



*Review*

## Tissue interactions, cell signaling and transcriptional control in the cranial mesoderm during craniofacial development

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**Abstract:** The cranial neural crest and the cranial mesoderm are the source of tissues from which the bone and cartilage of the skull, face and jaws are constructed. The development of the cranial mesoderm is not well studied, which is inconsistent with its importance in craniofacial morphogenesis as a source of precursor tissue of the chondrocranium, muscles, vasculature and connective tissues, mechanical support for tissue morphogenesis, and the signaling activity that mediate interactions with the cranial neural crest. Phenotypic analysis of conditional knockout mouse mutants, complemented by the transcriptome analysis of differentially enriched genes in the cranial mesoderm and cranial neural crest, have identified signaling pathways that may mediate cross-talk between the two tissues. In the cranial mesenchyme, *Bmp4* is expressed in the mesoderm cells while its signaling activity could impact on both the mesoderm and the neural crest cells. In contrast, *Fgf8* is predominantly expressed in the cranial neural crest cells and it influences skeletal development and myogenesis in the cranial mesoderm. WNT signaling, which emanates from the cranial neural crest cells, interacts with BMP and FGF signaling in monitoring the switch between tissue progenitor expansion and differentiation. The transcription factor *Twist1*, a critical molecular regulator of many aspects of craniofacial development, coordinates the activity of the above pathways in cranial mesoderm and cranial neural crest tissue compartments.

**Keywords:** Craniofacial development; Cranial mesoderm; Cranial neural crest; *Twist1*; Mesenchymal maintenance; Transcriptional regulation

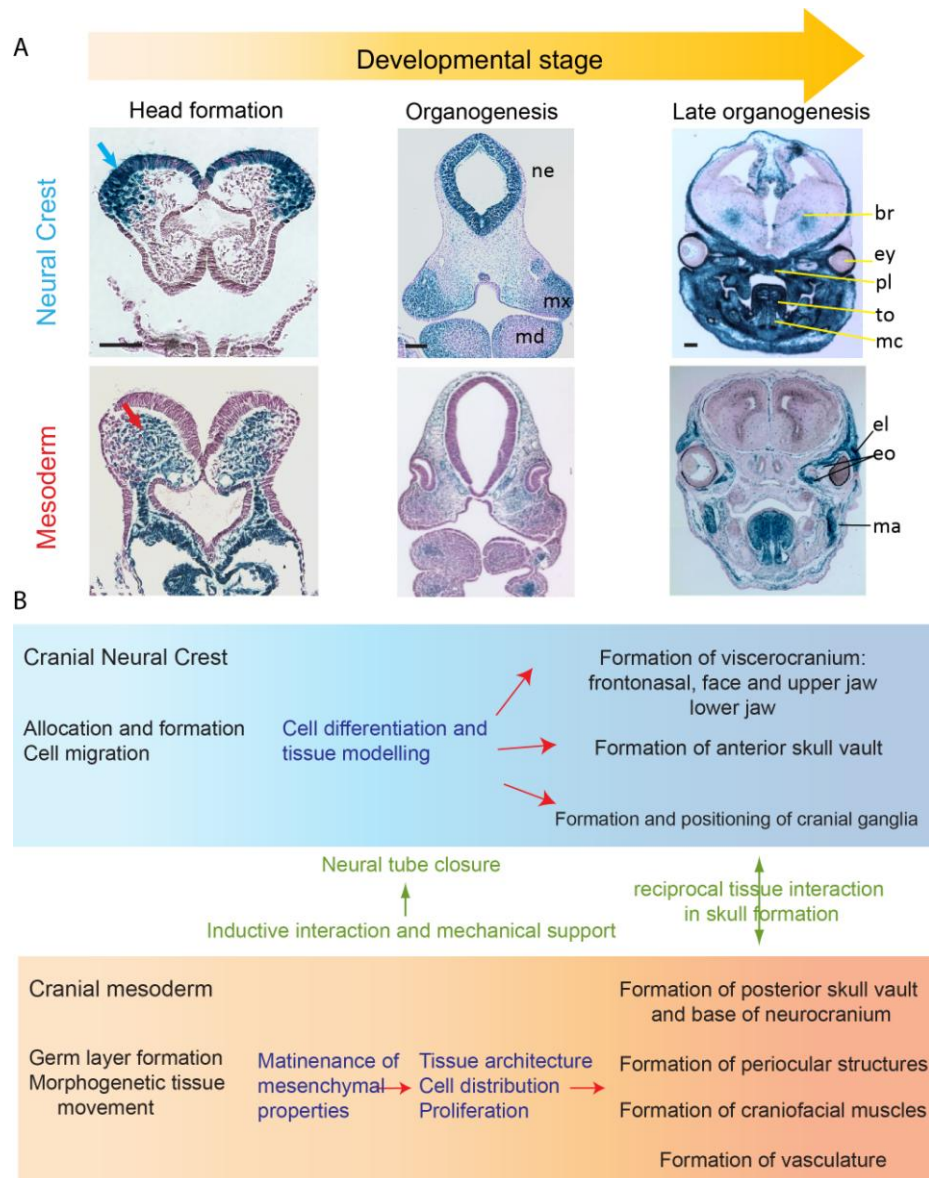
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## 1. Introduction

The embryonic head contains two skeletogenic mesenchymal cell populations that are derived separately from the cranial neural crest (CNC) and cranial mesoderm (CM) [1]. The CNC gives rise to the rostral-most tissues, including the frontal bone, nasal bones and cartilage, as well as the pigmented cells and contributes to the innervation of the head and face. The CM is the source of tissues of the posterior skull, all the head musculature and endothelial cells of the vasculature. The tissue contributions of the CNC and the functions of individual genes in its development have been extensively studied, with the widespread use of CNC-specific CRE drivers such as *Wnt1-Cre* that can effectively generate embryos with tissue-specific loss of function mutations (Table 1). In contrast there are few studies that focus on gene function in the CM and the role of the CM in craniofacial development (Table 1). A greater understanding of the development of the CM is of particular importance given its significant contribution to craniofacial skeleton and musculature, its influence on CNC migration, and the likely role of the CM as a supportive tissue for the morphogenesis of the cranial neural tube (Figure 1). Despite their distinct tissue origin, the two cell types establish an intimate spatial and interactive relationship during tissue morphogenesis [2-4] (Figure 1).

## 2. Tissue origins of the skull and face

The tissue contributions of the CNC and CM in the mouse have been studied using reporters of tissue-specific CRE recombinase activity and clonal analysis. Initially, the cranial mesenchyme is entirely derived from CM (head fold formation stage E 8.5–9.5; Figure 1). The CNC migrates into the lateral regions, subjacent to the ectoderm and populates the frontonasal mesenchyme and the superficial mesenchymal tissue domain of the branchial arches (BAs). The CM contributes to the tissue in the core of the branchial arches and the pre-chordal mesenchyme beneath the forebrain. The CNC and the CM, which will form the mandible and the branchiomic muscles respectively, migrate and co-localise in the first branchial arch (BA1) [7] (Organogenesis E 9.5–E 11). In the skull base, most of the skeletal tissues anterior to the basioccipital are derived from the CNC, with those posterior being derived from the CM. The acrochordal cartilage, which lies between the basioccipital and the basisphenoid shows dual tissue origin [5]. The exception is the hypochiasmatic cartilage, which lies adjacent to the orbital cartilage. Although surrounded by CNC- derived elements, this cartilage is derived from the CM [5], and is closely associated with the extraocular muscles, which are likely to be derived from a combination of the mesoderm from the first branchial arch and pre-chordal mesoderm [6] (Late organogenesis stage E 11+, Figure 1). The skull vault and frontonasal tissues display a similar rostral-caudal division in tissue origin, with the division lying between the frontal and parietal bones [7].



