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Molecular mechanisms of cranial neural crest cell migration and patterning in craniofacial development

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Summary

During vertebrate craniofacial development, neural crest cells (NCCs) contribute much of the cartilage, bone and connective tissue that make up the developing head. Although the initial patterns of NCC segmentation and migration are conserved between species, the variety of vertebrate facial morphologies that exist indicates that a complex interplay occurs between intrinsic genetic NCC programs and extrinsic environmental signals during morphogenesis. Here, we review recent work that has begun to shed light on the molecular mechanisms that govern the spatiotemporal patterning of NCC-derived skeletal structures – advances that are central to understanding craniofacial development and its evolution.

Key words: Cranial neural crest, Craniofacial skeleton evolution, Homeodomain factors, Signalling molecules

Introduction

The neural crest cells (NCCs) (see Glossary, Box 1) are a migratory cell population specific to vertebrates that originates from the dorsal part of the developing neural tube. Following induction, NCCs delaminate and migrate to different regions of the embryo, where they differentiate into a broad range of cell types, including peripheral and enteric neurons, glia, melanocytes and smooth muscle (Gammill and Bronner-Fraser, 2003; Le Douarin and Kalcheim, 1999; Morales et al., 2005; Steventon et al., 2005). Moreover, in the cranial region, NCCs contribute to most of the cartilage and bone of the skull, facial and pharyngeal skeletons. Rostral cranial NCCs extensively contribute to the frontonasal skeleton and the membranous bones of the skull, whereas more posterior cranial NCCs fill the pharyngeal arches (PAs) (see Glossary, Box 1), where they form the jaw, middle ear, hyoid and thyroid cartilages (Couly et al., 1993; Köntges and Lumsden, 1996; Noden, 1983) (for reviews, see Gross and Hanken, 2008; McBratney-Owen et al., 2008; Santagati and Rijli, 2003). Although the initial patterns of NCC segmentation and migration are mainly conserved between species, the wide diversity of vertebrate craniofacial morphologies indicates that cranial NCC subpopulations are able to generate distinctive, species-specific skeletal structures. A full understanding of the underlying molecular mechanisms involved is central to advancing our understanding of craniofacial biology and evolution.

Here, we bring a recent perspective on the complex interplay that occurs between the intrinsic NCC genetic program and the extrinsic environmental signals to which distinct NCC subpopulations are exposed during coordinated craniofacial morphogenesis. First, we focus on the molecular mechanisms that establish the segmental pattern of NCC organization when these cells colonize the craniofacial and pharyngeal regions. We then describe how cranial NCCs acquire and maintain their positional identity, and how environmental signals influence NCC transcription activity to achieve spatially and temporally coordinated craniofacial morphogenesis. Finally, we briefly discuss the modification of the shape and function of selected orofacial structures during vertebrate evolution in the context of recent findings that have identified the molecular pathways underlying such changes.

Molecular mechanisms of migrating cranial NCC segregation

Cranial NCCs follow stereotypical migratory pathways that are conserved among vertebrate species. NCCs that originate from the diencephalon and anterior mesencephalon (see Glossary, Box 1) migrate into the frontonasal process (FNP) (see Glossary, Box 1), whereas at a more caudal level, NCCs from the posterior mesencephalon and hindbrain, which is transiently subdivided into neuroepithelial segments called rhombomeres (r), colonize the PAs, another series of metameric structures (Couly et al., 1996; Couly et al., 1993; Johnston, 1966; Köntges and Lumsden, 1996; Le Lievre, 1978; Le Lievre, 1974; Noden, 1983; Osumi-Yamashita et al., 1994) (Fig. 1A). The NCC subpopulations that target individual PAs migrate in stereotypical streams (Fig. 1A) (Birgbauer et al., 1995; Kulesa and Fraser, 2000; Lumsden et al., 1991; Sechrist et al., 1993; Serbedzija et al., 1992; Shigetani et al., 1995; Trainor et al., 2002b). Maintaining the spatial segregation of such streams has an important impact on craniofacial pattern. Indeed, NCCs from different rhombomeres remain spatially segregated through to late developmental stages, and contribute to skeletal elements of multi-rhombomeric origin forming cryptic intraskeletal interfaces (Köntges and Lumsden, 1996). Moreover, NCC-derived connective muscle tissues from a given axial level are anchored to skeletal domains derived from NCCs that originate from the same axial level (Köntges and Lumsden, 1996; Matsuoka et al., 2005). Thus, the early anteroposterior (AP) pattern of NCC generation and migration is fundamental to establishing an underlying segmental pattern upon which the pharyngeal region of the vertebrate head is built (Graham, 2008; Kuratani et al., 1997).

In most vertebrates, hindbrain NCC migratory streams are separated by crest-free regions lateral to r3 and r5 (Lumsden et al., 1991; Golding et al., 2002; Graham et al., 1993). Environmental signals force each stream into well-defined pathways. For example, the mesenchyme lateral to r3 and r5 inhibits NCC migration (Farlie et al., 1999; Trainor et al., 2002b), in part through the non-cell autonomous functions of the receptor tyrosine kinase Erbb4, which is expressed in r3 and r5 (Golding et al., 2000) (Table 1). Also involved in sculpting the NCC migratory streams are the repulsive interactions between the Eph tyrosine kinase receptors and their ephrin ligands, and between the transmembrane neuropilin (Nrp) receptors and their secreted semaphorin (Sema) ligands (see Table 1 for references; Fig. 1). For example, in zebrafish, NCCs targeting PA1-3 express *nrp2a* and *nrp2b*, and avoid cells expressing *sema3f*

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Box 1. Glossary

Diencephalon
The most posterior of the two subdivision of the forebrain (the anterior subdivision being the telencephalon).

Foregut endoderm
The endodermal 'cul-de-sac', which covers internally the pharyngeal arches and from which pharyngeal pouches form.

Frontonasal process
The midline unpaired embryonic structure that develops into the forehead, between the telencephalon, the stomodeum and the nasal pits.

Incus, malleus and stapes
The mammalian middle ear ossicles that transfer the sound vibrations from the eardrum to the inner ear. The incus and malleus are derived from the first pharyngeal arch, whereas the stapes derived from the second pharyngeal arch.

Mesencephalon
The middle one of the three vesicles that arise from the neural tube during brain development, also known as midbrain. Located between the anterior forebrain and the posterior rhombencephalon (or hindbrain).

Neural crest
The region at the border between the neural plate and the non-neural ectoderm, from which the neural crest cells arise.

Neural crest cells (NCCs)
A multipotent vertebrate-specific migratory cell population that originates from the neural crest and migrates to different regions of the embryo, giving rise to diverse cell types, including melanocytes, endocrine and para-endocrine cells, sensory and autonomic neurons, and glia. In the head, NCCs also generate odontoblasts, cartilage, bone, connective tissue and smooth muscle cells.

Pharyngeal arches
Also called branchial arches, pharyngeal arches are segmentally repeated structures that arise in an antero-posterior order ventral to the embryonic vertebrate head region. They develop as a series of endodermal outpockets to the left and right of the developing pharynx, and are filled with ectomesenchymal cells derived from mesodermal and cranial neural crest cells. They are covered by surface ectoderm and they give rise to numerous facial and visceral structures, including skeletal, muscular and neural elements.

Selector genes
Genes that are required to determine the identity of a specific developmental field or organ (Garcia-Bellido, 1975).

Stomodeum
The primitive oral cavity, which forms between the frontonasal process and first pharyngeal arch.

and *sema3g* located in the NCC-free zones lateral to r3 and r5 (Yu and Moens, 2005). Moreover, in addition to the mechanisms involved in sculpting the migratory streams, cranial NCCs need also to be provided with specific directional guidance to their final destination (see Box 2 and Table 1 for references). *Twist*, which is expressed in the pharyngeal mesenchyme, and *Tbx1* (T-box 1), which is expressed in the mesodermal core of the PAs and in the endodermal pouches, are additionally required for the proper segregation of PA1 and PA2 NCC streams (Moraes et al., 2005; Soo et al., 2002; Vitelli et al., 2002).

In summary, to allow their appropriate positioning in the PA and, therefore, the proper assembly of skeletal structures, NCC subpopulations are guided by complex sets of cues to which they respond locally during their journey. Such positional cues are translated into stereotypical directional migratory behaviours, depending on the positional identity of NCC subpopulations. This raises questions about how cranial NCCs acquire their positional identity and how their response to several simultaneous extrinsic inputs may be integrated at the transcriptional level.

Transcriptional programs underlying cranial NCC positional identity

The positional identity of NCC subpopulations is established by combinatorial sets of homeodomain (HD) transcription factors, the expression of which is induced and maintained in NCCs through later developmental stages by signals from the surrounding environment. Recent work has begun to shed light on these intrinsic transcriptional mechanisms and their involvement in craniofacial and pharyngeal morphogenesis.

AP patterning of NCC derived head mesenchyme

In *Drosophila*, the head region is patterned by two HD transcription factors: *empty spiracle* (*ems*) and *orthodenticle* (*Otd*). The mouse orthologues of *ems* and *Otd* are the *Emx1/Emx2* and *Otx1/Otx2* genes, respectively (Finkelstein and Perrimon, 1991). *Otx2* is expressed in forebrain NCCs that colonize the frontonasal region and in midbrain NCCs that colonize the distal, mandibular, region of PA1 (Kimura et al., 1997; Kuratani et al., 1997). *Otx2* is required in cranial NCCs during a specific time window [between E8.5 and E10.5 in the mouse (Fossat et al., 2006)] and its regulation is conserved between mouse and zebrafish (Kimura et al., 1997). While *Otx2* homozygous mouse mutants lack head structures, *Otx2* heterozygotes have developmental defects in frontonasal and distal mandibular elements, but no structural anomalies in the rhombencephalic NCC-derived structures or the bones of the skull vault (Matsuo et al., 1995). Inactivation of histone deacetylase (HDAC) 8 in NCCs induces severe skull vault defects, in part due to the upregulation of *Otx2* (Haberland et al., 2009). This finding indicates that normal skull vault development requires epigenetic-mediated repression of *Otx2*. Despite the variability in the defects induced by *Otx2* haplo-insufficiency, no homeotic transformation has been described, suggesting that, as in *Drosophila*, *Otx2* does not act as a selector gene (see Glossary, Box 1) (Matsuo et al., 1995).

