



Published in final edited form as:

Curr Top Dev Biol. 2015 ; 115: 235–269. doi:10.1016/bs.ctdb.2015.07.001.

Zebrafish Craniofacial Development: A Window into Early Patterning

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Abstract

The formation of the face and skull involves a complex series of developmental events mediated by cells derived from the neural crest, endoderm, mesoderm, and ectoderm. Although vertebrates boast an enormous diversity of adult facial morphologies, the fundamental signaling pathways and cellular events that sculpt the nascent craniofacial skeleton in the embryo have proven to be highly conserved from fish to man. The zebrafish *Danio rerio*, a small freshwater cyprinid fish from eastern India, has served as a popular model of craniofacial development since the 1990s. Unique strengths of the zebrafish model include a simplified skeleton during larval stages, access to rapidly developing embryos for live imaging, and amenability to transgenesis and complex genetics. In this chapter, we describe the anatomy of the zebrafish craniofacial skeleton; its applications as models for the mammalian jaw, middle ear, palate, and cranial sutures; the superior imaging technology available in fish that has provided unprecedented insights into the dynamics of facial morphogenesis; the use of the zebrafish to decipher the genetic underpinnings of craniofacial biology; and finally a glimpse into the most promising future applications of zebrafish craniofacial research.

1. ZEBRAFISH CRANIOFACIAL ANATOMY

The skull of an adult zebrafish consists of 43 bones derived from a cartilage template and 30 directly ossifying intramembranous bones (Fig. 1D–F; Cabbage & Mabee, 1996), significantly exceeding the approximately 22 skull bones of an adult mammal. However, many of these bones, particularly those forming the cranial vault, develop and ossify long after the embryonic period, such that the skull of a zebrafish larva that is just acquiring the capacity to feed (~5 days postfertilization (dpf)) is, by contrast, quite simple (Fig. 1A) compared with that of newborn mammals. At this stage, the craniofacial skeleton consists of a neurocranium, which provides support for the brain and sensory systems, and the viscerocranium, which serves as the feeding and respiratory apparatus (Fig. 1A and B). Both are primarily composed of cartilage elements that are later replaced by bone, as well as a few intramembranous bones such as those that support the gill covering. Larval cartilages are relatively simple, often only one or a few cell layers thick, and can be easily visualized in fixed individuals by staining with Alcian blue (Fig. 1A and B) or in living larvae using fluorescent transgenic reporters expressed in chondrocytes (Fig. 3C).

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The layout of the larval zebrafish craniofacial skeleton has been extensively described in anatomical studies (Cubbage & Mabee, 1996; Piotrowski et al., 1996; Schilling & Kimmel, 1997; Schilling et al., 1996). The viscerocranium is derived from cranial neural crest cells (NCCs) that populate the seven pharyngeal arches of the zebrafish embryo, with multiple skeletal elements forming along the dorsal–ventral axis of each arch (Fig. 1C). The first, or mandibular, arch gives rise to the ventrally positioned bilateral Meckel’s cartilages, which join at the midline and serve as the lower jaw in the larva, as well as the more dorsal palatoquadrates, which articulate with the posterior end of Meckel’s to form the jaw joint. The pterygoid processes are anterior extensions of the palatoquadrate that contact the ethmoid plate, the most anterior part of the neurocranium, to form the larval upper jaw. The second, or hyoid, arch also forms two large cartilages, the dorsal hyosymplectics and the ventral ceratohyals, with a smaller interhyal cartilage linking the two as part of the hyoid joint. The ceratohyals each contact the unpaired basihyal cartilage at the midline. The hyosymplectic, a fusion of the plate-like hyomandibula with the symplectic rod, is connected to the neurocranium via the anterior part of the otic cartilage and thereby secures the jaw skeleton to the rest of the head. Moving posteriorly, arches 3–7 produce paired ventrolateral rod-like ceratobranchial cartilages affixed to small hypobranchials, which in turn attach to unpaired basibranchials at the ventral midline. The ceratobranchials support the densely branched, delicate gill tissues. Unique to the larval viscerocranium, the fifth pair of ceratobranchials, from arch 7, forms several ossified pharyngeal teeth. The larval viscerocranium also contains several directly ossifying bones, the most notable of which is the fan-shaped opercle bone that projects off of the dorsal hyomandibula. This bone grows dramatically in size during the first weeks of life (Kimmel, DeLaurier, Ullmann, Dowd, & McFadden, 2010) and functions to support and protect the gills.

