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Review article

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Shaping faces: genetic and epigenetic control of craniofacial morphogenesis

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Abstract

Major differences in facial morphology distinguish vertebrate species. Variation of facial traits underlies the uniqueness of human individuals, and abnormal craniofacial morphogenesis during development leads to birth defects that significantly affect quality of life. Studies during the past 40 years have advanced our understanding of the molecular mechanisms that establish facial form during development, highlighting the crucial roles in this process of a multipotent cell type known as the cranial neural crest cell. In this Review, we discuss recent advances in multi-omics and single-cell technologies that enable genes, transcriptional regulatory networks and epigenetic landscapes to be closely linked to the establishment of facial patterning and its variation, with an emphasis on normal and abnormal craniofacial morphogenesis. Advancing our knowledge of these processes will support important developments in tissue engineering, as well as the repair and reconstruction of the abnormal craniofacial complex.

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Introduction

The face comprises the vital organs that allow us to breathe, see, hear, smell, eat and communicate. Facial features also provide a gateway to social interactions and a criterion for mate selection, as well as conveying our thoughts and emotions to other individuals[1,2]. In vertebrates, the process of face formation during development involves a conserved series of molecular and morphogenetic events that generate a bilaterally coordinated pattern of species-specific cartilaginous and bone structures, each with distinct morphologies, which assemble with precision into a 3D face[3]. Facial morphogenesis involves a specialized multipotent cell type of neuroepithelial origin, the neural crest cell (NCC), which is the main source of craniofacial mesenchyme[4]. Cranial neural crest cells (CNCCs) detach from the neural epithelium (known as delamination) and migrate away in distinct streams to various regions of the embryo, where they differentiate into cartilage and bone under the influence of local signalling. CNCCs contribute to the formation of most craniofacial structures, including the skull vault, frontonasal prominence (FNP), upper jaw (also known as maxilla), lower jaw (also known as mandible), external ear, middle ear and hyoid bone[5–7].

The initial patterns of CNCC generation, segmentation and migration are mainly conserved between species[8]. However, the variety of facial morphologies that exist in different species indicates that a complex interplay occurs between the intrinsic genetic programmes of CNCCs and the environmental cues to which distinct CNCC subpopulations are exposed during craniofacial morphogenesis. Recent studies have defined the genes, gene regulatory networks (GRNs) and epigenetic landscapes that direct the life cycle of multipotent CNCCs[9,10]. A large number of review articles have selectively focused either on genetic and regulatory control of neural crest development and evolution[8,11] or on the behaviours of NCCs, including specification, epithelial-to-mesenchymal transition (EMT), migration and differentiation[12–15], and neural crest pathology[16,17]. Each of these topics has been covered comprehensively elsewhere.

Here, we address a conspicuous gap in the literature – linking genes, GRNs and epigenetic regulation to face morphogenesis during development. Specifically, we discuss the contribution of these regulatory mechanisms to facial anatomy, morphology and their variation. We describe the effects of these interconnected levels of regulation on CNCC-directed morphogenesis in both animal models and humans. We further discuss the underpinnings of normal morphological variation in humans, which results in a wide range of facial features, as well as the evolutionary variation that underlies changes in craniofacial morphology across different species. Lastly, we review abnormalities of CNCC development in select craniofacial birth defects.

Craniofacial morphogenesis

Morphogenesis of the mammalian face requires a complex choreography of tightly regulated and coordinated embryonic events. Most of these processes are remarkably similar in humans and mice, occurring from the 5th to the 8th week of gestation in humans[18], and from embryonic day 9.5 (E9.5) to E11.5 in mice[19,20]. The first major partition of the developing craniofacial tissues occurs early, during the 4th week of embryonic development in humans and between E9.5 and E10.5 in mice, with the separation of the FNP from the branchial arches. Subsequently, during the 5th week of gestation in humans (E10.5 in mice), the embryonic mandibular prominences (MdPs) merge at the midline to form the lower jaw, chin and lower lip (Fig. 1). Furthermore, the FNP separates into the medial nasal prominence (MNP) and the lateral nasal prominence (LNP), which then fuse with the maxillary prominence (MxP) at approximately the 7th week of gestation in humans (E11.5 in mice), leading to the formation of the central structures of the nose, upper lip and primary palate, rostral to the stomodeum[20,21] (Fig. 1; details in legend). The three-way anatomical seam wherein the three facial prominences coalesce is termed the lambdoidal junction[22]. All developing facial prominences comprise more than one type of tissue – mainly, a core of CNCC-derived mesenchymal cells surrounded by tall, tightly associated epithelial cells that form an external sheet. These structures grow and mature during the remaining weeks of pregnancy (Fig. 1). Consistent with the crucial roles of CNCCs in craniofacial development, abnormal CNCC-directed morphogenesis leads to birth defects in humans, including neurocristopathies[17,23]. Birth defects that affect facial features are among the most common congenital diseases; they have marked impact on quality of life and require substantial resources from health-care systems.

Patterning and morphogenesis of craniofacial structures are regulated by the combinatorial activity of sequence-specific transcription factors[24], through both cell-autonomous and extrinsic signal-responsive mechanisms. Major signalling pathways that transmit environmental cues to CNCCs during craniofacial morphogenesis include those involving bone morphogenetic proteins (BMPs), Sonic hedgehog (SHH), fibroblast growth factors, WNT proteins and transforming growth factor-β (TGFβ)[25–28]. Complex interactions between CNCCs and other cell types – such as the surface cephalic epithelium, which covers the facial prominences[29], and the cranial mesoderm – are essential for the proper morphogenesis of the craniofacial structures[30–37]. Here, however, we focus exclusively on genetic and epigenetic regulatory mechanisms involved in CNCC-dependent facial morphogenesis.

Genetic regulation of CNCCs

The positional identity of CNCC subpopulations and their patterning are established in large part by the combinatorial expression of genes that encode homeodomain transcription factors. Their expression is induced and maintained in CNCCs through later developmental stages by signals from the surrounding environment. Here, we focus on the roles of three important classes of homeodomain transcription factor that have long been viewed as key regulators of CNCC positional identity and patterning during craniofacial development – the HOX, PBX and DLX families.

Hox genes

Anterior–posterior positional identity of CNCC progenitors in the branchial arches is established by the nested and combinatorial expression of Hox genes in a collinear manner, with the exception of branchial arch 1 (BA1), which is devoid of Hox expression. The 39 mammalian Hox gene family members are subdivided into 13 paralogous groups[38]. The role of Hox genes in establishing CNCC anterior–posterior positional identity was first demonstrated by the targeted inactivation of *Hoxa2* in mice[39,40]. Loss of *Hoxa2* resulted in the mirror-image homeotic transformation of rhombomere 4-derived BA2 (hyoid arch) CNCC structures, including the stapes of the middle ear ossicles, into BA1-like skeletal elements, such as the proximal part of Meckel's cartilage and the other middle ear ossicles, the incus and the malleus, which are normally derived from CNCCs of rhombomeres 1 and 2 (ref. 41) (Fig. 2). Interestingly, *Hoxa2* downregulation in *Xenopus* and zebrafish also resulted in BA2-to-BA1-like homeotic transformation, underscoring a conserved role for *Hoxa2* in BA2 patterning across species[5]. In chicken, frog and mouse models, high levels of ectopic *Hoxa2* expression in HOX-free CNCCs induced severely hypoplastic facial structures, whereas