**Figure 1. Contribution and differentiation of cranial neural crest and cranial mesoderm to craniofacial structures during development.** (A) Coronal sections of the embryonic head, showing distribution of *Wnt1-Cre* (neural crest); *Rosa26R* and *Mesp1-Cre* (mesoderm); *Rosa26R* positive cells (indicated by colored arrows) at head formation (E 8.5–9.5), organogenesis (E 9.5–11) and late organogenesis stages (E 11.0 +). Abbreviations: br, brain; ey, eye; el, eyelid; eo, extraocular muscles; mc, Meckel's cartilage; ma, masseter muscle; md, mandible; mx, maxilla; ne, neural epithelium; pl, palate; to, tongue. Scale bar = 100  $\mu$ m. (B) Early development and interactions of CNC and CM. From head formation to organogenesis stage, CNC cells undergo highly regulated migration and differentiation to form a variety of tissues. In the cranial mesoderm, mesenchymal architecture and proliferative capacity influences CNC migration and neural tube closure. Normal neural tube morphogenesis offers the morphogenetic options for the CNC and the mesoderm to localize to the sites of the skull vault. The formation of the bones in the skull vault is also dependent on the reciprocal interactions of these two cell types.

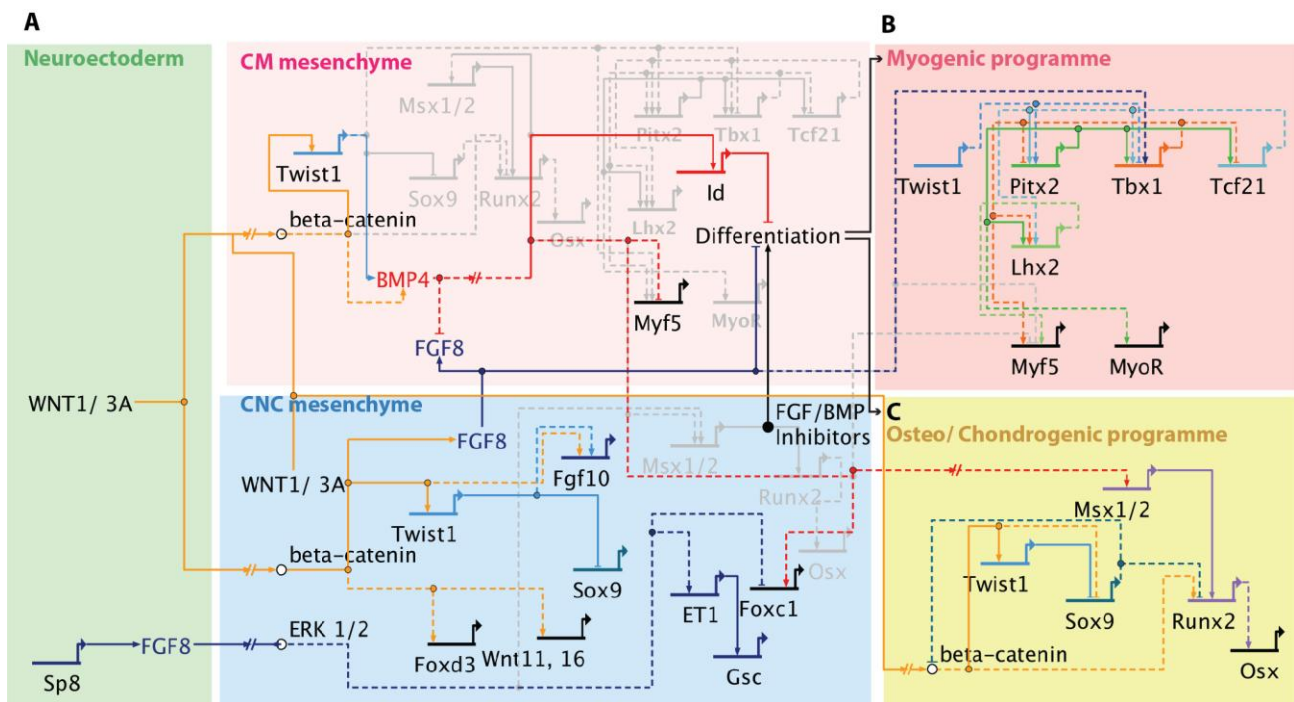
### 3. The cranial mesoderm and the cranial myogenic program

The mesoderm that is derived from the epiblast during gastrulation forms the musculature, vascular system and the majority of the posterior cranial skeleton [2,8,9]. The molecular profile of the CM is distinct from the trunk mesoderm, despite a lack of physical boundary between the two, as revealed by marker analysis in chick embryos [10]. Another unique feature of head mesoderm is the lack of clear segmentation. Metameric structures within the cranial paraxial mesoderm (the somitomeres) have been identified in the avian and mouse embryos [11,12]. Somitomeres are visible as concentric arrangements of the superficial cells in the CM, which can be observed when the overlying ectoderm is removed. However, unlike the somites in the trunk mesoderm, somitomeres do not correlate with lineage propensity, spatial properties of the cell, nor marker gene expression [2]. The developmental significance of somitomeres is therefore uncertain. Molecular markers marking the di-mesencephalon territories are a more reliable indicator of regionalization within the cranial mesoderm [10].

Most studies of mesoderm development in the head have focused on the development of the musculature. The myogenic program in the head is significantly delayed compared to the trunk and is under the direction of a unique group of myogenic factors (MRF) [13-15]. In the trunk mesoderm, *Pax3* initiates the myogenic program and upregulates MRFs that promote differentiation of myotubes. In the CM, where *Pax3* is not expressed, *Tbx1* and *Pitx2* specify the muscle in the head [16,17]. *Pitx2* shares an expression domain in the first branchial arch with *Tbx1*, yet it is uniquely required for formation of the extraocular muscles [18-20]. They cooperatively induce the early myogenic factors *Tcf21* (*Capsulin*), *Msc* (*MyoR*), and *Lhx2*, which subsequently induces the expression of *Myf5* and *Myod1* [15,17] (Summarized in Figure 2B). Embryonic expression pattern of genes discussed in this review can be found in EMAGE gene expression database [21] (<http://www.emouseatlas.org/emage/>).

Mesodermal *Tbx1* is necessary and sufficient to induce MRF and generate the brachiomeric muscles in BA1 and BA2 [22] (Table 1, Figure 2B). Impairment of brachiomeric muscle seen in *Tbx1* deficient embryos can be fully rescued by *Mesp1-Cre* mediated re-expression of *Tbx1*, confirming a tissue-autonomous requirement in the CM. *Tbx1* also has a non-cell autonomous role in guiding the migration of CNC [22,23]. Hypoplastic mandibular bone and missing cartilage including the ear and stapes occur as a result of the loss of BA2 localization of CNC mesenchyme. In the absence of mesodermal *Tbx1*, CNC cells from rhombomere 4 are misplaced into BA1, and BA2 fails to form [23]. Deregulation of FGF and BMP target genes in the *Tbx1* mesoderm-specific mutant might explain the non-cell autonomous disruption of the formation of the CNC-derived mandible.

*Twist1* has been shown as a negative regulator of myogenesis in cell cultures [24-26]. However, in mesoderm-specific *Twist1* mutant embryos, there is no increase in muscle mass, despite the upregulation of the cranial muscle specifier *Tbx1* [27]. Instead, the extraocular muscles are specifically affected and the upper eyelids are missing and the posterior cranial muscles are mis-patterned in mesoderm-specific loss-of-function mutants [27,28]. Consistent with the extraocular muscle defect, expression of *Pitx2* and *Myf5* is reduced in the periocular tissue of mesoderm-specific *Twist1* mutants. In summary, *Twist1* acts upstream of *Tbx1* and *Pitx2* and facilitates extraocular muscle formation by inducing *Pitx2* while repressing *Tbx1* (Figure 2B).



**Figure 2. Mouse cranial mesenchyme gene regulatory network (GRN).** A GRN model that maps hierarchical gene regulatory interactions in the mouse cranial neural crest (CNC) and cranial mesoderm (CM) during craniofacial development. (A) Tissue origin, effects and interactions of signaling pathways in undifferentiated CM and CNC mesenchyme are highlighted, with neuroectoderm as a source of WNT and FGF ligands. Differentiation programmes (in grey), which favour cell proliferation, are inhibited by high levels of signaling activity until differentiation is facilitated by antagonists of the signals. (B) Myogenic and (C) osteo/chondrogenic programmes in CM and CNC are summarised in sub-regions. Solid lines: direct regulatory interaction; dashed lines: putative direct regulatory interaction; broken lines: putative indirect interactions; Bubble nodes: protein-protein interaction. Linkage and regulatory interactions are inferred from data from promoter and *cis*-regulatory analysis and gene perturbation experiments in the mouse. The model was built using BioTapestry software [29].