AP patterning of hindbrain NCCs: the inter-arch Hox code

The hindbrain-derived NCCs that colonize the mandibular region of PA1 give rise to the Meckel's cartilage and to the middle ear ossicles incus and malleus (see Glossary, Box 1), whereas NCCs migrating into PA2 generate the middle ear ossicle stapes and part of the hyoid bone. NCC AP positional identity is thought to be acquired at the pre-migratory stage (Couly et al., 1998; Hunt et al., 1991b; Hunt et al., 1998; Noden, 1983; Prince and Lumsden, 1994; Saldivar et al., 1996; Trainor et al., 2002a). However, this identity is not irreversible but displays some degree of plasticity: intrinsic NCC molecular programs can be switched to new programs when NCCs are exposed to ectopic environmental cues (Couly et al., 1998; Hunt et al., 1998; Ishikawa and Ito, 2009; Prince and Lumsden, 1994; Schilling et al., 2001; Trainor and Krumlauf, 2000a; Trainor et al., 2002a).

NCC AP positional identity in pre-migratory progenitors is established by the same molecular mechanisms that control segmentation and patterning of the rhombomeres from which they delaminate; namely, by the nested and combinatorial

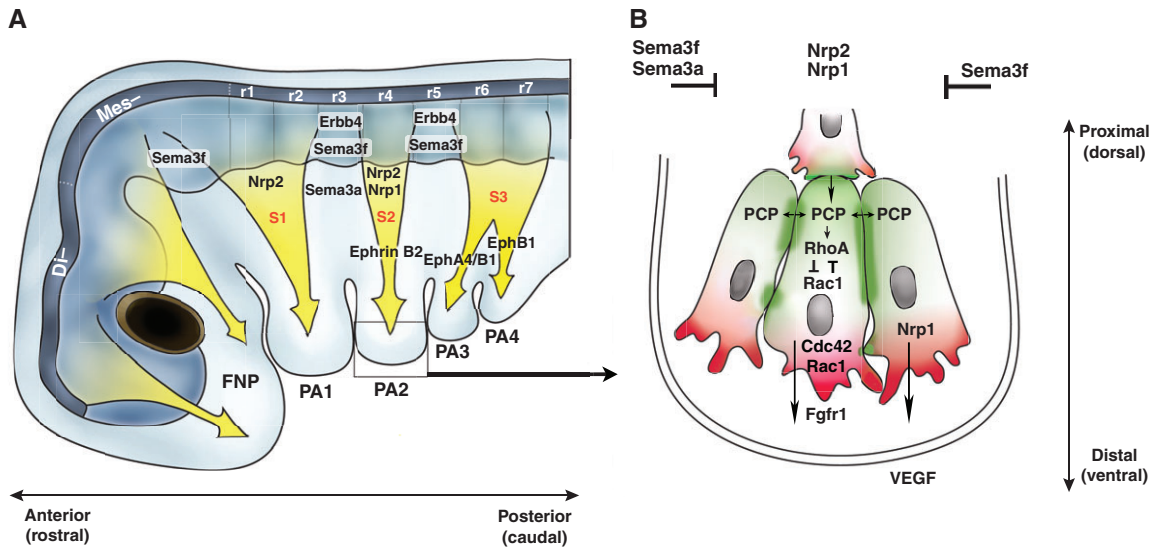

Fig. 1. Segmental and directional migration of cranial neural crest cells. (**A**) Segmental migration of cranial neural crest cells (NCCs) in a representative vertebrate embryo. The yellow arrows represent the patterns of migration of diencephalic (di-), anterior and posterior mesencephalic (mes-), and rhombencephalic NCCs into the frontonasal process (FNP) and pharyngeal arches 1-4 (PA1-4). The NCCs migrate in three individual streams: S1, S2 and S3. NCCs from the posterior mesencephalon, rhombomere 1 (r1) and r2 fill the first pharyngeal arch (PA1), whereas NCCs from r4 fill the second pharyngeal arch (PA2). In the post-otic hindbrain, NCCs from the r6-r8 region colonize indifferently PA3-6, with PA3 being mainly contributed by r6 NCCs (Kulesa and Fraser, 2000; Lumsden et al., 1991; Serbedzija et al., 1992; Trainor et al., 2002b). Some of the molecular mechanisms involved in establishing and maintaining the migration of segmentally restricted NCC streams into the PAs are also shown. The spatial expression patterns of Erbb4 (v-erb-a erythroblastic leukemia viral oncogene homologue 4), neuropilin/semaphorin 3 (Nrp/Sema3) and ephrin B2/EphA4 (Eph receptor A4)/EphB1 in the neural tube, the NCCs and their surrounding mesenchyme correspond to those described in chick, mouse and *Xenopus* (Gammill et al., 2007; Golding et al., 2004; Golding et al., 2000; Schwarz et al., 2008; Smith et al., 1997). (**B**) Directional migration of NCCs. The drawing shows the inset in A at a higher magnification, highlighting the NCC stream that migrates into PA2 and some molecular mechanisms involved in this directional migration. Cell-cell contacts between NCCs induce the localized activation of planar cell polarity (PCP)/Ras homologue gene family member A (RhoA) signalling (dark green) (Carmona-Fontaine et al., 2008). RhoA in turn inhibits Rac1 (Ras-related C3 botulinum substrate 1, light green) (Matthews et al., 2008). A mutual inhibition between RhoA and Rac1 is established (Rottner et al., 1999). Cell protrusions form at the leading edge (red) in response to the coordinated action of the small Rho GTPases [RhoA, Rac1 and Cdc42 (cell division cycle 42 homologue)] (Mayor and Carmona-Fontaine, 2010). To highlight the PCP signalling pathways induced in response to contact inhibition of locomotion, and for simplicity, a large part of the cell bodies are shown in contact. However, filopodia-like contacts between NCC are sufficient to promote this mechanism (Carmona-Fontaine et al., 2008; Teddy and Kulesa, 2004). The NCC chemoattractant roles of Fgfr1 and Nrp1 are represented by arrows (McLennan and Kulesa, 2007; Trokovic et al., 2003). For simplicity, Fgfr1 has been represented distally in PA2. Above the inset, the Nrp/Sema repulsive signals that limit the stream laterally. Abbreviations: r1-r7, rhombomeres 1-7; VEGF, vascular endothelial growth factor.

expression of the HD transcription factors of the Hox (homeobox) gene family (Hunt et al., 1991a; Hunt et al., 1991b; Lumsden and Krumlauf, 1996). The maintenance of Hox gene expression in migrating NCCs and developing rhombomeres is regulated by independent Hox gene enhancers, sometimes resulting in expression differences between NCC subpopulations and their rhombomere of origin (Hunt et al., 1991a; Hunt et al., 1991b; Maconochie et al., 1999; Trainor and Krumlauf, 2000b; Tumpel et al., 2008; Tumpel et al., 2002). For example, the *Hoxa2* expression domain has its anterior limit at the boundary between r1 and r2, whereas r2-derived NCCs migrating into PA1 are devoid of Hox gene expression (Krumlauf, 1993; Prince and Lumsden, 1994). The NCCs that contribute to the second and more posterior arches instead express specific combinations of Hox genes (Fig. 2A).

The involvement of Hox genes in establishing the AP positional identity of NCCs was first demonstrated by the targeted inactivation of mouse *Hoxa2* (Gendron-Maguire et al., 1993; Rijli et al., 1993), which resulted in the homeotic transformation of PA2 into PA1-like skeletal elements (Fig. 3B). Rhombomere 4-derived PA2 elements are transformed into proximal PA1-like structures (Gendron-Maguire et al., 1993; Rijli et al., 1993), such as the proximal part of Meckel's cartilage, incus and malleus (Fig. 3B), which are normally mainly contributed by r1-r2-derived NCCs (Köntges and Lumsden, 1996). This supports the idea that distal mandibular and maxillary PA1 structures require AP specification from a Hox-independent patterning system, partially mediated by *Otx2* (see above) (Kuratani et al., 1997). *Hoxa2* downregulation in *Xenopus* (Baltzinger et al., 2005) and zebrafish (Hunter and Prince, 2002) also results in second-to-first arch homeotic changes, underscoring a conserved role for *Hoxa2* in second (hyoid) arch patterning. Some differences exist, however, among vertebrates concerning the involvement of *Hoxb2*, the only *Hoxa2* paralogue, in PA2 NCC patterning. Indeed, in contrast to mouse and *Xenopus*, *Hoxb2* expression in zebrafish PA2 is maintained where it genetically interacts with *Hoxa2* (Baltzinger et al., 2005; Barrow and Capecchi, 1996; Davenne et al., 1999; Hunter and Prince, 2002). Irrespective of such differences, these experiments show that paralogue group 2 (PG2) Hox genes are required to select a PA2-specific mode of development. Moreover, the outcome of PG2 Hox gene inactivations indicates

that NCCs contributing to the proximal region of PA1 and PA2 share an underlying Hox-free ground (default) patterning molecular program (Rijli et al., 1993). This has been further supported by complementary gain-of-function experiments, showing that the ectopic expression of *Hoxa2* in the Hox-free PA1 of chick and frog, or of *Hoxa2/Hoxb2* in the Hox-free PA1

Table 1. Factors involved in segmental and directional NCC migration

Chemoattractant/repellant factors involved in segmental and directional NCC migration	Expression domains	Role	References
Receptor tyrosine kinase Erbb4	**Mouse:** r3 and r5. **Chick:** r1, r3 and r5.	Maintenance of the r3-adjacent NCC-free zone.	Golding et al., 2000; Golding et al., 2004
Nrp/Sema	**Zebrafish:** *nrp2a* and *nrp2b* receptors are expressed in migrating NCCs targeting PA1-3. *sema3f* and *sema3g* ligands are expressed in the NCC-free zones lateral to r3 and r5.	Segregation between NCC streams that colonize PA1-3.	Yu and Moens, 2005
	Chick: *Sema3a* and *Sema3f* are expressed by r1, r3 and r5.	Segregation between NCC streams that colonize PA1-3.	Osborne et al., 2005
	Mouse: *Nrp2* and *Nrp1* are expressed in migrating NCCs targeting PA1-2 and PA2, respectively. *Sema3f* is expressed in the caudal midbrain, r3 and r5. *Sema3a* is expressed in the r3 NCC-free zone.	Sema3a/Nrp1 and Sema3f/Nrp2 act synergistically to prevent the intermingling between PA1 and PA2 NCC streams.	Gammill et al, 2007; Schwarz et al., 2008
Eph/ephrin	***Xenopus*:** *EphA4* and *EphB1* receptors are expressed in the mesoderm and NCCs of PA3 and PA3-4, respectively. The e*phrin B2* (*efnb2*) ligand is expressed in the PA2 NCC stream and mesoderm.	Inhibition of fusion between PA2 and PA3 NCC streams. Role in targeting PA3 NCCs to their correct destination.	Smith et al., 1997
	Chick: *EphA3*, *EphA4*, *EphA7*, *EphB1*, *EphB3* and e*phrin B2* are expressed by migrating NCC streams. e*phrin B1* and *EphB2* are expressed by cells bordering the streams of cranial NCCs.	Maintaining the NCC streams segregated.	Mellott and Burke, 2008
	Mouse: Ephrin B1 (*Efnb1*) is expressed in migrating NCCs.	Ephrin B1 acts as a receptor in migrating NCCs, where it activates a reverse signalling cascade to target NCC streams to PA3 and PA4.	Davy et al., 2004
	EphA4 and *EphB1/EphB3* are expressed in the NCC streams targeting PA2-3 and PA1-3, respectively. Ephrin B2 is expressed in the cleft between PA1 and PA2.	Ephrin B2 acts as a ligand to target the NCC stream into PA2.	Adams et al., 2001
Sdf1b/Cxcr4a	**Zebrafish:** *Sdf1b* receptor is expressed in pharyngeal arch endoderm, its ligand *Cxcr4a* is expressed in NCCs.	Cranial NCC directional migration.	Olesnicky Killian et al., 2009
Npn1/VEGF	**Chick:** *npn1* receptor is expressed in migrating NCCs. Its *vegf* ligand is expressed in the PA2 ectoderm.	The attractive interactions between Npn1 and Vegf allow migrating NCCs to invade PA2.	Eickholt et al., 1999; McLennan and Kulesa, 2007; McLennan et al., 2010
Fgfr1	**Mouse:** *Fgfr1* is expressed in the environment surrounding the NCCs.	Creation of a permissive environment allowing NCCs to invade PA2.	Trokovic et al., 2003

