



RESEARCH ARTICLE

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

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**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.

**Open Peer Review**

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**REVISED Amendments from Version 1**

We would like 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 by taking her input into account.

The whole text of the manuscript has been reformulated to clarify that we are not formulating any hypothesis but rather providing evidence for the 'Hinge and Caps' hypothesis. 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. We have changed the title and several sentences of the paper referring now to "*Dlx5/6* epithelial precursors" to make this point even clearer.

**See referee reports**

## Introduction

The skull of most vertebrates is characterized by the presence of articulated, asymmetric jaws which support the function of a muscularized oral cavity<sup>2,3</sup>. During embryonic development, the upper and lower jaws derive from the maxillary and mandibular processes of the first pharyngeal arch (PA1). Most cartilaginous and dermato-cranial derivatives of PA1 are formed by Cranial Neural Crest Cells (CNCCs)<sup>4-9</sup>. During migration, signals emanating from the endoderm and possibly other PA1 components instruct the CNCCs to unfold the morphogenetic process of the jaws<sup>8,10,11</sup>. 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<sup>2,12</sup>. 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*<sup>13,14</sup> 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<sup>15-19</sup>. Interestingly it has been observed<sup>13,14</sup> 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<sup>1,3,20</sup>. 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*<sup>+/-</sup>; 3 *Dlx5/6*<sup>+/-</sup>; 12 *B6.129S4-Gt(ROSA)26Sor<sup>tm1Sor</sup>/J*; 5 *B6(Cg)-Dlx5<sup>tm1(cre/ERT2)Zjh</sup>/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<sup>lacZ/+</sup>* knock-in mice were maintained on a mixed B6/D2 genetic background<sup>21</sup>. Double *Dlx5* and *Dlx6* (*Dlx5/6*) mutant mice were maintained and genotyped as reported<sup>22</sup>. The inducible Cre driver strain *B6(Cg)-Dlx5<sup>tm1(cre/ERT2)Zjh</sup>/J* (designed by Z. J. Huang<sup>23</sup>), and the Cre reporter strain *B6.129S4-Gt(ROSA)26Sor<sup>tm1Sor</sup>/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<sup>21,24</sup>. Vehicle (corn oil) injection in double heterozygous mice did not yield leaking β-galactosidase activity.

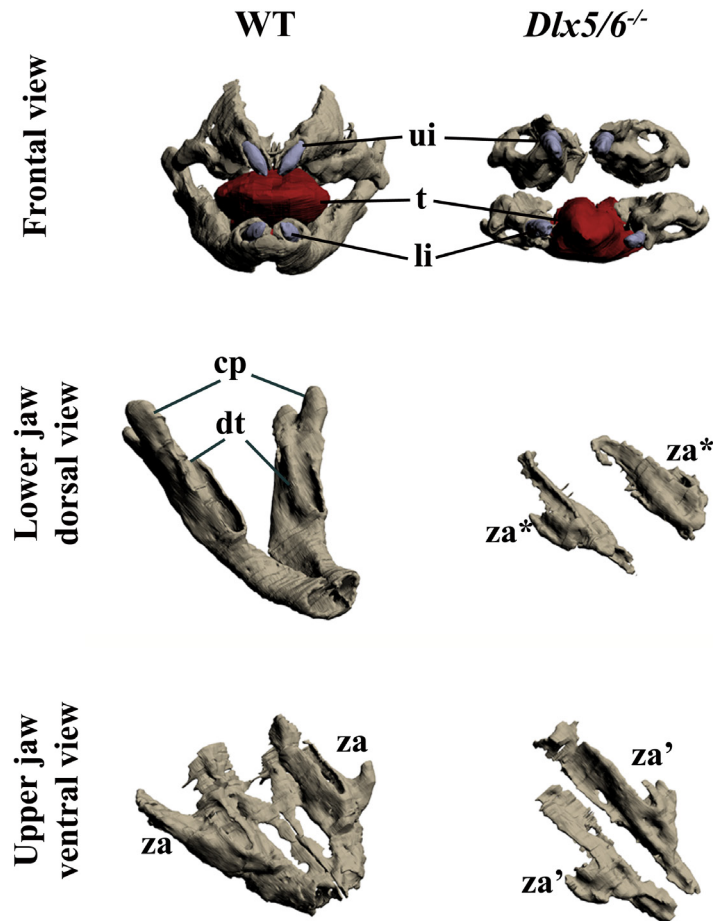
### Histology and 3D reconstruction

Heads from 18.5dpc (days post coitum) *Dlx5/6*<sup>-/-</sup> 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<sup>19</sup> and photographed (Nikon Digital Site DS-F11). 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](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<sup>13,14</sup>. 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)