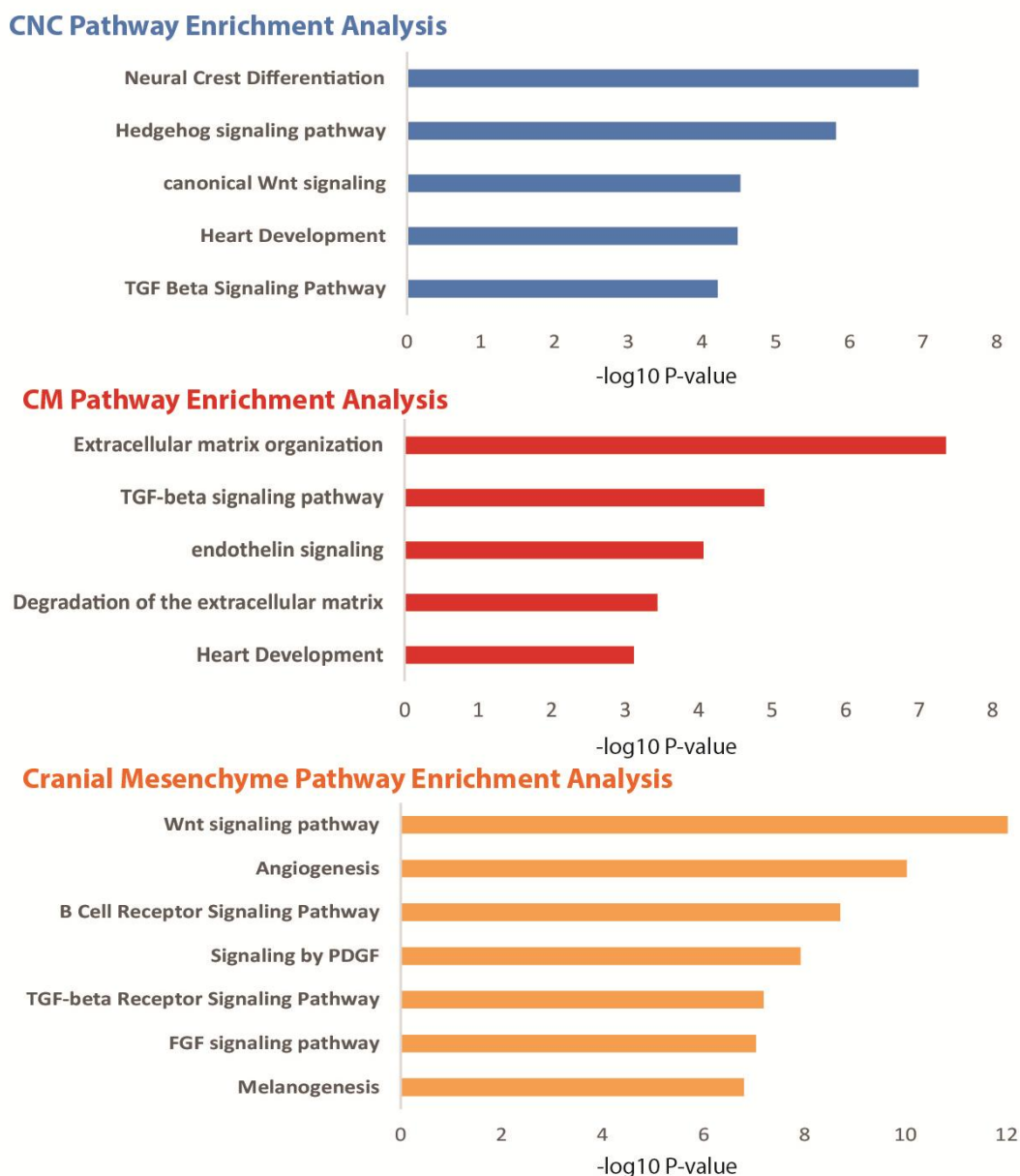
The pre-myogenic mesodermal mesenchyme grows in close proximity to the CNC, as well as the pharyngeal ectoderm and endoderm. It is subject to signaling from these surrounding tissues, including WNT from the neural tube and BMP both from neural ectoderm and the CM. Myogenic induction is facilitated by repression of these signals by BMP and WNT inhibitors from the neural crest cells [13].

#### 4. Transcriptomic comparisons between CNC and CM reveal regional signaling interactions

We have conducted a microarray-based comparison of the gene expression profiles of the CNC and CM, using cells isolated from E 9.5 head tissues, sorted for the expression of a GFP-reporter activated by *Wnt1-Cre* and *Mesp1-Cre* respectively (H. Bildsoe et al, unpublished). The list of differentially expressed genes was filtered to include only those that are known to be associated with developmental craniofacial abnormalities. ToppGene (<http://toppgene.cchmc.org>) was used to detect functional enrichment. As expected, the CNC-enriched pathways are well-studied processes

important for the differentiation and patterning of CNC tissues, including NC differentiation, canonical WNT signaling, SHH signaling and TGF $\beta$  signaling (Figure 3). In the CM, genes involved in extracellular matrix (ECM) organisation are over-represented. Also identified is TGF $\beta$  signaling, which regulates ECM organization and also myogenic and skeletogenic programmes in the CM [30,31]. Endothelin signaling, which is likely to be associated with vascular development was also enriched in the mesoderm. The presence of heart development genes in the lists points to a conservation of developmental programmes for the head and heart mesenchyme [32]. Among the pathways that are enriched in both head mesenchyme cell populations are TGF $\beta$ -related, FGF and WNT signaling (Figure 3). In this review, we will concentrate on how these pathways are utilised in the head mesenchyme, and the impact of the interaction between the CM and the CNC and neighbouring ectoderm and endoderm tissues on craniofacial development. Signaling pathways exert their influences at multiple stages of development. Here, we will concentrate on signaling that affects the early craniofacial organogenesis (nominally E9.5–E11.5 in the mouse, Figure 1), in which perturbation of pathways that affect cell survival, proliferation and specification as well as tissue patterning and differentiation are likely to have dramatic effects later on development of muscular and skeletal structures. The studies referred to in Table 1 are primarily concerned with gene ablation in progenitor cells (such as *Wnt1-Cre* for early migratory CNC and *Mesp1-Cre* for early anterior mesoderm as it exits the primitive streak). Although the primary defects of mouse mutants are likely to be due to early events, later functions of target genes during tissue differentiation cannot be discounted.

In addition, when interpreting data derived from *Cre*-driven conditional mutations, some consideration must be given to the fidelity of *Cre* expression and the possibility that the effects of the *Cre* transgene are not neutral. For example, although the activity of the original and widely used *Wnt1-Cre* transgene appears to reflect the expression of the endogenous *Wnt1* gene in the dorsal neural tube, resulting in efficient deletion of target genes in the neural crest, other neural crest-expressed *Cre* transgenes (eg: *HtPA-Cre*) show highly variable activity [9]. Recently, Lewis and colleagues showed that the original *Wnt1-Cre* transgene ectopically activates *Wnt1* in the midbrain, leading to enlargement of the midbrain, which is associated with ectopic WNT signalling [33]. An alternative reporter, *Wnt1-Cre2*, which recapitulates *Wnt1*-restricted CRE recombinase activity but not the ectopic *Wnt1* expression, was recommended. However, conditional mutants in the Hippo Signaling pathway generated using the two reporter lines displayed similar lethality and craniofacial phenotypes, except for the neural tube closure phenotype [34]. Our experience with *Mesp1-Cre* as a driver for mesodermal *Cre* activity demonstrated the expected temporal and spatial specificity in *Cre* expression, as assessed by both *Mesp1-Cre*-driven lacZ reporter staining and *Cre* transcript expression [27]. In conclusion, artefacts in *Cre* reporter lines do exist, but they are still valid tools in dissecting the tissue-specific gene functions, especially when multiple reporter lines can be assessed in parallel.



**Figure 3. Pathway analysis of craniofacial genes.** CM and CNC cells were separated by flow sorting, from mouse embryos expressing either *Wnt1-Cre* (for CNC) or *Mesp1-Cre* (for CM) and a GFP reporter of *Cre* activity. Labeled RNA was hybridized to Illumina Mouse WG-6 v2 arrays. Differential gene expression was analysed using LIMMA, implemented in GenePattern, on quantile normalized, log2 transformed data. ToppGene was utilized to perform functional enrichment of 840 cranial mesenchyme genes associated with abnormal craniofacial phenotypes. Genes were categorized as being significantly enriched specifically in CM, CNC, or the whole cranial mesenchyme, based on transcriptomic comparison. The raw expression data was generated from microarray analysis from mouse E 9.5 embryos, by isolating *Wnt1-Cre* and *Mesp1-Cre* expressing GFP-labeled cells from the head. Significantly enriched pathways relevant to the biological context are presented along with the *P*-value.