Expression patterns and the role of chemoattractant/repellant factors involved in the segmental and directional migration of cranial neural crest cell (NCC) streams in different animal models. Erbb4 and the repulsive interactions of neuropilin/semaphorins and Eph/ephrin are involved in NCC stream segregation, whereas Fgfr1 and the attractive interactions of Sdf1b/Cxcr4a and Nrp1/Vegf are involved in NCC directional migration.
Abbreviations: Cxcr4a, chemokine receptor 4a; Erbb4, v-erb-a erythroblastic leukemia viral oncogene homologue 4; Fgfr1, fibroblast growth factor receptor 1; Nrp, neuropilin; PA, pharyngeal arch; r, rhombomeres. Sdf1b, stroma cell-derived factor 1b; Sema, semaphorin; VEGF, vascular endothelial growth factor.

in zebrafish, is sufficient to induce PA2-like derivatives in place of PA1-like derivatives (Grammatopoulos et al., 2000; Hunter and Prince, 2002; Pasqualetti et al., 2000).

Hoxa2 performs its main patterning function in post-migratory NCCs, as assessed by its temporal inactivation in the mouse, and is thus directly involved in the morphogenesis of PA2 structures (Fig. 3B) (Santagati et al., 2005). Conversely, temporal induction of *Xenopus XHoxa2* ectopic expression at post-migratory stages in PA1 is sufficient to induce homeotic transformation of mandibular elements towards hyoid morphology (Pasqualetti et al., 2000). These complementary experiments demonstrate that: (1) the skeletal pattern of mandibular and hyoid crest is not irreversibly committed before migration but NCC positional identity must be maintained through post-migratory stages in order to provide information about shape, size and orientation of PA2 skeletal elements; and (2) *Hoxa2* is a selector of hyoid fate, representing an integral component of the PA2 NCC morphogenetic program (Santagati et al., 2005). At the molecular level, *Hoxa2* participates in PA2 morphogenesis by modulating the competence of post-migratory NCCs to respond to local skeletogenic signals such as fibroblast growth factor (Fgf) (Bobola et al., 2003). This results in direct or indirect negative regulation of relevant transcription factors normally expressed in PA1 [including *Pitx1* (paired-like homeodomain transcription factor 1), *Lhx6* (LIM homeobox protein 6), *Six2* (sine oculis-related homeobox 2 homologue), *Alx4* (aristaless-like homeobox 4), *Bapx1* (*Nkx3-2* – Mouse Genome Informatics) and *Barx1* (BraH-like homeobox 1)] (Fig. 3A) (Bobola et al., 2003; Kanzler et al., 1998; Kutejova et al., 2005; Kutejova et al., 2008; Minoux et al., 2009; Santagati et al., 2005).

The inactivation of Hox PG3 genes, particularly of *Hoxa3*, induces malformations of PA3 and PA4 NCC-derived skeletal elements (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). More severe phenotypes are observed in compound mutants, indicating synergistic interactions between members of the same paralogue group (Condie and Capecchi, 1994; Manley and Capecchi, 1997). However, no homeotic transformations of NCC-derived elements are seen in either single or compound Hox PG3 or PG4 mutants (Boulet and Capecchi, 1996; Chisaka and Capecchi, 1991; Condie and Capecchi, 1994; Horan et al., 1995; Manley and Capecchi, 1995; Manley and Capecchi, 1997; Ramirez-Solis et al., 1993). By contrast, selective deletion of the Hoxa cluster in NCCs results in the partial homeosis of PA3 and PA4 derivatives into morphologies that are characteristic of PA1-derived structures, in addition to the PA2 homeosis induced by the lack of *Hoxa2* (Minoux et al., 2009). Thus, pre- and post-otic NCCs may share the same Hox-free PA1-specific ground patterning program (Fig. 3A). By modifying this common ground patterning molecular program in an arch-specific manner, Hox genes provide each PA with unique rostrocaudal identities (Fig. 2A, Fig. 3A) resulting in the formation of arch-specific skeletal elements that precisely connect with arch-specific subsets of muscles (Köntges and Lumsden, 1996; Noden, 1983).

Furthermore, the results of Minoux et al. (Minoux et al., 2009) rule out a simple 'posterior prevalence' model (Duboule and Morata, 1994) in which Hox PG3 or PG4 expression would provide positional identity to the NCCs of PA3 or PA4, respectively. Indeed, the NCC-specific Hoxa cluster deletion has uncovered a role for *Hoxa2* in the patterning of PA3 and PA4 that only becomes apparent in the absence of *Hoxa3*, supporting a prevalent role for *Hoxa3* over *Hoxa2* in patterning PA3 and PA4 (Minoux et al., 2009). The concomitant removal of *Hoxa2* and *Hoxa3* induces homeosis of PA3- and PA4-derived structures, unveiling synergistic genetic interactions between *Hoxa2* and *Hoxa3* (Minoux et al., 2009). Differences in *Hoxa2* and *Hoxa3* relative expression levels and/or target specificity might underlie the distinct effects of their inactivation. Moreover, expression of Hox genes in PA epithelia might further contribute to establish PA-specific patterning. The additional removal of the Hoxd cluster in a NCC-specific Hoxa-deleted background does not increase the extent of the homeotic phenotype (Minoux et al., 2009). Thus, the Hoxa cluster may have a primary role in the biology of skeletogenic NCCs (Duboule, 2007; Minoux et al., 2009), whereas Hoxb and Hoxd genes could provide a 'quantitative backup' (Rijli and Chambon, 1997) that might become functionally relevant only in the absence of Hoxa genes.

The maintenance of Hox gene expression in NCCs is under epigenetic control. In the absence of the zebrafish histone acetyltransferase Moz (monocytic leukemia zinc finger) or its partner Brpf1 (bromodomain and phd finger containing 1), both of which are involved in chromatin remodelling, the expression of Hox PG1 to PG4 genes in NCCs is initiated but not maintained, resulting in anterior homeotic transformations of PAs similar to those observed in the Hoxa cluster deletion mutants (Laue et al., 2008; Miller et al., 2004). The nuclear matrix protein special AT-rich binding protein 2 (SATB2) represses *Hoxa2* in the FNP and developing calvarial bones (Dobreva et al., 2006), whereas Hox gene repression in PA1 NCCs may be influenced by Fgf8-mediated signalling from the mid-hindbrain boundary (Irving and Mason, 2000; Trainor et al., 2002a). Indeed, Hox gene expression is

Box 2. Cranial NCC directional migration

Interactions between neural crest cells (NCCs) and their local environment are crucial in cranial NCC directional migration (Kulesa and Fraser, 2000). Directional migration is not pre-determined, as NCCs can move around barriers introduced into their migration paths and re-target their direction (Kulesa et al., 2005). Migrating NCCs are polarized in the direction of their migration; they have a tail at the back of the cell and large lamellipodia and filopodia at their leading edge, which form in response to small GTPases [e.g. Ras homologue gene family member A (RhoA), RAS-related C3 botulinum substrate 1 (Rac1) and cell division cycle 42 homologue (Cdc42)] activity (Mayor and Carmona-Fontaine, 2010) (Fig. 1B). The non-canonical Wnt (wingless-related MMTV integration site) planar cell polarity (PCP) signalling pathway and cell-cell contact are involved in controlling the polarity of migrating NCCs. When the PCP pathway interacts with syndecan 4 (Syn4), Rac1 is inhibited at the back of the cell, with the consequent formation of cell protrusions at the front only; thus, directional migration occurs (Matthews et al., 2008). Moreover, when two migrating NCCs make contact, they retract their protrusions and change their direction (Carmona-Fontaine et al., 2008; Teddy and Kulesa, 2004) through a mechanism known as contact inhibition of locomotion (Abercombie, 1979). This cell-cell contact between NCCs induces the localized activation of RhoA, leading to the inhibition of cell protrusions in this region (Carmona-Fontaine et al., 2008) (Fig. 1B). Thus, through PCP signalling activation, cell-cell contacts coordinate the orientation of cells protrusions and thus the direction of NCC migration (Carmona-Fontaine et al., 2008). As a migrating NCC stream is laterally limited by repulsive signals (see main text and Table 1), contact inhibition results in NCCs only moving forwards, towards a NCC-free zone. This process also highlights the need for NCCs to move in groups, with close contact between each other, in order to maintain their polarity and directional migration.

