; 3 *Dlx5/6+/-*; 12 *B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J*; 5 *B6(Cg)-Dlx5$^{tm1(cre/ERT2)Zjh}$/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 form Charles River France and were maintained in the MNHN mouse facility which is officially certified by the French National Animal well being committee.

Dlx5$^{lacZ/+}$ knock-in mice were maintained on a mixed B6/D2 genetic background[18]. Double *Dlx5* and *Dlx6* (*Dlx5/6*) mutant mice were maintained and genotyped as reported[19]. The inducible Cre driver strain *B6(Cg)-Dlx5$^{tm1(cre/ERT2)Zjh}$/J* (designed by Z. J. Huang[20]), and the *lacZ* Cre reporter strain *B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J (R26R-lacZ)* 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 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[18,21]. Vehicle (corn oil) injection in double heterozygous mice did not yield leaking ß-galactosidase activity.

Histology and 3D reconstruction

Heads from 18.5 dpc (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[22] and photographed (Nikon Digital Site DS-FI1). 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[15,16]. To better visualize the jaw phenotype of *Dlx5/6* mutants, we performed 3D reconstruction 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. 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 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.

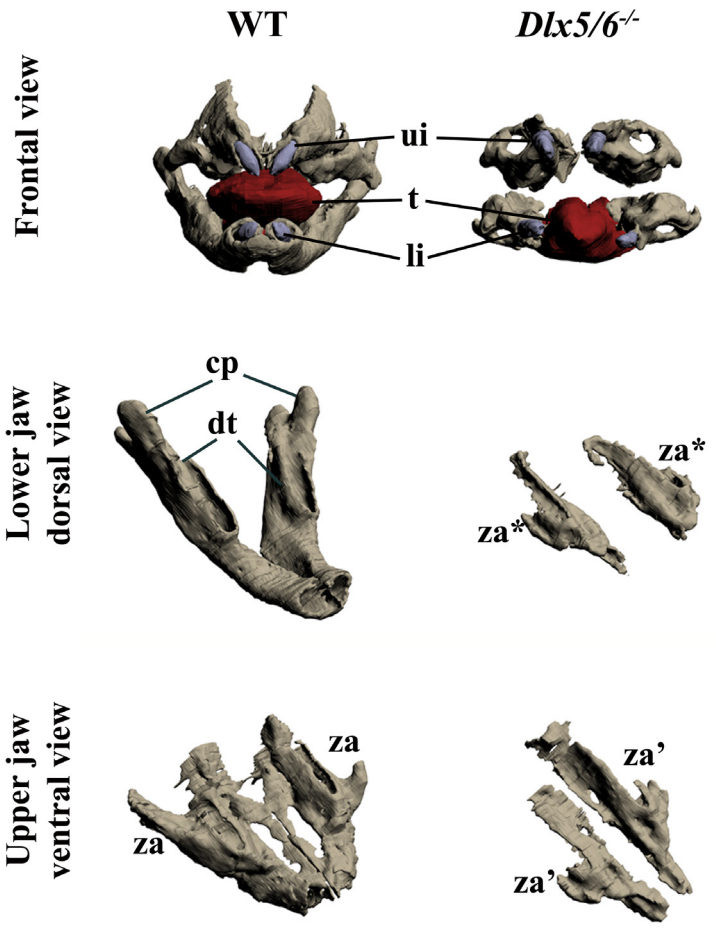

Figure 1. Three-dimensional reconstruction of the dentary and maxillary bones of 18.5 dpc wild type and *Dlx5/6*$^{-/-}$ mouse embryos. Upper row: Frontal view of WT and *Dlx5/6*$^{-/-}$ oral apparatus. Skeletal elements are grey, the tongue is red and incisors are purple. Middle row: Dorsal view of the dentary bone of WT and *Dlx5/6*$^{-/-}$ 18.5 dpc mice. Lower row: Ventral view of the maxillary components of WT and *Dlx5/6*$^{-/-}$ 18.5 dpc mice. Note that the inactivation of *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.