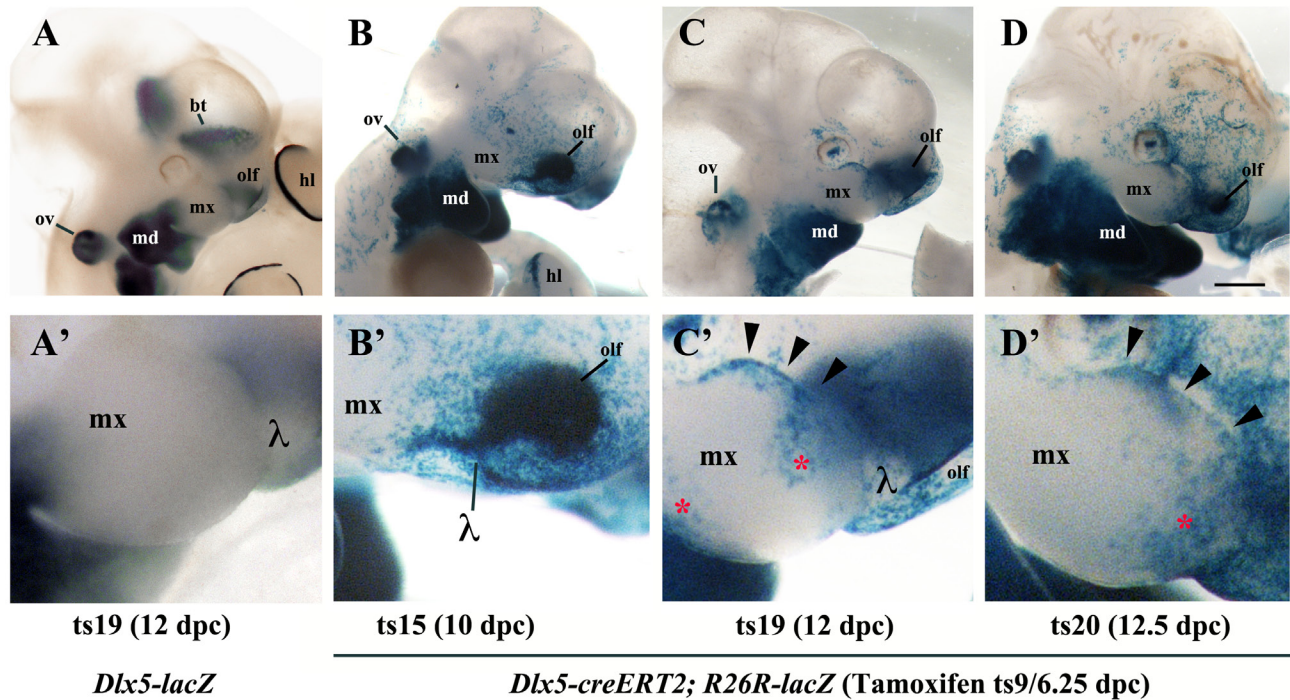
**Figure 1. Three-dimensional reconstruction of the dentary and maxillary bones of 18.5dpc wild type and *Dlx5/6*<sup>-/-</sup> mouse embryos.** Upper row: Frontal view of WT and *Dlx5/6*<sup>-/-</sup> oral apparatus. Skeletal elements are grey, the tongue is red and incisors are violet. Middle row: Dorsal view of the dentary bone of WT and *Dlx5/6*<sup>-/-</sup> 18.5dpc mice. Lower row: Ventral view of the maxillary components of WT and *Dlx5/6*<sup>-/-</sup> 18.5dpc 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.

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.

#### 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  $\beta$ -Gal reporter activity from ts15 (10 dpc) to ts20 (12.5 dpc). 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<sup>1,25</sup>, 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').