**Table 1. Craniofacial phenotypes of mouse mutants in key pathway components.**

<b>Mutant genotype</b> ( <i>f/f</i> = floxed/floxed; GOF = gain of function)	<b>Pathway/ component</b>	<b>CNC tissues affected</b>	<b>CM tissues affected</b>	<b>Reference</b>
<i>β-catenin f/f; Foxg1-Cre</i> (facial ectoderm and neuroepithelium)	WNT signaling effector	Loss of most facial bones; mandible and tongue remains	N/A	Wang et al. 2011
<i>β-catenin f/f; Crect-Cre</i> (early ectoderm)	WNT signaling effector	Loss of most facial bones	Bones are present, with minor defect	Reid, 2010
<i>β-catenin f/f; Wnt1-Cre</i> <i>or Sox10-Cre (CNC)</i>	WNT signaling effector	Lost almost all craniofacial structure; Meckel's cartilage remained; impaired melanocyte and sensory neuron specification	Loss of most bones; ectopic smooth muscle	Brault et al 2001; Hari et al., 2002
<i>β-catenin f/-; En1-Cre</i> (subset of CNC and CM)	WNT signaling effector	Loss of most bones; ectopic cartilage	Loss of most bones; ectopic cartilage	Goodnough, 2012
<i>β-catenin f/f; Twist2-Cre</i> (subset of CNC and CM)	WNT signaling effector	Reduced most bones; ectopic cartilage; synovial joint fusions	Loss of most bones; ectopic cartilage	Day et al., 2005
<i>β-catenin ex3/ex3</i> (GOF); <i>Foxg1-Cre</i>	WNT signaling effector	Absent nasal prominences and maxillary prominence	N/A	Wang et al. 2011
<i>β-catenin ex3/ex3</i> (GOF); <i>Prx1-Cre</i> (CM mesenchyme)	WNT signaling effector	Loss of posterior frontal bones	Greatly reduced parietal bone	Goodnough, 2012
<i>Wls f/f; Crect-Cre</i>	WNT ligand transporter	Lost most bones; ectopic cartilage	Lost most bones; ectopic cartilage	Goodnough, 2014
<i>Lrp6 -/-</i>	WNT receptor	Facial hypoplasia; cleft palate	N/A	Song et al., 2009
<i>Col2a1-Wnt14</i> overexpression	WNT ligand	Expanded oestrogenic zone	Expanded osteogenic zone	Day et al., 2005



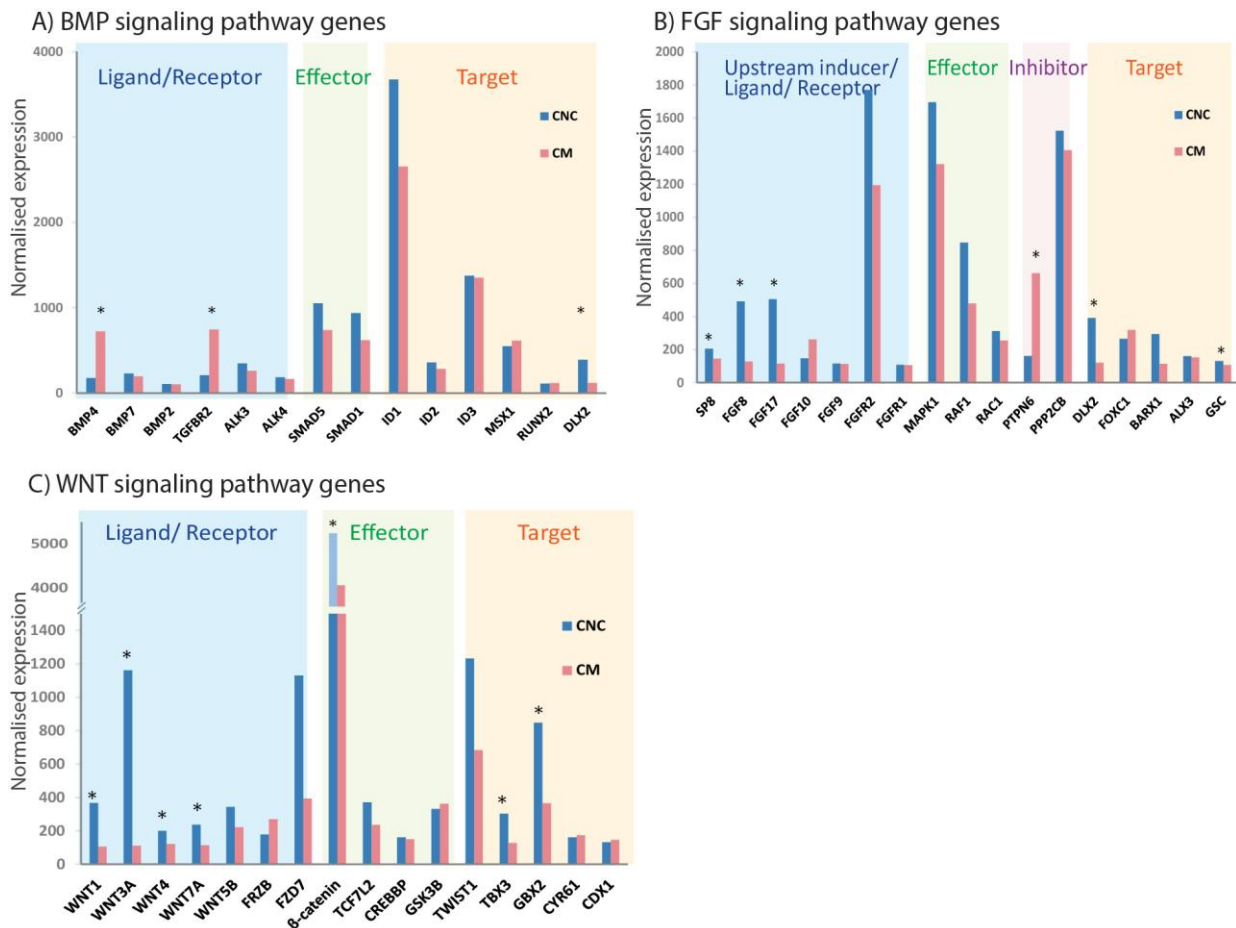
<i>Apc f/f; P0-Cre</i> (CNC- peripheral nervous system, facial mesenchyme)	WNT inhibitor	Loss of frontonasal bones; malformed maxilla and mandible	Reduced CM derived skull vault	Hasegawa et al 2001
<i>Foxd3 f/f; Wnt1-Cre;</i>	WNT target	Loss of frontal nasal bone and cartilage; short maxilla and mandibular bone; cleft face and palate	Reduced interparietal, parietal, basal occipital bone	Teng et al 2008
<i>Sox9 f/f; Wnt1-Cre;</i>	WNT target (inhibitory)	Reduced size of all facial bones; loss of Meckel's cartilage , nasal capsule, inner ear ossicles and endochondral bones;	Loss of basisphenoid and presphenoid	Mori-Akiyama et al 2003
<i>Bmp4 f/f; Nestin-Cre</i>	BMP ligand	Cleft lip (low penetrance); delayed palatal fusion	N/A	Liu, 2005
<i>Alk2 f/f; Wnt1-Cre</i>	BMP receptor	Absent anterior Meckel's cartilage and posterior part of zygomatic bone; reduced frontal bone, mandible; cleft palate	N/A	Dudas et al 2004
<i>Alk2 f/f; Nestin-Cre</i> (first branchial arch specific)	BMP receptor	Cleft lip and palate; arrested development of palatal shelf and maxillary molar	N/A	Liu, 2005
<i>Alk5 f/f; Wnt1-Cre:</i>	BMP receptor	Skull vault defect, maxilla defects	Skull vault defect	Dudas et al 2006, Zhao et al 2007
<i>Smad4 f/f; Wnt1-Cre</i>	BMP signaling effector	Severely affected first branchial arch, lack of midline fusion in mandible and maxilla	N/A	Ko et al 2007
<i>Msx1 f/f; Msx2 f/f; Wnt1-Cre;</i>	BMP targets	Cleft palate; foreshortened mandible and maxilla; enlarged frontal bone	Reduced parietal bones; clefting of interparietal bone	Roybal, et al., 2010
<i>Fgf8 neo/-</i>	FGF ligand	Severely reduced maxilla, mandible and palates; missing Merkel's cartilage	Hypoplastic branchial arches	Abu-Issa et al., 2002; Griffin et al., 2013
<i>Fgf8 f/f; Nestin-Cre;</i>	FGF ligand	Loss of most BA1 structures: mandible, maxilla and Merkel's cartilage; only reminiscent distal structures left	Loss of most BA1 structures; slightly reduced parietal and interparietal bones	Trumpp et al., 1999
<i>Fgfr1f/f; Wnt1-Cre;</i>	FGF receptor	Cleft lip, palate	N/A	Wang et al.,2013a