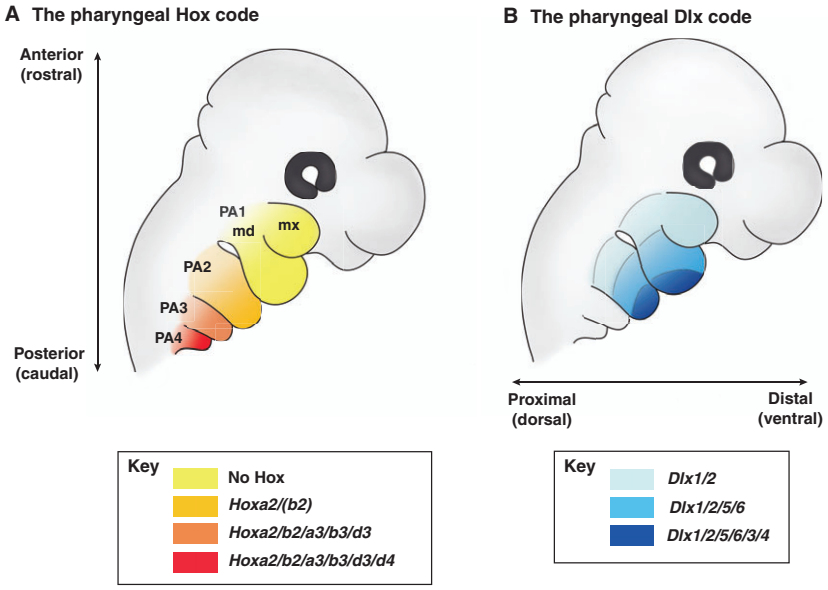

Fig. 2. Intrinsic transcriptional programs underlying cranial NCC positional identity. Schematics of a developing head and pharyngeal regions of a mouse embryo at E10.5. (**A**) The homeobox (Hox) code provides spatial identity (inter-arch identity) along the AP (anteroposterior, rostrocaudal) axis to cranial neural crest cells (NCCs) colonizing the pharyngeal arches (PAs). Each PA is represented by a different colour (see Key) representing its specific Hox expression code. PA1 is devoid of Hox gene expression. The Hox-free molecular program of the PA1 mandibular (md) process represents the PA ground (default) patterning program (see Fig. 3). In mouse, *Hoxb2* is downregulated in PA2 post-migratory NCCs, and *Hoxb3* and *Hoxd3* are only weakly expressed in PA3. (**B**) The Dlx code provides spatial identity (intra-arch identity) to cranial NCCs along the DV (dorsoventral, proximodistal) axis of PAs. Key: DV-nested expression patterns of Dlx genes in NCCs. Abbreviations: md, mandibular process of PA1; mx, maxillary process of PA1.

incompatible with jaw and craniofacial development (Couly et al., 1998; Creuzet et al., 2002). The elucidation of the molecular mechanism(s) involved in Hox gene cluster repression in the first arch will be of particular interest in the future and will be crucial for understanding jaw development and evolution.

Altogether, the above findings underscore the importance of establishing the rostrocaudal positional identity of NCCs at pre-migratory stages and maintaining it through late post-migratory stages for the metameric assembly of the pharyngeal region of the vertebrate head. Indeed, a skeletogenic NCC-specific molecular ground pattern, shared by all PAs, is modified in an arch-specific manner to select appropriate segment-specific skeletal morphologies. This is achieved by the integration of local signals (see below) with intrinsic combinatorial programs of Hox gene function that, in turn, regulate directly or indirectly downstream effector genes through the distinct phases of NCC development. However, in addition to Hox-dependent AP positional addresses that distinguish the segmental identity of each arch from that of its neighbours, NCCs need also to be provided with dorsoventral positional information to establish intra-arch identity.

DV patterning of hindbrain NCCs: the intra-arch Dlx code

A distal-less homeobox (*Dlx*) code provides cranial NCCs with patterning information and intra-arch polarity along the dorsoventral (DV) (i.e. proximodistal in the mouse) axis (Fig. 2B). The vertebrate Dlx genes are HD transcription factor homologues of *Drosophila Distal-less*. Mammals have six Dlx genes (*Dlx1-Dlx6*) (Stock et al., 1996), which exhibit nested expression patterns. In each PA, *Dlx1/2*, *Dlx5/6* and *Dlx3/4* transcripts overlap distally but display offset proximal expression limits, with *Dlx1/2* extending more proximally than *Dlx5/6*, which in turn extends more proximally than *Dlx3/4* (Fig. 2B). In PA1, *Dlx1* and *Dlx2* are expressed in both the maxillary (upper jaw) and mandibular (lower jaw) processes. *Dlx5* and *Dlx6* are expressed only in the mandibular process; their expression extends close to the position of the future hinge region between the upper and lower jaws, which in mammals includes both the primary (incudo-malleal) and secondary (dentary-squamosal) jaw articulations (Depew et al., 2005). *Dlx3* and *Dlx4* expression domains are further restricted to the distal-most end of the mandibular process (Depew et al., 1999; Depew et al., 2002; Jeong et al., 2008; Qiu et al., 1997). In posterior PAs, the nested DV expression domains of Dlx genes intersect with the AP Hox code in NCCs (Santagati and Rijli, 2003) (Fig. 2).

The role of Dlx genes has been mainly investigated by loss-of-function mutations in the mouse. [A detailed account of the craniofacial alterations of such mutants is outside the scope of this review (Depew et al., 2005).] Here, we briefly discuss the functional importance of the Dlx code in PA patterning. As *Dlx5/6* control *Dlx3/4* expression (Depew et al., 2002; Jeong et al., 2008) (Fig. 4A), the partitioning of PA1 is mainly achieved with two Dlx combinations: *Dlx1/2* for the maxillary and *Dlx1/2/5/6* for the mandibular process. Single or compound *Dlx1/Dlx2* mutants selectively affect the development of upper jaw elements and upper components of the hinge region (Depew et al., 2005; Qiu et al., 1997; Qiu et al., 1995). Conversely, inactivation of *Dlx5* results in proximal lower jaw and hinge region abnormalities (Acampora et al., 1999; Depew et al., 1999). The simultaneous inactivation of *Dlx5* and *Dlx6*, although having no effect on *Dlx1/2* expression, results in a homeotic transformation of lower jaw into a mirror image of upper jaw derivatives, thus generating a mouse with two upper jaws facing each other with their associated soft tissue, including vibrissae (Beverdam et al., 2002; Depew et al., 2002) (Fig. 4B). Such an outcome provides the best evidence that a Dlx combinatorial code in NCCs establishes intra-arch polarity (Figs 2, 4). Conceptually, it may be similar to the above-mentioned Hox code for inter-arch patterning. Indeed, intra-arch polarity is also achieved by modifying an underlying *Dlx1/2*-positive ground (default) patterning program, corresponding to the PA1 maxillary process molecular program, upon which *Dlx5/6* select a mandibular identity (Fig. 4). However, it is unclear at the moment whether this Dlx ground patterning program also extends to more posterior PAs, as for the Hox genes (Minoux et al., 2009). Even though PA2 and PA3 skeletal elements are affected by the double *Dlx5/Dlx6* inactivation, such malformations cannot be assigned to clear homeotic transformations (Depew et al., 2002; Depew et al., 2005). Similar to the Hox code, synergistic interactions between distinct Dlx paralogue groups, namely *Dlx1/2* and *Dlx5/6*, are observed, indicating both qualitative and quantitative features of the code (Depew et al., 2005). For example, when *Dlx1* or *Dlx2* are

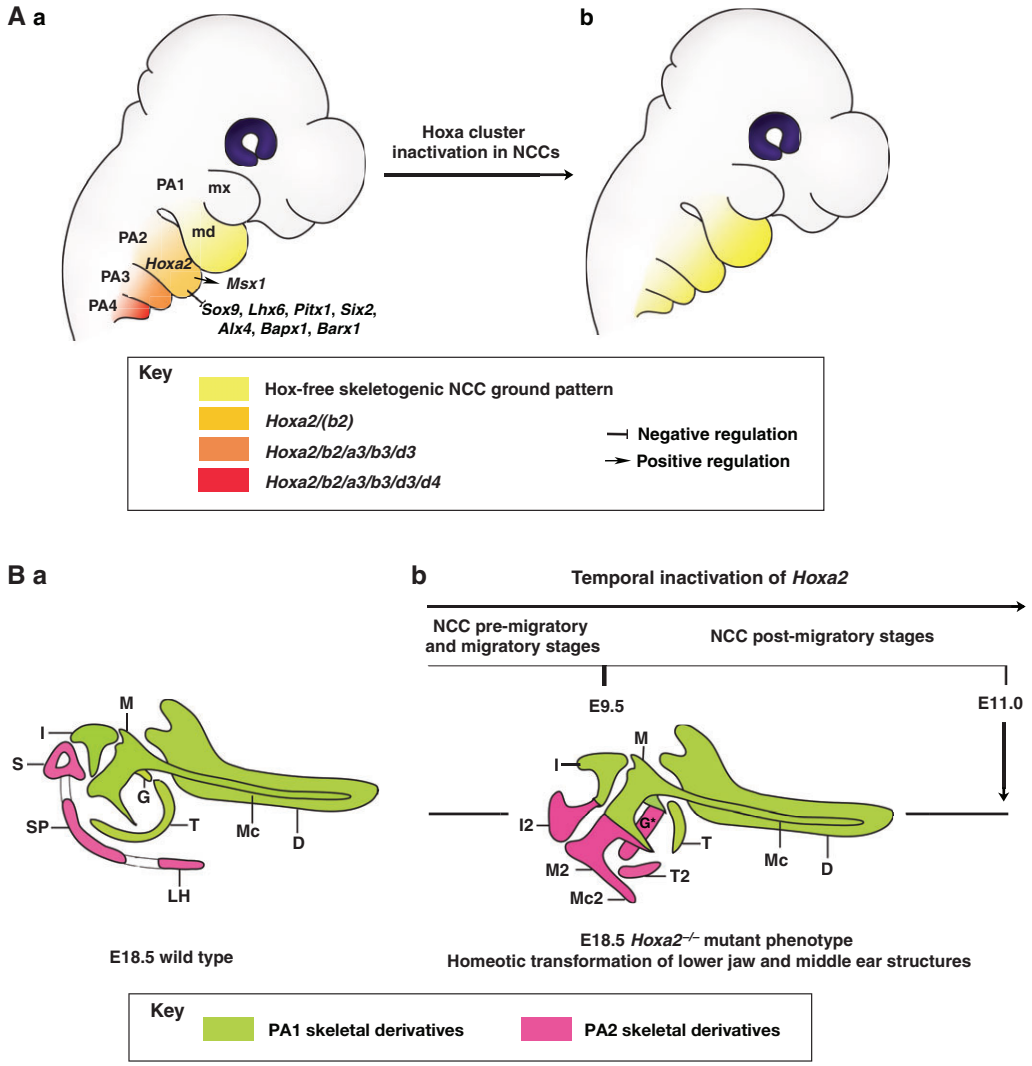

Fig. 3. Hox gene requirement for arch patterning and skeletogenic cranial NCC ground pattern. (**A**) Schematic of a developing head and pharyngeal regions of a mouse embryo at E10.5 (rostral is uppermost). A homeobox (Hox)-free ground pattern is shared by skeletogenic neural crest cells (NCCs) contributing to pharyngeal arches (PAs). (**a**) Each PA is endowed with a specific Hox code, except for PA1, which is devoid of Hox expression (see Key). The molecular targets of *Hoxa2* in PA2 are shown. (**b**) The conditional inactivation of Hoxa genes in NCCs reveals that rostral and caudal pharyngeal arches (PAs) share the same Hox-free skeletogenic ground patterning program, corresponding to the molecular program of the mandibular region of PA1 (all mutant arches are depicted in yellow) (Minoux et al., 2009). (**B**) A schematic representing the effect of *Hoxa2* temporal inactivation on the identity of PA2 skeletal derivatives in the mouse. (**a**) Lower jaw and middle ear structures in a wild-type newborn mouse. PA1 and PA2 NCC-derived skeletal elements are stained in green and pink, respectively (see Key). (**b**) The conditional inactivation of *Hoxa2* from pre-migratory up to late post-migratory stage (E11.0) always reproduces the full skeletal second-to-first arch homeotic transformation observed in *Hoxa2*-null mutants, i.e. PA2 skeletal elements acquire a PA1-like identity (Santagati et al., 2005). Abbreviations: *Alx4*, aristaless-like homeobox 4; *Bapx1*, *Nkx3-2* – Mouse Genome Informatics; *Barx1*, BraH-like homeobox 1; D, dentary bone; G, gonial bone, G*, modified gonial bone, I and I2, incus and its duplicated counterpart; LH, lesser horns of the hyoid bone; *Lhx6*, LIM homeobox protein 6; *Pitx1*, paired-like homeodomain transcription factor 1; M and M2, malleus and its duplicated counterpart; Mc and Mc2, Meckel's cartilage and its duplicated counterpart; *Msx1*, muscle segment homeobox like 1; *Six2*, sine oculis-related homeobox 2 homologue; *Sox9*, SRY box containing gene 9; SP, styloid process; S, stapes; T and T2, tympanic bone and its duplicated counterpart.