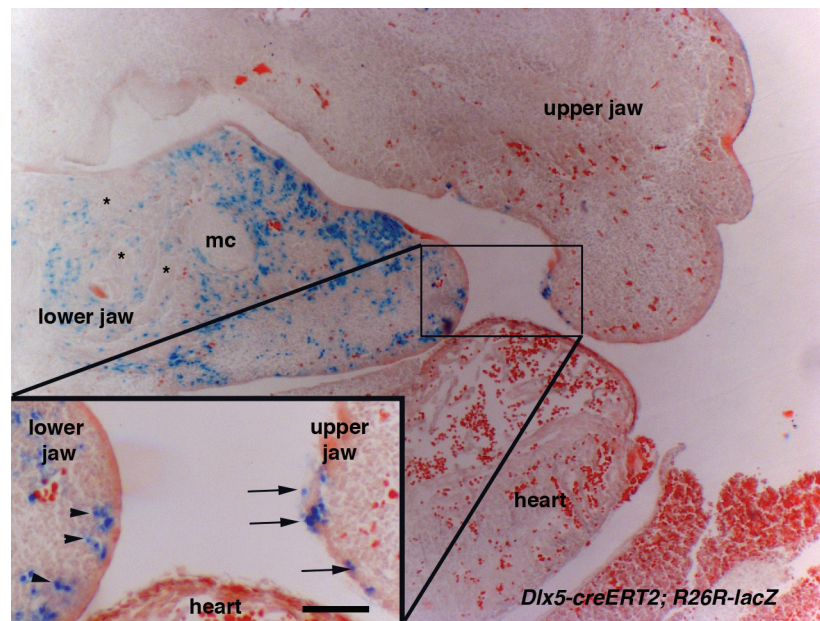
**Figure 2. Lineage of *Dlx5*-expressing cells in the maxillary arch.**  $\beta$ -Galactosidase activity in the cephalic region of *Dlx5-lacZ* (**A,A'**) and *Dlx5-creERT2; R26R-lacZ* mouse embryos (**B-D'**). In all cases pregnant dams were treated with tamoxifen at 7dpc/Theiler stage 9 (ts9) and embryos 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 ( $\lambda$ ) 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;  $\lambda$ , lambdoidal junction; red asterisk/black arrowheads, territories of the maxillary arch colonized by derivatives of *Dlx5*-expressing progenitors. Bar: **A-D** 1mm; **A'-D'** 250 $\mu$ m.

To determine more precisely the tissue distribution of craniofacial derivatives of *Dlx5*-positive cells, we performed serial paraffin sections of *Dlx5-creERT2; R26R-lacZ*  $\beta$ -Gal-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  $\beta$ -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*.

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<sup>1,20,25</sup>. These derivatives of *Dlx5*-positive cells have lost *Dlx5* expression as seen by *Dlx5 in situ* hybridization (see for example<sup>14,21,26</sup>) and by *lacZ-Dlx5* knock-in<sup>(21)</sup>, and Figure 2A'). We have shown that early *Dlx5* and *Dlx6* expression in the anterior neural fold is essential for nasal capsule patterning<sup>27</sup>; our present findings suggest that the same population of cells could also contribute to maxillary patterning. This 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 (unpublished observation). This observation fits with the prediction of the 'Hinge and Caps' model<sup>1,3,20</sup>, and suggests that 'cap' signals could originate from derivatives of *Dlx5*-expressing frontonasal progenitors. Even if, after migration in the maxillary arch, these



**Figure 3. Derivatives of *Dlx5*-expressing progenitors in the upper and lower jaw primordia.** Sagittal section of *Dlx5-creERT2; R26R-lacZ* through the cephalic region of a 12.5 dpc/Theiler stage 22 (ts22) mouse embryo. The pregnant dam was treated with tamoxifen at 7dpc/ts9.  $\beta$ -Galactosidase activity is found in CNCCs derivatives of the lower jaw (arrowheads). In contrast, in the upper jaw positive cells are only present in the overlying epithelium (arrows). mc, Meckel's cartilage; asterisks, lower jaw muscles. Scale bar 50 $\mu$ m in insert, 150 $\mu$ m in the main figure.

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.

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

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# Open Peer Review

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Version 2

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**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't 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 *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.
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 (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 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 (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.

Referee Report 02 June 2014

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**Jennifer Fish**

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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.

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Version 1

Referee Report 10 February 2014

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**Jennifer Fish**

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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 primordia 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 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.

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 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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