1.1 Zebrafish Pharyngeal Arches as Models of the Mammalian Jaw and Middle Ear

The pharyngeal arches are transient and morphologically complex embryonic structures positioned posterior to the eye and below the ear. In all vertebrates, the arches consist of cylinders of cranial NCCs surrounding a core of mesoderm, lined externally by ectoderm and internally by pharyngeal endoderm. The arches are extensively patterned by signals from the surrounding epithelia, as well as by NCC-intrinsic information carried over from their neuroepithelial origins; integration of this information determines the major anterior–posterior (Hox genes) and dorsal–ventral axes (Endothelin-1, Jagged-Notch, Bmp) as well as fine-tunes exactly what parts of each arch need to produce cartilage or bone (Fgf, Hh) to establish domain-specific skeletal bauplans. The general functions of these pathways in patterning the pharyngeal arches prior to skeletal development have proven to be highly conserved across vertebrates (reviewed by Medeiros & Crump, 2012), despite the differences in the actual shapes and numbers of arch-derived skeletal elements in different animals. For example, in mice and zebrafish, mutations in the Endothelin-1 pathway result in drastic changes specifically to the anterior lower face, manifesting as reductions and transformations of Meckel’s and the ceratohyals in zebrafish *edn1* mutants (Miller, Schilling, Lee, Parker, & Kimmel, 2000), extensive abnormalities including a severely reduced mandible in *Edn1* mouse mutants (Kurihara et al., 1994) and a homeotic transformation of the lower jaw to an upper jaw, complete with whiskers, in mice mutant for *Dlx5/6*, two key *Edn1* target genes (Depew, Lufkin, & Rubenstein, 2002). This deep

conservation across vertebrates in early pharyngeal arch patterning has been demonstrated repeatedly and justifies the use of the zebrafish as a model to identify additional genes and better understand their functions in this process.

Skeletal elements in the fish skull do not always have recognizable homologs in mammals (or vice versa), but there are some that clearly do. In a fascinating evolutionary story, the middle ear bones of mammals have their origins in the bones that form the jaw and connect the viscerocranium to the ear in fish (Fig. 2A; reviewed by Anthwal, Joshi, & Tucker, 2012; Gould, 1990). Anatomical, developmental, and evolutionary analyses have confirmed that the posterior end of Meckel's cartilage and part of the palatoquadrate are evolutionarily homologous to the first arch-derived malleus and incus of the mammalian middle ear, with the second arch-derived hyomandibula representing the stapes homolog (Fig. 2A; Liem & Walker, 2001). The connection between the hyomandibula and the otic cartilage in fish is reminiscent of the stapes' attachment to the oval window in the otic capsule housing the mammalian inner ear. This homology between the mammalian middle ear and the fish jaw means that we can exploit the powerful genetic tools available in the zebrafish system to identify new genes that may shape these skeletal homologs in both systems. This represents an important opportunity because while a number of mouse models with middle ear abnormalities have been described, the developmental basis of the skeletal defects remains unresolved in most instances, and there are many reported clinical cases of hearing loss where one or more of the middle ear bones present with abnormal morphology or are entirely absent (e.g., Park & Choung, 2009; Park, Choung, Shin, & Hong, 2007; Rahbar, Neault, & Kenna, 2002).

1.2 The Fish Neurocranium as a Model for the Mammalian Palate

Unlike the viscerocranium, the larval neurocranium is of mixed cranial neural crest and mesoderm origins: the anterior part of the neurocranium, including the ethmoid plate and paired trabeculae, and parts of the bilateral otic capsules are formed by cranial NCCs, while the posterior and medial components that develop around the cranial end of the notochord are derived from mesoderm (Fig. 2B; Kague et al., 2012; Mongera et al., 2013). The anterior neurocranium, particularly the ethmoid, has emerged as a genetically tractable model of the mammalian hard palate (Fig. 2B). Both the ethmoid and the mammalian secondary palate derive from bilateral groups of anterior maxillary cranial NCCs (termed the "palatine shelves" in mammals) that migrate medially to fuse at the midline and form the roof of the mouth. Impairments in the migration and midline fusion processes result in cleft palate in mammals (Bush & Jiang, 2012). Comparable phenotypes have been observed in zebrafish "midline" mutants, ranging from a complete absence of the anterior neurocranium to the formation of two unfused, parallel rods or a single rod instead of a plate (Eberhart et al., 2008; Eberhart, Swartz, Crump, & Kimmel, 2006; Kimmel, Miller, & Moens, 2001; Swartz, Sheehan-Rooney, Dixon, & Eberhart, 2011; Wada et al., 2005). Importantly, zebrafish mutant for *Pdgf*, *Shh*, or *Bmp* pathway components, or deficient in *irf6*, all show cleft or midline phenotypes resembling those of their mammalian counterparts (Table 1; Dougherty et al., 2013; Eberhart et al., 2008, 2006; Swartz et al., 2011; Wada et al., 2005). These findings indicate that a conserved genetic network regulates the formation of this part of the face across vertebrates and support the use of the zebrafish anterior neurocranium as a

model for the mammalian hard palate. However, there are significant morphogenetic differences between the two systems, particularly the fact that the mammalian palatal bone directly ossifies from mesenchymal cells while the zebrafish anterior neurocranium passes through a cartilaginous template, and so caution is advised when extrapolating zebrafish findings to mammals (Bush & Jiang, 2012). Nonetheless, zebrafish will likely be a good model for early events regulating the growth and midline fusion of the palatine shelves, whereas later processes such as ossification and development of the lip and soft palate may prove more difficult to model in fish.