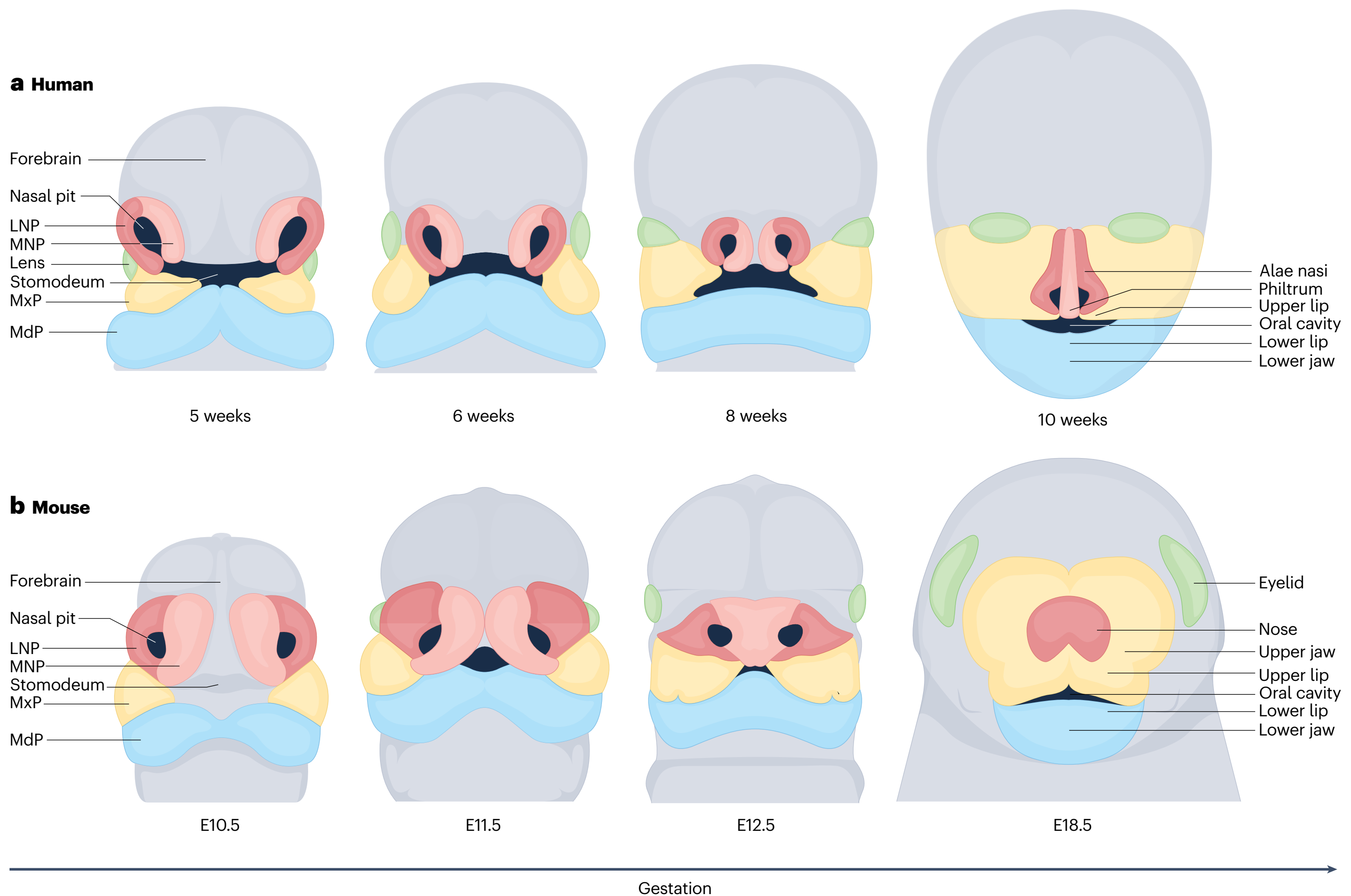

Fig. 1 | Facial development in humans and mice. Comparison of human (panel **a**) and mouse (panel **b**) facial development, depicting human embryos from 5 to 10 weeks of gestation, and mouse embryos from embryonic day 10.5 (E10.5) to E18.5. All stages are portrayed as anterior views. Equivalent gestational stages between humans and mice are shown. At 5 weeks of gestation in humans (E10.5 in mice), the medial nasal prominence (MNP) and lateral nasal prominence (LNP) are formed; they comprise a downward-facing 'horseshoe' around the nasal pit. At this developmental stage, the stomodeum (future oral cavity) is surrounded caudally by the growth of the maxillary prominence (MxP) and mandibular prominence (MdP), which derive from branchial arch 1. At 5 weeks of gestation in humans, the lens (future eye) is also visible. The subsequent merging of the LNP and the MxP (by 6–7 weeks of gestation in humans and by E11.5 in mice) will give rise to the wings of the nose (alae nasi), the midline groove in the upper lip (philtrum) and the upper lip. During early gestation (5–8 weeks in humans, E10.5–E12.5 in mice), facial morphogenesis is strikingly similar in humans and mice, with facial features progressively diverging during later gestational stages to generate the distinct facial appearances of humans and mice.

moderate levels of ectopic *Hoxa2* had a smaller effect on facial development and allowed for homeotic repatterning of (part of) HOX-free CNCCs from BA1 into BA2-like structures[5,42,43]. In summary, evidence from various animal models demonstrates the evolutionary conservation of *Hoxa2* function, being both necessary and sufficient for the morphogenesis of BA2-derived CNCC structures.

Moreover, CNCC-specific deletion of the Hoxa gene cluster in mice induced a more extensive phenotype than that observed in mice with single loss-of-function mutations in either *Hoxa2* or *Hoxa3*. Conditional mutant mice with deletion of the Hoxa gene cluster selectively in CNCCs have multiple sets of structures reminiscent of BA1-like skeletal elements[44]. This phenotype could be interpreted as resulting from the localized ectopic activation of chondro-osteogenic progenitor differentiation, which is partially repressed by Hox genes in posterior branchial arches. However, BA1-specific molecular markers were ectopically induced in BA3 and BA4 of embryos with CNCC-specific deletion of the Hoxa gene cluster, which suggests that in the absence of Hoxa genes, BA3 and BA4 CNCCs adopt a partial BA1 identity. Concomitantly, a homeotic transformation was induced in BA2 by the absence of *Hoxa2*. These findings suggest that an underlying anterior–posterior ground patterning programme of BA1 (MdP) to BA4 CNCCs is executed in the absence of Hox gene expression and generates a series of MdP-like metameric elements[40,44]. The additional removal of the Hoxd gene cluster in a CNCC-specific Hoxa cluster-deleted background did not increase the extent of the homeotic phenotype[44], whereas deletion of the Hoxb cluster suggested that Hoxb genes may fine-tune some of the processes controlled by Hoxa genes[45]. Thus, the Hoxa cluster has a primary role in skeletogenic CNCC fates.

Of note, in humans, *HOXA2* haploinsufficiency causes bilateral microtia (small external ear) associated with an abnormally shaped

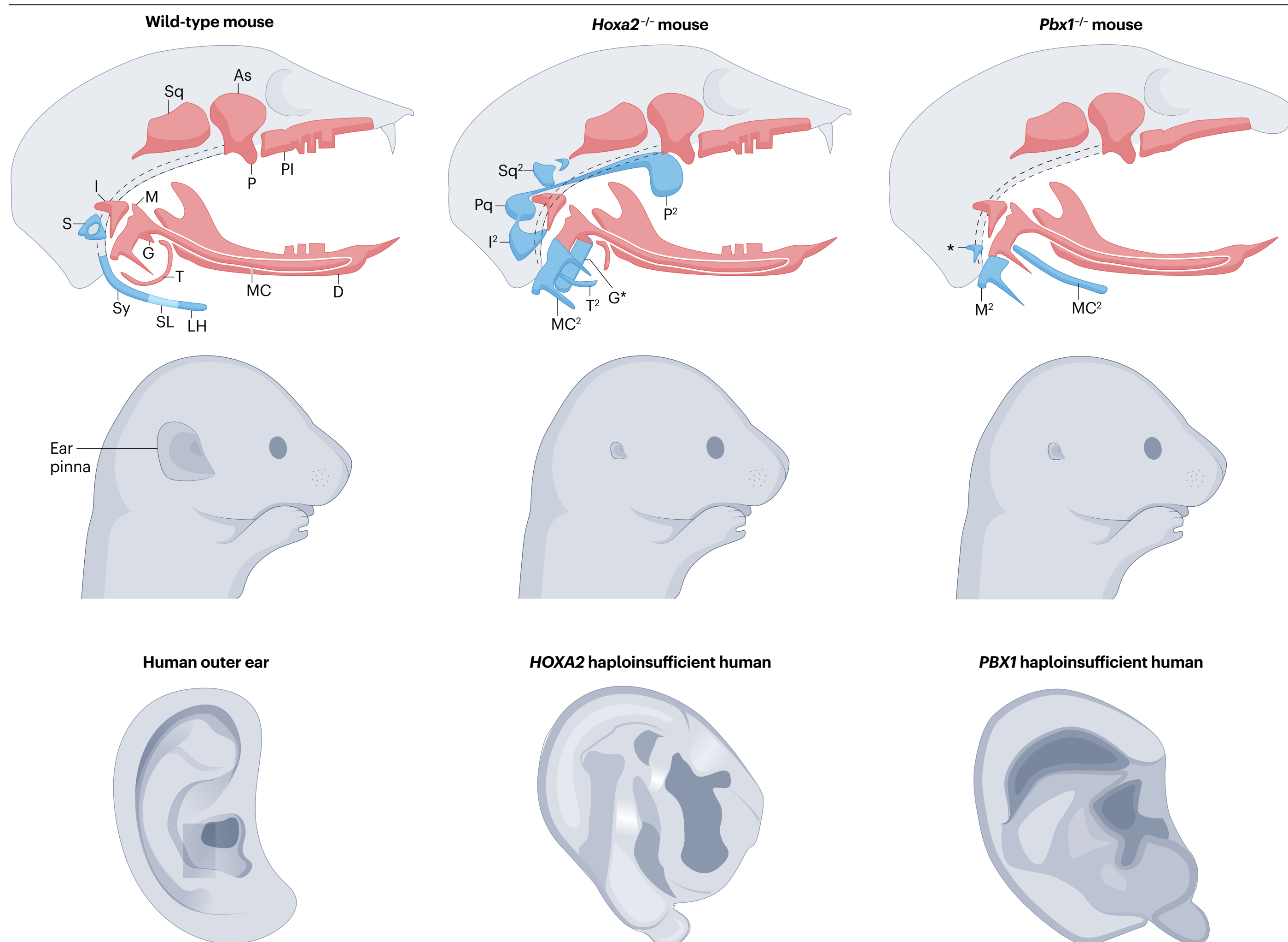

Fig. 2 | Genetic regulation of cranial neural crest cells and conservation of homeodomain gene function. The top row depicts crania from wild-type (left), *Hoxa2*-null (middle) and *Pbx1*-null (right) mouse embryos. Cranial neural crest cell (CNCC)-derived skeletal elements of branchial arch 1 (BA1) (in red) and of BA2 (in blue) are shown. *Hoxa2*-null and *Pbx1*-null mouse embryos exhibit homeotic transformations that affect CNCC-derived craniofacial structures. In *Hoxa2*-null embryos, BA2 CNCCs are homeotically transformed and generate a mirror image, duplicated set of BA1-like (proximal) lower jaw and middle ear structures[40]. In *Pbx1*-null embryos, the BA2-derived styloid process (Sy) and lesser horn of hyoid bone (LH) acquire BA1-like morphological features resembling a shortened Meckel's cartilage (MC)[55]. Although HOX and PBX proteins can work together as cofactors, they also have independent functions in BA2, as the morphological transformations in the two mouse mutants are not a phenocopy. The middle row depicts mice of the same genotypes as indicated in the top row. *Hoxa2*-null mice and *Pbx1*-null mice show loss of or highly hypoplastic ear pinnae, respectively. The bottom row depicts the normal human outer ear (left), compared with the outer ears of *HOXA2* haploinsufficient (middle) and *PBX1* haploinsufficient (right) individuals. *HOXA2* haploinsufficient and *PBX1* haploinsufficient individuals have outer ears that are hypoplastic and similarly dysmorphic, demonstrating conservation of gene function. As, alisphenoid; D, dentary; G, gonial; I, incus; M, malleus; P, pterygoid; Pl, palatine; Pq, palatoquadrate; S, stapes; SL, stylohyoid ligament; Sq, squamosal bone; T, tympanic ring. Partially duplicated structures are indicated by a superscript '2' (for example, Sq and Sq^2). Dysmorphic transformed structures are indicated by an asterisk (*). In *Hoxa2*-null mouse embryo, Pq represents the duplication of an atavistic structure[40].

and hypoplastic ear pinna (the visible part of the outer ear) and hearing loss[46]. In mice, temporally induced *Hoxa2* inactivation at early gestational stages results in external auditory canal (EAC) duplication and complete absence of the ear pinna, whereas *Hoxa2* loss later in gestation results in a microtic pinna, mimicking the human *HOXA2* haploinsufficient condition[47] (Fig. 2). Genetic fate mapping revealed that the mouse ear pinna derives from *Hoxa2*-expressing CNCC-derived mesenchyme of BA2 (ref. 48). Furthermore, conditional ectopic *Hoxa2* expression in HOX-free CNCCs was sufficient to induce duplication of the pinna and EAC loss, suggesting that BA1 CNCC-derived mesenchyme lining the EAC is transformed into an ectopic pinna. *Hoxa2* partly controls pinna morphogenesis through BMP signalling and expression of *Eya1*, the homologue of which is involved in branchio-oto-renal syndrome in humans[48]. Overall, *Hoxa2* loss-of-function and gain-of-function experiments in mice provide a unique model to investigate the molecular aetiology of human microtia and ear pinna duplication,

as well as underscoring conserved functions for Hox genes in other species, including humans.