inactivated on *Dlx5*- or *Dlx6*-deficient backgrounds, the mandibular process derivatives are reduced in size and/or transformed into structures that resemble maxillary elements, with disruption of the hinge region partially reproducing the *Dlx5/Dlx6* mutant phenotype (Depew et al., 2005; Jeong et al., 2008).

In PA1, *Dlx5/6* induce and/or maintain the expression of several genes involved in the development of the mandibular process, including *Dlx3/4*, *Hand1/2* (heart and neural crest derivatives expressed transcripts), *Alx3/4*, *Pitx1*, *Gbx2* (gastrulation brain homeobox 2), *Bmp7* (bone morphogenetic protein 7) and *Evf2* [*Dlx6os1* – Mouse Genome Informatics; Dlx6 opposite strand transcript 1; itself involved in *Dlx6* regulation (Feng et al., 2006)], while repressing other genes, the expression of which is normally restricted to the maxillary process and is under the control of *Dlx1/2*, including *Pou3f3* (POU domain, class 3, transcription factor 3), *Foxl2* (forkhead box L2) and *Irx5* (Iroquois-related

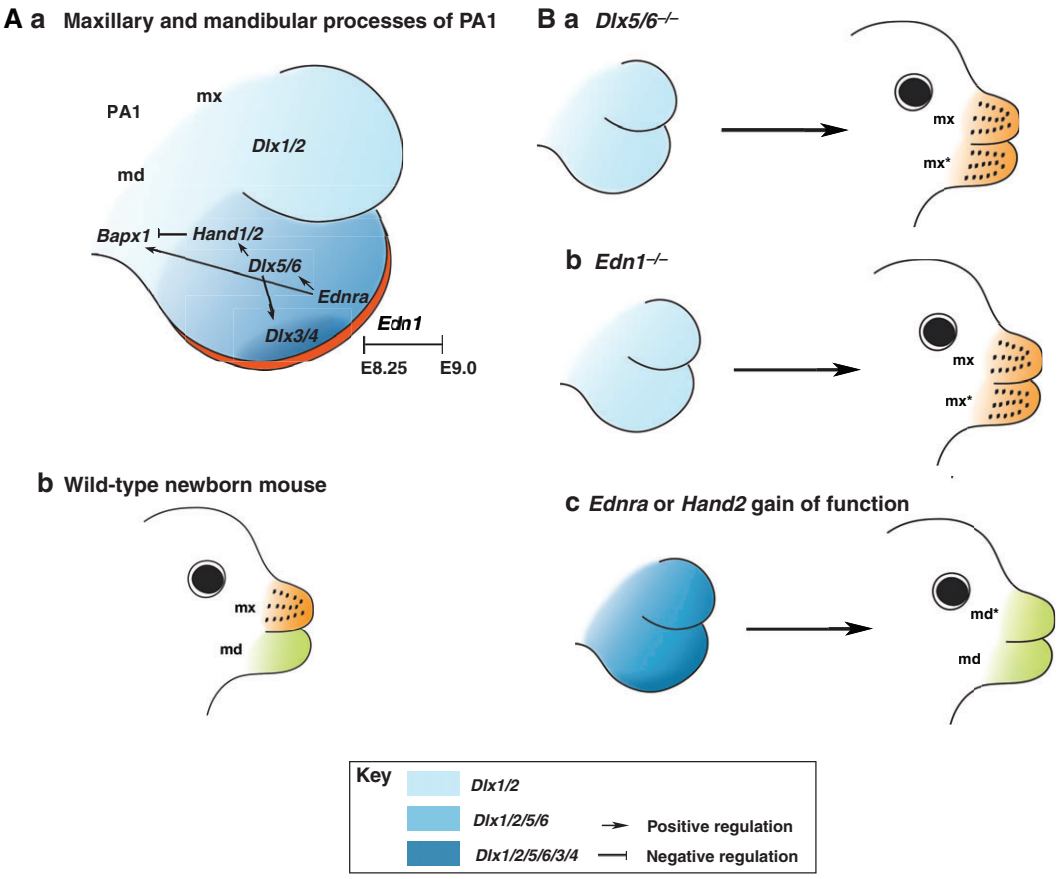

Fig. 4. *Edn1/Ednra→Dlx5/6→Hand2* pathway and mandibular process identity. (**A**) (**a**) Endothelin 1 (*Edn1*) pathway in the first pharyngeal arch (PA1) between E8.25 and E9.0 in a wild-type mouse embryo. The maxillary (mx) and mandibular (md) processes of PA1, and distal-less (Dlx) gene expression code (see Key) are shown. Edn1 secreted by surface ectoderm is shown in red (Edn1 is also secreted by the mesoderm and the endoderm of PAs; not shown for simplicity). Molecular targets of *Edn1* and of the endothelin A receptor (*Ednra*) are shown in the md process, summarizing data obtained in mouse and zebrafish. (**b**) External appearance of a wild-type newborn mouse. The maxillary and mandibular derivatives are shown in orange and green, respectively (dots represent vibrissae). (**B**) Phenotypic effects of modifications in the *Edn1/Ednra→Dlx5/6→Hand2* (heart and neural crest derivatives expressed transcript 2) signalling pathway. (**a**,**b**) *Dlx5/Dlx6* and *Edn1* mutant phenotypes. In both, the lower mouse jaw derivatives are homeotically transformed into a mirror image of upper jaw derivatives (mx*, orange) (Beverdam et al., 2002; Depew et al., 2002; Ozeki et al., 2004; Ruest et al., 2004). (**c**) The constitutive activation of *Ednra* throughout the head and pharyngeal mesenchyme, or the misexpression of *Hand2* in the *Ednra*-expressing domain, causes maxillary components to be replaced by a second set of mandibular derivatives (md*, green) (Sato et al., 2008b). Abbreviation: *Bapx1*, *Nkx3-2* – Mouse Genome Informatics.

homeobox 5) (Beverdam et al., 2002; Depew et al., 2002; Jeong et al., 2008). Interestingly, *Dlx1/2* and *Dlx5/6* also act partially redundantly in the mandibular process, where they upregulate mandibular and repress maxillary process-specific genes, such as *Pou3f1*. *Dlx1/2* have therefore opposing roles in specifying the mandibular versus maxillary processes of PA1 (Jeong et al., 2008), perhaps through differential interactions with distinct co-factors. Further studies will be required to resolve this issue.

Thus, the molecular information provided at the intersection of AP and DV positional values is converted into NCC patterning and differentiation programs that are appropriate for each spatial coordinate, prompting the question ‘how are such intrinsic programs influenced by the local cues to which NCCs are exposed?’.

Intrinsic program and environmental signals crosstalk

In recent years, considerable effort has been made to identify the environmental signals that are involved in craniofacial development. Here, we discuss recent insights into the signalling systems that establish and maintain the spatiotemporal identity of NCCs, and how they impinge on the NCC transcriptional program to form structures of the appropriate shape, size and orientation.

Endothelin 1 signalling

Endothelin 1 (*Edn1/ET1*) is secreted by the surface ectoderm [where it performs its main role (Nair et al., 2007)], and by the mesoderm and endoderm of PAs. It acts by binding to its G protein-coupled endothelin A receptor (Ednra), which is expressed in NCCs (Clouthier et al., 1998; Dettlaff-Swiercz et al., 2005; Ivey et al., 2003; Kurihara et al., 1994; Miller et al., 2000; Nair et al., 2007; Nataf et al., 1998; Offermanns et al., 1998; Sato et al., 2008a) (Fig. 4A). In mouse, the targeted inactivation of *Edn1*, endothelin receptor A (*Ednra*) or of endothelin converting enzyme 1 (*Ece1*, which cleaves Edn1 to form an active peptide) induces severe malformations of ventral PA elements, whereas dorsal structures are less affected (Clouthier et al., 1998; Kurihara et al., 1994; Yanagisawa et al., 1998). This Edn1-ventralizing activity is

particularly evident in PA1 where most of the mandibular process-derived structures are homeotically transformed into a second set of maxillary-like elements, thus mimicking the compound *Dlx5/Dlx6* mutation (Ozeki et al., 2004; Ruest et al., 2004) (Fig. 4B). Indeed, *Dlx5*, *Dlx6* and some of their molecular targets, such as *Hand1/2* and *Dlx3*, are downstream effectors of the *Edn1/Ednra* pathway. Thus, *Edn1/Ednra* establish a mandibular process identity partly by initiating a *Dlx5/Dlx6*-dependent transcriptional program (Charite et al., 2001; Clouthier et al., 2000; Ozeki et al., 2004; Thomas et al., 1998; Yanagisawa et al., 2003) (Fig. 4B). Conversely, constitutive activation of *Ednra* throughout the pharyngeal mesenchyme induces the replacement of the maxillary components by a second set of mandibular elements (Sato et al., 2008b) (Fig. 4B). A similar homeotic transformation is also induced by the ectopic expression of *Hand2*, thus indicating that, within PA1, the *Edn1/Ednra→Dlx5/Dlx6→Hand2* signalling pathway works as a genetic switch that is responsible for the choice of NCCs to select a mandibular, instead of a maxillary, process molecular program (Sato et al., 2008b) (see above). Nonetheless, the mandibular process molecular program also requires an *Ednra*-independent pathway (Ruest et al., 2004; Sato et al., 2008a).