1.3 Conservation of Cranial Sutures from Fish to Man

Though the bulk of research on zebrafish craniofacial biology has focused on the embryonic and early larval periods, this system can also be utilized to model other processes that are more relevant to adult stages, particularly the formation and maintenance of sutures (Grova et al., 2012; Holmes, 2012). As in mammals, the skull vault of adult zebrafish is formed by thin intramembranous bony plates separated by sutures of dense connective tissue (Fig. 2C; Quarto & Longaker, 2005). In fish, these bones progressively ossify over the brain during the second month of life (Quarto & Longaker, 2005), well after the onset of ossification in the cartilaginous templates of the viscerocranium and neurocranium starting at 5–6 dpf. In mammals, the coronal suture, which lies between the frontal and parietal bones, delineates the border between anterior, neural crest-derived bones and posterior, mesoderm-derived bones. In fish, as appears to be the case in avians (Evans & Noden, 2006; Noden, 1984), this border between neural crest and mesoderm actually exists as a cryptic boundary within the frontal bone, with the bones flanking the coronal suture both derived from mesoderm (Fig. 2C; Kague et al., 2012; Mongera et al., 2013). Despite this important difference in embryological contributions, the coronal sutures of zebrafish and mammals have similar anatomical structures (Quarto & Longaker, 2005).

Adult zebrafish continually grow throughout their lifetime, and their cranial sutures remain patent to allow for skull expansion (Quarto & Longaker, 2005), similar to rodents (Opperman, 2000) but unlike sutures in humans, which fuse in adulthood (Meindl & Lovejoy, 1985). Premature or ectopic suture fusion, or craniosynostosis, can occur in fish as well as mammals and leads to gross distortions in cranial shape. Though the potential of zebrafish as a model for suture formation and maintenance has only begun to be explored, recent pioneering studies have reported new unmapped adult zebrafish mutants with cranial bone fusions (Andreeva et al., 2011), genes with conserved suture expression patterns (*illIra*; Keupp et al., 2013), and craniosynostosis phenotypes in fish mutant for a gene required for suture maintenance in mammals (*cyp26b1*; Laue et al., 2011), indicating conservation of gene function throughout evolution.

2. IMAGING CRANIOFACIAL DEVELOPMENT

The ease with which living embryos and larvae can be accessed and imaged gives an enormous advantage to the zebrafish system. Relative to mouse and chick, zebrafish embryos are transparent, develop both rapidly and externally, and can be repeatedly anesthetized and subjected to confocal imaging for extended periods while continuing to develop without major abnormalities or delays. Furthermore, both the pharyngeal arches and

the later-arising skeletal elements of the zebrafish face are significantly smaller and contain many fewer cells than the corresponding structures in mouse and chick (Yelick & Schilling, 2002), making this system particularly suitable for high-resolution imaging to deconstruct skeletal shaping at the level of individual cells.

2.1 Fate-Mapping Facial Development

Fate-mapping analyses allow researchers to trace cell lineages to better understand how cells of one developmental stage (e.g., premigratory NCCs) are related to those of another (e.g., NCCs in the pharyngeal arches; Fig. 3B). Numerous tools have been used for short-term fate-mapping analyses in the zebrafish pharyngeal arches, including microinjection of fluorescent dyes into single cells (Crump, Swartz, Eberhart, & Kimmel, 2006; Crump, Swartz, & Kimmel, 2004; Kimmel et al., 1998; Wada et al., 2005) and laser-induced local activation of photoconvertible proteins (e.g., kaede; Dougherty et al., 2012; Eberhart et al., 2006) or kikGR (Balczerski et al., 2012; Swartz et al., 2011). These tools have allowed zebrafish researchers to push past the limitations of the avian and mouse models to perform extremely high-resolution fate-mapping studies of both pre- and postmigratory NCCs.

These fate maps have revealed that a cell's *pre migratory* position along the anterior–posterior axis is related to both its migratory route and destination in the arches and that a *post migratory* cell's location within its arch determines what part of the skeleton it will make. Within the first stream of neural crest, the initial, premigratory rostral–caudal position alongside the brain determines whether NCCs will migrate over or behind the eye (Dougherty et al., 2012; Wada et al., 2005), as well as whether their progeny will contribute to the anterior neurocranium or the upper or lower jaw (Dougherty et al., 2013, 2012; Eberhart et al., 2006; Wada et al., 2005). Additional analyses demonstrated that NCCs at certain anterior–posterior and mediolateral coordinates within each arch reproducibly contribute not only to particular skeletal elements but also to specific domains of said elements (Fig. 3B; Balczerski et al., 2012; Crump et al., 2006, 2004; Eberhart et al., 2006; Swartz et al., 2011; Wada et al., 2005), implying that the complex skeletal shapes of the face form as amalgamations of multiple subpopulations of arch NCCs that may be independently regulated.

These fate maps have also been particularly useful for deconstructing the developmental basis of skeletal phenotypes of mutant or transgenic lines: researchers have used these maps to hone in on the specific population of arch NCCs known to give rise to the affected element and then determine what aspect of their developmental trajectory (e.g., survival, migration, chondrocyte, or osteoblast differentiation) has been derailed in the mutant to produce the observed phenotype (Balczerski et al., 2012; Crump et al., 2006, 2004; Eberhart et al., 2006; Wada et al., 2005). For example, in the *moz* mutant, which forms a set of mirror-image first-arch elements within the second arch (Miller, Maves, & Kimmel, 2004), the fate map of the second arch is completely reorganized, such that cells that normally contribute to the anterior hyomandibula no longer make any cartilage at all (Crump et al., 2006).

