Jaw transformation with gain of symmetry after Dlx5/Dlx6 inactivation: mirror of the past ?

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ARTICLE

Jaw Transformation With Gain of Symmetry After *Dlx5/Dlx6* Inactivation: Mirror of the Past?

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Summary: In modern vertebrates upper and lower jaws are morphologically different. Both develop from the mandibular arch, which is colonized mostly by Hox-free neural crest cells. Here we show that simultaneous inactivation of the murine homeobox genes *Dlx5* and *Dlx6* results in the transformation of the lower jaw into an upper jaw and in symmetry of the snout. This is the first homeotic-like transformation found in this Hox-free region after gene inactivation. A suggestive parallel comes from the paleontological record, which shows that in primitive vertebrates both jaws are essentially mirror images of each other. Our finding supports the notion that *Dlx* genes are homeotic genes associated with morphological novelty in the vertebrate lineage. genesis 34: 221–227, 2002. © 2002 Wiley-Liss, Inc.

Key words: distal-less; mouse; loss-of-function; homeosis; craniofacial skeleton

INTRODUCTION

The skull is one of the most complex parts of the vertebrate body. Anatomically it is subdivided into the neurocranium, consisting of the vault and skull base, and the viscerocranium, comprising the entire branchial arch-derived skeleton. The posterior part of the head skeleton, including part of the otic capsule, the occipital bone, part of the sphenoid bone, and the postorbital bones, is derived from somitic and cephalic mesoderm (Couly *et al.*, 1993). Most other bones, including the entire viscerocranium, are derived from cranial neural crest cells, which originate from fore-, mid-, and anterior hindbrain regions (Couly *et al.*, 1993). Early during embryogenesis, these cells migrate into the frontonasal process and the branchial arches (BA1-6) and give rise to the nasal capsule, upper and lower jaws, and tongue skeleton.

Cranial neural crest cells derive from two distinct areas: a rostral Hox-negative and a caudal Hox-positive domain. Correct patterning of the Hox-negative region depends on signaling factors derived from the endoderm (Couly *et al.*, 2002). This domain contributes to the frontonasal process and BA1. The more caudal Hox-positive domain requires expression of *Hox* genes for correct patterning and generates the crest of the more posterior branchial arches (Köntges and Lumsden, 1996). For example, *Hoxa2* is expressed up to the second rhombomere. When *Hoxa2* is inactivated, the neural crest cells of BA2 behave like their Hox-negative counterpart of BA1 and form pieces of the lower jaw skeleton (Rijli *et al.*, 1993; Gendron-Maguire *et al.*, 1993). On the other hand, ectopic expression of *Hoxa2* in BA1 causes it's neural crest to adopt a second arch fate, resulting in homeosis of jaw elements (Grammatopoulos *et al.*, 2000; Pasqualetti *et al.*, 2000).

In insects, *Distal-less (Dll)* is required for correct morphogenesis of the distal portion of the legs, antennae, and mouth parts (Cohen *et al.*, 1989; O'Hara *et al.*, 1993). Based on sequencing comparison, it is thought that during the evolution of chordates an initial gene duplication occurred, followed by several cluster duplications and selective gene losses (Stock *et al.*, 1996; Ruddle, 1997; Zerucha and Ekker, 2000). *Dll* homologs have been isolated from vertebrate species like lamprey, zebrafish, newt, *Xenopus,* mouse, and human (see Neidert *et al.*, 2001, and references therein). They constitute a highly conserved family of homeobox genes, which are thought to act as transcription factors.

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In the mouse, six *Dll*-related (*Dlx*) genes were isolated. They are arranged as three convergently transcribed pairs. Each pair is located in proximity of a Hox cluster (*Dlx1* and -2 near *HoxD, Dlx3* and -7 near *HoxB* and *Dlx5* and *-6* near *HoxA).* During murine craniofacial development, *Dlx1*, *Dlx2*, and *Dlx3* are expressed in the mesenchyme of the first and second branchial arches beginning at E9.5. Mice in which *Dlx1* and -2 are inactivated have abnormalities in the proximal first and second arch-derived structures (Qiu *et al.*, 1995, 1997). *Dlx5* and *Dlx6* are expressed in all four branchial arches from around E9.0 onwards. While in BA1 strong expression is detected in the mandibular process with an onset at E9.0, no expression is observed in the maxillary process up to E10.5. Later, both the maxillary and mandibular processes strongly express *Dlx5* and *Dlx6* (Acampora *et al.*, 1999; Depew *et al.*, 1999; Charité *et al.*, 2001). *Dlx5* homozygous mutants have inner ear defects and many abnormalities in the craniofacial skeleton (Acampora *et al.*, 1999; Depew *et al.*, 1999).