<i>Fgfr2ff; Wnt1-Cre;</i>	FGF receptor	No anticipated mid-facial defects	N/A	Valverde-Franco et al., 2004
<i>Fgfr2ff; Twist2-Cre;</i>	FGF receptor	Domed-shaped skull	Open occipital arch; Failed closure of midline suture	Yu et al., 2003
<i>Fgfr2s252w f/+; Mesp1-Cre;</i>	FGF receptor	Foreshortened frontal and facial bones	Coronal craniosynostosis; retroflexion of skull base; Ectopic cartilage at interparietal foramen	Holmes, 2012
<i>Fgfr2s252w f/+; Wnt1-Cre;</i>	FGF receptor	Foreshortened frontal, facial bones, palate; Synostosis of major facial bones	N/A	Holmes, 2012
<i>Erk2 ff; Wnt1-Cre</i>	FGF signaling effector	Mandibular and maxilla truncated; cleft palate	Absent tongue	Newbern et al 2008
<i>Sp8 ff; Pax3-Cre</i> (Anterior neural ridge, facial mesenchyme)	FGF upstream inducer	Loss of frontal bones; severe midfacial defects; exencephaly; cleft palate	Loss of parietal bones	Kasberg et al. 2013
<i>Sp8 ff; Wnt1-Cre</i>	FGF upstream inducer	No cranial phenotype	No cranial phenotype	Kasberg et al. 2013
<i>Sp8 ff; Mesp1-Cre</i>	FGF upstream inducer	No cranial phenotype	No cranial phenotype	Kasberg et al. 2013
<i>Spry1 OE; Wnt1-Cre</i>	FGF inhibitor	Loss of maxilla; reduced and malformed mandible; facial clefting	Hypoplasia of skull vault elements	Yang et al 2010
<i>Tbx1 -/-</i>	Transcription factor	N/A	BA1 myogenesis defects	Kelly et al
<i>Tbx1 ff; Mesp1-Cre</i>	Transcription factor	Cranial Nerve: fusion of trigeminal nerve to facial nerve; hypoplasia and aberrant patterning of branchial arches	Aberrant patterning and hypoplasia of tissues derived from first and second branchial arches	Zhang, 2006
<i>Tbx1 ff; T-Cre</i>	Transcription factor	Reduced mandibles; loss of coronoid process and a hypoplastic angular process; missing 2 <sup>nd</sup> branchial arch and stapes	Missing 2 <sup>nd</sup> branchial arch	Aggarwal, 2010

<i>Pitx2</i> <i>-/-</i>	Transcription factor	N/A	Extraocular muscle dysgenesis; thickening of mesothelial layer of cornea	Kitamura et al 1999, Diehl et al 2006, Dong, 2006
<i>Pitx2 f/f; Mesp1-Cre</i>	Transcription factor	N/A	Masseter muscle deficiency; defective myogenesis of branchial arch derivatives	Dong, 2006
<i>Pitx2 f/f; Nestin-Cre</i> (BA1)	Transcription factor	Loss of mandibular elements; fused mouth	N/A	Sclafani et al 2006
<i>Twist1 f/f; Wnt1-Cre</i>	Transcription factor	Lost most bones, but mandible including Meckel's cartilage remained; cranial nerve patterning defect; ectopic cartilage in maxilla	Loss of supraoccipital; Reduced parietal and interparietal	Bildsoe et al 2009
<i>Twist1 f/f; En1-Cre</i>	Transcription factor	Loss of maxilla and frontonasal bones; skull vault defects	Loss of anterior parietal and interparietal bones; ectopic cartilage in posterior skull	Goodnough et al., 2012
<i>Twist1 f/f; Mesp1-Cre</i>	Transcription factor	Greatly reduced all CNC-derived bones; reduced nasal cartilage	Loss of most bones and cartilage, except basioccipital; loss of extraocular muscle and upper eyelid; irregular patterns of posterior head muscle	Bildsoe et al., 2013
<i>Twist1 f/f; Twist2-Cre</i>	Transcription factor	Loss of most bones	Reduced and malformed bones; loss of extraocular muscle	Goodnough et al., 2015
<i>Pdgfra f/f; Wnt1-Cre</i>	Transcription factor	Midline cleft, maxilla abnormal, skull base defects	Reduced size of skull vault	Tallquist and Soriano 2003

## 5. Cranial mesoderm is a source of BMP signaling in craniofacial development

BMPs are members of the TGF $\beta$  superfamily of signaling molecules with diverse roles in development, including tissue patterning, germ layer formation and skeletal development. *Bmp4* and *Bmp7* are able to replace neural ectoderm for the induction of CNC cells [35], indicating that the neural ectoderm is an important source of BMP signaling to the CNC. *Bmp4* is required for the maintenance, migration and differentiation of CNC cells [36]. Our data suggest that during early organogenesis, *Bmp4* is also expressed in the CM-derived cranial mesenchyme at a significantly higher level than in the CNC, whereas other ligands, including *Bmp2*, 5, 7, are expressed at low to almost undetectable levels in both CNC and CM (Figure 4A). Expression of BMP receptors and target genes is at similar level in CM and CNC-derived cells (Figure 4A), suggesting that both populations are capable of responding to BMP signaling. CM-derived *Bmp4* may therefore act autonomously on the CM and non-autonomously on the CNC. The expression of *Bmp4* in CM suggests the possibility that the CM is another BMP4 signaling centre that influences CNC development, in addition to the ectoderm (Figure 2A).



**Figure 4. Expression of BMP, FGF and WNT pathway components in CM and CNC cells.** Normalized expression level of important components of signaling pathways encompassing ligand, receptor, intracellular effectors, inhibitors and transcriptional target in head development are presented. Gene expression level was assayed using Illumina mouse gene expression chips. Asterisks mark genes that were differentially expressed (false discovery rate <0.05, determined by analysis with LIMMA).

BMP signaling acts autonomously in the CM to regulate myogenic and skeletogenic programs, as revealed by conditional mutagenesis of *Bmp4* or downstream signal transduction factors [37-42] (Table 1; Figure 4A). Evidence for the osteogenic effects of *Bmp4* also comes from studies of cultured progenitor cells from cranial sutures, in which application of exogenous *Bmp4* protein is sufficient to induce *Msx* genes and ossification [43]. Although these experiments model a later stage of development, they illustrate the ability of BMP signaling to influence the fate of mesenchyme progenitor cells.

Unfortunately, studies on conditional knockout or over-activation of BMP signaling in the mesoderm are lacking. It has been shown that *Msx1* and 2 are transcriptional targets of the *Bmp4*-activated signaling pathway [43,44]. Loss of function of *Msx1* and 2 from the CNC reproduced the hypoplastic jaw structures seen in BMP signaling mutants [42]. Downstream of *Msx* genes is *Runx2*, which activates an osteogenic transcriptional program [42] and *Bmp4* itself, further amplifying the signal.

BMP signaling is inhibitory to cranial muscle formation, in contrast to its function for trunk muscles. Expansion of *Bmp4* expression at the beginning of organogenesis in the brachial arch coincides with the loss of facial muscles at later stages, in mice lacking the muscle specifiers, *Tbx1* and *Pitx2* [19,22,23,45]. BMP4 signaling may curtail the myogenic process by targeting the myogenic regulatory factors. A plausible mechanism of myogenic repression of *Bmp4* is through direct induction of *Id* genes [42], which compete with muscle-specific basic helix-loop-helix factors for dimerization with E-proteins and thus prevent their activity as myogenic factors [46,47].