Some evidence in zebrafish indicates that within the arch epithelium, and the mesodermal core, *edn1* is regulated by Tbx1 (Piotrowski et al., 2003). To specify mandibular process identity, Ednra/Edn1 interaction is only necessary during a narrow temporal window between E8.25 and E9.0 of mouse embryogenesis (Fig. 4A). If Ednra-mediated signalling is inactivated after this period, mandibular structures develop normally and *Dlx5/Dlx6* expression is no longer downregulated, indicating that Ednra signalling is mainly required for the initiation of a mandibular process patterning program in NCCs (Fukuhara et al., 2004; Ruest and Clouthier, 2009; Ruest et al., 2005). The molecular mechanisms involved in the maintenance of *Dlx5/Dlx6* expression, as well as the potential later roles of these genes in craniofacial development, remain to be elucidated.

The *Ednra/Edn1* pathway is conserved in jawed vertebrates (Kempf et al., 1998; Miller et al., 2000; Walker et al., 2006; Walker et al., 2007). In zebrafish, the *edn1* defective mutant *sucker* displays severely reduced ventral PA cartilages that are fused with dorsal cartilages of the same arch (Kimmel et al., 1998; Miller et al., 2000; Piotrowski et al., 1996). Moreover, *edn1* may act as a morphogen to pattern specific skeletal elements along the entire DV axis (Kimmel et al., 2003; Miller et al., 2003). In PA2, high Edn1 levels induce the formation of ventral dermal bones (the branchiostegal rays), whereas low levels of Edn1 specify the dorsal bones (the opercles) (Kimmel et al., 2003). In PA1, *edn1* is involved in the correct positioning of the primary articulation between upper and lower jaws by regulating the expression of *bapx1*, while also inducing *hand2* ventrally (through *dlx* function, see above), which in turn represses *bapx1* (Miller et al., 2003) (Fig. 4A). As a result, the expression domain of *bapx1*, which regulates growth differentiation factor 5 (*gdf5*) and *chordin* expression, is restricted to a subset of NCCs at an intermediate DV position in PA1, defining where the jaw joint will form (Miller et al., 2003). Edn1 may additionally repress dorsal fate in ventral skeletal precursors by inhibiting Jagged-Notch signalling, which ensures dorsal identity of mandibular and hyoid skeletal components (Zuniga et al., 2010). In contrast to mouse, zebrafish possess two Ednra genes: *ednra1* and *ednra2*. The knockdown of *ednra1* alone disrupts the jaw joint, whereas the knockdown of both eliminates the ventral arch skeletal elements, thus reproducing the *edn1* mutant phenotype (Nair et al., 2007). These data indicate that higher levels of Edn1 are required to pattern joint cells compared with ventral cartilages (Nair et al., 2007).

Notably, in lamprey (a jawless vertebrate), homologues of Edn1 and Ednra are expressed as in jawed vertebrates, indicating the involvement of this signalling pathway in craniofacial patterning early in vertebrate evolution (Kuraku et al., 2010). However, the expressions of Dlx or *bapx1* genes are not spatially restricted in lamprey PAs (Kuraku et al., 2010), suggesting that the elaborate genetic program leading to the Dlx code is likely to have been acquired uniquely in jawed vertebrates (see below).

FGF and BMP signalling

FGF and BMP molecules have multiple roles during craniofacial and pharyngeal skeletal morphogenesis. For example, FGFs are key NCC survival factors (Abu-Issa et al., 2002; Frank et al., 2002; Macatee et al., 2003; Szabo-Rogers et al., 2008; Trumpp et al., 1999; Walshe and Mason, 2003). In the mouse, *Fgf8* conditional inactivation in PA1 ectoderm induces massive NCC apoptosis, resulting in the absence of most PA1 skeletal elements (Trumpp et al., 1999). Conversely, Fgf8-soaked beads placed in presumptive PA1 ectoderm causes r3-derived NCC proliferation, overcoming the failure of facial development induced by removing NCCs from di-, mes- and rhombencephalon down to r2 (Creuzet et al., 2004). In zebrafish, endodermal *fgf3* expression also exerts a trophic effect on NCCs. Early *fgf3* downregulation, or preventing its maintenance through later stages, causes apoptotic elimination of PA3 and PA4 NCCs (David et al., 2002; Nissen et al., 2003). *fgf8* and *fgf3* expressed in the mesoderm and hindbrain can also act as chemoattractants to promote the lateral migration of endodermal cells, which is required for the segmentation of the pharyngeal endoderm into pouches and, in turn, for the correct patterning of NCC-derived skeletal elements (Crump et al., 2004; Graham, 2008). Moreover, FGFs are involved in directing NCCs to adopt the ectomesenchymal fate (Blentic et al., 2008).

FGF signalling is also involved in specification of NCC spatial identity and in establishing the AP and DV polarity of PAs. The mouse mandibular process is subdivided into oral (anterior) and aboral (posterior) domains. This AP polarity is established by Fgf8, which diffuses from the oral epithelium and controls, in a concentration-dependent manner, the differential expression of HD transcription factors in the underlying mesenchyme (Grigoriou et al., 1998; Tucker et al., 1999), such as *Lhx6* and *Lhx7*, which are expressed in the oral domain in response to high Fgf8 levels, and goosecoid (*Gsc*), which remains restricted to the aboral mesenchyme (Grigoriou et al., 1998; Tucker et al., 1999) (Fig. 5A). *Gsc* expression, which is required for patterning aboral skeletal elements (Rivera-Perez et al., 1995; Yamada et al., 1995), is also controlled by the Edn1 pathway (Clouthier et al., 1998; Clouthier et al., 2000), underscoring that distinct pathways may interact and converge on the same targets to specify NCC spatial identity. In this respect, in chick PA1, Fgf8- and Bmp4-mediated signalling also contribute to the correct positioning of the *Bapx1*-positive jaw joint (Wilson and Tucker, 2004), probably by interacting with the Edn pathway (Fig. 5A). In addition, *Edn1* expression is downregulated in the *Fgf8* conditional knockout mouse (Trumpp et al., 1999).

At early developmental stages, Fgf8 and Bmp4 pattern the presumptive maxillo-mandibular region and define it from the pre-mandibular domain (Shigetani et al., 2000). Before mesencephalic NCCs arrive at their destination, ectodermal *Fgf8* prefigures the prospective oral cavity (Haworth et al., 2004; Shigetani et al., 2000). This *Fgf8* expression domain, which is induced by sonic

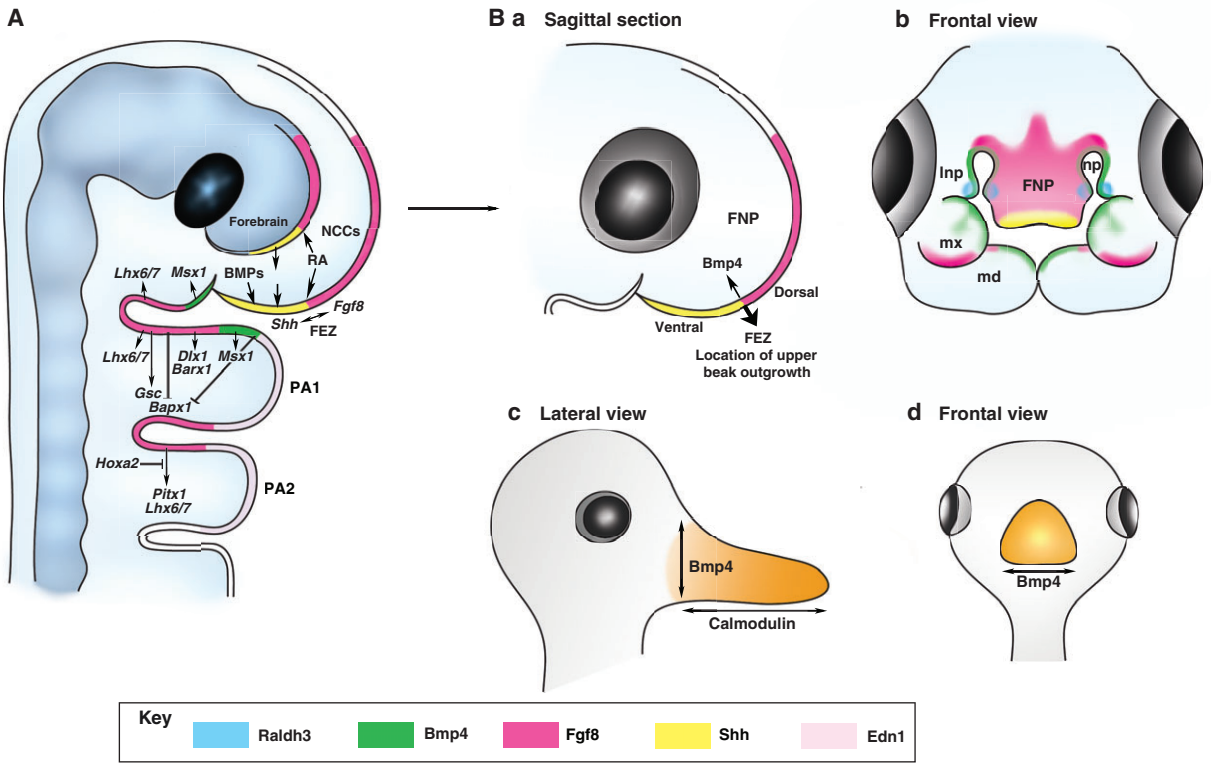

Fig. 5. Environmental signals and patterning of craniofacial and pharyngeal structures. (**A**) A schematic section through a vertebrate embryo along an imaginary mid-sagittal plane at the level of the frontonasal process (FNP), and along a parasagittal plane at the level of the first and second pharyngeal arches (PA1 and PA2, respectively) (rostral is uppermost) depicting the signals involved in craniofacial and pharyngeal morphogenesis. The indicated genetic interactions are representative of mouse, chick or zebrafish data. The arrows represent positive regulations. Some differences might exist in these pathways in different species, see main text for details and references. The juxtaposition of *Fgf8* (fibroblast growth factor 8, pink), *Shh* (sonic hedgehog, yellow), *Bmp4* (bone morphogentic protein 4, green) and *Edn1* (endothelin 1, pale pink) expression domains is arbitrary, except at the level of the frontonasal ectodermal zone (FEZ) and in the adjacent neuroectoderm (the signalling cascade initiated by *Edn1* is shown in Fig. 4). At the level of the FNP, the stage of development corresponds to the initiation of the FEZ. Abbreviation: NCCs, neural crest cells. (**B**) Schematics of the influence of the FEZ and *Bmp4*/calmodulin signalling in regulating upper beak formation and shape. (**a**,**b**) Drawing of a HH20 stage chick embryo (a) sectioned through the FEZ and (b) a frontal view. In addition to the FEZ, the expression patterns of different signalling molecules that sculpt the face are shown (see the main text for details). (**c**,**d**) A drawing of a duck head in (c) lateral and (d) ventral views, showing that *Bmp4* is involved in determining the width and depth of the beak, whereas calmodulin is involved in determining its length. Abbreviations: *Bapx1*, *Nkx3-2* – Mouse Genome Informatics; *Barx1*, BraH-like homeobox 1; Gsc, goosecoid; Lhx, LIM homeobox protein; lnp, lateral nasal prominence; md, mandibular process; Msx, muscle segment homeobox like; mx, maxillary process; np, nasal pit; Pitx1, paired-like homeodomain transcription factor 1; Raldh3, retinaldehyde dehydrogenase 3.

hedgehog (Shh) signalling from the endoderm (Haworth et al., 2004; Haworth et al., 2007), is delimited by *Bmp4* expressed on both sides of the adjacent *Fgf8*-expressing ectoderm (Shigetani et al., 2000). Ectodermally derived Fgf8 and Bmp4 control, in turn, the regionalization of incoming NCCs through the activation of specific patterning genes: Fgf8 induces the expression of *Dlx1* and *Barx1*, while Bmp4 induces *Msx1* (muscle segment homeobox like 1) expression in the underlying mesenchyme (Fig. 5A). These epithelio-mesenchymal interactions are crucial for specifying the identity of the pre-mandibular and maxillo-mandibular regions (Shigetani et al., 2000). In addition to its role in establishing AP and DV PA identity, *Fgf8* is required for left-right symmetry of the craniofacial skeleton (Albertson and Yelick, 2005; Albertson and Yelick, 2007).

