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Dlx5 and *Dlx6* expression in the anterior neural fold is essential for patterning the dorsal nasal capsule

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SUMMARY

Morphogenesis of the vertebrate facial skeleton depends upon inductive interactions between cephalic neural crest cells (CNCCs) and cephalic epithelia. The nasal capsule is a CNCC-derived cartilaginous structure comprising a ventral midline bar (mesethmoid) overlaid by a dorsal capsule (ectethmoid). Although Shh signalling from the anterior-most region of the endoderm (EZ-I) patterns the mesethmoid, the cues involved in ectethmoid induction are still undefined. Here, we show that ectethmoid formation depends upon *Dlx5* and *Dlx6* expression in a restricted ectodermal territory of the anterior neural folds, which we name NF-ZA. In both chick and mouse neurulas, *Dlx5* and *Dlx6* expression is mostly restricted to NF-ZA. Simultaneous *Dlx5* and *Dlx6* inactivation in the mouse precludes ectethmoid formation, while the mesethmoid is still present. Consistently, siRNA-mediated downregulation of *Dlx5* and *Dlx6* in the cephalic region of the early avian neurula specifically prevents ectethmoid formation, whereas other CNCC-derived structures, including the mesethmoid, are not affected. Similarly, NF-ZA surgical removal in chick neurulas averts ectethmoid development, whereas grafting a supernumerary NF-ZA results in an ectopic ectethmoid. Simultaneous ablation or grafting of both NF-ZA and EZ-I result, respectively, in the absence or duplication of both dorsal and ventral nasal capsule components. The present work shows that early ectodermal and endodermal signals instruct different contingents of CNCCs to form the ectethmoid and the mesethmoid, which then assemble to form a complete nasal capsule.

KEY WORDS: Dlx5, Dlx6, Nasal capsule, Olfactory placode, Neural crest

INTRODUCTION

The formation of craniofacial structures results from interactions between Hox-negative cephalic neural crest cells (CNCCs) and adjacent instructive epithelia (Bee and Thorogood, 1980; Couly et al., 2002). The vertebrate nose builds upon an embryonic cartilaginous frame, the nasal capsule, an evolutionary-conserved structure composed of ventral and dorsal components: the mesethmoid and ectethmoid cartilages, respectively. In avians, the mesethmoid supports upper beak formation, whereas the ectethmoid comprises elements of the olfactory system, including the lamina cribosa, the crista galli apophysis and the conchae.

Ablation and grafting experiments have demonstrated the importance of signalling from the endoderm to CNCCs for the specification and patterning of different craniofacial elements (Couly et al., 2002; Couly et al., 1993). In particular, we have shown that Shh-*Gli1* signalling from the anteriormost territory of the endoderm, which we named 'EZ-I' (Benouaiche et al., 2008) to rostroventrally migrating CNCCs is required for the edification of the chick mesethmoid cartilage. By contrast, the molecular and cellular mechanisms driving ectethmoid formation have not been elucidated.

In the chick embryo, the ectethmoid is formed by CNCCs emigrating from the prosencephalic ridges of the neural plate. These cells follow preferentially a subectodermal route, contact the presumptive olfactory ectoderm at Hamburger Hamilton stage 10 (HH10) and remain in its close proximity up to HH18 (Couly and Le Douarin, 1990; Couly et al., 1993; Le Douarin et al., 2007). Then, neural crest-derived chondrogenic ectomesenchyme accumulates within the nasal prominences. CNCC depletion at the neurula stage prevents any craniofacial chondrogenesis, including that of the nasal capsule. The observation that CNCCs are not specified before migration and constitute an equivalence group (Couly et al., 1992) implies that instructive signals are also required for ectethmoid formation. As the ectethmoid develops in the absence of the endoderm (Benouaiche et al., 2008; Couly et al., 2002; Couly et al., 1998; Ruhin et al., 2003), the patterning instructions may reside in other territories neighbouring CNCCs, such as the prospective olfactory ectoderm.

Starting at the end of gastrulation in amniotes, the presumptive olfactory ectoderm expresses the homeobox genes *Dlx5* and *Dlx6*, which play an important role in olfactory-fate specification of placodal precursors (Acampora et al., 1999; Bhattacharyya and Bronner-Fraser, 2008; McLarren et al., 2003; Pera and Kessel, 1999; Pera et al., 1999; Yang et al., 1998). In mouse, genetic lineage tracing using *lacZ* reporter activity shows persistent *Dlx5* expression throughout nasal differentiation (Acampora et al., 1999). Maintenance of *Dlx5* expression is necessary for the acquisition of olfactory fate in both mouse and chick embryos (Bailey et al., 2006; Bhattacharyya et al., 2004; Bhattacharyya and Bronner-Fraser, 2004; Bhattacharyya and Bronner-Fraser, 2008; Depew et al., 1999; Long et al., 2003; McLarren et al., 2003; Price et al., 1991; Quint et al., 2000; Simeone et al., 1994; Solomon and Fritz, 2002; Szabo-Rogers et al., 2008; Szabo-Rogers et al., 2009). Furthermore, *Dlx5*-null neonates display nasal capsule hypoplasia, which is often asymmetric (Acampora et al., 1999; Depew et al., 1999; Levi et al., 2003; Long et al., 2003). The double inactivation of *Dlx5* and *Dlx6* further accentuates nasal capsule defects, but the

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phenotype has not been yet described in detail (Beverdam et al., 2002; Depew et al., 2002; Levi et al., 2003; Robledo et al., 2002). Noticeably, nasal defects in *Dlx5/6*$^{-/-}$ heads are not shared with either single or compound mutations of other *Dlx* family members (Depew et al., 2005).