For longer-term lineage tracing of arch NCCs, the only Cre lines available to date are *Sox10*-based constructs that are broadly expressed in all neural crest derivatives (Kague et al.,

2012; Mongera et al., 2013). While these lines have been extraordinarily useful for determining what components of the skeleton arise from neural crest versus mesoderm, the field will need to develop more specific Cre lines that label only subsets of premigratory or arch NCCs in order to characterize, for example, the origins of skeletal elements that develop after the early larval period.

2.2 Visualizing Morphogenesis and Signaling in Real-Time

Each element of the facial skeleton has a unique three-dimensional shape and position within the skull. How cells in nascent skeletal elements coordinate with each other to attain these precise shapes is a long-standing question that is still far from being fully answered, even in the simplified zebrafish larval face. However, the ability to perform extended time-lapse imaging of craniofacial development in the zebrafish embryo has brought researchers closer than ever to grasping the complexity of facial morphogenesis at a cellular level.

Several transgenic reporters, most notably *sox10:EGFP* (Wada et al., 2005) and *fli1a:EGFP* (Lawson & Weinstein, 2002), are expressed in arch NCCs as well as the forming skeleton and have been widely used to image transitions between key developmental stages, as well as for side-by-side comparisons of mutant and control embryos (Crump et al., 2006, 2004; Eames et al., 2013; Eberhart et al., 2008, 2006; Le Pabic, Ng, & Schilling, 2014; Wada et al., 2005). These movies were captured at high spatial and temporal resolution, providing unprecedented access to the cellular processes that underlie the formation of the larval craniofacial skeleton (McGurk, Lovely, & Eberhart, 2014). One such process that was illuminated by time-lapse imaging in fish is the active condensation of mesenchymal NCCs (Fig. 3A). Arch NCCs form very tightly packed condensations at several locations throughout the arches, including around the oral epithelium and posterior to the first pharyngeal pouch. Disruption of signaling between these epithelia and the NCCs in *smo* and *itga5* mutants, respectively, causes the disintegration of these condensations, readily visualized by time-lapse imaging, resulting in losses of the specific cartilage subdomains that form from these cells (Crump et al., 2004; Eberhart et al., 2006). Another process important for shaping craniofacial cartilages, revealed at a slightly later stage of development, is convergence-extension (Fig. 3D). Both time-lapse movies and dye-based fate maps have demonstrated that cell intercalation rather than proliferation accounts for the elongation of bulky ventral precartilaginous condensations into long, rod-shaped cartilages consisting of stacks of polarized disc-shaped chondrocytes (Kimmel et al., 1998; Le Pabic et al., 2014). This process was recently shown to be regulated by the Fat/Daschous planar cell polarity pathway (Le Pabic et al., 2014).

Live imaging also offers the opportunity to correlate morphogenesis with intercellular signaling. Fluorescent transgenic lines that provide transcriptional readouts of Bmp (*BRE:EGFP*; Alexander et al., 2011), Fgf (*dusp6:EGFP*; Molina, Watkins, & Tsang, 2007), Notch (*Tp1:EGFP*; Parsons et al., 2009), Wnt (*7xTCF:EGFP*; Moro et al., 2012), and Hedgehog (*Gli-d:mCherry*; Schwend, Loucks, & Ahlgren, 2010) signaling activities can be used to identify the specific sets of NCCs that receive a given signal. For example, strong expression of *BRE:EGFP* was detected in the NCCs of the ventral first and second arches (Alexander et al., 2011), adjacent to the ventral ectoderm that expresses *bmp4* (Zuniga,

Rippen, Alexander, Schilling, & Crump, 2011). The conclusion that these cells were responding to a Bmp signal was supported by the phenotype of embryos transgenic for a dominant-negative Bmp receptor that presented malformations in skeletal elements that form from ventral arch NCCs (Alexander et al., 2011). Other useful reporters for live imaging include fusions between a fluorescent protein and signaling transducers like Disheveled (Choe et al., 2013) or mediators such as the immunoglobulin-domain protein Alcama (Choe et al., 2013), which change their cellular localization upon the receipt of a particular signal. Live imaging of these signaling reporters in conjunction with reporters that label all NCCs can reveal how signaling dynamics align with the many morphogenetic processes that transform the pharyngeal arches into the craniofacial skeleton.

2.3 Imaging Skeletal Differentiation

Time-lapse imaging of zebrafish craniofacial development also offers the opportunity to visualize the onset of differentiation of specific populations of NCCs into chondrocytes, osteoblasts, tendons/ligaments, and connective tissues (Fig. 3C). In addition to the transgenic reporters expressed in both arch NCCs and the early skeleton, the field has also developed a number of transgenic markers that are activated upon the differentiation of specific skeletal cell types, including *sox9a:EGFP* (Eames et al., 2013) and *col2a1a:EGFP* (Dale & Topczewski, 2011) for chondrocytes and *sp7:EGFP* (DeLaurier et al., 2010) and *RUNX2:EGFP* (Knopf et al., 2011) for osteoblasts. The utility of the osteoblast lines has been amply demonstrated; for example, time-lapse analyses have demonstrated that growth of the opercular bone occurs through Indian hedgehog-mediated recruitment of proliferating, *sp7:EGFP*-negative NCC precursors (Huycke, Eames, & Kimmel, 2012). In the future, a larger toolbox of differently hued fluorescent reporters should allow researchers to visualize specific stages of osteoblast and chondrocyte differentiation in the intact embryo, which when combined with mutant analyses should provide precise insights into how craniofacial genes regulate distinct stages of cartilage and bone differentiation.