Deletion of the coding and intergenic regions of *Dlx5* and *Dlx6* with a single targeting event in the mouse results in perinatal death and in a limb malformation reminiscent of the human ectrodactyly, Split Hand Foot Malformation type I (Merlo *et al.*, 2002; Robledo *et al.*, 2002). In this study, we describe the craniofacial lesion present in *Dlx5/6* double mutant mice. This is characterized by a homeotic-like transformation of the lower jaw into an upper jaw and in gain-of-symmetry of the snout. A suggestive parallel comes from the paleontological records, which show that in primitive bony fishes and early land vertebrates the upper and lower jaws are essentially mirror images of each other (Romer, 1940). Our findings support the notion that *Dlx5* and *-6* are homeotic genes essential for anteroposterior patterning of BA1 in modern mammals.

RESULTS

Generation of *Dlx5/6* Double Mutants

We have deleted the coding and intergenic regions of *Dlx5* and *Dlx6* in the mouse with a single targeting event (Merlo *et al.*, 2002). Homozygous mutant mice die shortly after birth. They have hindlimb malformations and exencephaly or anencephaly together with a unique craniofacial lesion. Both upper and lower jaws are severely affected and seem mirror-images of each other, causing their snouts to be symmetric both along the right–left and antero–posterior planes (Fig. 1a). Strikingly, whiskers pads with vibrissal follicles are visible both on the upper and the lower jaws (Fig. 1b,d). *Bmp4* expression, a marker for the dermal papillae, confirms this observation in E12.5 control and double mutant embryos (Fig. 1c,d; St-Jacques *et al.*, 1998). Moreover, structures resembling palatine rugae, a series of ridges associated with the inner surface of the palatal shelves, are present on the inner surface of both upper and lower jaws (data not shown).

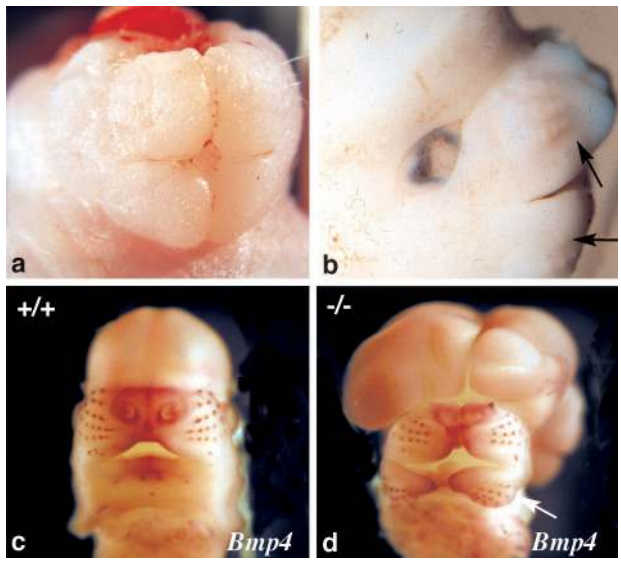

FIG. 1. BA1 phenotype of *Dlx5/6* double mutants. Whole-mount view of *Dlx5/6* double mutant snouts at birth (**a**) and at E12.5 (**b**). In situ hybridization with *Bmp4* probe on E12.5 normal (**c**) and *Dlx5/6* mutant (**d**) embryos. Note the presence of whisker pads (**b**) and *Bmp4*-expressing dermal papillae (**c,d**) both in the upper and in the lower jaw of the mutant (arrows in **b** and **d**).