A recent study has demonstrated that the olfactory pit plays a late role in controlling the morphogenesis of skeletal elements of the avian nasal capsule (Szabo-Rogers et al., 2009). These data were obtained from embryos in which CNCCs had already migrated into the developing olfactory territory and do not provide information about the initial interactions directing CNCCs towards ectethmoid formation. As the olfactory pit derives from the *Dlx5/Dlx6*-positive anterior part of the neural fold, we decided to explore the possibility that this territory could be endowed with early inductive capacities. Using mouse mutants and surgical and molecular analyses of chick embryos, we demonstrate that an anterior region of the neural fold ectoderm (NF-ZA) is the territory responsible for ectethmoid induction through a *Dlx5/Dlx6*-dependent mechanism.

We conclude that the complete nasal capsule results from the juxtaposition of two independent structures formed by CNCCs competent to respond to different endodermal or ectodermal cues.

MATERIALS AND METHODS

Avian and mouse embryos

All animal procedures were approved by national and institutional ethical committees. Fertilised eggs were obtained from Morizeau Farms, France (chicken, *Gallus gallus*) or Cailles de Chanteloup Farms, France (quail, *Coturnix coturnix japonica*) and incubated at 38°C in a humidified atmosphere to reach the appropriate Hamburger Hamilton (HH) developmental stage (Hamburger and Hamilton, 1992; Teillet et al., 1998).

The genotypes of *Dlx5;Dlx6* mutant mice and embryos were determined by PCR using the *Dlx5;Dlx6*-mutant allele-specific primers as reported previously (Beverdam et al., 2002). Homozygous mutant embryos were obtained by breeding *Dlx5;Dlx6* heterozygous parents. The developmental stage of embryos was determined according to the detection of vaginal plugs, considering noon of the day of plug observation as E0.5.

Embryo processing

All products were from Sigma-Aldrich (France) unless otherwise stated. Embryos were collected and dissected at room temperature in phosphate-buffered saline (PBS). Immunoperoxidase detection was performed as previously described (Couly et al., 2002). Quail nuclei were detected with the quail-specific monoclonal antibody QCPN at 1/500 (obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). Dlx5 was immunodetected using the anti-DLX5 HPA005670 rabbit polyclonal antibody from Sigma diluted 1:100 in PBS gelatin 0.2%. Whole-mount skeletons were visualised according to standard staining protocols using Alcian Blue for cartilage and Alizarin Red for bone.

Identification of different skeletal cartilaginous components of the nasal capsule in both mouse and chick embryos was performed on the basis of morphological features (overall shape and size; presence or absence of nasal cavities and turbinates; presence of the olfactory epithelium associated with the ectethmoid; presence of the olfactory nerve) and positional relations with respect to surrounding structures such as the nasal or the premaxillary bones.

Tissue dissections

Experiments were carried out in ovo on windowed chick embryos at neurula stages (Couly et al., 1998). Sub-blastodermal injections of India Ink (Pelikan, France) or Fast Green were used to delineate the embryos. Unilateral or bilateral excisions were first performed on each sides of the neurectodermal ridge. The NF-ZA extends for 150 μm anteroposteriorly, starting at 150 μm from the rostralmost terminus of the embryo (anterior neural ridge or presumptive territory of the adenohypophysis). At the five-somite stage, the NF-ZA is 150 μm rostral to the cephalic neural crest-producing domain (see Fig. S1 in the supplementary material). Operated embryos were then incubated up to 13 days (until HH40) for chondroskeletal analyses.

Tissue grafts

Grafts of NF-ZA were obtained from five-somite quail embryos following the same dissection procedure described for NF-ZA ablation in the chick. NF-ZA grafts were transplanted into chick embryos at the five-somite stage in ovo after performing a unilateral ectodermal incision at the appropriate axial level. The graft was placed either in the anteroventral mesenchyme (below the level of the prosencephalon) or into the presumptive mesenchyme of the first pharyngeal arch (PA1). In some cases, a simultaneous graft of NF-ZA and EZ-I (Benouaiche et al., 2008) was transplanted into the prosencephalic domain of the mesenchyme. Embryos were fixed and sectioned 24 hours after operation (HH14) to detect quail nuclei using the QCPN antibody, or at HH35-40 for chondroskeletal preparations.

siRNA-mediated knockdown

siRNA were designed against chicken *Dlx5* and *Dlx6* transcripts (sequences upon request). For each gene, sets of three siRNA were synthesized (Sigma-Aldrich), including one with a fluorescein (*Dlx5*) or rhodamin (*Dlx6*) tag. The siRNA were prepared according to manufacturer instructions, pooled to yield a final 50 μM *Dlx5/6* siRNA mix, supplemented with 1 μg/ml Fast Green tracer dye (Sigma-Aldrich) in water. A scrambled siRNA was used as a control. Two microlitres of siRNA solution were slowly deposited under the vitelline membrane in the anterior domain of the developing neurula. Gold electrodes connected to a BTX 830 (Harvard Apparatus) were laid 3 mm apart at various positions above the vitelline membrane along the anterior neural folds. Four pulses, 500 ms apart, of 20 V for 50 ms were delivered. A 10 μl drop of tyrode was applied to cool down the embryo, then the egg was taped and reincubated.