3. GENETIC ANALYSES OF CRANIOFACIAL DEVELOPMENT

3.1 Forward Genetic Screens for Craniofacial Mutants

Because zebrafish can be maintained at high densities for relatively low cost and are capable of producing hundreds of offspring on a regular basis, their potential for large-scale forward genetic screens was quickly recognized in the early days of zebrafish research (Grunwald & Streisinger, 1992; Kimmel, 1989). The first large-scale screens for mutations affecting zebrafish larval craniofacial development were conducted in the Nusslein-Volhard lab at Tübingen (Piotrowski et al., 1996; Schilling et al., 1996) and the Driever lab at Harvard (Driever et al., 1996; Neuhauss et al., 1996). Here, potential mutants were identified based on the gross morphology of the head at larval stages and only later stained to illuminate cartilage morphology, so these screens favored mutants with severe morphological abnormalities over those with more subtle patterning defects. A later screen for craniofacial defects within a set of embryonic lethal insertional mutant lines in the Hopkins lab at MIT (Amsterdam et al., 1999) identified additional loci that, by definition, also had severe craniofacial and/or pleiotropic effects that precluded the detection of milder phenotypes (Nissen, Amsterdam, & Hopkins, 2006). These screens were nevertheless extraordinarily

fruitful, identifying more than 80 loci (some redundantly) required for the development of cartilage elements in the viscerocranium and/or neurocranium, or more generally for chondrocyte differentiation and organization. Subsequent screens performed at the University of Oregon using direct staining of cartilage and bone with Alcian blue and Alizarin red, respectively, identified additional mutants with craniofacial patterning phenotypes, including many that are likely too subtle to have been detected in the initial screens (e.g., Cox et al., 2012; Crump et al., 2004; Eames et al., 2011; Eberhart et al., 2008; Miller et al., 2004; Zuniga, Stellabotte, & Crump, 2010).

Draft versions of the zebrafish genome first became available in the early 2000s (Jekosch, 2004), facilitating ongoing mapping efforts. To the delight of the community, many of the first mutant loci to be mapped were in genes or pathways previously identified in mice or humans as critical for early neural crest (e.g., *tfap2a*; Barrallo-Gimeno, Holzschuh, Driever, & Knapik, 2004; Knight et al., 2003) or craniofacial development (e.g., the Endothelin-1 pathway; *edn1*, Miller et al., 2000; *plcb3*, Walker, Miller, Swartz, Eberhart, & Kimmel, 2007; *furina*, Walker, Miller, Coffin Talbot, Stock, & Kimmel, 2006; *mef2ca*, Miller et al., 2007) and the Sonic Hedgehog pathway (*shh*, Schauerte et al., 1998; *smo*, Varga et al., 2001; *disp1*, Nakano et al., 2004; *gli1*, Karlstrom et al., 2003; *gli2*, Karlstrom, Talbot, & Schier, 1999). This impressive degree of conservation in itself validates the use of zebrafish as a model for investigating the genetic control of mammalian craniofacial development. Other loci were mapped to genes not previously recognized as being important for neural crest or skeletal patterning (e.g., the integrin *itga5*, Crump et al., 2004; the Notch ligand *jag1b*, Zuniga et al., 2010; and the variant histone *h3f3a*, Cox et al., 2012), opening entirely new avenues of research in the field. Another mutant, which entirely lacked endoderm (*sox32*), also failed to develop any jaw cartilages, revealing a crucial nonautonomous role for endoderm in facial cartilage formation (David, Saint-Etienne, Tsang, Schilling, & Rosa, 2002). As many of the mutants identified in these screens have still not been mapped to a specific genetic locus and are represented by only a single allele (suggesting a lack of screen saturation), the utility of the forward mutagenesis approach for identifying new genes required for early craniofacial development has yet to be fully realized.

While initial screens focused on mutations affecting early larval cartilage and bone, more recent screens have begun to identify a number of mutations affecting later development of the skull, including in adults (Andreeva et al., 2011; Harris et al., 2008; Huitema et al., 2012). As opposed to larval lethal mutants, these viable adult craniofacial mutants may better replicate the types of nonlethal birth defects seen in humans. For example, loss of the ectodysplasin A receptor (*edar*) in adult zebrafish was found to result in a loss of fins as well as scales, pharyngeal teeth, and bony gill rakers (Harris et al., 2008), mimicking the ectodermal organ defects (e.g., defective teeth, hair, sweat glands) seen in human patients with *EDA* or *EDAR* mutations (Kere et al., 1996; Monreal et al., 1999). Another common class of viable craniofacial zebrafish mutants involves misregulated ossification of cartilage-derived elements (Apschner, Huitema, Ponsioen, Peterson-Maduro, & Schulte-Merker, 2014; Eames et al., 2011; Huitema et al., 2012). In particular, a number of mutations in enzymes that add sugar groups to extracellular matrix proteins (e.g., *xyll1*, *fam20b*) have revealed important roles for cartilage-derived proteoglycans in suppressing premature

osteoblast differentiation within the periosteum (Eames et al., 2011). Continued analyses of these viable craniofacial mutants will provide new insights into the development of bones and cartilage that develop after the larval period, as well as how the craniofacial complex maintains its structure during growth and aging.