Pbx genes

Vertebrate Pbx genes (*PBX1–PBX4*) encode homeodomain transcription factors of the TALE (three-amino acid loop extension) superclass[49,50]. With the exception of PBX4, which is detectable mainly in the testes, PBX proteins are present in most vertebrate embryonic tissues[50]. PBX transcription factors are essential developmental regulators in various embryonic contexts, being involved in GRNs that direct crucial patterning and morphogenetic processes in vertebrate organogenesis. PBX proteins were long thought to be simply cofactors for HOX proteins; however, these transcription factors can also function as upstream regulators of Hox genes[51], can interact with non-HOX proteins and work independently of HOX[35,50], and can behave as potential pioneer factors during early morphogenetic processes[52]. Of note regarding the proposed role of PBX proteins as pioneer factors, recent models suggest that this function could simply reflect the spatiotemporal affinity of interaction between PBX proteins and DNA[53,54].

PBX transcription factors act as cofactors for HOX proteins in distinct craniofacial domains. Indeed, certain craniofacial phenotypes of *Pbx1*-null embryos[55] resemble, at least in part, abnormalities subsequently described in mice with CNCC-specific deletion of the Hoxa gene cluster, as discussed above[44]. Specifically, in *Pbx1*-null embryos, the BA2-derived lesser horn of hyoid bone acquires BA1-like morphological features that resemble Meckel's cartilage (Fig. 2). Together, these observations confirm the long-held notion that PBX proteins function as cofactors for HOXA proteins encoded at the 3′ end of the Hoxa gene cluster to instruct the developmental programmes that shape BA2 and posterior branchial arches, where Pbx and 3′ Hoxa genes are co-expressed[55]. As further support for this model, HOXA2 DNA-binding profiles overlap with those of PBX1 in BA2 (ref. 56). However, PBX1 and 3′ HOXA proteins must also have independent functions in BA2, given that the morphological transformations and craniofacial defects observed in the two respective mouse mutants are not a phenocopy.

Pbx1-null embryos also have hypoplastic and dysmorphic ear pinnae[55], closely resembling the ear pinna phenotype of mouse embryos with *Hoxa2* loss induced at late gestational stages (Fig. 2). Furthermore, patients with de novo haploinsufficient sequence mutations in *PBX1* present with a pleiotropic syndrome that includes abnormal development of branchial arch derivatives, heart malformations, diaphragmatic hernia, renal hypoplasia and ambiguous genitalia, all of which are phenotypes that have been reported in *Pbx1*-null mouse embryos[49]. Patients with *PBX1* mutations also have ear pinna abnormalities of varying expressivity and severity[57], mimicking the ear defects reported in humans with *HOXA2* haploinsufficiency. These findings from both mice and humans support a conserved role for PBX proteins as HOX cofactors in BA2 CNCC-derived mesenchyme, wherein *Hoxa2* and *Pbx1* are co-expressed.

Interestingly, in mice with CNCC-specific deletion of *Pbx1* on a *Pbx2*-deficient background, secondary palate morphogenesis is affected, resulting in cleft palate only (CPO) in mutant embryos, which also have broadening of the midface[37]. By contrast, *Pbx1* conditional loss selectively in the surface cephalic epithelium, on a *Pbx2*-deficient or *Pbx3*-deficient background, causes cleft lip and cleft palate through perturbation of a GRN that controls both apoptosis and EMT at the lambdoidal junction where MNP, LNP and MxP fuse[35,36]. Note that *Pbx4* is not expressed in craniofacial tissues[49,50]. These findings underscore that, in addition to CNCCs, the cephalic epithelial layer has crucial roles in craniofacial morphogenesis.

Dlx genes

In addition to the HOX–PBX-dependent anterior–posterior positional information that characterizes the segmental identity of each branchial arch from that of its neighbours, CNCCs also require dorsoventral (or proximo-distal) positional information to establish intra-arch identity. A CNCC transcriptional code for intra-arch polarity is provided by the vertebrate Dlx genes. Mammals have six Dlx genes (*DLX1–DLX6*) with nested expression patterns[58]. Loss-of-function mutations in mice have provided insight into the roles of Dlx genes in craniofacial development. Based on the expression pattern of Dlx genes, the partitioning of BA1 is achieved in large part by two Dlx combinations: *Dlx1* and *Dlx2* for the MxP, and *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* for the MdP. Single or compound loss of *Dlx1–Dlx2* in mice affects the development of upper jaw elements and upper components of the hinge region[58]. Conversely, inactivation of *Dlx5* results in abnormalities of the proximal lower jaw and hinge region[59,60]. The compound loss of *Dlx5* and *Dlx6*, although it has no effect on *Dlx1* or *Dlx2* expression, generates a homeotic transformation of the lower jaw into a mirror image of upper jaw components[61,62].

The findings discussed above provide evidence that a Dlx combinatorial code is required in CNCCs to establish intra-arch polarity in BA1, similar to the Hox code for inter-arch patterning. However, it is unclear whether the Dlx ground patterning programme extends to more posterior branchial arches, as it does for Hox genes[44]. Although BA2 and BA3 skeletal elements are affected by the compound loss of *Dlx5* and *Dlx6*, the resulting malformations are not clear homeotic transformations[58,62]. One possibility is that the Hox-dependent inter-arch patterning programmes modify an underlying Dlx-dependent intra-arch ground pattern. In this respect, it would be interesting to evaluate the effect of *Dlx5* and *Dlx6* inactivation in the context of Hoxa gene cluster deletion in CNCCs, and to test whether serial transformations, even partial, of lower jaw to upper jaw, might occur in posterior branchial arches. Similar to the Hox code, there are interactions between paralogous groups of Dlx genes, pointing to both qualitative and quantitative features of the Dlx code. For example, when *Dlx1* or *Dlx2* is inactivated on a *Dlx5*-deficient or *Dlx6*-deficient background, MdP-derived structures are reduced in size and/or transformed into elements that resemble MxP derivatives, which mimics the *Dlx5* and *Dlx6* mutant phenotype[63].

Together, these findings relating to the roles of Hox, Pbx and Dlx genes in regulating CNCC positional identity and patterning prompt the question of how such intrinsic, cell-autonomous genetic programmes are affected by the local cues to which CNCCs are exposed during their life cycle, an important theme that is discussed below.

Regulation of CNCCs by GRNs

Multi-omics approaches have led to the unbiased identification of new factors involved in the development of CNCCs and their interconnected GRNs in the neural crest (Fig. 3), comprising regulatory enhancer elements that function as 'switches' to integrate inputs from upstream signalling and transcription factors and to drive the transcription of downstream targets. In the chick embryo, the enhancers *SOX10E1* and *SOX10E2* control expression of *SOX10*, a neural crest master regulator[64–66], and SOX9, ETS1 and MYB proteins are transcriptional regulators for endogenous *SOX10* expression (Fig. 3). Comparative transcriptomics of avian trunk NCC and CNCC populations, coupled with functional validation assays, identified a cranium-specific GRN in migratory CNCCs comprising *BRN3C–LHX5–DMBX1* in the neural plate border and *SOX8–TFAP2–ETS1* in premigratory CNCCs[67]. Overexpression of transcription factor-encoding genes that characterize premigratory CNCCs in trunk