Electroporation efficiency was monitored using a fluorescence binocular microscope, showing fluorescence-filled cells in the anterior neural fold (data not shown). Subsequent immunodetection of Dlx5 at various stages post-electroporation verified the reduction of targeted gene expression (see Fig. S2 in the supplementary material).

Embryos were collected at various stages and processed for either paraffin sectioning or whole-mount skeletal preparation as described earlier.

Mouse embryo sectioning and histological staining

For paraffin sectioning, embryos and foetuses were fixed in Bouin's solution (75% saturated picric acid, 8% formol, 5% acetic acid) for 5 days (E14.5 and E18.5). After fixation, E18.5 foetuses were decalcified by immersion in Jenkin's solution (48 hours in 4% hydrochloric acid 37%, 3% acetic acid, 10% chloroform, 10% deionised H_2O, 73% ethanol). Fixed embryos were then dehydrated in ethanol, equilibrated in SafeSolv (Labonord, Templemars, France), embedded in paraffin and oriented in blocks. Paraffin blocks were then sectioned at 8-12 μm using a Leica RM2235 microtome. Serial sections of E18.5 foetuses were subjected to Mallory trichromic staining for anatomical analysis (Sato et al., 2008).

RESULTS

The dorsal nasal capsule is absent in *Dlx5/6*$^{-/-}$ mouse embryos

The nasal capsule of *Dlx5/6*$^{-/-}$ mouse embryos is reduced to an anterior cartilaginous bar (Beverdam et al., 2002; Robledo et al., 2002). To further analyze the early contribution of *Dlx5* and *Dlx6* to nasal development, we re-examined chondroskeletal and dermatoskeletal preparations of these mutants. As previously reported, the simultaneous inactivation of *Dlx5* and *Dlx6* results in the transformation of mandibular into maxillary structures (Beverdam et al., 2002; Depew et al., 2002) with embryos displaying symmetric jaws. From E14.5 onwards, the vestigial nasal capsule is reduced to a symmetrical medial cartilage shaft spanning the whole length of the snout (Fig. 1). This long structure results from the