Gradual Transformation of the Lower Jaw Into an Upper Jaw Depending on *Dlx5/Dlx6* Gene Dosage

To study the skeletal phenotype of *Dlx5/6* double mutants we performed skeletal stainings on E14.5 and newborn *Dlx5* and *-6* single and compound mutant mice. Cartilage skeletal preparations of E14.5 embryos (Fig. 2a–d) show that in *Dlx5/6* homozygous double mutants Meckel's cartilage is almost totally absent, yet in some embryos a small rudiment of a Meckel's cartilage-like structure can be found in the distalmost part of the lower jaw. Skeletal staining of newborn mice lacking two ($Dlx5^{+/-}/Dlx6^{+/-}$ and $Dlx5^{-/-}/Dlx6^{+/+}$), three ($Dlx5^{-/-}/Dlx6^{+/-}$), or four ($Dlx5^{-/-}/Dlx^{-/-}$) alleles of the cluster and dissection of their jaw regions shows that, besides being strongly malformed, the maxillae and mandibles gradually acquire a near to identical shape, depending on the gene dosage (Fig. 3). Loss of two *Dlx* alleles (either in $Dlx^{+/-}/Dlx6^{+/-}$ or $Dlx5^{-/-}/Dlx6^{+/+}$) leads to the loss of the coronoid process, to a reduction of the angular process and to a shortening of the mandible. Removal of three alleles leads to the complete loss of the coronoid, condylar, and angular processes and to a severe shortening of the mandible. Finally, in mutants lacking all four alleles ($Dlx5^{-/-}/Dlx6^{-/-}$), the general structure of the maxillary group of bones remained identifiable, albeit profoundly distorted. In contrast, the mandible became unrecognizable and was transformed in a structure indistinguishable from the deformed maxillary bone complex. This symmetry is particularly evident when the snout skeleton of $Dlx5^{-/-}/Dlx6^{-/-}$ newborn mice is observed from the front (Fig. 3b). Moreover, the transformed lower jaw seemed to articulate with structures

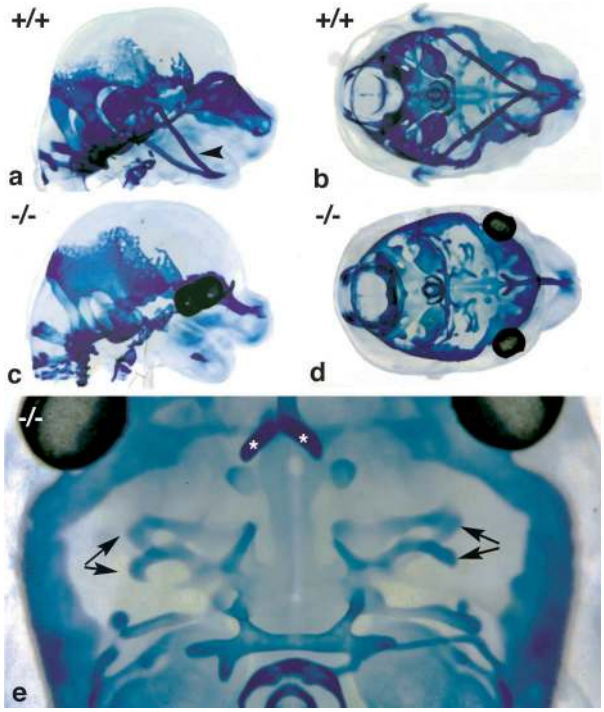

FIG. 2. Cartilage staining of normal and *Dlx5/6* double mutant E14.5 embryos. Cartilage skeletons of normal (**a,b**) and E14.5 *Dlx5/6* double mutant (**c,d,e**) embryos. Meckel's cartilage (arrowhead in **a**) is reduced to a rudiment at the tip of the lower jaw of double mutant animals (asterisks in **e**). Most derivatives of the first, second, and third branchial arch are distorted and fused and the ala temporalis is duplicated (arrows in **e**).

that may be interpreted as distorted and duplicated pterygoid processes, rather than with the squamosal bone in normal skeletons.

Abnormalities in Vaults, Skull Base, and Hyoid Skeleton of *Dlx5/6* Double Mutant Mice

Besides the abnormalities in the first arch-derived skeleton, also second and third arch-derived structures were affected in *Dlx5/6* homozygous double mutants. In double mutants at E14.5 and at birth, fusions were detected between the hyoid bone and the pterygoid processes of the sphenoid bone and the superior horns of the thyroid; the stylohyoid ligament was often chondrified. Additional craniofacial abnormalities were detected in the skull plates, which were virtually absent, and in the anterior skull base. The basisphenoid bone was distorted and severely bent to allow articulation of the pterygoid process with the transformed lower jaw. The presphenoid was strongly reduced and the alisphenoids were duplicated: the two copies lie on different planes and are connected, as illustrated for its precursor, the ala temporalis, in E14.5 skeletons in Figure 2e. The occipital bone, which is derived from presomitic mesoderm, remained relatively unaffected by the mutation.