Shh signalling

Blocking Shh signalling in chick, mouse and zebrafish induces severe head skeleton abnormalities, including holoprosencephaly and cyclopia, which result from defects in NCC survival, proliferation and patterning (Ahlgren and Bronner-Fraser, 1999; Chen et al., 2001; Chiang et al., 1996; Cordero et al., 2004; Hu and Helms, 1999; Jeong et al., 2004; Washington Smoak et al., 2005). Several sources of Shh, such as the foregut endoderm, the neuroepithelium and the facial ectoderm, are crucial in these processes. The foregut endoderm (see Glossary, Box 1) is a major source of the patterning signals that provide NCCs with information about the size, shape and orientation of the skeletal elements that are generated in the first and more posterior PAs (Couly et al., 2002; Graham, 2008; Ruhin et al., 2003). Recent studies have identified Shh as one such signal (Benouaiche et al., 2008; Brito et al., 2006; Brito et al., 2008). Its absence in the ventral foregut endoderm prevents the development of Meckel's cartilage and associated PA1 structures because of massive NCC apoptosis (Brito et al., 2006). By contrast, providing an extra source of Shh to the presumptive PA1 induces the formation of two supernumerary Meckel's cartilages that develop in a mirror-image to the normal one. The formation of these structures is preceded by *Bmp4*, *Fgf8* and *Shh* ectopic expression in the caudal ectoderm of

PA1 (Brito et al., 2008). The Shh patterning effect on PA1 is not seen in PA2 (Brito et al., 2008), suggesting that other signals act independently, or additionally, to Shh in more posterior PAs. In zebrafish, *fgf3* downregulation in the pharyngeal pouch endoderm leads to the formation of a hyoid cartilage with an inverted AP polarity (David et al., 2002), suggesting that *fgf3* expressed in the dorsal half of the pouch polarizes PA2 skeletal structures.

In addition to its role in foregut endoderm, Shh signalling arising from the ventral brain primordium is required in zebrafish for the proper specification of the roof of the stomodeum (see Glossary, Box 1), which in turn promotes NCC condensation to form the anterior neurocranium and upper jaw cartilages (Eberhart et al., 2006). This early function of Shh is followed by a later role in NCCs to promote their differentiation into cartilages (Eberhart et al., 2006; Jeong et al., 2004; Wada et al., 2005). Moreover, Shh plays a role in establishing skeletal polarity along the mediolateral axis of the embryo (Eberhart et al., 2006; Wada et al., 2005).

Shh signalling from the facial ectoderm is also involved in the specification of NCC spatial identity. In the ectoderm overlying the FNP, a signalling centre called the frontonasal ectodermal zone (FEZ), defined by the juxtaposition of *Fgf8* and *Shh* expression domains, regulates the growth and DV polarity of the upper beak in birds (Hu et al., 2003) (Fig. 5). When grafted ectopically, the FEZ can reprogram the developmental fate of the underlying NCCs, inducing upper beak duplications with a DV polarity that reflects the orientation of the grafted tissue. However, mandibular NCCs form a supernumerary lower (and not upper) beak when in contact with an ectopic FEZ, and Hox-positive NCCs are unresponsive to FEZ cues (Hu et al., 2003). Thus, the epithelial-mediated patterning instruction is not absolute, but is interpreted by the NCCs according to their relative AP positional identity. The same observation holds also true for signals arising from the endoderm (Couly et al., 2002).

At early stages, Shh emanating from the forebrain acts on the NCCs, which in turn induce *Shh* expression in the FEZ (Marcucio et al., 2005) (Fig. 5A). Part of this signalling network could include BMPs (Foppiano et al., 2007) (Fig. 5A). Once the FEZ is established, reciprocal inhibitory interactions contribute to the maintenance of the transient *Fgf8*/*Shh* molecular boundary, which marks the dorsal tip of the upper beak (Abzhanov et al., 2007) (Fig. 5A). In avian embryos, the FEZ spans the entire FNP, whereas in mouse, two FEZ are present – in the left and right median nasal processes (Hu and Marcucio, 2009b). If the *Shh* pathway is ectopically activated in the forebrain of chick embryos, two instead of one FEZ are induced in the facial ectoderm, mimicking the mouse organization (Hu and Marcucio, 2009a). Thus, modifications in the molecular organization of the FEZ might in turn generate distinct patterns of growth in the upper face and contribute to morphological diversity among species (Hu and Marcucio, 2009a; Hu and Marcucio, 2009b).

Retinoic acid signalling

In mouse, the double inactivation of the retinoic acid (RA)-synthesizing enzymes *Raldh2* and *Raldh3* (*Aldh1a2* and *Aldh1a3* – Mouse Genome Informatics), which are expressed in the forebrain and facial ectoderm leads to a partial lack of skeletal structures derived from the FNP, reproducing the compound retinoic acid receptor (RAR) α/RARγ mutant phenotype (Halilagic et al., 2007; Lohnes et al., 1994). Whereas RARα and RARγ act cell-autonomously in NCCs, RARβ is dispensable (Dupe and Pellerin, 2009). A local source of RA in the chick rostral head coordinates FNP morphogenesis through *Fgf8* and *Shh* induction in the forebrain and facial ectoderm (Schneider et al., 2001) (Fig. 5A). RA is also required for FNP spatial patterning. Increasing RA levels in the presumptive maxillary field, while inhibiting BMP signalling, transforms the side of the beak (derived from the maxillary process) into a second set of midline structures (normally derived from the FNP) (Lee et al., 2001). This suggests that an endogenous source of RA is necessary to pattern the FNP and its derivatives: the pre-nasal and pre-maxillary skeletal elements. Accordingly, the nasal pit is an important RA source (Dupe et al., 2003) that, together with *Fgf8* and *Bmp4*, coordinates local cell survival around the nasal placode, thus contributing to the shaping of the face (Song et al., 2004; Szabo-Rogers et al., 2008) (Fig. 5B).

The morphogenesis of the pharyngeal region also requires RA signalling (Dupe et al., 2003; Lohnes et al., 1994; Mark et al., 2004; Mendelsohn et al., 1994; Niederreither et al., 1999; Vermot et al., 2003; Wendling et al., 2000). The double inactivations of RARα and RARγ, or RARα and RARβ, induce PA hypoplasia and NCC-derived cartilage malformations (Dupe et al., 1999; Lohnes et al., 1994; Mendelsohn et al., 1994; Wendling et al., 2000). However, the endoderm could be the primary target of RA action in mediating morphogenesis of PA2 and more posterior arches (Matt et al., 2003), as RARs are mostly dispensable in cranial NCCs (Dupe and Pellerin, 2009). Not only RA deficiency, but also exogenous RA excess during pregnancy or the inactivation of the cytochrome P450 (Cyp) RA-degrading enzymes induce teratogenesis in the craniofacial region (Abu-Abed et al., 2001; Maclean et al., 2009; Mulder et al., 2000; Reijntjes et al., 2007; Uehara et al., 2007). Hence, a tight balance between the production and degradation of RA is necessary for correct NCC-derived craniofacial skeleton morphogenesis.

Craniofacial evolution and NCC spatial identity

Recent findings support the view that heterochronic (in time), heterotopic (in place) or quantitative changes in the expression of key signalling molecules in the face epithelia and/or NCCs might underlie the evolution and variation of facial morphology. Modifications in the level and/or spatiotemporal distribution of the HD transcription factors that modulate the intrinsic NCC ground patterning molecular programs could also lead to the morphological evolution of skeletal elements. Such mechanisms could modify the positional identity of cranial NCCs, thus potentially resulting in species-specific skeletal elements of different size and/or shape.

Quantitative changes of signalling molecule expression

Variation in beak size and shape among birds is one example of craniofacial morphological evolution that is well studied. The exchange of NCCs between the presumptive beak regions of quails and ducks generates quail-like beaks in duck hosts, and vice versa (Schneider and Helms, 2003). NCCs from donors respond to local signalling from the host environment, while partially maintaining their original molecular programs, and regulate expression in adjacent host tissues to specify the morphology of the beak in a species-specific manner (Schneider and Helms, 2003; Tucker and Lumsden, 2004). The spatial organization and activity of proliferation zones in the FNP or between the FNP, maxillary and nasal prominences may also result in species-specific beak shape (Wu et al., 2004; Wu et al., 2006). *Bmp4* mediates the activity of such proliferation zones, and its expression level in the FNP is crucial for final beak