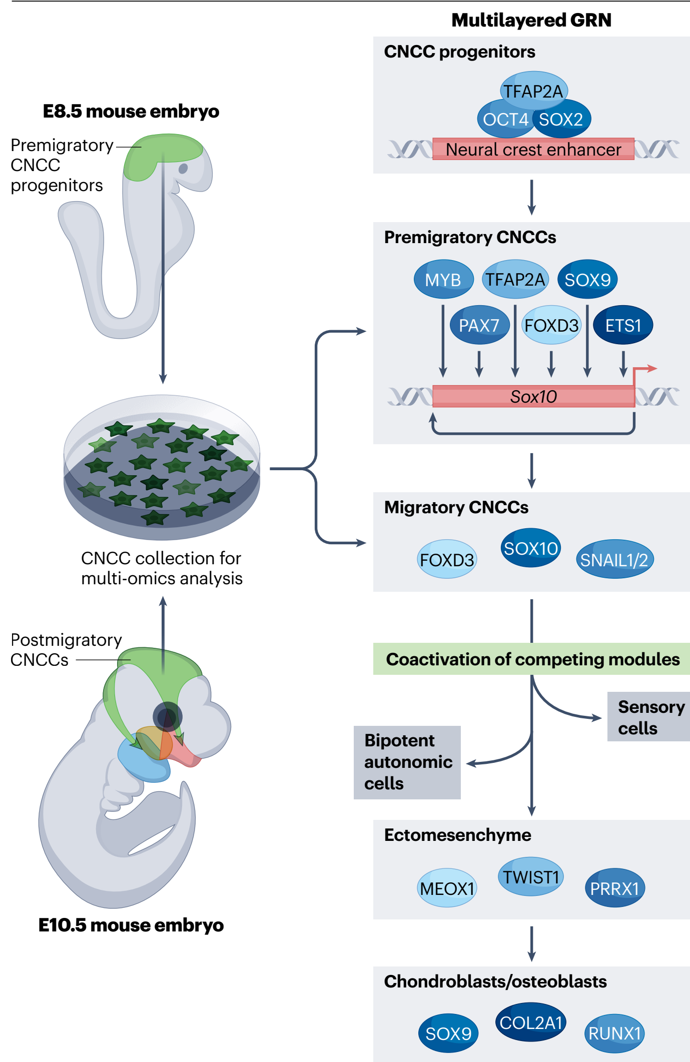

Fig. 3 | Identification of multilayered gene regulatory networks in cranial neural crest cells using multi-omics approaches. Premigratory cranial neural crest cell (CNCC) progenitors and postmigratory CNCCs (depicted in green) are collected from mouse embryos at embryonic day 8.5 (E8.5) and E10.5, respectively, for genome-wide explorations using multi-omics approaches to obtain system-level data sets. The integration of gene modules derived from these data sets enables the construction of a multilayered gene regulatory network (GRN) of CNCCs. A dimer of the pluripotency factors OCT4 and SOX2 in CNCC progenitors establishes a multipotent neural crest epigenomic signature that is subsequently lost upon neural crest fate commitment[84]. The OCT4–SOX2 targets in neural crest differ from those of embryonic stem cells, indicating context-specific functions of this dimer. Binding of OCT4–SOX2 to neural crest enhancers requires TFAP2A, which physically interacts with the dimer to modify its genomic targets[84]. Select transcription factors are constituents of gene modules representing CNCCs at different stages of their differentiation. In premigratory CNCCs, specific upstream transcription factors converge on the activation of *Sox10* transcription[8,10,24]. SOX10 integrates inputs from many neural crest specification genes and uses a positive-feedback loop to maintain neural crest identity[64–66]. Migratory CNCCs become sensory cells, bipotent autonomic cells or ectomesenchymal cells through sequential, binary lineage decisions, comprising the initial coactivation of competing transcriptional modules, before cells acquire fate-specific phenotypic traits, followed by gradual shifts towards commitment[87]. Lastly, the ectomesenchyme differentiates into head chondroblasts and osteoblasts.

NCCs led to reprogramming of their identity into that of CNCCs and, accordingly, resulted in upregulated expression of chondrocyte markers such as *RUNX2* and *ALX1* (ref. 67). The skeletogenic potential of avian trunk NCCs was previously shown also by their exposure to appropriate extrinsic signals in culture; moreover, when placed in the cranial environment, avian trunk NCCs contributed to skeletal structures[68]. Thus, NCCs from all axial levels might have, although to different extents, an intrinsic capability to generate the full repertoire of neural crest derivatives, including the ability to realize their skeletogenic potential when exposed to the appropriate environmental cues. Indeed, in other vertebrates, such as turtles, trunk NCCs contribute to skeletogenic structures[69]. Nonetheless, these case studies highlight the crucial roles of transcription factors and enhancers in controlling the spatiotemporal expression of developmentally regulated neural crest genes, and they have led to genome-wide profiling and identification of large sets of CNCC enhancers both in vitro and in embryos of various species (Box 1).

Epigenomic profiling of neural crest enhancers

Genome-wide epigenomic mapping of enrichment profiles for histone marks, and binding of the enhancer-associated transcriptional coactivator p300, led to the identification of more than 4,300 active enhancers in human embryonic stem cells (ESCs) differentiated in vitro into NCCs[70]. Binding motif and chromatin immunoprecipitation followed by high-throughput sequencing (ChIP–seq) analyses further revealed the occupancy of a major neural crest specifier, TFAP2A, at about 30% of all putative enhancers in human NCCs[70]. Moreover, studies in mouse ESCs highlighted the mechanisms by which FOXD3, another neural crest specifier, functions to decommission enhancers by recruiting chromatin remodelling factors[71,72]. In vivo studies suggested that FOXD3 has a dual function, as a pioneer factor to prime genes for NCC specification and as a repressor by decommissioning enhancers during migration and differentiation[73] (Fig. 3). In the chick embryo, two distant enhancers, *NC1* and *NC2*, regulate *FOXD3* expression, and the transcription factors PAX7, MSX1, ETS1 and ZIC1 are part of a *FOXD3*-driven GRN in trunk NCCs and CNCCs[74].

ChIP–seq analysis of p300 binding in mouse embryos identified more than 4,000 putative craniofacial enhancers, of which 200 were further characterized and shown to drive complex gene expression patterns during mouse craniofacial development[75]. Three of these enhancers were deleted in mice, which resulted in subtle but important variations of craniofacial morphology. Similarly, a series of rearrangements was engineered over the mouse genomic region syntenic to the human 640 kb non-coding DNA interval at chromosome 8q24, which is associated with increased susceptibility to non-syndromic cleft lip with or without cleft palate (CL/P) in humans. Epigenomic profiling of the syntenic mouse genomic region led to the identification of a long-range, *cis*-acting medionasal enhancer region containing several putative regulatory elements. Deletion of the medionasal enhancer interval resulted in slight, although penetrant, facial dismorphology and, sporadically, in CL/P, revealing that enhancers in this region collectively control *Myc* expression levels in the face of the mouse embryo[76]. Overall, these findings indicate that variations in distant enhancer sequences and/or accessibility may contribute to the diversity of mammalian facial morphology and abnormal craniofacial morphogenesis.

Regulation of CNCC multipotency

A long-standing debate in the field has centred around the multipotent nature of NCCs[77,78]. Recent reports have revived this topic and proposed distinct models of CNCC multipotency. One study[79] concluded that

Box 1

Non-model organisms for the study of craniofacial morphogenesis

Classical mammalian 'model' organisms that can be genetically altered, such as mice, have led to fundamental discoveries regarding gene function in craniofacial morphogenesis, which cannot be recapitulated in cell culture because of the complexity of the craniofacial development process. The recent availability of less expensive sequencing methods, such as next-generation sequencing, that enable genome annotations of a large number of species, combined with improved CRISPR–Cas genome editing technology[137,138], has made possible the use of so-called 'non-model' organisms[144] for studies of craniofacial development[145]. Investigations of cranial neural crest cells (CNCCs) and their derivatives can now be carried out using developmental biology techniques and multi-omics approaches in multiple 'non-model' organisms, including *Ciona intestinalis*, lamprey, sharks, corn snakes, geckos, Darwin's finches and pigs[146–152]. This has led to new comparisons of craniofacial trait evolution across species[141]. For example, the resemblance of lamprey CNCCs to amniote trunk neural crest cells (NCCs) suggests that complex gene regulatory networks (GRNs) are progressively acquired in the neural crest during vertebrate evolution, with trunk-like circuits being established first[153]. Moreover, by examining CNCC transcriptional profiles, additional GRNs that are unique to the lamprey were identified[146]. Other lamprey regulatory elements, such as the *SoxE1* enhancer, are also active in CNCC-derived craniofacial structures of jawed vertebrates, underscoring the conservation of transcription factor–enhancer interactions in the GRNs of the neural crest during vertebrate evolution[146]. Similarly, dissection of neural crest GRNs in the proto-vertebrate *C. intestinalis* led to the identification of an ancestral regulatory module comprising Six, Msx and Pax genes that is shared between cranial placodes and neural crest in vertebrates[147].

Although they enable novel discoveries in craniofacial development of different species, these non-model systems do have limitations. A major drawback is that not all of the findings obtained in animals can be confirmed in humans. For example, modelling of human haploinsufficient disorders is not feasible in mice, which show tolerance to haploinsufficiency of transcription factor-encoding genes[154]. To overcome this and other limitations of model and non-model organisms to study craniofacial abnormalities, methods for the directed differentiation of embryonic stem cells and induced pluripotent stem cells[142] make it possible to obtain large numbers of cells of the desired type, such as CNCCs, from human controls and from individuals with neurocristopathies[125,131] and other craniofacial birth defects.

in mice the broad developmental potential of CNCCs is achieved in precursor cells by the transient re-acquisition of molecular markers of ESC pluripotency. Single-cell RNA sequencing (RNA-seq) analysis identified a neuroepithelial precursor subpopulation characterized by transient expression of genes that encode canonical pluripotency transcription factors, including *Oct4*, *Nanog*, *Klf4* and *Sox2*. Lineage analysis of *Oct4*-expressing cells showed that they form facial structures. In addition, loss of *Oct4* resulted in loss of craniofacial elements in mice, underscoring that transient expression of this factor is required for the formation of head ectomesenchyme. Moreover, open chromatin landscapes of *Oct4*-expressing CNCC precursors as assessed by assay for transposase-accessible chromatin using sequencing (ATAC-seq) resembled those of epiblast stem cells[79].

A few caveats related to this model are worth mentioning here. First, the transient reactivation of pluripotency factors seems to be restricted to the formation of CNCCs[79], leaving open the question of how trunk NCCs acquire their multipotency. Second, a large number of data from heterotopic neural fold grafting experiments indicate that rostro-caudal populations of Hox-negative premigratory CNCCs behave as a 'group of equivalence' with similar broad developmental potential[42]. By contrast, in the study described above[79], transient reactivation of the pluripotency programme seems to follow an anterior–posterior progression in the cranial region, which could make it difficult to reconcile both observations. However, regional identity and pluripotency markers were shown to be erased during CNCC delamination[79], resulting in a transcriptional 'group of equivalence' of delaminating CNCCs. Third, the above-discussed study[79] did not include long-term lineage tracing experiments in vivo, which would unequivocally test the true potency of CNCCs that transiently re-express the pluripotency programme and their contribution to the full extent of CNCC-derived lineages. It is possible that select core pluripotency factors could be repurposed during CNCC specification, conferring the potential to generate only a limited set of cell lineages (see below). In addition, select core pluripotency factors, such as OCT4 and NANOG, could be necessary for the proper deployment of subsequent developmental programmes, as recently shown for the onset of Hox gene cluster activation[80].

The model for CNCC multipotency based on the transient re-acquisition of pluripotency factors in mice[79] is in contrast to findings reported in *Xenopus*, which suggest that the pluripotency programme is retained in NCCs from very early stages throughout NCC lineage progression[81]. This model proposes that a subset of cells at the neural border remain pluripotent. Accordingly, the retention of pluripotency long after other cell types have become fate-restricted would endow the neural crest with its unique capacity to contribute a wide range of cell types to vertebrate embryos. However, single-cell RNA-seq analysis in *Xenopus* could not provide evidence that NCCs retain pluripotency from the blastula stage[82]. Moreover, if early expression of *ventx2* (the *Xenopus* orthologue of *Nanog*) is driven experimentally to persist in *Xenopus* from the late blastula stage to the neurula stage, the expression of blastula-specific pluripotency genes is retained but neural crest is not formed[83]. By contrast, when *ventx2* is activated in *Xenopus* after neural border formation has been initiated, *ventx2* is required to stimulate multipotent neural crest formation[83].