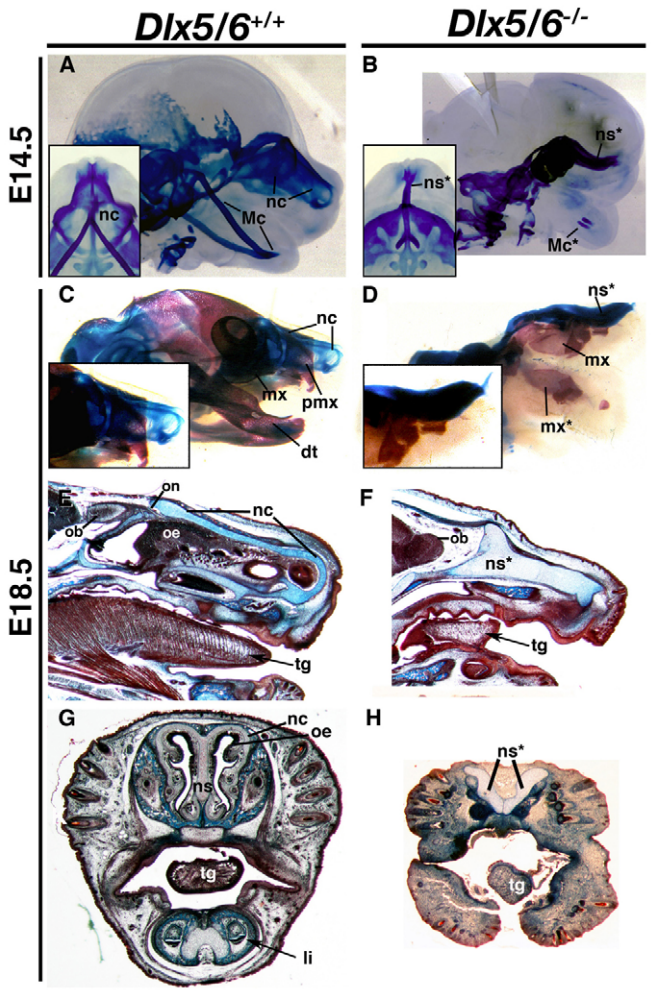

Fig. 1. Defective nasal capsule development in *Dlx5/6*$^{-/-}$ mice. (**A**,**B**) Lateral and ventral (insert) views of chondroskeletal preparations of E14.5 wild-type (A) and *Dlx5/6*$^{-/-}$ embryos (B). In the mutant, the nasal capsule (nc) is reduced to a medial cartilage bar reminiscent of the nasal septum (ns*), lower jaw cartilages are transformed (Beverdam et al., 2002; Depew et al., 2002) and only a distal vestigial region of the Meckel's cartilage (Mc*) is maintained. (**C**,**D**) Lateral views of dermatoskeletal preparations of E18.5 wild-type (C) and *Dlx5/6*$^{-/-}$ (D) embryos. The dentary bone is transformed in a maxillary-like structure (mx*), the premaxillary bone (pmx) and the dorsal nasal capsule are absent, and only a large nasal septal-like cartilage (ns*) is present. (**E**-**H**) Mallory trichromic staining of parasagittal (E,F) and frontal (G,H) sections of E18.5 wild-type (E,G) and *Dlx5/6*$^{-/-}$ (F,H) embryos. In the mutant, the dorsal nasal capsule is absent and only a cartilagineous structure that is reminiscent of an enlarged nasal septum (ns*) is present. In *Dlx5/6*$^{-/-}$ embryos, nasal cavities and turbinates are absent and, as a consequence, the olfactory epithelia (oe) and the olfactory nerve (on) are missing; a small olfactory bulb (ob) is maintained in the mutant, but it lacks rostral innervation. dt, dentary; li, lower incisor; Mc, Meckel's cartilage; mx, maxillary bones; nc, nasal capsule; ns, nasal septum; ob, olfactory bulb; oe, olfactory epithelium; on, olfactory nerve; pmx, premaxillary bone; tg, tongue. Asterisks indicate transformed structures in *Dlx5/6*$^{-/-}$ embryos.

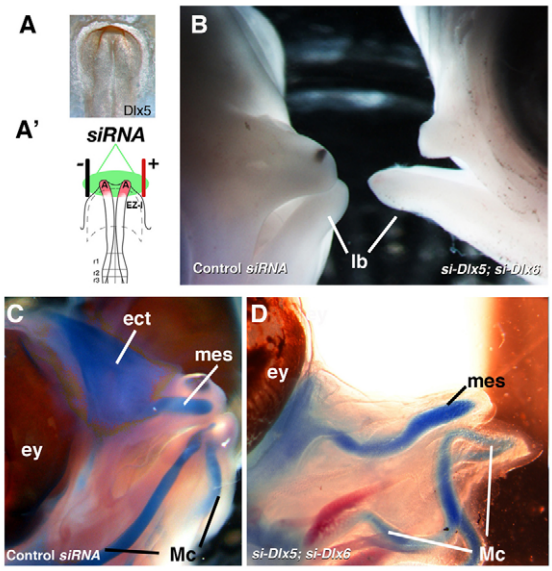

Fig. 2. siRNA-mediated simultaneous knockdown of *Dlx5* and *Dlx6* in the anterior region of the chick neurula prevents ectethmoid formation. (**A**) Immunodetection of Dlx5 protein in the three-somite chick neurula. Expression is limited to the anterior neural ectoderm (brown area at the top of the picture). (**A'**) Procedure used for electroporation of siRNA deposited under the vitelline membrane. Electrodes are placed in order to ensure maximal current delivery within the anterior neural ectoderm. The red domain in the anterior neural fold represents the territory of *Dlx5* expression that coincides with the NF-ZA (A). (**B**) HH35 embryos electroporated 9 days earlier (HH7) with either scrambled siRNAs (left) or *si-Dlx5/si-Dlx6* (right). The latter embryo displays facial hypoplasia and a short upper beak. (**C**,**D**) Skeletal preparation of the electroporated embryos pictured in B, showing cartilages in blue and bones in red. Note the absence of a differentiated ectethmoidal cartilage (ect) in the *si-Dlx5/si-Dlx6* electroporated embryo (D), and the presence of a fully developed mesethmoid (mes) in both specimen. Meckel's cartilage (Mc) develops properly, despite a distorted shape in the *si-Dlx5/si-Dlx6* skeletal preparation. A, NF-ZA; ect, ectethmoid; ey, eye; EZ-I, endoderm zone I; lb, lower beak; Mc, Meckel's cartilage; mes, mesethmoid.

midline apposition of two lateral flat cartilages as seen in frontal section of E18.5 embryos (Fig. 1H). The nasal cartilage of *Dlx5/6*$^{-/-}$ mouse embryos is devoid of any cavity (Fig. 1F,H), and both sensory and mucosal epithelia are absent. The olfactory nerve is missing while a small olfactory bulb is still present (Fig. 1E,F). The midline position of the cartilaginous rod (Fig. 1E-H) and the absence of cavity renders this structure similar to the nasal septum, which also derives from the midline juxtaposition of two hemisepta (Wealthall and Herring, 2006). Thus, in *Dlx5/6*$^{-/-}$ mouse embryos, the dorsal nasal capsule (ectethmoid) is absent, while the ventral, mesethmoid-derived, nasal septum is still present.

The ectethmoid fails to develop upon *Dlx5* and *Dlx6* simultaneous knock-down in the chick embryo