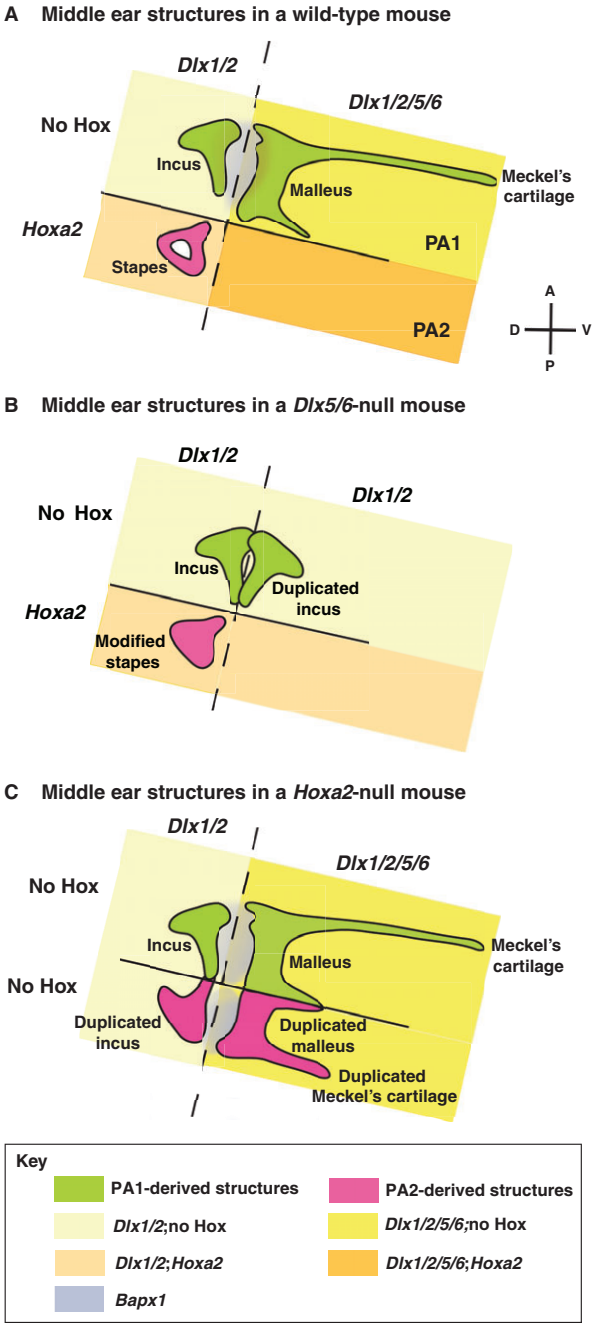

Fig. 6. Relationship between rostrocaudal and dorsoventral neural crest cell transcriptional programs and middle ear structures and primary jaw joint positioning. Quadrant-specific colour codes represent the orthogonal integration of the distal-less (Dlx) and homeobox (Hox) codes that establish the positional identity of neural crest-derived mouse middle ear structures. (**A**) The middle ear of a newborn wild-type mouse showing the Meckel's cartilage and the middle ear incus, malleus and stapes. In the first pharyngeal arch (PA1), the *Bapx1* (*Nkx3-2*, NK3 homeobox 2; grey) expression domain identifies the position of the incudo-malleal articulation (corresponding to the primary jaw joint), at or near the proximal border of *Dlx5/Dlx6* expression domain. (**B**) In *Dlx5/6*-null mutants, the incus is (partially) duplicated along the PA1 dorsoventral (DV) axis, whereas in the PA2, the stapes is present but lacks its foramen (Depew et al., 2002). (**C**) In *Hoxa2*-null mutants, the incus, malleus and the *Bapx1* expression domain are ectopically duplicated in PA2, as mirror images of their normal PA1 counterpart (Gendron-Maguire et al., 1993; Rijli et al., 1993). A, anterior; P, posterior; D, dorsal; V, ventral.

morphology (Abzhanov et al., 2004; Wu et al., 2004). Indeed, Darwin's finch species with deeper and broader beaks express *Bmp4* in their beak prominences at higher levels and at earlier stages than do species with narrow and shallow beaks (Abzhanov et al., 2004). In chick embryos, Bmp4 overexpression in the FNP mesenchyme induces a significant enlargement of the beak width and depth, while Noggin-mediated inhibition induces its reduction (Abzhanov et al., 2004). Recently, calmodulin (CaM), a molecule involved in mediating Ca^{2+} signalling, has been suggested to control beak length in Darwin's finch. In chick, the upregulation of the CaM-dependent pathway in the FNP leads to an elongated upper beak, reproducing the beak morphology of Darwin's cactus finches (Abzhanov et al., 2006). Thus, two different pathways, Bmp4- and CaM-dependent signalling, act to shape the beak along different axes: CaM controls the length, whereas Bmp4 controls the width and depth (Fig. 5B). This provides evidence that variations in Bmp4 and/or CaM expression levels may have contributed to species-specific beak morphologies (Abzhanov et al., 2004; Abzhanov et al., 2006).

Heterochronic changes in signal expression

Temporal variations of signalling molecule activity could also underlie morphological evolutionary changes. *Fgf8* and *Shh*, which are expressed in the FEZ of the facial epithelia, promote cartilage outgrowth, in part, by inducing *Bmp4* expression in the underlying NCCs (Abzhanov and Tabin, 2004; Hu and Marcucio, 2009b) (Fig. 5B). Hence, modifications in the timing of their expression could induce shape diversity. Accordingly, it has been proposed that the persistence of *Fgf8* expression in the facial ectoderm of duck, but not of chick, contribute to the distinct morphology of the duck beak, inducing it to produce more cartilage (Wu et al., 2006). In quail-duck chimaeric embryos, bone and cartilage formation are controlled by the NCCs that also control the stage-specific size and shape of skeletal structures, in part by temporally regulating their own expression of *Bmp4* (Eames and Schneider, 2008; Merrill et al., 2008).

Heterotopic changes of epithelial-mesenchymal interactions

In the jawless lamprey, the oral apparatus consists of a lower lip and a velum, derived from the mandibular region, and an upper lip, derived from the pre-mandibular region. By contrast, in gnathostomes (jawed vertebrates), the lower jaws, the primary jaw joint and part of the upper jaws are derived from the mandibular region (Shigetani et al., 2002; Takio et al., 2004) (but see Cerny et al., 2004; Lee et al., 2004). Hence, phenotypically similar protrusions, i.e. the lips in lampreys and the jaws in gnathostomes, originate from non-equivalent cell populations (Shigetani et al., 2002). A heterotopic (posterior) shift of epithelial-mesenchymal interactions could underlie these changes, placing jaws as evolutionary innovations rather than as simple modifications of the ancestral oral apparatus (Shigetani et al., 2002).

NCC ground patterning programs and morphological evolution

One feature of the bilaterian body plan is its organisation in successive metameric units along the AP axis. Segment-specific morphology is achieved by modifying underlying ground (default) patterning molecular programs that are shared by all segments of a given series, to provide successively more posterior positional identities. In the hindbrain and pharyngeal regions of vertebrate embryos, such ground patterning programs correspond to the molecular programs of the Hox-free rostralmost elements of the series, namely rhombomere 1 and PA1, respectively (see above) (Minoux et al., 2009; Rijli et al., 1993; Waskiewicz et al., 2002) (Fig. 3A). Such a strategy may be widely conserved in animal evolution because in the short germ band beetle *Tribolium*, the complete inactivation of Hox genes induces the transformation of all embryonic segments into antennae (Brown et al., 2002).

The morphological diversity of NCC-derived skeletal elements among vertebrates could arise by modifying the level, timing and/or spatial expression of HD factors involved in the specification of spatial identity, or by modifying the expression pattern of genes involved in establishing the molecular ground patterning program. This is illustrated by the dramatic morphological and molecular modifications that have occurred in PA1 during the transition from jawless to jawed vertebrates, yielding the appearance of the primary jaw articulation between upper and lower jaw processes. In gnathostomes, this is likely to be accompanied by the modification of the PA1 ground pattern by a Dlx code, which generates DV polarity in the NCC-derived skeletal elements (Beverdam et al., 2002; Depew et al., 2002; Depew et al., 2005) (Fig. 4). It is noteworthy that lampreys possess multiple Dlx genes; however, their expression is not nested within PAs (Kuraku et al., 2010; Neidert et al., 2001). Focal expression of *bapx1* is also a gnathostome feature (Kuraku et al., 2010). Both *dlx5/6* and *bapx1* expression is under the control of *edn* signalling (Fig. 4A), which might therefore be involved in the evolutionary changes resulting in the appearance of the jaw joint (Miller et al., 2003). A further modification in the PA1 DV patterning program may have subsequently occurred in tetrapods because, in mouse (unlike in zebrafish), *Bapx1* is unable to regulate *Gdf5* and *Gdf6*, two genes that are essential for the formation of the jaw joint (Tucker et al., 2004). Therefore, the regulatory cascade initiated by *bapx1* in fish is not maintained in mouse, thus potentially contributing to species-specific morphologies.

The existence of a PA1 molecular ground patterning program shared by all segments predicts that any genetic change modifying DV patterning information in PA1 would be integrated into the basic genetic program of all PAs and, in turn, would be modified by segment-specific Hox codes along the AP axis to yield segment-specific structures. Indeed, the inactivation of *Hoxa2* in post-migratory PA2 NCCs results in the ectopic induction of *Bapx1* in the mutant PA2 (Santagati et al., 2005) (Fig. 6). This indicates that the DV molecular changes leading to the appearance of the jaw joint in PA1 of jawed vertebrates have also been fixed in the molecular ground pattern of PA2, where they undergo PA2-specific repression in response to the post-migratory expression of *Hoxa2* in NCCs. This additionally suggests that a late function of *Hoxa2* in PA2 NCCs is to interfere with *Edn*-mediated induction of *Bapx1* and to prevent the formation of a jaw joint in PA2. Such a function appears to be conserved, as *Hoxa2* overexpression in *Xenopus* PA1 after NCC migration is sufficient to downregulate *Xbap* and cause loss of articulation and mandibular-to-hyoid transformation (Pasqualetti et al., 2000). Changes in the levels and/or local spatial distribution of *Hoxa2* in PA2 of different species might therefore differently modulate the underlying molecular ground patterning program and the response of downstream targets to endothelin signalling, resulting in the generation of species-specific elements along the PA2 DV axis (e.g. the mammalian sound-conducting stapes or fish hyomandibular skeleton). Thus, interactions between AP ground patterning and DV molecular programs might provide the molecular framework to link the DV morphological evolution of PA1 to the coordinated morphological changes in the other elements of the rostrocaudal series (Fig. 6). Further work is required to support such a speculative model and to address potential genetic interactions between the Hox and Dlx codes.

Conclusions

Many birth defects are associated with craniofacial malformations. It is becoming increasingly clear that these craniofacial abnormalities could be attributed to defects in the generation, proliferation, migration and differentiation of cranial NCCs. Thus, a full comprehension of craniofacial development will rely on a complete dissection of the molecular processes that underlie NCC differentiation and patterning. Significant progress has been made recently in revealing conserved gene regulatory modules that are crucial for patterning the cranial neural crest and signalling pathways that regulate tissue interactions during craniofacial development. The next challenge is that of achieving a comprehensive systems biology understanding of how collections of genes integrate into functional networks to generate normal or defective complex craniofacial phenotypes. It will be important to reconstitute the molecular aspects that make up the signalling mechanisms of epithelial-mesenchymal interactions and the spatiotemporal epigenetic regulation of NCC transcriptional programs of differentiation at the single and cell population levels. Integrating information on genetic, cellular and systems levels will hopefully provide insights into the mechanisms that underlie complex craniofacial phenotypes.

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