In conclusion, most affected craniofacial structures are neural crest-derived. This correlates well with the expression patterns of *Dlx5* and *-6* in neural crest-derived mesenchyme in all branchial arches. Malformation of cephalic or presomitic mesoderm derived cranial structures most likely is secondary to the failure of neural tube closure or to the primary skull defects in the viscerocranium. Table 1 is a summary of the cranial structures affected by this mutation.

Transformation of the Mandibular Process Is First Visible Around E10.5/E11.0 and Is Confirmed by Expression of Mandibular Marker Genes

The striking morphological similarity of the upper and the lower jaw of *Dlx5/6* double mutants suggests a transformation of the mandibular process into a maxillary process early during craniofacial development. To determine the time point of onset of the branchial arch abnormalities in *Dlx5/6* double mutant embryos, we isolated E9.5 to E14.5 embryos. While the exencephalic phenotype was already clearly present at E9.5, the branchial arch abnormalities become first visible around E10.5–11.0. At this stage *Dlx5* and *Dlx6* are normally predominantly expressed in the mandibular process of BA1. In E10.5–11.0 *Dlx5/6* double mutant embryos the mandibular processes have failed to fuse and are somewhat increased in size. This defect becomes even more evident in later stage double mutant embryos (see Fig. 4e,f for an E11.5 embryo).

To determine the molecular identity of the mandibular process of *Dlx5/6* double mutant embryos, we analyzed the expression of marker genes with an asymmetric anteroposterior distribution in BA1 (Fig. 4). In E10.5 embryos, *PitX1* is normally expressed in the mesenchyme of the mandibular process and in the ectoderm of the stomodeum (Lanctôt *et al.*, 1997). In E10.5 and E11.0 double mutant embryos, *PitX1* expression was present in the ectoderm of the mandibular and the maxillary process, but was completely absent from the mandibular mesenchyme (see Fig. 4a–c), suggesting that it may be a *Dlx5/6* downstream target gene during patterning of the mandibular process. Moreover, *dHAND,* which in E10.5 pharyngeal regions is usually expressed in the mandibular process and is activated by *Dlx6* (Charité *et al.*, 2001), is almost silenced in the branchial arch of the double mutant (Fig. 4d). Finally, whereas in normal E11.5 embryos *Dlx1* is uniformly expressed in the maxillary process and only proximally in the mandibular process, in the *Dlx5/6* double mutant the expression domain of *Dlx1* in the mandibular process has extended to more distal regions, causing a "mirror-image pattern of expression" in the upper and lower jaws (Fig. 4e,f; Qiu *et al.*, 1995, 1997). These molecular data corroborate the hypothesis that the mandibular process has acquired a maxillary identity.

DISCUSSION

The simultaneous inactivation of *Dlx5* and *Dlx6* in the mouse results in severe malformations in the skull vault and base and in all branchial arch derivatives. Most strikingly, in these mice lower jaws are gradually transformed into upper jaws, depending on the gene dosage