An advantage of forward screens is its ability to identify partial loss-of-function alleles (hypomorphic alleles) and dominant alleles. For example, in cases where null alleles are early lethal, hypomorphic alleles can reveal dose-dependent effects of gene loss on craniofacial development, and dominant alleles can disrupt multiple members of a gene family (e.g., the *h3f3a^{db1092}* mutation, which disrupts multiple redundant H3.3 histones during neural crest specification; Cox et al., 2012). On the other hand, forward screens will often fail to uncover gene function in cases of functional redundancy. A complication of teleost fish is that they experienced a whole-genome duplication following their divergence from the lineage that gave rise to tetrapods (reviewed by Braasch & Postlethwait, 2012), and thus zebrafish often possess two copies of what exists as a single-copy gene in mammals. However, this issue of “genome duplication” is often exaggerated, with current estimates suggesting that only 20–30% of mammalian genes have two paralogs in zebrafish (Postlethwait, Amores, Cresko, Singer, & Yan, 2004; Postlethwait et al., 2000; Woods et al., 2005). In addition, many of these paralogs are expected to have diverged in expression pattern and/or function throughout evolution, a process referred to as “subfunctionalization” (Braasch & Postlethwait, 2012; Glasauer & Neuhauss, 2014). For example, although mammalian *Jag1* has both *Jag1a* and *Jag1b* paralogs in zebrafish (Zecchin, Conigliaro, Tiso, Argenton, & Bortolussi, 2005), loss of *jag1b* is sufficient to cause patterning defects of the upper face in zebrafish, with *jag1a* expression being lost from the pharyngeal arches during teleost evolution (Zecchin et al., 2005; Zuniga et al., 2010). Nonetheless, some paralog pairs or related members of a gene family may still function redundantly, such that a phenotype only manifests when both genes are mutated. These loci would therefore be difficult to uncover in forward screens.

3.2 Reverse Genetics to Test Candidate Craniofacial Genes

In the early 2000s, two new approaches were developed that allowed for targeted mutagenesis (reverse genetics) or gene knockdown in zebrafish: Targeting Induced Local Lesions in Genomes (TILLING) and morpholinos. With TILLING, researchers screen chemically mutagenized zebrafish genomes with sensitive PCR-based methods to detect point mutations in genes of interest (Wienholds et al., 2003). Enhanced TILLING methods, combined with next-generation sequencing, have been employed by the Zebrafish Mutation Project at the Sanger Institute, with the goal of identifying nonsense or essential splice-site mutations in the vast majority of predicted genes (Kettleborough et al., 2013).

Stable morpholino oligonucleotides (MOs) that interfere with splicing or mRNA translation were also developed as a novel knockdown approach (Nasevicius & Ekker, 2000). The great appeal of MOs rested in the speed of their applications, allowing researchers to rapidly test the function of candidate genes for craniofacial development. While many MOs were predicted to yield only hypomorphic phenotypes based on their knockdown efficiency, several were shown to effectively phenocopy their corresponding null mutants, including

those targeting *edn1* (Miller & Kimmel, 2001), *sox9a* (Yan et al., 2002), *tfap2a* (Arduini, Bosse, & Henion, 2009), and *dlx5a* (Talbot, Johnson, & Kimmel, 2010). However, it was also recognized that MOs can cause nonspecific artifacts due to toxicity or off-target effects related to the great excess of MO injected relative to the amount of endogenous RNA in the embryo (reviewed by Schulte-Merker & Stainier, 2014); researchers thus employed a number of controls (e.g., coinjecting a MO against p53 to block cell death, Robu et al., 2007, or rescuing the phenotype by coinjecting wild-type RNA) to improve the validity of their findings. Dozens of studies have since identified craniofacial phenotypes in different morphants, ranging from general reductions in the size or elongation of the viscerocranium to defects in cartilage differentiation or malformations of specific cartilage elements. Unfortunately, subsequent identification of null alleles in the same genes has revealed that the vast majority of morphant phenotypes (>80% or more; Kok et al., 2015) may be due to nonspecific toxicity effects and/or reductions of more than just the target gene. In some cases, MOs cause the same phenotype even when the endogenous target gene is deleted (Kok et al., 2015), a strong argument for nonspecific toxicity. In more worrying cases, even when morphant phenotypes are properly controlled for by rescue with nontargeted mRNA, they still fail to be replicated in true null alleles (Law & Sargent, 2014; van Impel et al., 2014). While there is still much to be learned about the nature of these discrepancies, great caution must be taken when interpreting morphant phenotypes in the absence of a genetic mutant. Indeed, given the recent ability to rapidly generate null alleles in virtually any gene (see below), there appears to be little justification to continue to use MOs in zebrafish research.