The above studies[79,81] support the view that neural crest multipotency is related to the stem cell pluripotency network of the embryo. However, a study in avian embryos[84] offers an alternative model, indicating that OCT4–SOX2 pluripotency factors carry out

functions in the multipotent neural crest that are distinct from those they carry out in ESCs. This model suggests that OCT4–SOX2 pluripotency factors are co-opted from the ESC circuit and repurposed during neural crest development to generate distinct chromatin landscapes. This is achieved by interaction with the neural crest-specific TFAP2A pioneer transcription factor, which physically interacts with the OCT4–SOX2 dimer and induces epigenomic remodelling at specific *cis*-regulatory regions, distinct from those in ESCs, that contribute to the induction of a neural crest GRN (Fig. 3). Lastly, a recent preprint using single-cell multiplex spatial transcriptomics with RNA-seq reports the maintenance of stem cells expressing NANOG, OCT4, POUV and KLF4 that span the entire ectoderm during neurulation in both chick and mouse embryos[85]. By the end of neurulation, high levels of ectodermal stemness, as evaluated by co-expression of the core pluripotency genes, are restricted to the dorsal neural tube at all axial levels, conferring enhanced multipotency to the forming NCCs[85].

Overall, the above findings underscore that regulation of CNCC multipotency is still highly controversial and that our understanding will benefit from additional studies in different vertebrate systems to reach a unifying model that survives the test of time.

Fate restriction and lineage progression

A rapid and abrupt transition resulting from the activation of a specific GRN that drives EMT has classically been viewed as a fundamental step required for NCC delamination, migration and subsequent differentiation[86]. However, recent findings[87] do not support this model. Indeed, a sequence of transcriptional states has been reported surrounding NCC delamination in mice, establishing that pre-EMT NCCs express genes that are closely related to neural plate border and neural tube identity. Later, more differentiated NCCs downregulate the expression of neural tube genes and upregulate NCC-specific genes. These results suggest that the transition from premigratory to migratory NCCs is more gradual and complex than initially thought.

An important conclusion from the above-discussed study[87] relates to the importance of the sequential steps involved in NCC differentiation. Indeed, this research establishes that progenitor cells undergo binary choices between possible fates owing to prior cell-autonomous and non-cell-autonomous events. At the single-cell transcriptional level, progenitor cells first coactivate gene expression programmes, normally thought to be mutually exclusive, that instruct competing cellular fates. Following a decision point (bifurcation), they upregulate one programme and downregulate the other to transition stably towards a specific fate. Moreover, several transcription factors that are thought to be ‘master regulators’ for select cell lineages are not even expressed at the time of the specific bifurcations[87]. These findings suggest that the activation of specific gene expression programmes at the bifurcation point is elicited by environmental cues, including chemical and/or mechanical factors[88,89]. The first bifurcation identified in CNCC differentiation separates sensory progenitors from progenitors of the autonomic and mesenchymal lineages, which is followed by a bifurcation that separates autonomic neuronal fates from ectomesenchymal differentiation[87] (Fig. 3).

This model challenges the long-held view of early fate restriction of delaminating NCC progenitor cells that irreversibly activate one of multiple alternative cell fate programmes. The revised view of in vivo NCC differentiation in mammals is similar to differentiation models proposed in the past for other cell types, such as haematopoietic cells[90], and is in conflict with the paradigm that a single precursor NCC can directly differentiate into many different cell types. A recent model[91] potentially reconciles early fate restriction with late retention of multipotency. Accordingly, NCCs repeatedly transit, in a cyclical manner, through a series of transcriptionally biased states. By doing so, NCCs are temporarily biased to adopt a distinct fate, possibly through competing transcriptional programmes, while retaining multipotency for an extended period. A possible epigenetic mechanism underlying this process might be provided by dynamic chromatin priming and/or poising, which might maintain cryptic multipotency and transcriptional plasticity of CNCCs in vivo during an extended time window of competence (discussed below).

This model of cyclical fate restriction and potency retention is consistent with many lineage tracing, transplantation and genetic studies. However, a key question concerns the mechanisms that ultimately allow cells to exit the cycle at a given state, prevent them from re-entering the cycle and thus enable commitment to a specific fate. To become committed, mitotic cells may need to integrate qualitative and quantitative responses to fate-specifying environmental cues above a certain threshold through a sufficient period of time. These events are likely required to induce irreversible transcriptional changes and/or resolve competition between transcription factor activities.

Epigenetic regulation of CNCCs

Central to head morphogenesis is the question of how CNCC subpopulations acquire their morphogenetic positional identity, in other words, how they are instructed to generate the differently patterned craniofacial elements in the right place. Craniofacial abnormalities that involve positional swapping of facial structures are rare, which suggests that positional identity is robustly assigned and is an integral part of CNCC fate specification. How and when skeletogenic CNCCs acquire their positional identity during craniofacial development has been debated for the past four or five decades[3,5,92].

Prepatterning versus plasticity

The debate about CNCC prepatterning versus plasticity has been long-standing, in terms of how much intrinsic patterning information is carried by CNCC subpopulations as opposed to local, position-specific, patterning information that is received after migration into the developing facial processes. Experiments in the chick embryo[93] about 40 years ago suggested that CNCC premigratory progenitors are prespecified with respect to their morphogenetic potential before leaving the neural tube. However, a later study[94] revisited those findings and supported instead the morphogenetic plasticity of those cells. Moreover, graft experiments in the chick embryo[42] showed that the HOX-free premigratory CNCC progenitors that contribute to the FNP, MxP and MdP are equally capable of replacing each other and of regenerating an entire facial skeleton. These findings indicated that premigratory CNCC progenitors have similar intrinsic, broad developmental potential and patterning competence and that their positional identity is not irreversibly established.

Postmigratory CNCCs can generate fully patterned craniofacial elements when exposed to cues from grafted ectodermal or endodermal tissue. For example, if a stripe of ectoderm that overlies the chick embryonic FNP, known as the frontonasal ectodermal zone, is ectopically grafted, this is sufficient to induce an ectopic upper beak[95]. Moreover, endothelin 1 (EDN1), which is present in the epithelium and mesodermal core of the MdP, is a primary signal that establishes the positional identity of CNCCs within the MdP. The EDN1–endothelin receptor type A signalling pathway is crucial for induction of *Dlx5*, *Dlx6* and *Hand2* expression in postmigratory CNCCs in the MdP.

In *Edn1*-null mice, the lower jaw is morphologically transformed into an upper jaw-like structure[62,96,97]. Conversely, EDN1 signalling is sufficient for the transformation of the upper jaw to a lower jaw-like identity[98]. Furthermore, removal of specific portions of foregut endoderm in chick embryos prevents facial bone development in the adjacent CNCC mesenchyme[42]. Importantly, distinct ectopically grafted portions of chick endoderm not only induce supernumerary jaw elements but also can influence their position and orientation[42]. In summary, CNCC positional identity is instructed by the surrounding environment, but some species-specific facial morphological features can be intrinsically informed by CNCC progenitors[99]. Indeed, when quail and duck CNCC progenitors of the presumptive beak region were exchanged, the chimeric embryos appeared as ducks with a quail beak (duails) and quails with a duck beak (quacks), respectively[99]. However, although the beak phenotype resembled that of the donor species owing to the donor-specific intrinsic developmental programme, the beak transformation was not complete, supporting the importance of extrinsic cues from the host species environment.

Together, the above experiments underscore a remarkable plasticity of positional identity and morphogenetic potential of CNCC subpopulations that contribute to most of the craniofacial structures. Notably, positional plasticity is retained throughout migration, while cells are being transcriptionally biased to specific lineages, as discussed above.

Poised chromatin domains maintain plasticity

CNCCs maintain plasticity of positional identity and developmental potential throughout migration, until they respond to local cues. Thus, each individual CNCC subpopulation, before reaching its final destination, should be competent and poised to induce potentially all of the postmigratory MdP, MxP and FNP morphogenetic programmes, so that it can activate the transcriptional programme appropriate for its final position when instructed by the local environment.

How can such morphogenetic plasticity be achieved at the molecular level? About 15 years ago, a bivalent, Polycomb-dependent, repressive chromatin signature associated with gene transcriptional poising was discovered in ESCs[100]. The promoters of key lineage specifying and developmental genes, not expressed in ESCs, are maintained in a poised transcriptional state by being accessible and bivalently marked with both the Polycomb-dependent repressive modification of histone H3 lysine 27 trimethylation (H3K27me3) and the Trithorax-dependent activating modification of H3K4me2/3. Recently, a similar bivalent poised chromatin organization was discovered in vivo by which distinct CNCC subpopulations maintain the required plasticity[101]. Differentially silenced CNCC genes – genes that are not expressed in some postmigratory CNCC subpopulations but are expressed in others at different positions – have accessible putative distal enhancer elements and promoters marked by H3K27me3 and H3K4me2 (ref. 101) (Fig. 4). Interestingly, transcripts for chondrogenic markers, including *Col2a1*