To examine the role of *Dlx5* and *Dlx6* in chick nasal capsule formation, we downregulated their expression by simultaneous electroporation of siRNAs against *Dlx5* and *Dlx6* (see Fig. S2 in the supplementary material) in HH6/7 embryos. At this stage, *Dlx5* and *Dlx6* are expressed primarily in the anterior neural ectoderm spanning the presumptive olfactory territory (NF-ZA), as revealed by immunostaining (Fig. 2A) and in situ hybridization [data not shown (Bailey et al., 2006)], highlighting the likelihood that the knockdown of *Dlx5/6* activity is stage and tissue specific. Furthermore, CNCCs had yet to undergo delamination and

migration, and therefore they were not likely to be targeted by siRNA transfection. Nine days later, at HH35, we analyzed skeletal preparations of the resulting surviving specimens (23/42). Inhibition of *Dlx5* and *Dlx6* results in a hypomorphic upper beak (Fig. 2B) characterized by a reduced or absent ectethmoid (Fig. 2C,D); other dorsal nasal capsule structures, including the olfactory epithelium, were not observed. By contrast, the mesethmoid and Meckel's cartilages are present in their normal position. To further confirm the importance of *Dlx5* and *Dlx6* expression in NF-ZA, we performed homotopic (23) and heterotopic (26) grafts of NF-ZA dissected from siRNA-transfected donors. Unfortunately, the failure of these grafted embryos to survive up to stages where it was possible to analyze skeletal morphology has precluded any meaningful interpretation of the outcome.

Ectethmoid formation in the chick embryo requires NF-ZA

To gain insight into the formation of the nasal capsule, we then undertook a series of experimental manipulations of the Dlx5/6-rich NF-ZA in the avian embryo. We first determined the contribution of NF-ZA to the development of the olfactory system by performing unilateral homotopic substitution of five-somite chick NF-ZA with stage-matched quail NF-ZA (Fig. 3A). Seven days after operation, in surviving embryos (8/15), a cartilaginous nasal capsule develops normally and quail-derived cells can be identified by QCPN staining in territories previously reported to derive from NF-ZA (Couly and Le Douarin, 1985). Donor cells are present within the olfactory bulb and the olfactory epithelia, but not in the surrounding ectethmoid cartilages (Fig. 3B,C). Bilateral ablation of NF-ZA (Fig. 3D) results in the strong reduction of ectethmoid cartilages (Fig. 3E,F), while the median mesethmoid is still present in surviving embryos (17/87). Heterotopic grafts of territories neighbouring NF-ZA (neural fold zone B; presumptive adenohypophysis; presumptive forebrain; non-neural ectoderm) cannot compensate for its absence (see Fig. S1 in the supplementary material and data not shown).

NF-ZA supports the formation of an ectopic olfactory system in the chick

We then unilaterally grafted supplementary NF-ZAs at the level of the presumptive first pharyngeal arch (PA1) and examined chondroskeletal and dermatoskelatal structures at HH35 and HH38 (Fig. 4). In whole-mount skeletal preparations of all surviving embryos (35/78), we consistently observed large ectopic cartilaginous or bony structures reminiscent of ectethmoidal conchae, apophysa crista Galli and associated dermatocranial elements (Fig. 4B), in contrast to the unoperated contralateral side (Fig. 4B′). Surrounding structures, including Meckel's cartilage, develop normally.

To determine the cellular origin of the supernumerary structures, we first substituted the chick host mesencephalic CNCCs with stage-matched CNCCs from quail donor embryos; then we ectopically grafted chick NF-ZA onto the same host (Fig. 4C). The resulting surviving chimerae (2/8) were analyzed at HH35 for quail cell lineage. QCPN-positive cells are present in all cartilages, including the ectopic ectethmoid (Fig. 4D,E). This observation suggests that the ectopic NF-ZA recruits CNCCs from the mesencephalic region to induce the ectopic ectethmoid. Histological analysis of serial sections of HH28 grafted embryos showed that the ectopic ectethmoid is lined by an internal olfactory epithelium derived from the chick grafted NF-ZA (Fig. 4E). NF-ZA grafts failed to survive and did not induce any ectopic structure when implanted in posterior Hox-positive territories (data not shown).

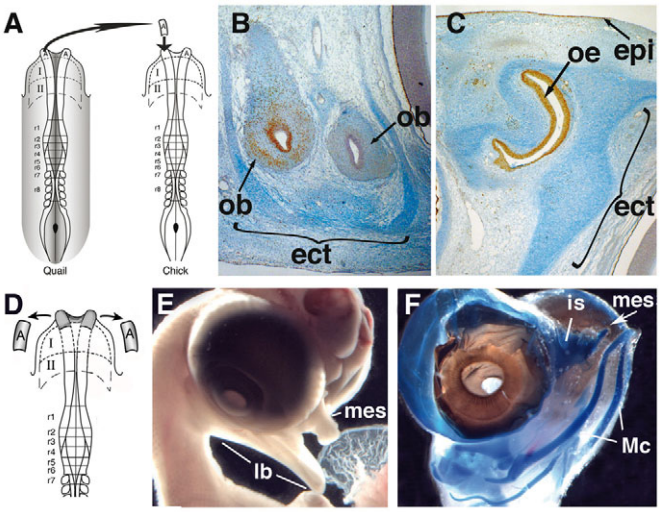

Fig. 3. NF-ZA gives rise to the sensory olfactory system and is required for ectethmoid formation. (**A**) Homotopic unilateral substitution of a five-somite chick NF-ZA with a quail stage-matched equivalent territory. (**B**,**C**) Coronal sections of resulting HH28 embryos stained for the quail-specific QCPN antigen (brown) and Alcian Blue (cartilages). The olfactory epithelium (oe) and the olfactory bulb (ob) derive from the graft, while the surrounding ectethmoid (ect) cartilage is of host origin. As previously shown (Couly and Le Douarin, 1985), the nasal epidermis (epi) and some mesenchymal cells also derive from NF-ZA. (**D-F**) Bilateral ablation of NF-ZA from a five-somite chick embryo results in severe reduction of the dorsal nasal capsule. The mesethmoid (mes) and the infraorbital septum (is) are still recognizable. ect, ectethmoid; epi, nasal epidermis; is, infraorbital septum; lb, lower beak; Mc, Meckel's cartilage; mes, mesethmoid; ob, olfactory bulb; oe, olfactory epithelium.