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 (6.25 dpc). We monitored ß-gal reporter activity from ts15 (10 dpc) to ts20 (12.5 dpc). At ts15 we observed a stream of ß-gal-positive cells extending from the lambdoidal junction, which joins the olfactory pit with the distal maxillary arch[17,23], 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').

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. 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 arch[17,23]. These derivatives of *Dlx5*-positive cells have lost *Dlx5* expression as seen by *Dlx5 in situ* hybridization (see for example Depew *et al.* (2002)[16], Acampora *et al.* (1999)[18] and Depew *et al.* (1999)[24]) and by *lacZ-Dlx5* knock-in[18], and Figure 2A'. We have shown that early *Dlx5* and *Dlx6* expression in the anterior neural fold is essential for nasal capsule patterning[25]; our present findings suggest that the same population of cells could also contribute to maxillary patterning. This cell contingent might well exert a patterning role upon the maxillary arch providing either epithelial or mesenchymal cues. This observation fits with

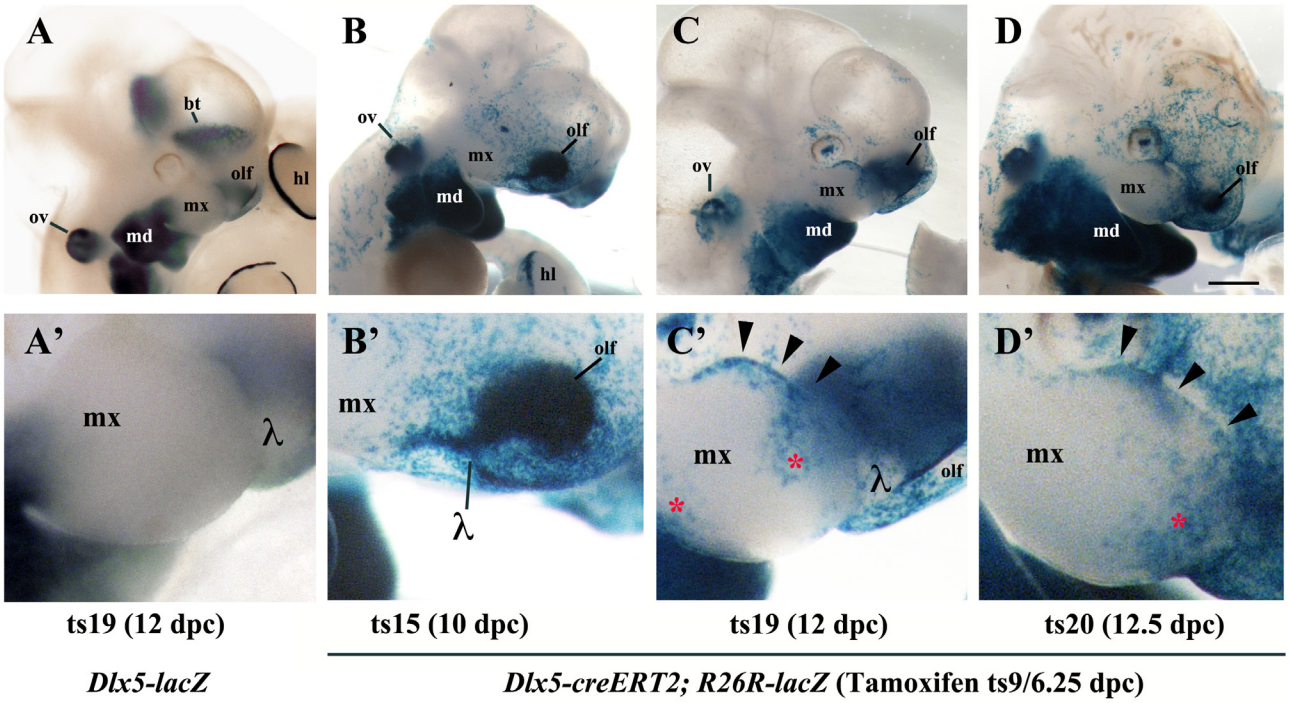

Figure 2. Lineage of *Dlx5*-expressing cells in the maxillary arch. ß-Galactosidase activity in the cephalic region of *Dlx5-lacZ* (**A**,**A'**) and *Dlx5-creERT2; R26R-lacZ* mouse embryos (**B**–**D'**). In all cases pregnant dams were treated with tamoxifen at 6.25 dpc/Theiler stage 9 (ts9) and embryos 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 basal telencephalon (bt) 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.

the prediction of the 'hinge and caps' model[17], and suggests that 'cap' signals could originate from derivatives of *Dlx5*-expressing progenitors. Even if after migration in the maxillary arch these cells lose *Dlx5* expression, it is still possible that the early expression of *Dlx5* confers on them the capacity to pattern maxillary arch CNCCs, which do not themselves express *Dlx5* and *Dlx6*. In contrast, in the lower jaw *Dlx5* and *Dlx6* are expressed by CNCCs; it appears, therefore, that *Dlx5* and *Dlx6* pattern the upper and lower jaw through very different mechanisms, which must be coordinated to generate the asymmetric, articulated, muscularized jaws of vertebrate predators.

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. 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
2. 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
3. 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
4. Kontges G, Lumsden A: **Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny.** *Development.* 1996; **122**(10): 3229–42.