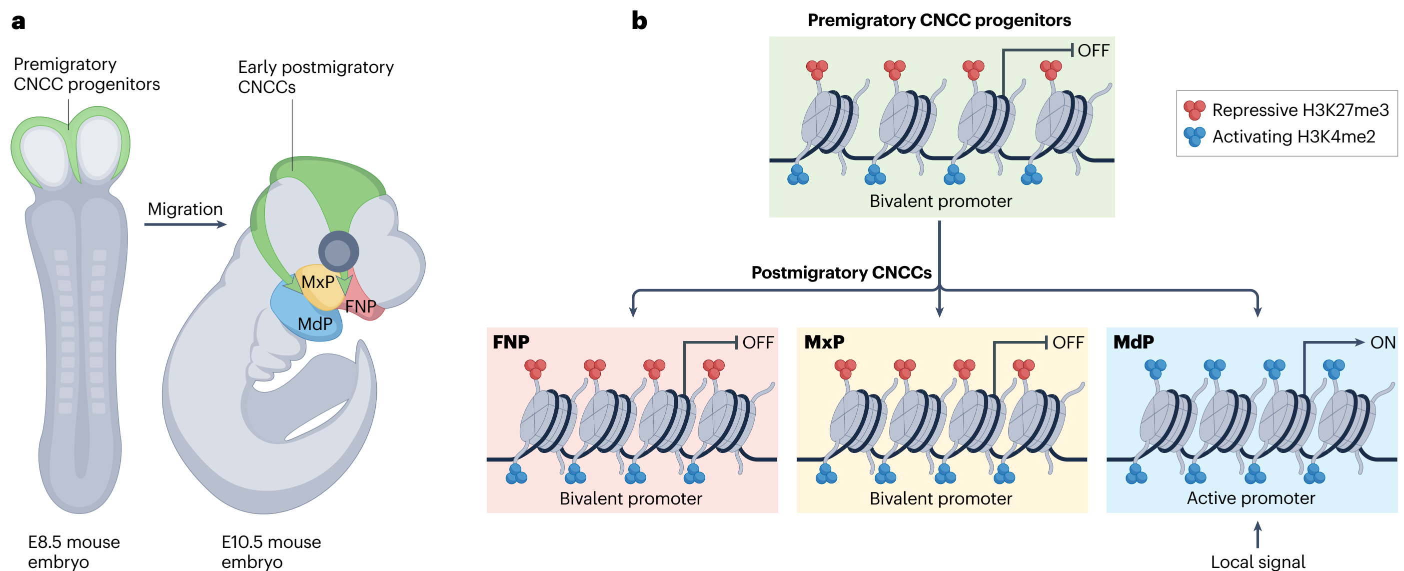

Fig. 4 | Epigenetic regulation of the positional plasticity and identity of cranial neural crest cells. a, In the mouse embryo at embryonic day 8.5 (E8.5), HOX-free premigratory cranial neural crest cell (CNCC) progenitors (green) behave as a 'group of equivalence', being endowed with equivalent patterning potential[42]. In the E10.5 mouse embryo, frontonasal prominence (FNP), maxillary prominence (MxP) and mandibular prominence (MdP) positional identities of CNCCs are established at migratory or postmigratory stages by extrinsic environmental cues[42,94–98]. **b**, A chromatin pattern of accessible bivalent, poised promoters – Polycomb-dependent repressive histone H3 lysine 27 trimethylation (H3K27me3) and Trithorax-dependent activating H3K4me2 – is present in premigratory CNCC progenitors at genes that, after CNCC migration into distinct facial prominences, become differentially expressed and are required to establish subpopulation-specific morphogenetic identity. In FNP, MxP and MdP, postmigratory CNCC subpopulations interact with specific environmental cues (such as the local signal depicted in MdP). As a result, accessible bivalent promoters can switch from a poised repressed (OFF) transcriptional state to an active (ON) transcriptional state, establishing transcriptional identities specific to a distinct prominence (as shown for MdP). In those prominences where the signal is not available, the same gene (or genes) remains in an OFF state (as shown for FNP and MxP). Therefore, an accessible, transcriptionally poised, repressive chromatin pattern of gene regulation maintains the broad developmental potential and positional plasticity of CNCC (pre)migratory progenitors[101]. The chromatin pattern of CNCCs can be differentially modulated and transcriptionally resolved by specific signals in distinct facial prominences, triggering the expression of key genes to form the right facial structures in the right place.

Box 2

Divergent regulation of cranial neural crest cells during evolution

The craniofacial complex (comprising head, face and oral cavity) and teeth provide an archive of changes that can be tracked over time, illustrating the evolution of species and the emergence of human-specific facial traits. Recently, the ability to compare the human genome with that of our closest relative, the chimpanzee, has provided new avenues to link genetic, regulatory and epigenetic changes to phenotypic variation in the evolution of the human brain and craniofacial complex[141,155]. Comprehensive comparative epigenomic profiling of in vitro-derived cranial neural crest cells (CNCCs) has been used to explore how the distinct facial features of humans and chimpanzees, whose genomes differ by less than 2%, might be driven by *cis*-regulatory changes[141]. Human and chimpanzee induced pluripotent stem cells (iPSCs) were differentiated into CNCC lines to generate genome-wide maps of transcription factor binding, p300 coactivator binding, chromatin accessibility and histone modifications. This approach led to the identification of ~14,500 predicted *cis*-regulatory elements across the genomes of chimpanzees and humans, with ~13% of the predicted regulatory elements exhibiting functional divergence between the two species and species-specific bias of enrichment for histone H3 lysine 27 acetylation. Testing putative species-biased CNCC enhancers by transient transgenesis in mouse embryos showed their differential activity in embryonic head and face domains[141]. Genes located near species-biased enhancers, many of which are enriched for craniofacial functions, exhibited differential expression. This study suggested that species-biased divergence of gene expression between humans and chimpanzees results from differences in enhancer sequences and the *cis*-regulation of key CNCC factors, instead of from differences in *trans*-regulatory environments of CNCCs between the two species. These findings are consistent with an older model positing that the evolution of morphological diversity is steered by *cis*-regulatory mutations that affect expression patterns of developmental genes[156]. Using tetraploid hybrid cells generated by fusion of human and chimpanzee iPSCs, which enables the separation of *cis*-regulatory effects from *trans*-regulatory effects, another study addressed whether *cis*-regulation or *trans*-regulation has a primary role in determining human-specific traits[157]. The study showed that the Hedgehog (HH) signalling pathway mediates, in part, the divergence in craniofacial features between humans and chimpanzees, with the regulatory divergence of HH signalling resulting in lower activity of *EVC2*, which encodes a protein in primary cilia, in humans compared with chimpanzees. In humans, homozygous loss-of-function mutations in *EVC2* cause Ellis–van Creveld syndrome, a ciliopathy characterized by skeletal defects, including high forehead and retracted midface[158]. The HH pathway represents a recurrent target of selection, given its known dosage-dependent effects that enable fine-tuning of organismal morphology[25,159–162]; future research based on interspecific hybrids may uncover novel signalling pathways divergently regulated between humans and chimpanzees that underpin facial morphological variation.

and *Sox9*, were detected in all CNCC subpopulations, indicating that the isolated postmigratory CNCCs are already engaged in an ectomesenchymal chondro-skeletogenic programme while still keeping a plastic positional identity[101].

These poised chromatin configurations at promoters and enhancers were inherited from premigratory CNCC progenitors. Thus, genes associated with positional identity are maintained in a transcriptionally repressed but poised state throughout CNCC migration, irrespective of the final position of the CNCC subpopulation. The bivalent chromatin state is regulated by *Ezh2*, which encodes a subunit of the Polycomb repressive complex 2 that adds methyl groups to H3K27 (ref. 101). Conditional targeted deletion of *Ezh2* in mouse CNCC premigratory progenitors resulted in faceless newborn mice[101,102], and mutations in *EZH2* in humans cause Weaver syndrome, which presents with facial dismorphology[103]. Epigenetic chromatin poising may allow CNCCs to adapt rapidly to local variations in environmental signalling, thus potentially contributing, in addition to other (epi)genetic mechanisms, to subtle changes in facial shape and morphology between individuals, or even to larger morphological differences between species.

CNCC-dependent morphological variation

Strong genetic control of facial features is evident in identical twins and in the similarities of facial appearance between family members. In addition, individuals with the same genetic syndrome, such as Down syndrome, have shared dysmorphic facial characteristics. Recent studies have focused on documenting genetic and epigenetic factors that drive facial trait variation in extant and extinct human and non-human primate populations throughout evolution (Box 2) and animal domestication (Box 3). Nevertheless, our knowledge of the genetic architecture and mechanisms that underlie normal-range human facial variation, such as face length, distance between the eyes and nose shape, is still rudimentary. Although craniofacial variation is continuous, and normal and syndromic populations overlap to varying degrees, abnormal variation specifically relates to craniofacial birth defects. Advances in imaging and morphometrics, when coupled with modern approaches based on human genetics, statistics and high-throughput analyses, including genome-wide association studies (GWAS), have led to mapping of genetic variants that influence human facial shape[104–115] (Table 1).

GWAS variants underlying craniofacial morphology

Two GWAS from 2012 reported a small number of genes associated with facial variants, one of which, *PAX3*, was reproduced in both studies[105,106]. Three additional studies published in 2016 confirmed roles for *PAX3*, *CACNA2D3*, *C5orf50* and *PRDM16*, and reported new associations of facial variants with *SCHIP1*, *PDE8A*, *PAX1*, *RUNX2* and *EDAR*[107–109]. However, many of the associations described in the 2012 GWAS were not replicated in the 2016 studies. There was also limited overlap of signals between the 2016 studies, which may be explained by differences in study design and in population background. Follow-up research applied an innovative facial phenotyping method to leverage the wealth of

information contained in 3D facial surface images[110]. Focusing on a sample of 2,329 individuals of European ancestries, the authors reported 38 loci involved in face shape variation, 15 of which were replicated in an independent sample of 1,719 individuals of European ancestries. Importantly, at least 11 of these loci had been previously reported in GWAS of qualitative facial features in a large study of 23andMe volunteers[111]. The authors subsequently showed enrichment in CNCCs of the histone H3 lysine 27 acetylation (H3K27ac) active enhancer mark[116,117] in the vicinity of the highest 15 peak single-nucleotide polymorphisms (SNPs), as compared with more than 30 other cell types.

In recent publications, the same global-to-local facial segmentation approach was applied to larger cohorts. In a GWAS meta-analysis of 8,246 individuals of European ancestries, collaborative efforts identified more than 200 independent signals with genome-wide significance that have an impact on normal-range facial variation in humans[112]. Notably, 89 signals of genome-wide significance overlapped with the results of prior analyses by GWAS of normal-range facial phenotypes. A total of 61 significant peaks were located at loci harbouring craniofacial genes that had been implicated in human malformations or craniofacial defects in animal models (such as WNT and TGFβ signalling pathways) but that had not yet been identified in GWAS for normal-range facial morphology. In addition, 53 genome-wide significant peaks were found at loci harbouring genes with unknown function in facial development or disease. The facial segments comprising the nose and upper lip emerged as ‘hot spots’ for genomic signals. Follow-up analyses indicated that the regions surrounding these signals were enriched for enhancer activity in CNCCs and developing craniofacial tissues[112]. Not surprisingly, the genes located within 500 kb of the genome-wide significant lead SNPs were enriched for processes and phenotypes associated with craniofacial development and morphogenesis in humans and mice. The top human phenotype that emerged was orofacial clefting (OFC), suggesting that there is overlap between the genes involved in normal facial variation and those responsible for the most common human craniofacial birth defect[118]. Additional research using this approach was then carried out on a trans-ethnic GWAS of human cohorts from different continents[113,114]. A 2021 GWAS reported ‘facial endophenotypes’ — distinctive facial features displayed by unaffected relatives of individuals with non-syndromic OFC[115] — which suggests that these traits may reflect underlying genetic susceptibility to OFC in larger unselected populations. Of the 29 loci emerging from this analysis that were significantly associated with at least one of the endophenotypic facial traits that were tested, 22 were in the proximity of loci previously associated with normal-range facial variation and 18 were near genes that showed significant association with OFC. Overall, this study supports the presence of a shared genetic architecture of normal facial development and susceptibility to OFC.