NF-ZA and EZ-I induce a complete nasal capsule

We then analyzed the effect of simultaneous ablation of NF-ZAs and of EZ-I (Fig. 5A). In surviving embryos (2/9), both components of the nasal capsule are absent (Fig. 5B-C′). Other CNCCs chondroskeletal derivatives of PA1, including Meckel's cartilage, develop normally. Next, we simultaneously grafted NF-ZA and EZ-I in the presumptive PA1 (Fig. 5D); care was taken to maintain the relative orientation of all elements. Analysis of the chondroskeleton of HH35 surviving embryos (5/10) shows a supernumerary nasal capsule consisting of a cartilaginous bar corresponding to the mesethmoid associated to an ectethmoid-like cartilage (Fig. 5E,E′). This supernumerary structure is remarkably similar to the neighbouring nasal capsule, although slightly reduced in size. First and second arch derivatives develop normally.

DISCUSSION

The development of sensory systems of the craniate head results from dynamic interactions between cephalic neural crest cells and sensory placodes. Here, we show that the pre-placodal olfactory territory of the neural plate (NF-ZA) is necessary and sufficient to induce CNCCs to form the dorsal nasal capsule (ectethmoid). As NF-ZA further gives rise to the olfactory placodes and their derivatives, this anterior ectodermal region is therefore the organizer of the olfactory system.

NF-ZA is characterized, in both the avian and the mammalian embryo, by high levels of *Dlx5* and *Dlx6* expression (Acampora et al., 1999; Bhattacharyya and Bronner-Fraser, 2008; McLarren et

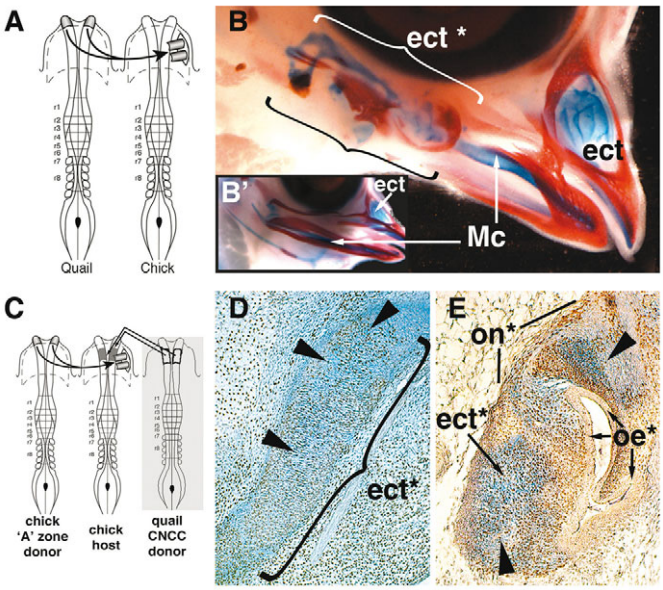

Fig. 4. NF-ZA instructs CNCCs to form the ectethmoid. (**A**) Ectopic graft of quail NF-ZAs in the presumptive first pharyngeal arch (PA1) region of a stage-matched chicken host. (**B**,**B'**) In a resulting chimera (HH35), ectopic structures (ect*) morphologically resembling the endogenous dorsal nasal capsule (ect) are present in the operated region (B), but not in the contralateral unoperated side (B'). (**C-E**) To determine the origin of ectopic nasal structures, a chick donor NF-ZA from a stage-matched embryo was first grafted ectopically in the presumptive host PA1 region, then the presumptive host mesencephalic CNCCs were substituted with CNCCs from a stage-matched quail donor. (D,E) Coronal sections of resulting HH28 embryos stained for QCPN antigen (brown) and Alcian Blue. The cartilagineous ectethmoid (ect*) derives from the quail grafted CNCCs, while the ectopic olfactory epithelium (oe*) derives from the chick NF-ZA. The ectopic olfactory nerve (on*) is composed of cells deriving both from the chick and quail. ect, ectethmoid; Mc, Meckel's cartilage; ob, olfactory bulb; oe, olfactory epithelium; on, olfactory nerve. Arrowheads indicate QCPN-positive cells. Asterisks indicate supernumerary structures.