PubMed Abstract
5. Noden DM: **Vertebrate craniofacial development: novel approaches and new dilemmas.** *Curr Opin Genet Dev.* 1992; **2**(4): 576–81.
PubMed Abstract | Publisher Full Text
6. 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
7. 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): 239–46.
PubMed Abstract | Publisher Full Text
8. 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
9. Merlo GR, Zerega B, Paleari L, *et al.*: **Multiple functions of Dlx genes.** *Int J Dev Biol.* 2000; **44**(6): 619–26.
PubMed Abstract
10. 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
11. 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
12. Ozeki H, Kurihara Y, Tonami K, *et al.*: **Endothelin-1 regulates the dorsoventral branchial arch patterning in mice.** *Mech Dev.* 2004; **121**(4): 387–95.
PubMed Abstract | Publisher Full Text
13. 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
14. 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
15. 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
16. Depew MJ, Lufkin T, Rubenstein JL: **Specification of jaw subdivisions by Dlx genes.** *Science.* 2002; **298**(5592): 381–5.
PubMed Abstract | Publisher Full Text
17. Depew MJ, Compagnucci C: **Tweaking the hinge and caps: testing a model of the organization of jaws.** *J Exp Zoolog B Mol Dev Evol.* 2008; **310**(4): 315–35.
PubMed Abstract | Publisher Full Text
18. Acampora D, Merlo GR, Paleari L, *et al.*: **Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5.** *Development.* 1999; **126**(17): 3795–809.
PubMed Abstract
19. Merlo GR, Paleari L, Mantero S, *et al.*: **Mouse model of split hand/foot malformation type I.** *Genesis.* 2002; **33**(2): 97–101.
PubMed Abstract | Publisher Full Text
20. 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
21. Gitton Y, Cohen-Tannoudji M, Wassef M: **Specification of somatosensory area identity in cortical explants.** *J Neurosci.* 1999; **19**(12): 4889–98.
PubMed Abstract
22. Sato T, Kurihara Y, Asai R, *et al.*: **An endothelin-1 switch specifies maxillomandibular identity.** *Proc Natl Acad Sci U S A.* 2008; **105**(48): 18806–11.
PubMed Abstract | Publisher Full Text | Free Full Text
23. 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
24. 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
25. 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

Open Peer Review

Current Referee Status:

Version 1

Referee Report 10 February 2014

doi:10.5256/f1000research.3104.r3486

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 mesenchepalic 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.