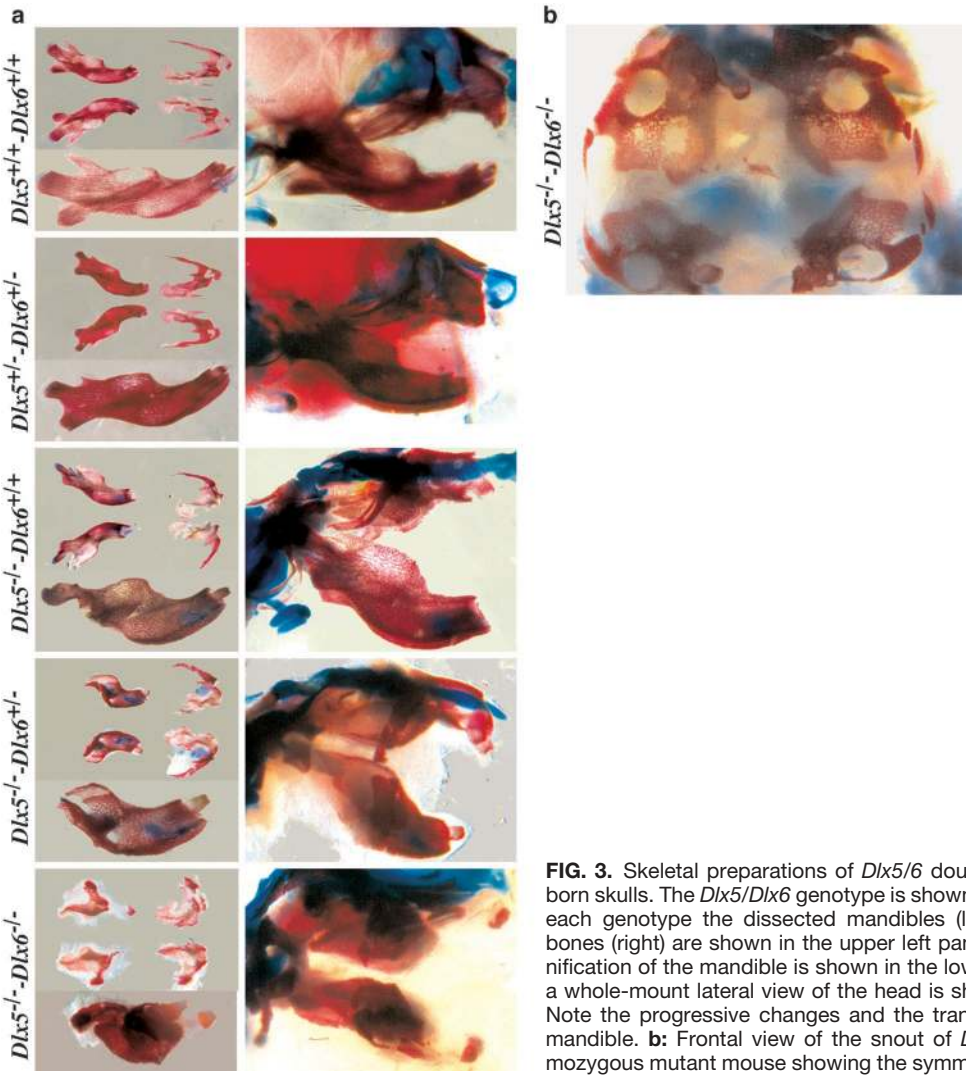

FIG. 3. Skeletal preparations of *Dlx5/6* double mutants newborn skulls. The *Dlx5/Dlx6* genotype is shown on the left. **a:** For each genotype the dissected mandibles (left) and maxillary bones (right) are shown in the upper left panel, a higher magnification of the mandible is shown in the lower left panel, and a whole-mount lateral view of the head is shown on the right. Note the progressive changes and the transformation of the mandible. **b:** Frontal view of the snout of *Dlx5/6* double homozygous mutant mouse showing the symmetry of the mouth.

and resulting in a symmetric snout in mutants lacking all four alleles. Their mandibular processes gives rise to a structure, which is the mirror image of that derived from the maxillary portion ofBA1.

Homeotic-Like Transformation of Lower Jaw Into an Upper Jaw in a Hox-Free Region

Altogether, both morphological observations and molecular data support the hypothesis that combined inactivation of *Dlx5* and *Dlx6* results in a transformation of the lower jaw into an upper jaw. Using the initial definition of homeosis given by Bateson (1894) as a phenomenon in which "something has been changed into the likeness of something else," we could interpret this as a homeotic-like transformation of the mandibular into a maxillary portion of BA1.

Both the upper and the lower jaws derive from BA1, which is colonized by neural crest cells arising from the mesencephalic neural fold and the segmented anterior hindbrain (Köntges and Lumsden, 1996; Couly *et al.*, 1996). It has been shown that these crest cells do not express *Hox* genes and get patterning clues from the endoderm (Couly *et al.*, 2002). Homeosis of jaw elements has previously been shown only after inactivation and forced expression of *Hox* genes in postmigratory neural crest (Rijli *et al.*, 1998; Pasqualetti *et al.*, 2000). Our findings support the notion that *Dlx5* and *Dlx6* can act as homeotic genes essential for anteroposterior patterning of BA1 in modern mammals. These data pave the way for further studies on the origin and molecular nature of the signals involved in BA1 patterning.