## 6. Differential activities of FGF signaling in CM and CNC

### 6.1. Tissue-specificity of signaling function

FGF signaling was identified as a pathway enriched globally in the cranial mesenchyme (Figure 4B). Signaling activity is regulated at multiple levels, including the availability of ligand and receptors, the presence of inhibitory factors and upstream inductive or repressive signals. Closer analysis of the data reveals that most FGF ligands, receptors and intracellular effectors are expressed at similar levels in two tissues of interest, yet *Fgf8* and the closely related ligand *Fgf17* are preferentially expressed in the CNC (Figure 4B). Some known downstream targets that are expressed in the head have higher expression level in the CNC, including *Dlx2* and *Gsc*.

*Fgf8* and *10* encode ligands that are critical for the development of the facial primordia and the skull, and their expression is frequently perturbed in mutants of other craniofacial genes [14,15,48,49]. Whole mount *in situ* hybridization (WISH) analysis indicates that *Fgf8* is highly expressed in the facial ectoderm initially, and subsequently becomes restricted to nasal and oral edges [50]. Our microarray data suggest that *Fgf8* is also expressed in the CNC at E9.5 (Figure 4B). *Fgfr1* and 2 transcripts are found to be overlapping in the cranial mesenchyme and ectoderm [51]. In agreement with these findings, we detected high levels of *Fgfr2* in both CM and CNC derived head tissues in E9.5 embryos (Figure 4B).

### 6.2. Impact on skeletogenesis

The level of FGF signaling activity needs to be finely controlled. Dosage-dependent abnormalities related to the level of *Fgf8* have been reported [52,53]. *Fgf8*<sup>neo/neo</sup> (hypomorphic) mutants display moderate nasal and basal capsule defects. *Fgf8*<sup>neo/-</sup> mutants, presumably expressing less *Fgf8*, showed more severe hypoplasia of frontonasal and maxilla bone and cartilage, with almost

fully penetrant midfacial clefting [52]. When *Fgf8* function is completely abolished, development is arrested at gastrulation. Removal of *Fgfr1* from the CNC leads to cleft palate and lip, accompanied by a proliferation defect in these regions [54]. CNC-specific ablation of the *Fgf8* upstream factor *Sp8* or overexpression of the FGF antagonist *Spry1* has generated disruption similar to *Fgf8* and *Fgfr1* mutants [55,56]. Notably, CNC-specific knockout of *Sp8* also impairs parietal bone development

CM-derived skull elements are less affected by the disruption of FGF signaling than the CNC-derived structures. Mesoderm-specific knockout mutants display only mild abnormalities of CM-derived bones [57]. Intriguingly, constitutive activation of *Fgfr2* in the mesoderm does not affect growth of bones of mesoderm origin, yet it induces severe shortening in all CNC-derived skull structures and craniosynostosis through an unknown mechanism [58]. Over-activated FGF signaling in the CNC itself induces a similar shortening of facial bones due to arrested osteogenesis [58]. The lack of response of CM tissues to loss or gain of FGF receptor activities suggests that the FGF signaling has lower activity level in the CM compared to CNC, as also indicated in our transcriptome analysis. The inhibitory factor that keeps the signaling activity in check is likely to be downstream of receptor activation. BMP signaling is shown to antagonize FGF signaling in BA derived tissues [59-61]. The high level of *Bmp4* expression in the cranial mesoderm might indicate a strong repression of FGF activity by BMP signaling (Figure 2A).

### 6.3. Impact on myogenesis

In cell cultures and chick myoblasts, FGF delays differentiation while promoting proliferation [61,62]. Chick studies have shown that FGFs emanating from the neural ectoderm keeps the mesoderm-derived myoblasts in a proliferative state [14]. In mouse branchial arch muscle development, *Fgf8* also supports the proliferative state over differentiation after myoblast specification [63], whereas blocking pERK nuclear transport (and consequently the response to FGF signaling) in mouse branchial arch myoblast cell culture is sufficient to reduce cell proliferation while initiating myogenic differentiation [14,64]. In support of this, excess FGF signaling activity in *Spry1/2* mutant myoblasts results in an expansion of the proliferative population when compared with wild-type counterparts [14,64]. However, loss of *Fgf8* at early organogenesis effectively abolishes the formation of *Tbx1* expression zone and BA that later forms the facial muscles, indicating an earlier role of *Fgf8* in patterning of facial primordial [52,65]. Ablation of the CNC by removal of the dorsal neural tube in mouse embryos results in increased proliferation and delayed differentiation in the BA mesoderm, which is associated with increased *Fgf8* expression in the ectoderm [66]. This suggests that the CNC influences myogenic differentiation of the CM indirectly, via regulation of *Fgf8* expression in the overlying ectoderm.

## 7. The WNT signaling pathway interacts with BMP signaling and TWIST1

WNT signaling plays critical roles in the generation, migration, and proliferation of CNC cells [67]. CNC cells are enriched in WNT signaling components and WNT signaling also stands out as the most enriched pathway in head mesenchyme in general (Figure 3). The expression of major components in this pathway (Figure 4C) shows preferential activation of most WNT ligand genes, the effector  $\beta$ -catenin and some target genes in the CNC, but there is no significant difference in receptor expression between CNC and CM. This suggests that the CM is capable of responding to WNT signaling mediated by the ligands sourced from the surrounding CNC and surface ectoderm.

Conditional mutation in WNT components results in the most drastic loss of cranial bones among mutants of the three signaling pathways [1,68-72] (Table 1). CNC mutants display the most

severe defects, including the absence of almost all head structures, followed by various degrees of skeletal defects in ectoderm and CM mutants [1,68-72] (Table 1). *Wntless* (WNT ligand transporter) conditional knock out in the ectoderm leads to loss of majority of the skull [70]. As indicated above, the ectoderm is the major source of WNT ligands, which acts on the adjacent CNC-derived mesenchyme. Signaling activity in the CM might be limited by the availability of ligand from surrounding tissues.

The size of the FGF signaling domain is tightly correlated with level of WNT activity. The expression domain of FGF ligands is diminished in the facial ectoderm of WNT loss of function mutants and expanded in mutants with overactivated WNT signaling [71,73]. Supporting this, Tcf/Lef binding sites are present in the *Fgf8* gene [71,74]. Following diminished FGF signaling, severe cell death and facial deformation, frequently seen in FGF mutants, was also found in WNT mutants at the beginning of organogenesis.

In addition to loss of bones, ectopic cartilage forms following WNT inactivation. Osteogenic progenitors are diverted into chondrogenesis in the absence of WNT signals [69,70,75]. In contrast, exogenous *Wnt3a* or overexpression of  $\beta$ -catenin in chondrogenic cell lines or limb mesenchyme blocks cartilage formation [76]. In addition, WNT antagonists, *Sfrp2* and *Sfrp3*, are upregulated in the pre-chondrogenic mesenchyme [77,78], further supporting the notion that WNT signaling antagonizes chondrogenesis. WNT signals repress chondrogenesis by counteracting *Sox9*, the principal regulator of chondrogenesis. Overexpression of *Sox9* inhibits osteogenesis in vivo, most likely via down-regulation of *Runx2* [79-81]. *Sox9* physically interacts with *Runx2* and  $\beta$ -catenin and triggers their degradation [79,82]. At the same time, the interaction of  $\beta$ -catenin with *Sox9* leads to the degradation of *Sox9* itself. In addition, the WNT signaling responsive transcription factor *Twist1* is able to repress *Sox9* expression by targeting chromatin in the *Sox9* 3' UTR in chromatin [69].

As well as blocking the chondrogenic pathway, WNT signaling promotes bone morphogenesis, as demonstrated by its requirement in expression of osteogenic differentiation markers *Msx2*, *Runx2* and *Osx* [70,75]. This might be an indirect effect of WNT induction of BMP ligands. *Bmp4* is downstream of the WNT pathway in cranial and heart mesenchyme [70,73]. The two signaling pathways might be bridged partially by *Twist1*, a direct target of  $\beta$ -catenin [69,28]. Osteogenic calvaria explants deprived of *Twist1* function lose *Wnt3a* responsiveness and osteogenesis is impaired. Further, loss of *Twist1* in the CM and/or the CNC recapitulated the loss of bone defects of WNT mutants [9,27,69,70] (Figure 5). Underlying the skull defects is loss of the BMP target *Msx2*, and *Runx2* in the facial bones [9]. Ting and colleagues [83] described abnormal BMP signaling activity in *Twist1*<sup>+/-</sup> mutants skull suture, revealed by reduced but more dispersed pSmad 1/5/8 staining at the osteogenic boundary. ChIP-seq experiments in a human mammary cell line further indicate that *Twist1* directly activates BMP4 [84].