In the last few years, newer tools for targeted mutagenesis of candidate genes, including zinc-finger nucleases (ZFNs; reviewed by Porteus & Carroll, 2005), TALENs (Christian et al., 2010), and CRISPR/Cas9 (Cong et al., 2013), have begun to supersede the use of morpholinos. Both ZFNs and TALENs are chimeric proteins consisting of sequence-specific DNA-binding domains fused to DNA endonucleases, while the CRISPR/Cas9 system targets the Cas9 endonuclease to specific sites in the genome using specially designed guide RNAs. All of these techniques produce short indels at their target sites, which, if located in an exon, can disrupt the reading frame and lead to a nonfunctional protein. Stable lines carrying these purported null mutant alleles can be constructed within two fish generations, though phenotypes can sometimes also be observed in larvae injected with highly efficient TALENs or CRISPRs (e.g., Bedell et al., 2012; Dahlem et al., 2012). CRISPR/Cas9, the most recently developed technology, is far more cost-effective and efficient than the proprietary ZFN technology and even easier and more amenable to high-throughput analyses than TALENs, making it the current system of choice for targeted mutagenesis in zebrafish.

As described above, zebrafish researchers are increasingly finding that many of these new targeted mutant lines do not exhibit the same phenotypes as observed in the corresponding morphants (Kok et al., 2015; Schulte-Merker & Stainier, 2014). One concern is that the small lesions generated by TALENs and CRISPRs could lead to residual translated protein, for example, by alternative mRNA splicing or translational frameshifting. However, in the few cases in which this has been directly examined using antibodies against the gene product, residual protein translation has not been found to account for the lack of a

phenotype previously described for morphants (Kok et al., 2015; Law & Sargent, 2014). Nonetheless, due to concerns about residual gene function, some researchers are choosing to use pairs of CRISPRs to delete all or most of the open reading frame, thus ensuring that no residual functional protein is made.

With all of the new mutant lines now available, zebrafish are increasingly being used for complex genetic experiments not technically feasible in any other vertebrate model. A pair of zebrafish can produce a hundred embryos at a time on a near-weekly basis, so sufficient numbers of those rare individuals homozygous for mutations in two (1/16 offspring), three (1/64), or even four genes (1/256) can be identified in a relatively short time. The ability to perform such complex crosses will allow researchers to test for redundancy among multiple members of a gene family, as well as to determine epistatic relationships between groups of key patterning factors and build gene interaction networks.

3.3 Transgenic Technology to Manipulate Developmental Patterning

Overexpression studies are often used to complement knockdown or mutant analyses of gene function. Embryo-wide overexpression can be achieved by injecting mRNAs into single-celled embryos, though in these cases early embryonic defects often cloud analyses of later craniofacial changes. Alternatively, DNA constructs can be used that allow transgenic expression of gene products under control of a heat shock-inducible promoter (e.g., *hsp70l:dnfgfR1*; Lee, Grill, Sanchez, Murphy-Ryan, & Poss, 2005), thus temporally restricting gene expression to craniofacial stages. While injected mRNAs are usually fairly even distributed among cells of the later embryo, DNA constructs are sparsely and mosaically distributed, which most often necessitates the creation of stable transgenic lines. However, use of the I-SceI (Grabher, Joly, & Wittbrodt, 2004) or Tol2 transposon systems (reviewed by Kawakami, 2007) makes the generation of stable transgenic lines in zebrafish a highly efficient process, with the major drawback being the 3-month generation time to generate stable lines.

The Gal4/UAS system has also been used to achieve tissue-specific or temporally regulated overexpression in fish. In these experiments, the Gal4 transcriptional activator is expressed under the control of a tissue-specific or heat shock-inducible promoter, where it binds to the UAS recognition sequence and activates the expression of the target gene in only the cell type or stage of interest. Gal4 drivers expressed in different subsets of arch tissues have been developed, including *sox10:GAL4VP16* (all NCCs; Das & Crump, 2012), *hand2:GAL4VP16* (ventral arch NCCs; Nichols, Pan, Moens, & Kimmel, 2013), and *nkx2.3:GAL4VP16* (pharyngeal endoderm) and *nkx2.5:GAL4VP16* (arch mesoderm; Choe et al., 2013). The primary problem with the Gal4/UAS system is that the UAS sequence is CpG-rich and thus becomes methylated and silenced over successive generations, interfering with transcriptional activation (Goll, Anderson, Stainier, Spradling, & Halpern, 2009). The Q transcriptional regulatory system, which relies on a non-CpG-rich activating sequence, has been proposed as an alternative (Subedi et al., 2014). Nonetheless, the Gal4/UAS technique has been particularly useful for manipulating signaling pathways in the arches, e.g., researchers can activate a pathway by overexpressing a ligand or a constitutively active version of a receptor or inhibit the same pathway by overexpression of a dominant-negative

receptor or competing ligand. For example, a key role for Bmp signaling in patterning the ventral-most elements of the viscerocranium was demonstrated by overexpressing Bmp4 with a heat shock-inducible Gal4 driving *UAS:Bmp4* and then blocking the pathway using Gal4-mediated activation of Grem2, a secreted BMP inhibitor (Alexander et al., 2011; Zuniga et al., 2011).