Dlx Genes as Evolutionary Tools to Generate Morphological Asymmetry

Apart from sharks, where the upper and lower teeth are relatively similar in shape and number, the upper and lower jaws of the extant jawed vertebrates (gnathostomes) generally differ in the shape and number of their teeth or tooth-bearing dermal bones. In bony fishes (osteichthyans), and land vertebrates (tetrapods), this difference disappears as one considers early, Paleozoic groups, whose upper jaw bones are almost a mirror

Table 1.
Summary of Craniofacial Structures Affected in Dlx5/Dlx6 *Double Mutant Mice at Birth*

		Bones/cartilages	*Dlx5−/−/Dlx6−/−*
Viscerocranium	Premandibular arch	Premaxilla	Affected
		Incisors	Present in most embryos
		Nasal capsule	Affected
		Presphenoid	Affected
	1st arch	Maxilla	Affected
		Zygomatic	Affected
		Palate	Affected
		Mandible	Transformed
		Condylar, angular and coronoid processes	Affected
		Incisors	Present in most embryos
		Malleus and incus	Affected
		Alisphenoid (partly)	Affected, duplicated
	2nd arch	Stapes	Affected
		Hyoid	Affected
		Stylohyoid	Chondrified
	3rd to 6th arch	Thyroid	Affected
Neurocranium	Cartilaginous neurocranium	Sphenoid bone	
		Basisphenoid (Partially NC der.)	Affected
		Alisphenoid (Partially NC der.)	Affected, duplicated
		Pterygoid processes (?)	Affected
		Occipital bone	
		basioccipital	Apparently normal
		exoccipital	Apparently normal
		supraoccipital	Apparently normal
	Membranous neurocranium	Nasal bones (NC derived)	Affected
		Frontal bones (NC derived)	Affected
		Parietal bones (Possibly NC derived)	Affected
		Interparietal bones (?)	Affected
		Squamosal (Partially NC derived)	Affected

image of those of the lower jaw. This curious symmetry was pointed out long ago by the American paleontologist A.S. Romer (1940) in early amphibians, but has never received any explanation other than merely functional. The generalized osteichthyan condition, in this respect, can be observed in a Devonian tristichopterid fish (*Eusthenopteron),* a close piscine relative to the tetrapods (Fig. 5). In living osteichthyans, this bone pattern is profoundly modified in most ray-finned fishes (actinopterygians) and, among tetrapods, in mammals, in which the lower jaw is represented by the dentary alone. But important modifications of the jaw bones, such as the loss of the maxillary or coronoids also occur in the living piscine sarcopterygians, i.e., the coelacanth and the lungfishes.

Conclusion

Our results show that *Dlx5/6* gene inactivation in the mouse leads to a homeotic-like transformation of the lower jaw into an upper jaw and generates a symmetric mouth. The transformed structure is, in a sense, reminiscent of the jaw pattern of early osteichthyans, including early tetrapods. This analogy is a hint for future investigations in the evolution of an asymmetric mouth. Our findings might imply, more generally, that *Dlx* genes were needed to allow asymmetry when required by the rise of complex anatomical structures during evolution. Our data reinforce the concept, already present in the literature that *Dlx* genes are associated with the appearance of morphological novelties in vertebrates (Neidert *et al.*, 2001).

MATERIALS AND METHODS

Locus Targeting

We previously reported the generation of mice with targeted disruption of *Dlx5* and *Dlx6* (Merlo *et al.*, 2002).