The constitutive activation of  $\beta$ -catenin also leads to a dramatic loss of bones, yet via a distinct mechanism [44,69,75]. The phenotype is partly attributed to the mis-specification of osteoprogenitor mesenchyme into sensory neurons [85]. Overall, these results imply that a moderate level of WNT activity is required for osteogenic differentiation and cell survival, while strong WNT activity inhibits lineage commitment of osteoprogenitors and favors the neuronal lineage.

## 8. TWIST1 is a mesenchymal maintenance factor in the cranial mesoderm

The importance of the ECM production and remodeling is underlined by the overrepresentation of genes that encode ECM components and ECM remodeling factors in CM cells (Figure 3). Nearly 20% of the CM-enriched genes are involved in some aspect of mesenchymal cell characteristics. Our previous work on *Twist1* has revealed a pivotal role of *Twist1* in mesenchymal cell maintenance *in vivo*. Our work on constitutive and conditional deletion mutants of *Twist1* described a unique craniofacial phenotype related to this process, apart from other head tissue formation defects aforementioned.

Constitutive loss of *Twist1* results in the failure to close the cephalic neural tube, hypoplasia of BA1 and demise of the embryo before E11.5 [86,87]. Since *Twist1* is expressed in CNC and CM-derived cells, the lineage-specific origins of this phenotype cannot be identified in null mutants. *Twist1* transcript is detectable in the mesoderm during gastrulation, but *Twist1* protein has not been detected until early somite stages [27,88]. *Twist1* is absent from the mesodermal core of the mandible, from which the majority of the cranial muscles are derived [27,66]. Expression persists in the CM that gives rise to the posterior skull and extraocular muscles [27]. *Twist1* is also detected in CNC cells and CNC-derived tissues in the frontonasal, maxilla and mandibular tissues.

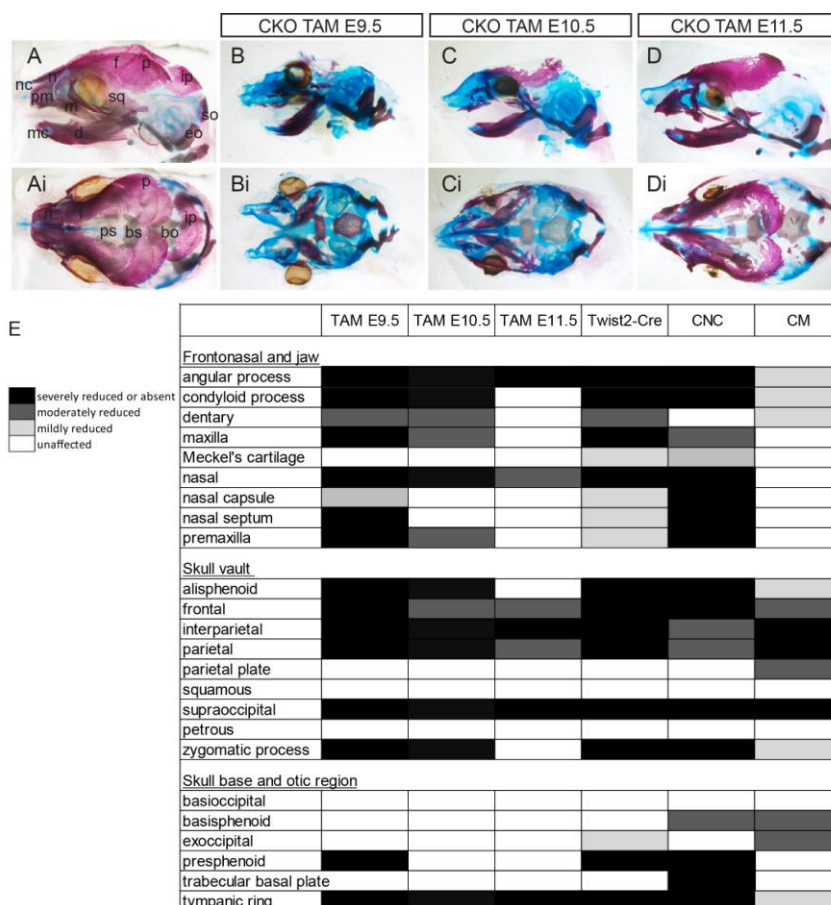
### 8.1. Role of *Twist1* in neural crest cells

*Twist1* has been conditionally inactivated in the CNC [9], specifically in the mandibular arch CNC [22], in the CM [27] and in both CNC and CM populations [28,89,90] (Figure 5). In CNC cells, *Twist1* is required for cell survival and to maintain the capacity to differentiate into bone and cartilage [9,28]. The consequence of these defects is a lack of neural crest-derived bones and cartilage, as well as the CNC-derived dura mater that underlies the skull, and other CNC-derived tissues such as the cornea. As a result, CM-derived skeletal elements, which require interactions with the dura, are also affected. Although the mandible is formed in the absence of *Twist1*, it is truncated at the proximal end, and articulation with the cranium is not established [9,91].

### 8.2. Role of *Twist1* in the mesoderm

When *Twist1* was specifically ablated in the CM, skull development was also severely impaired, but this was not due to excessive cell death [27]. Although *Twist1*-deficient CM cells display a slightly reduced rate of proliferation, the most pervasive defect was a change in the distribution of the CM cells. *Twist1*-deficient CM cells become unevenly spread throughout the mesodermal domain and form cyst-like clumps. Cell in these clumps display polarization of the actin cytoskeleton and express E-cadherin, both of which are characteristics of epithelial cells. This accompanies a reduction in the expression of mesenchyme-specific genes, including *Snai2* and *Pdgfra*. This requirement for *Twist1* in maintaining cells in a mesenchymal state, appears to be specific to the CM, since no disruption to mesenchymal cell phenotypes were observed in surviving *Twist1*-deficient CNC cells [9], or in limb-bud mesenchyme that lacks *Twist1*, apart from a moderate change in cell density [27].





**Figure 5. Skull phenotypes of *Twist1* conditional knockout embryos.** (A-D, Ai-Di). Alcian blue/alizarin red preparations of skulls from wild type and mutant embryos (*Twist1*<sup>del/flox</sup>; *Ubc-CreERT2*) collected at E17.5. *Ubc-CreERT2* enabled *Twist1* to be ablated throughout the embryo at different developmental time-points by administration of tamoxifen (TAM) to pregnant mothers at E9.5, E10.5 or E11.5 [90]. In TAM E9.5 embryos (B, Bi), the cartilage of the nasal capsule (nc) was split and the overlying nasal (n), premaxilla (pm), and maxilla (m) were reduced to small remnants, compared to wild type embryos (A, Ai). In TAM E10.5 embryos (C, Ci), the nasal capsule fused medially and formed a nasal septum, but the disruption to nasal, maxilla and pre maxilla development was similar to TAM E9.5 embryos. Frontonasal abnormalities in TAM E11.5 (D, Di) embryos were less severe. The bones of the skull vault, both CM and CNC-derived, were absent in TAM E9.5 injected embryos (B, Bi). In some TAM E10.5 embryos (C, Ci), some development of the lateral part of the frontal (f) and parietal (p) bones was seen, whereas in others the phenotype of the skull vault was similar to TAM E9.5 embryos (not shown). In TAM E11.5 (D, Di) embryos, frontal and parietal bone were formed, but interparietal and supraoccipital bones were absent. In the skull base, the basioccipital (bo) and basisphenoid bones (bs – visible through the skull vault) are unaffected even in TAM E9.5 embryos, whereas the presphenoid (ps) is absent. Like in neural crest CKO embryos, the dentary (d) was truncated at the proximal end in TAM E9.5 and TAM E10.5 embryos and did not form a proper articulation with the skull. (E) Summary of skull phenotypes of TAM E9.5–E11.5 embryos at E17.5 compared with cranial neural crest (CNC)-specific conditional knockout [9], cranial mesoderm (CM)-specific knockout [28] and *Twist2-Cre*-mediated knockout, which affects both CNC and

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CM [29]. The *Twist2-Cre* knockout shares characteristics of TAM E9.5 and TAM E10.5 embryos, indicating that deletion of *Twist1* occurs sometime between these time points. Abbreviations: mc, Meckel's cartilage; sq, squamosal bone.