GWAS variants with shared roles in face and brain shape

A recent study reported that 70 of the 400 genomic loci identified to influence human brain shape are also significantly linked to face shape. For example, association signals linked to features of the frontal lobe were most shared with those associated with forehead shape variation[119]. Shared loci included genes encoding transcription factors involved in neural crest formation and craniofacial development, such as DLX5, DLX6, SOX9, ZEB2, ZIC2, ZIC3 and TCF4. Also among the shared loci were genes that encode transcription factors thought to function primarily during neural crest development rather than brain development (for example, ALX1, ALX4, TWIST1, PAX3 and TFAP2B), mutations in which result in craniofacial defects[119]. This GWAS confirms the proposed interactions between brain and face architecture and the genes involved in pathways that mediate signalling from the developing brain to the face. Multiple studies have suggested that face and brain morphology are related, and human syndromic disorders comprising both neurological and craniofacial abnormalities support this idea[120]. A phrase describing these close correlations (‘the face predicts the brain’) was coined nearly six decades ago in patients with holoprosencephaly[121].

Box 3

The neural crest hypothesis for animal domestication

Domestication of wild animals is a major aspect of human civilization, and the scientific study of animal domestication has a long history, beginning with Charles Darwin. Darwin observed that domesticated animals evolve distinct morphological, physiological and behavioural traits. Directly related to the evolution of craniofacial traits and the roles of cranial neural crest cells (CNCCs) in this process, the ‘neural crest hypothesis’, which has long been proposed by evolutionary biologists, posits that selection for animal domestication resulted mainly from alterations to CNCCs during embryonic development[163,164]. These changes caused the appearance of most craniofacial features associated with domestication, comprising smaller cranial capacity, floppy ears, smaller teeth, loss of pigmentation, altered facial shape and a shorter muzzle. However, it is currently debated whether or not the differences in craniofacial elements between wild and domesticated species are generated by genetic alterations that affect CNCCs, specifically those that modify the process by which CNCCs give rise to their derived tissues, under the selection of domestication[165,166]. Interestingly, a recent study reported changes in DNA methylation in farm-bred sea bass compared with those present in wild sea bass, including marked epigenetic changes in multiple neural crest cell regulators[167]. Indeed, epigenetic regulatory mechanisms can respond to environmental changes, they can be maintained throughout the lifetime of an organism and they might also be transmitted across generations. Given the strong analogies between human evolution and domestication — indeed, *Homo sapiens* could be seen as a ‘self-domesticated’ animal[168,169] — we may learn more about the former by understanding the changes in genetic and epigenetic regulation of CNCCs during the latter. It is anticipated that the future identification of new variants in genes associated with craniofacial traits, the establishment of embryonic CNCC chromatin landscapes and further advances in CNCC transcriptomes at single-cell resolution will precisely define which transcription factors, enhancers and gene regulatory networks differ between the CNCCs of wild and domestic animals.

The highly collaborative GWAS projects described here have led to major advances in our understanding of normal variation of human facial morphology and its involvement in genetic susceptibility to craniofacial birth defects, as well as its connections to the shaping of the brain. However, despite how informative these studies have been, the epigenetic status of adults may not fully reflect the developmental regulation that occurs in the fetus. Accordingly, whereas most GWAS have analysed cohorts of adults, studying cohorts of newborns will be essential for further research. Indeed, identification of loci that contribute to facial shape variation may be more relevant in faces of newborns than in faces that are fully developed or undergoing age-related changes. Increasing knowledge of the genes involved in variation of facial morphology in healthy individuals will provide invaluable insights into the mechanisms that underlie abnormal facial morphogenesis.

Non-coding variants and birth defects

In the past few years, the importance of non-coding regulatory elements in the establishment of CNCC gene expression programmes during craniofacial morphogenesis and their involvement in human birth defects have been topics of intense research[122]. Recent studies have shown that disruption of gene enhancers can result in human disease affecting various organ systems through different mechanisms: structural variants can cause changes in 3D chromatin organization, and mutations can directly alter the enhancer sequences. Here, we focus on enhancer disruption in the context of neurocristopathies, which are caused by abnormal CNCC development and present with abnormal morphological variation of craniofacial features.

Neurocristopathy caused by structural variants

The branchio-ocular-facial syndrome (BOFS)[123] is a rare birth defect underpinned by heterozygous mutations in *TFAP2A*, which encodes a key regulator of CNCCs[124]. BOFS is characterized by ocular, ear and facial abnormalities, as well as defects in branchial arch-derived craniofacial structures. A comprehensive study described a patient with BOFS without mutation in the *TFAP2A* gene but carrying a balanced, 89 Mb, de novo heterozygous inversion in chromosome 6 (ref. 125). The 6p24.3 inversion breakpoint was located approximately 40 kb downstream of *TFAP2A*, within a topologically associating domain (TAD) that contains enhancers essential for *TFAP2A* expression in CNCCs, which suggests that the inversion may have a long-range regulatory effect on *TFAP2A* expression. Human induced pluripotent stem cells (iPSCs) derived from fibroblasts of control individuals and patients with BOFS were differentiated into CNCCs to investigate the enhancer profile within the *TFAP2A* TAD. In the inverted allele, the CNCC enhancers no longer contacted the *TFAP2A* promoter and, as a result, *TFAP2A* expression was decreased. *TFAP2A* total RNA levels were reduced by 50%, recapitulating haploinsufficiency in patients with BOFS harbouring heterozygous mutations in *TFAP2A*. Exploring the consequences of reduced levels of *TFAP2A* in CNCCs, the researchers identified multiple enhancers with decreased TFAP2A binding and reduced H3K27ac levels and linked these enhancers to genes that are downregulated in CNCCs with the inverted allele. Among these genes were those that encode the neural crest regulators ZIC1 and PAX3, mediators of EMT such as SNAI2 and TWIST1, and regulators of cell migration including SEMA3C[125]. The potential reversion of pathological structural variants to wild-type conditions in iPSCs, followed by differentiation into relevant cell types, holds great promise for cell therapy in the near future.

Table 1 | Genes associated with facial shape identified by GWAS

Cellular process or pathway	Genes	Associated facial traits	Refs.
Transcriptional regulation	*ALX1, ALX3, ALX4, BCL11B, DLX5/6, FOXL2, GLI3, GSC, HOXD, MEIS2, MSX1, MSX2, NFIA, PAX1/3, PRDM16, PRRX1, RUNX2, SATB2, SIX1/3/4, SOX9, TBX15, TBX22, TFAP2B, TP63, TRPS1, TWIST1, ZEB2, ZIC2/3*	Microphthalmia, intercanthal width, nose prominence, nose width, distance between eyes, forehead protrusion, eye–nasion distance, eye size, position of ears, size of teeth	104–107,109,110,112,119
Signalling	*BMP2/4, BMPER, DACT1, FGF2/13/18, FGFR2, PTCH1, ROR2, RSPO2, TGFB, WNT*	Shape and size of eyes, distance between eyes, midface size, size of upper jaw and lower jaw	104,112,114,119
Chromatin modification and remodelling	*ARID1B, HDAC8, KAT6B, KDM1A, STAG2*	Face width, upper lip size, position of ears, inter-eye width, position of hairline, protrusion of forehead and cheeks	104,107,110
Ion channel transport	*KCNJ2*	Size of lower jaw, distance between eyes, position of ears	104
Intracellular trafficking	*VPS13B*	Midface morphology, size of columella	104
Extracellular matrix, cell adhesion and proteolysis	*CDH18, DCHS2, FREM1, PKDCC, TASP1*	Height of central upper lip, mental fold, protrusion of cheeks	104,109,110,113
Intracellular structure	*COLEC10, COL17A1, SCHIP1, TNNI2*	Shape of eyebrows, size of lower jaw, size of nasolabial folds	105,108,110
Cell cycle regulation	*CDKN1C, CACNA2D3*	Forehead protrusion, size of lower jaw, position of ears	104,106,107,110
Ribosomal protein-mediated translation	*RPS12, RPS26*	Head size, distance between eyes, size of lower jaw	104,110
Post-translational modification	*DPH1*	Head size, size of forehead, position of ears	104
Metabolic pathways	*CYP26B1*	Facial symmetry, size and position of ears, size of lips, protrusion of chin	104

Examples of select genes reported by genome-wide association studies (GWAS) that are linked to variation of facial traits and have crucial roles in fundamental cellular processes and pathways. When multiple genes for a given cellular process or pathway are listed, the associated facial traits collectively refer to all genes within that category, with each individual gene being associated with only one or some of the traits.

Glossary

Branchial arches
Also known as pharyngeal arches. Segmented structures arising as a series of endodermal outpockets on the sides of the developing pharynx that are filled with ectomesenchymal cells derived from cranial neural crest and mesodermal cells. They give rise to multiple facial and visceral structures, including skeletal, muscular and neural elements.

Branchio-oto-renal syndrome
This syndrome is characterized by neck and external ear abnormalities, including hearing loss, and kidney defects. Symptom severity varies greatly from person to person.

Collinear
Refers to the physical gene order within each Hox cluster on the chromosome (telomeric to centromeric), which correlates with the serial activation of these genes along the anterior–posterior embryonic body axis.

Epithelial-to-mesenchymal transition
(EMT). Process by which epithelial cells lose their cell polarity and cell–cell adhesion, and gain migratory and invasive properties to become mesenchymal cells.

Frontonasal prominence
(FNP). Midline, unpaired embryonic structure that develops between the telencephalon, the forming oral cavity and the nasal pits, into the forehead.

Homeodomain
The DNA-binding homeobox domain (homeodomain) is encoded by a 180-bp homeobox DNA sequence, found within genes encoding transcription factors that are involved in pattern formation during development in animals, fungi, plants and numerous single-cell eukaryotes.