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed on E10.5 to E12.5 embryos essentially as described by Wilkinson (1992), with slight modifications. The *Dlx1* probe comprised 720 bp of the 3′ end of murine *Dlx1* cDNA and was linearized with *Bam*HI and transcribed with T7 RNA polymerase. The *Bmp4* probe, kindly provided by R. Zeller (Utrecht, The Netherlands), comprised 1.6 kb of *Bmp4* cDNA sequence and was linearized using *Acc*I and transcribed with T7 RNA polymerase. The *PitX1* probe, provided by P. Briata (Genova, Italy), comprised 950 bp of the 3′ end of the *PitX1* cDNA and was linearized with *Nco*I and transcribed with T3 RNA polymerase. The *dHAND* probe, kindly provided by E. Olson (Dallas, TX, USA), corresponded to 450 bp in the 3′ end of *dHAND* cDNA and

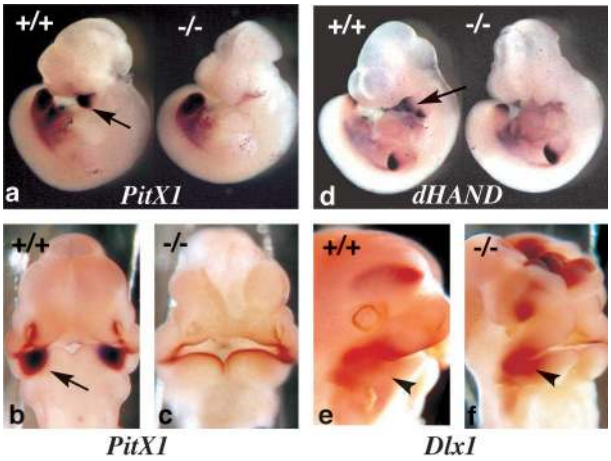

FIG. 4. In situ hybridization on normal and *Dlx5/6* double mutant embryos with *PitX1, Dlx1, dHAND* probes. *PitX1* (**a,b,c**) is normally expressed in the mesenchyme of the mandibular process (arrows) and in the oral epithelium, but is undetectable in the lower jaw of the mutant embryo both at E10.5 (**a**, lateral view) and E11.0 (**c**, frontal view). *dHAND* is normally expressed in a distal territory of BA1 and BA2 at E10.5, but is nearly absent in the *Dlx5/6* double mutant (**d**). *Dlx1* (**e,f**) is expressed in the entire maxillary process and in proximal regions of the mandibular process at E11.5 (**e**), while in the mutant it is detected with a more symmetric distribution (**f**) (arrow in **e** and **f**).

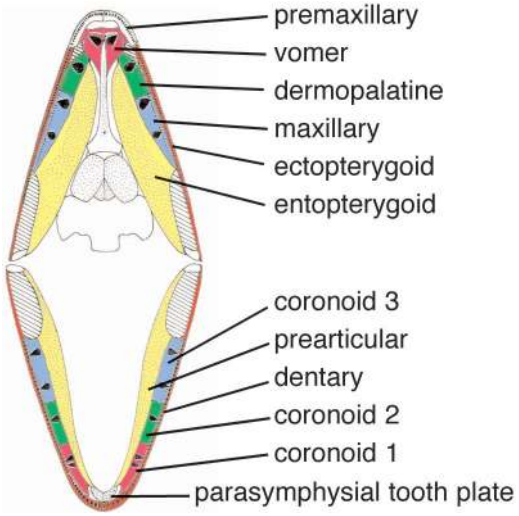

FIG. 5. Dermal bones of the upper and lower jaws in the 370-million-year-old sarcopterygian fish *Eusthenopteron,* showing their mirror-image pattern. The dermal bones of the lower jaw, surrounding the Meckelian bone, consist of a dentary (laterally), three coronoids (dorsally) and a prearticular (medially), and those of the upper jaw, surrounding the palatoquadrate, are the maxillary (laterally), a series of three dermal bones (the vomer, dermopalatine, and ectopterygoid, ventrally), and the entopterygoid (medially). In the upper jaw, the premaxillary, although a tooth-bearing bone, is not a dermal bone of the mandibular arch proper. It has no contact with the palatoquadrate but rests on the snout, and has no counterpart in the lower jaw. Teeth in black, denticle-bearing bone: dotted; choanae and adductor muscle fossae: obliquely hatched.

was linearized with *Eco*RI and transcribed with T7 RNA polymerase. Hybridization was detected with anti-DIG Fab and BCIP/NBT (Roche, Nutley, NJ).

Bone and Cartilage Staining

Cartilage staining of E14.5 embryos as well as bone and cartilage staining of newborn mice was carried out as previously described (Acampora *et al.*, 1999).

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