Transitory expression of *Dlx5* and *Dlx6* in maxillary arch precursors is essential for upper jaw morphogenesis [version 1; peer review: 1 approved with reservations]

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**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.
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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. 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. The six Dlx genes found in mammals are arranged in a clustered and highly conserved manner in the genome. Dlx1 and Dlx2 are expressed in the maxillary component of PA1, Dlx5 and Dlx6 in the mandibular component, and Dlx3, Dlx4, Dlx7 in both. While Dlx1 and Dlx2 are expressed in all embryonic tissues, the Dlx3, Dlx4, and Dlx7 genes are expressed only in the cranial neural crest cells (CNCCs) and their derivatives, the cranial and dermatocranial derivatives.

Results

Dlx5/6 inactivation results in 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 indicated that the upper jaw of these mice is not normal. 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 their normal counterparts.

β-galactosidase detection

For lacZ expression, embryos were fixed for 15–30 min in 4% paraformaldehyde. Vehicle (corn oil) injection in double heterozygous mice did not yield leaking β-galactosidase activity.
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. These derivatives of Dlx5-positive cells have lost Dlx5 expression as seen by Dlx5 in situ hybridization (see for example Depew et al. (2002) and Acampora et al. (1999) and Depew et al. (1999)) and by lacZ-Dlx5 knock-in, and Figure 2A'. We have shown that early Dlx5 and Dlx6 expression in the anterior neural fold is essential for nasal capsule patterning; 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

**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. These derivatives of Dlx5-positive cells have lost Dlx5 expression as seen by Dlx5 in situ hybridization (see for example Depew et al. (2002) and Acampora et al. (1999) and Depew et al. (1999)) and by lacZ-Dlx5 knock-in, and Figure 2A'. We have shown that early Dlx5 and Dlx6 expression in the anterior neural fold is essential for nasal capsule patterning; 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

**Transient Dlx5 expression in maxillary arch progenitors**

In Dlx5-lacZ heterozygous Théiler 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 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 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').

**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.
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.

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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 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, 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.

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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 mesencephalic 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).

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and 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.

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**Author Response 06 May 2014**

**Giovanni Levi**, Muséum National d'Histoire Naturelle, Paris, 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 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 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.
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