This primary effect of *Twist1* in regulating the mesenchymal characters in the CM extends to other aspects of cranial tissue morphogenesis, including neural tube closure, CM cell migration and response to the inductive signals for differentiation. In CM-specific *Twist1* mutant embryos, but not CNC-specific mutants, the cephalic neural tube fails to close. This points to a vital role of the CM in supporting neural tube morphogenesis. It is known that the cranial mesenchyme supports neural tube elevation through changes to the rigidity and volume occupied by the extracellular matrix [92].

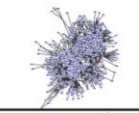
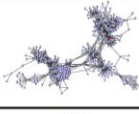
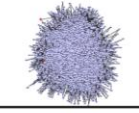
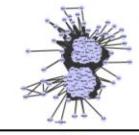
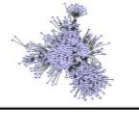
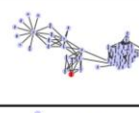
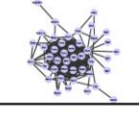
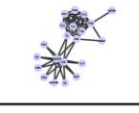
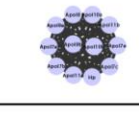
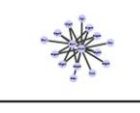
CM cells may also be impaired in their ability to migrate, as shown by the accumulation of CM-derived cells in the lateral part of the head, and the absence of the mesoderm-derived skull bones in CM-specific *Twist1* mutants [27]. Since *Mesp1-Cre* causes the deletion of *Twist1* at mid-gastrulation, this phenotype may be generated by the physical constraints posed by the open neural tube, or may reflect an impaired innate migratory capacity [27]. Failure to form skeletal elements even when *Twist1* is ablated after the closure of the neural tube suggests that *Twist1*-deficient CM cells are intrinsically impaired in their ability to migrate or differentiate, rather than being obstructed by the malformed neural tube (Figure 5).

It is established that, in tumor-derived cells and other cultured cells, overexpression of *Twist1* can drive cells to undergo an epithelial to mesenchymal transition (EMT) [93]. *Twist1*-dependent maintenance of mesenchymal properties has been observed in tumor cells, including synovial sarcomas [94]. Down-regulation of *Twist1* expression may, in fact, be a key step in the establishment of secondary tumors following metastasis [95]. This EMT function of *Twist1* is apparently not required in embryos, since mesoderm and neural crest, cell populations that express *Twist1* and that arise by EMT, are formed in *Twist1*-null mutant embryos [86,87].

Together, the information gleaned from phenotype studies and studies of tumor metastasis indicates that *Twist1* function is important for the maintenance of mesodermal progenitor cells and regulating their morphogenetic properties.

### 8.3. A gene regulatory network of *Twist1* transcriptional target genes in the cranial mesoderm

To decipher the molecular basis of *Twist1*-regulated processes, we have used microarrays to identify the changes in the transcriptome following the loss of *Twist1* function in the CM, and ChIP-Seq to identify the suite of *Twist1*-binding sites in the genome of mesenchymal cells that over-express *Twist1*. By combining these two types of data we have identified putative *Twist1* transcriptional targets in the CM (H. Bildsoe et al, unpublished). To explore the biological processes that are affected by *Twist1* and its transcriptional targets in the cranial mesoderm, we constructed a gene regulatory network based on third neighbor protein-protein interactions of these predicted transcriptional targets. Through applying a modularity based clustering algorithm to this densely connected network, functionally relevant sub-networks (modules) were identified. The modules reveal the connection of *Twist1* target genes with metabolic processes, ion and lipid transport, signaling and signal transduction, and organization of the cytoskeleton and ECM, among other functional processes (Figure 6 and Supplementary Material 1: lists of genes and the interaction in the functional modules).

Module	Visualization	GO term 1	GO term 2	GO term 3
1		lipid metabolic process monocarboxylic	cellular lipid metabolic process	acid metabolic process
2		hydrogen ion transmembrane transport	generation of precursor metabolites and energy	ATP metabolic process
3		positive regulation of biological process	cell surface receptor signaling pathway	positive regulation of cellular process
4		G-protein coupled receptor signaling pathway	signal transduction	single organism signaling
5		cellular nitrogen compound metabolic process	nitrogen compound metabolic process	organonitrogen compound biosynthetic process
6		extracellular matrix organization	extracellular structure organization	collagen fibril organization
7		muscle contraction	striated muscle contraction	muscle system process
8		complement activation	protein activation cascade	humoral immune response
9		lipoprotein metabolic process	lipid transport	lipid localization
10		protein glycosylation	macromolecule glycosylation	glycosylation

**Figure 6. Gene regulatory network of *Twist1* transcriptional targets in the cranial mesoderm.** Illumina microarrays were used to identify genes that are differentially expressed between wild-type and CM-specific *Twist1* mutant embryos. Labeled RNA from control and CM-specific *Twist1*-mutant embryos was hybridized to Illumina Mouse WG-6 v2arrays. Differential gene expression was analysed using LIMMA, implemented in GenePattern, on quantile normalized, log<sub>2</sub> transformed data. ChIP-Seq was carried out with a *Twist1*-specific antibody (Abcam) and chromatin extracts from *Twist1*-overexpressing MDCK cells, which adopt a mesenchymal phenotype. 50bp reads were aligned to the CanFam3 (dog) genome using Bowtie2 and Twist 1 binding sites were identified with MACS2. Equivalent binding sites in the mouse genome were determined with Liftover (NCBI) and annotated with ChipSeeker. Putative targets were determined by the overlap of these the ChIP-seq and microarray gene lists. The third neighbor networks were established based on protein interaction data from the STRING database [96], with an minimum interaction score threshold value of 900. The network was deconstructed into modules using the fast greedy modularity optimization algorithm implemented in the igraph R library [97]. The networks were visualized with Cytoscape

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and the top three GO Biological Process terms associated with the set of interacting proteins is shown. See Supplementary Material 1 for detail.

The functional connection of *Twist1* to the biology of the ECM and cytoskeleton is consistent with our finding that *Twist1* is required for the maintenance of mesenchymal attributes of the CM [27], and provides a link from *Twist1* to the biological processes that mediate cellular interactions. *Twist1* targets interact with the machinery for the metabolism of lipids and carboxylic acids (Figure 6). The compendium of interacting proteins further reveals interactions with WNT ligands, suggesting that Twist may contribute to the regulation of lipid modification of WNTs, which is required for extracellular transport of these proteins [98]. *Twist1* function is also connected to BMP, FGF, PDGF and WNT signaling pathways via its association with cell surface receptor signaling components, and to multiple signaling pathways via G-protein coupled signal transduction (Figure 6).

## 9. Conclusion

In essence, the protein network analysis shows that the key processes regulated by *Twist1* and its downstream targets include those related to the secretion, detection and response to intercellular signaling and the maintenance of the mesenchymal cell states. Mesodermal *Twist1* constitutes the node of a molecular hub through which multiple signaling pathways, including but not limited to BMP, FGF and WNT signaling. Through its downstream targets, *Twist1* translates the signals for the CM progenitors to proliferate and differentiate and the CM to provide signaling and mechanical support to the adjacent CNC-derived tissues.

CM and CNC contribute to different sets of craniofacial tissues and are of distinct embryological and evolutionary origins. Our microarray data also show that the transcriptomes of these two mesenchymal progenitor populations are distinct from each other. CNC and CM cells intermingle to a greater degree than previously appreciated in the branchial arches of avian embryos, despite their unique tissue contributions [99], suggesting that CNC and CM have intrinsically different responses to the same environmental cues. On the other hand, when avian CNC cells are ectopically located, they can form cartilage that is normally exclusively derived from CM [100]. This suggests that cell fates of the CNC are plastic and influenced by the local signaling environment. Further studies are needed to clarify how much of the observed differences between the CNC and CM cell populations are intrinsic differences and how much can be accounted for by the environment and local interactions that influence them.

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## Conflict of interest

All authors declare no conflicts of interest in this paper.

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