Homeotic transformation
Morphological variation in body plan in which one structure is changed into the likeness of another structure, arising from loss-of-function or gain-of-function mutations of the developmentally crucial homeotic genes.

Hox genes
Also known as homeotic genes. A subset of homeodomain genes that specify the morphology of the distinct structures of the body plan of an embryo along the anterior–posterior (head-to-tail) body axis. Mammals have 39 Hox genes, organized into four clusters of 9–11 paralogous genes (some clusters lack select paralogues), resulting from successive evolutionary duplications.

Hyoid bone
Horseshoe-shaped bone situated in the anterior midline of the neck between the base of the lower jaw and the thyroid cartilage that provides an attachment structure for the tongue. The greater horns of the hyoid bone arise from branchial arch 3, whereas the lesser horns originate from branchial arch 2.

Induced pluripotent stem cells
(iPSCs). Pluripotent stem cells that can be generated directly from a somatic cell by the introduction of specific transcription factor genes (*MYC*, *OCT3*, *OCT4*, *SOX2* and *KLF4*).

Lateral nasal prominence
(LNP). Ectoderm-covered swelling filled with mesenchymal cells of cranial neural crest origin that separates the embryonic olfactory pit from the developing eye. The wings of the nose (alae nasi) develop from the LNP.

Mandibular prominences
(MdPs). Caudal prominences formed by bifurcation of embryonic branchial arch 1. Each MdP fuses antero-ventrally with the MdP on the other side of the embryonic face to form the lower jaw.

Maxillary prominence
(MxP). Rostral prominence formed by bifurcation of embryonic branchial arch 1, which joins with the ipsilateral medial nasal prominence to form the upper jaw.

Meckel's cartilage
Bilaterally paired, rod-like, cartilaginous ventral component of the lower jaw, within the branchial arch 1-derived mandibular prominences of vertebrate embryos.

Medial nasal prominence
(MNP). Ectoderm-covered swelling filled with mesenchymal cells of cranial neural crest origin that lies medial to the olfactory pit in the embryo. The nasal tip and philtrum (midline groove) of the lip (in humans) develop from the MNP.

Middle ear ossicles
The incus, malleus and stapes, which transfer vibrations from the eardrum to the inner ear. The incus and malleus are derived from branchial arch 1, whereas the stapes is derived from branchial arch 2.

Multi-omics
Branch of biological science comprising various experimental approaches, such as genomics, transcriptomics, proteomics, metabolomics and phenomics. The goal of multi-omics is the combined characterization and quantification of large data sets that translate into the structure and function of an organism.

Neurocristopathies
A class of human disorders that result from abnormal expression, migration, differentiation or death of neural crest cells during embryonic development.

Paralogous
Genes related to each other through a gene duplication event. A paralogous gene in the same organism gains novel regulation and function, but also often keeps redundant functions with its paralogues. An example of paralogous genes is provided by genes in similar linear positions in the distinct Hox clusters.

Rhombomere 4
The rhombomeres (up to eight in total) are transient compartments of neuroepithelial precursor cells formed in the developing hindbrain of vertebrate embryos. They appear as a series of swellings with meristic organization in the early developing neural tube.

Stomodeum
The primitive oral cavity, which forms between the frontonasal process and branchial arch 1.

Topologically associating domain
(TAD). Self-interacting genomic region of approximately 1 Mb. DNA sequences within a TAD are likely to interact physically with each other more frequently than with sequences outside the TAD.

Long-range enhancer mutation in neurocristopathy

Pierre Robin sequence (PRS)[126] is a birth defect characterized by hypoplasia of the lower jaw (micrognathia), which leads to secondary retraction of the tongue (glossoptosis), obstruction of the airway and a distinctive U-shaped cleft palate with incomplete penetrance[126]. These abnormalities result in feeding and breathing problems and failure to thrive. PRS is caused by non-coding mutations, including deletions and translocation breakpoints, at the centromeric far end of a large gene desert surrounding the TAD that encompasses the *SOX9* gene[127–130]. Leveraging a human stem cell differentiation model, two long-range

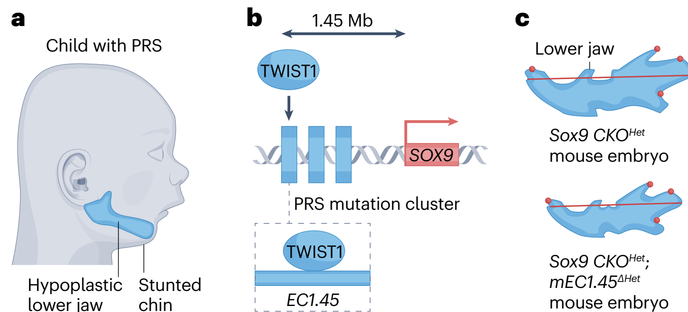

Fig. 5 | Loss of long-range enhancers in human cranial neural crest cells results in Pierre Robin sequence, a congenital craniofacial disorder. **a**, Illustration of the head of a child affected by Pierre Robin sequence (PRS), showing a hypoplastic lower jaw (in blue) and stunted chin (known as micrognathia). **b**, Long-range enhancer clusters (separated by 1.45 Mb from the *SOX9* gene) overlap with mutations found in patients with PRS (the PRS mutation cluster)[131]. These enhancers drive stage-specific *SOX9* expression in cranial neural crest cells (CNCCs). TWIST1 binds to the *EC1.45* enhancer in human CNCCs. **c**, Deletion of the mouse orthologue of the human *EC1.45* enhancer on a *Sox9* heterozygous background (*Sox9 CKO^{Het}*;*mEC1.45$^{\Delta Het}$*) leads to quantitative changes in lower jaw morphology and length (red line) in mouse embryos at embryonic day 18.5, which significantly exacerbate PRS-like phenotypes in embryos with conditional *Sox9* heterozygous loss in *Wnt1*-expressing CNCCs (*Sox9 CKO^{Het}*)[131]. Red dots indicate morphometric landmarks for the lower jaw of *Sox9 CKO^{Het}* and *Sox9 CKO^{Het}*;*mEC1.45$^{\Delta Het}$* mouse embryos.

enhancer clusters were identified within the PRS-associated region[131]. These enhancers regulate *SOX9* expression during a narrow window of CNCC progenitor development, becoming inactive after CNCC differentiation to chondrocytes. Deletion of the 1.45 Mb enhancer cluster in mice leads to moderate reduction in size of the lower jawbone and morphological changes, resulting from *Sox9* downregulation[131]. Although the PRS-associated enhancers have broad facial activity, PRS phenotypic specificity is surprisingly restricted to the lower jaw, suggesting that this structure has heightened sensitivity to *Sox9* dosage (Fig. 5).

An explanation for why the lower jaw is more sensitive than the upper jaw to changes in *Sox9* dosage could reside in the selective expression of genes encoding different transcription factors and signalling molecules in the upper jaw versus lower jaw during development. For example, *Dlx5*, *Dlx6* and *Hand2* are expressed in the developing lower jaw but not the upper jaw[62,132]. Therefore, spatially restricted morphogenetic programmes could be differentially sensitive to SOX9 activity, which in turn could lead to domain-specific and structure-specific effects. An alternative explanation could consist in differences in the trajectory of craniofacial skeletal development. Indeed, both the midfacial skeleton and lower jaw form via intramembranous ossification, independently of any cartilage anlage, but the lower jaw develops together with Meckel's cartilage, which provides support and also influences shaping of this structure. Therefore, if perturbation of *Sox9* expression in CNCCs affects the ability of Meckel's cartilage to form, it could account for the selective effect on lower jaw development. A recent study showed that in mice, the PRS regulatory region drives *Sox9* expression in CNCC-derived Meckel's cartilage but not in limb cartilage[133]. Moreover, intersection of enhancer signatures and chromatin topology led to the identification of more than 10,000 enhancers that function differentially in Meckel's cartilage versus limb cartilage. Such a specific regulatory effect underscores the importance of context-dependent chromatin topology in directing enhancer usage. The PRS-associated enhancers join a group of long-range regulatory sequences that direct precise transcription from a genomic distance of more than 1 Mb, including the *Shh* ZRS enhancer and the *Myc* BENC and MNE enhancers[76,134–136]. These studies demonstrate that PRS is an enhanceropathy and support the idea that small changes in gene expression in select structures can lead to facial morphological variation.

Concluding remarks and perspectives

In this Review, we address themes and debates regarding CNCC-directed craniofacial morphogenesis and its variation within a framework that links genes, regulatory networks and epigenetic landscapes. Despite the knowledge of CNCC gene function that has been gained using genetically engineered mice, and despite the arsenal of new genetic tools for genome editing of 'model' and 'non-model' organisms[137–139], many questions remain open. High-throughput multi-omics techniques with computational integration of genomics, transcriptomics and chromatin conformation data have led to a revolution in the study of CNCCs, generating new hypotheses and discoveries together with establishing new CNCC GRNs and epigenetic landscapes. However, we believe that omics technologies will not erode the importance of experimental design, unlike recently suggested[140], and that organism-level validations will continue to be important for the associations derived from analyses of large data sets gathered from in vitro studies. 'Cellular anthropology' has shown the strength of combining omics approaches in cultured CNCCs from different species with validation in animal models to understand the roles of CNCCs in evolution[141]. Similarly, GWAS have illuminated the polygenic nature of craniofacial features, but the mechanisms by which variants alter gene expression or function are still unknown, and differences in epigenetic regulation may also contribute to variation of human facial morphology[112,119]. Also, as we have discussed, although GWAS have so far analysed cohorts of adults, studies on newborns will be essential. Concomitantly, directed differentiation of ESCs and iPSCs[142] has allowed for the production of large numbers of CNCCs from children with craniofacial birth defects for mechanistic research, as showcased in the case of neurocristopathies[125,131]. Overall, new knowledge of the genetic, epigenetic and developmental processes that underlie facial morphogenesis will herald major advances in tissue engineering[143]. Refined repair and regeneration strategies will enable restoration of normal shape and function for CNCC-derived facial structures affected by congenital malformations, destructive trauma or tumour removal. Facing the future, craniofacial biologists, stem cell biologists and bioengineers will aid surgeons to implement new techniques and tools towards a flawless replacement of facial structures.

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