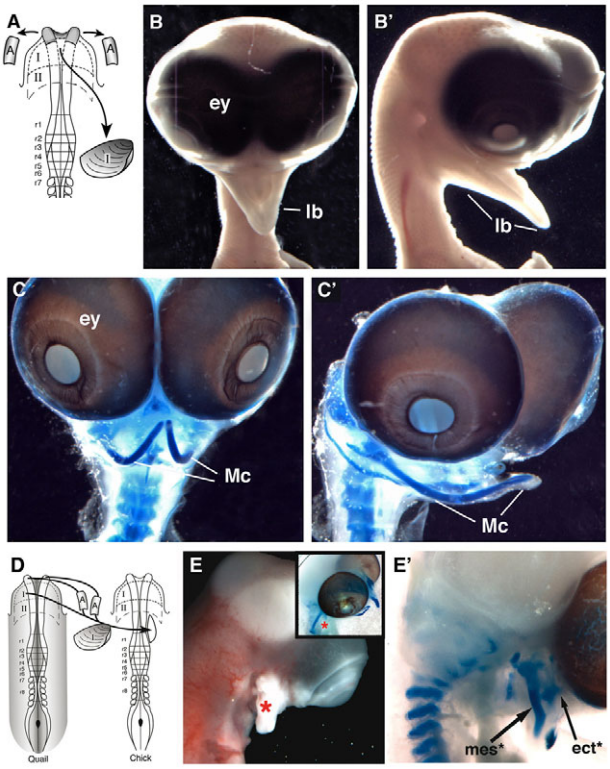

Fig. 5. Simultaneous ablation or grafting of NF-ZA and EZ-I results respectively in absence or duplication of the complete nasal capsule. (**A-C'**) Simultaneous ablation of NF-ZA and EZ-I from the five-somite chick embryo results in the complete absence of the nasal capsule. Note the synophtalmy and the properly developed Meckel's cartilage. (**D-E'**) Simultaneous heterotopic graft of a five-somite quail NF-ZA and EZ-I into a stage-matched chick presumptive PA1 region. At HH28, the resulting embryo shows ectopic structures (red asterisk, ect*, mes*) on the operated region. The morphology of these structures resembles to the endogenous nasal capsule, comprising both a mesethmoid-like cartilagineous bar (mes*) and an ectethmoid-like associated element (ect*). ect, ectethmoid; ey, eye; lb, lower beak; Mc, Meckel's cartilage; mes, mesethmoid. Asterisks indicate supernumerary ectopic structures.

al., 2003; Pera and Kessel, 1999; Pera et al., 1999; Yang et al., 1998). We show that the simultaneous invalidation of *Dlx5* and *Dlx6* in the mouse and their downregulation in the chick embryo specifically prevents the formation of the ectethmoid.

Our findings imply that both specification and patterning instructions for nasal capsule morphogenesis are already engraved in the pre-placodal presumptive olfactory territory (NF-ZA) at HH7. Our results extend to pre-placodal stages of development, with recent findings obtained at HH15-20 (Szabo-Rogers et al., 2008; Szabo-Rogers et al., 2009) showing that, in chick embryos, signalling from the nasal pits is involved in nasal capsule morphogenesis. This instructive potential of NF-ZA is present well before the onset of CNCCs migration.

In contrast to later manipulations of placodal territory (Szabo-Rogers et al., 2009), which did not induce nasal capsule-specific cartilage outside presumptive frontonasal territories, we show here that mesencephalic CNCCs migrating to PA1 are competent to respond to NF-ZA instructions. Furthermore, after induction, CNCCs give rise to a supernumerary ectethmoid, while the development of neighbouring PA1 CNCCs derivatives is not affected. Collectively, these findings suggest that, at HH15, CNCC-derived facial mesenchyme has become refractory to further ectodermal induction. Our observations thus fill the gap between the early activation of Dlx genes within pre-placodal progenitors and later roles exerted by their *Dlx*-positive derivatives in organizing the morphogenesis of the olfactory system.

The dorsal induction of the ectethmoid is concomitant with the ventral induction of the mesethmoid by endodermal EZ-I (Benouaiche et al., 2008). We suggest, therefore, that the interactions of CNCCs with the endoderm ventrally, and the pre-placodal ectoderm dorsally, give rise to two independent structures, the mesethmoid and the ectethmoid, respectively, which then assemble to form the nasal capsule (Fig. 6). Moreover, all information needed for final assembly lie within the three components (CNCCs, EZ-I and NF-ZA) because a complete nasal capsule can form ectopically upon simultaneous grafting of EZ-I and NF-ZA. We further show that the assembly paradigm of the nasal capsule stands true across species, as lack of *Dlx5/6* in the prospective olfactory domain specifically prevents ectethmoid formation in the nasal capsule of both mouse and avian embryos.

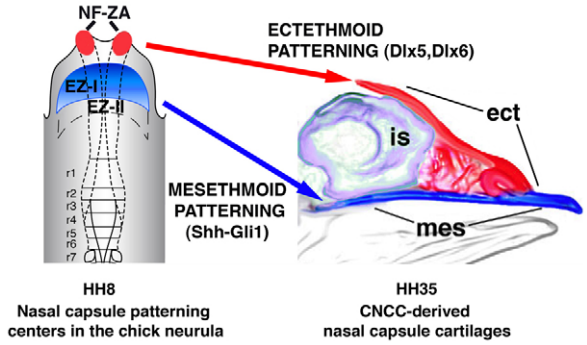

Fig. 6. Binary origin of nasal capsule patterning. Collectively, our present and previous work (Benouaiche et al., 2008) suggest that the nasal capsule is formed by the juxtaposition of two complementary CNCCs-derived cartilages, the mesethmoid ventrally (blue) and the ectethmoid dorsally (red). Mesethmoid and ectethmoid patterning depends upon two distinct inducing centres within the anterior-most endoderm (EZ-I) and the anterior-most neural ectoderm (NF-ZA), respectively. Activation of the Shh signalling pathway in the EZ-I and expression of *Dlx5* and *Dlx6* in NF-ZA are required for mesethmoid and ectethmoid patterning, respectively. ect, ectethmoid; EZ-I and EZ-II, endodermal zones I and II; is, interorbital septum; mes, mesethmoid; NF-ZA, neural fold zone A.

Dlx5 and *Dlx6* are expressed in olfactory precursors and derivatives through adulthood (Levi et al., 2003; Robledo et al., 2002). Mouse anterior ectodermal cells fated to express *Dlx5* and *Dlx6* persist, despite simultaneous invalidation of both genes (Depew et al., 2002). On the contrary, in both birds and mammals, frontonasal CNCCs and their nasal capsule chondrogenic derivatives never express *Dlx5* and *Dlx6* (Acampora et al., 1999; Bhattacharyya et al., 2004; Levi et al., 2003; Robledo et al., 2002). We find that both in mouse and chick nasal skeletal structures are present after *Dlx5* and *Dlx6* downregulation, suggesting that frontonasal CNCCs persist even in the absence of these gene products. Collectively, these observations support the hypothesis that the lack of ectethmoid in *Dlx5/6*$^{-/-}$ mutants results from disrupted interactions between pre-placodal precursors (NF-ZA) and CNCCs.

The question of which molecular signals mediate the interaction between NF-ZA and CNCCs remains still unanswered. Several signalling activities are present in the developing nasal-olfactory system and might account for these early instructive cues. Among those, Fgf ligands are expressed in mouse (Baker and Bronner-Fraser, 2001; Kawauchi et al., 2005; Minoux and Rijli, 2010) and chick (Abzhanov et al., 2007; Abzhanov and Tabin, 2004; Creuzet et al., 2004) neurulas within the presumptive olfactory domain. Alterations of olfactory structures have been reported in both species upon genetic or molecular invalidation of Fgf signalling (Bailey et al., 2006; De Moerlooze et al., 2000; Sjodal et al., 2007; Trokovic et al., 2003; Wang et al., 1999). In vitro studies have shown that Fgf is involved in determining the early lineage segregation of uncommitted pre-placodal precursors towards an olfactory or an optic fate (Bailey et al., 2006; Sjodal et al., 2007). Along these lines, Bmp activity favours lens placodal fate at the expanse of olfactory fate (Sjodal et al., 2007) and has a later role in promoting facial mesenchymal cells proliferation (Wu et al., 2004).

Collectively, these data and our present work support a two-step model for early morphogenesis of the olfactory system starting from a large Dlx3/5/6-positive preplacodal territory of the anterior neurula. First, as demonstrated in the chick (Bailey et al., 2006), interaction of preplacodal cells with CNCCs, mediated by Fgf signalling (Creuzet et al., 2006), restricts *Dlx5* expression to olfactory precursors which includes NF-ZA. Then, as we show here, the NF-ZA induces CNCCs to form an ectethmoid, which subsequently remains intimately associated with the NF-ZA-derived olfactory epithelia. This second step requires the expression of *Dlx5* and *Dlx6*. The nasal capsule is properly specified, but severely deformed in both *Dlx5*$^{-/-}$ and *Dlx5*$^{-/-}$; *Dlx6*$^{+/-}$ mice (see Fig. S3 in the supplementary material) and does not develop in the absence of both genes (Depew et al., 2005). Collectively, these data suggest that, as for the first pharyngeal arch (Vieux-Rochas et al., 2007; Vieux-Rochas et al., 2010), the allelic dosage of *Dlx5* and *Dlx6* controls the correct morphogenesis of the olfactory system.

In parallel with the dorsal induction of the ectethmoid, ventral *Shh* signalling from the anterior-most region of the endoderm instructs postmigratory CNCCs to form the mesethmoid cartilage (Benouaiche et al., 2008; Gitton et al., 2010).

The interaction between these two systems results into the juxtaposition of two independent modules that, together, give rise to a complete nose (Fig. 6). Consistently, we show that a complete and properly-patterned nose is induced by juxtaposed ectopic grafts of NF-ZA and EZ-I, suggesting that the ensemble of these inducing centres constitutes a robust and autonomous organizer.

Recently, it has been suggested that Fgf and Wnt signalling interact (Liu et al., 2010) to define the precise patterning of craniofacial cartilages. This observation provides a paradigm to explain craniofacial variations within and across species (Brugmann et al., 2007; Brugmann et al., 2006). Although *Fgf8* expression is retained by cultured explants and grafts of NF-ZA (data not shown), it seems unlikely that Fgf8 is the only signal involved in nose dorsoventral patterning as its frontonasal expression persists in *Dlx5/6*$^{-/-}$ mice (Depew et al., 2002) (data not shown).

The fact that the nose is formed through two independent signalling pathways might reflect the fact that it supports smell and respiration, two independent physiological functions that were acquired separately during evolution (O'Malley, 1923). Although variations in the chronology, topography and/or intensity of the interactions may have underlined craniofacial evolution (Minoux and Rijli, 2010), their alteration might as well account for the origin of human nasal defects. More specifically, this modular conception of the nose opens the possibility to adopt a new, much needed, classification method for human frontonasal malformations (Couly, 1981).

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Competing interests statement
The authors declare no competing financial interests.

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