Both standard and tamoxifen-inducible Cre lines have also been successfully established in zebrafish (Mosimann & Zon, 2011), including a Cre expressed under a human *SOX10* enhancer specific to the neural crest (Kague et al., 2012) and an ERT2-Cre regulated by the zebrafish *sox10* promoter, which labels both the neural crest lineage and all chondrocytes (Mongera et al., 2013). To date, these lines have only been used to recombine transgenic constructs for the purposes of lineage tracing or conditional expression of a transgene. Conditional knockouts have been hampered by the field's inability to efficiently insert loxP sites into endogenous loci, though many new methods for targeted insertions are currently under development (reviewed by Auer, Duroure, Concordet, & Del Bene, 2014), and floxed endogenous genes are certainly on the horizon.

4. FUTURE DIRECTIONS

4.1 Gene Editing to Model Human Craniofacial Birth Defects in Zebrafish

In the past two decades, many groups have used morpholinos or mutants identified in forward screens to develop new zebrafish models of human craniofacial birth defects. The speed of zebrafish research has made this model attractive for testing new loci identified by GWAS or patient genome sequencing. Demonstrating that a gene or noncoding sequence suspected to underlie a specific human condition has a conserved function in zebrafish can help to validate its status as a human disease gene. Furthermore, the myriad imaging and genetic tools available for zebrafish can facilitate efforts to determine the root molecular or developmental basis of the human craniofacial phenotype. There are now reports of zebrafish models of at least 35 human genetic diseases with craniofacial abnormalities (Table 1). In many of these cases, the zebrafish models phenocopied, at least in some respects, the human phenotype, exhibiting symptoms recognizable as cyclopia, cleft palate, craniosynostosis, etc. In other cases, particularly in the morpholino models, the affected fish showed minor or nonspecific skeletal abnormalities or losses that were more difficult to interpret as resembling the mammalian defect.

In the next few years, as tools for targeted gene editing become even more sophisticated, we will be able to very precisely alter the zebrafish genome to not only knock out genes of interest but also edit endogenous genes to match a patient-specific allele or single-nucleotide polymorphism. This will not only improve the accuracy of the disease models but will also reveal whether a particular amino acid substitution results in gain or loss of function of the affected protein, thereby providing *in vivo* validation of computational predictions of protein function. A current limitation is that precise homologous recombination to introduce polymorphisms, even when stimulated by TALEN/CRISPR-mediated double-strand breaks, remains fairly inefficient in zebrafish (e.g., Auer, Duroure, De Cian, Concordet, & Del Bene, 2014; Zu et al., 2013), although there is hope that rapid developments in the gene-editing field will lead to improvements in the near future. In addition, not all polymorphisms

found in human patients will affect regions conserved in fish, though, encouragingly, mutagenic lesions do tend to occur in protein residues and enhancers that are highly conserved throughout vertebrates. It therefore seems likely that zebrafish will become the first-pass organism of choice for testing the exponentially increasing number of putative disease alleles being identified by next-generation sequencing projects.

4.2 Adult Skeletal Homeostasis and Regeneration

Bone is a highly dynamic organ, constantly remodeling in response to mechanical forces and undergoing limited repair after fractures or minor injuries. In humans, defects in bone homeostasis can lead to osteoporosis, particularly in postmenopausal women, and birth defects such as Paget's disease have their underlying etiology in defective bone remodeling. As in mammals, larval cartilages in zebrafish are organized into growth plates, with zones of proliferative and hypertrophic chondrocytes surrounded by a distinct perichondrium and periosteum. However, unlike in mammals, zebrafish cartilage-replacement bone forms largely around the developing cartilage and not within it, and hence is best referred to as perichondrial rather than endochondral bone. Nonetheless, many of the pathways involved in mammalian endochondral ossification, such as *Ihh* and parathyroid hormone receptor-like signaling, appear to play analogous roles in fish perichondrial ossification (Bhattacharya, Yan, Postlethwait, & Rubin, 2011; Hammond & Schulte-Merker, 2009; Yan et al., 2012). Zebrafish also have abundant osteoclasts that promote bone remodeling through the degradation of existing bony matrix (Witten, Hansen, & Hall, 2001). While fish are not subjected to the same gravitational forces that tetrapod bones experience, load experienced by water current and respiratory and biting forces would be expected to similarly promote bone remodeling in adults, particularly in the jaw. Hence, as with developmental patterning, the relatively simple architecture of fish growth plates, combined with the likely remodeling of craniofacial bones through adulthood, makes zebrafish an interesting new model to visualize how interactions between chondrocytes, osteoblasts, and osteoclasts regulate bone remodeling in both normal and pathological contexts.

Another attractive feature of zebrafish is their remarkable regenerative ability, and the skeleton is no exception. Most work on skeletal regeneration has been conducted in the context of the fin, which in adults is able to fully regenerate all its tissues in about 1 week (reviewed by Poss, Keating, & Nechiporuk, 2003). Fin bone regeneration appears to proceed through at least two routes: dedifferentiation, proliferation, and redifferentiation of existing osteocytes and *de novo* osteoblast differentiation (Knopf et al., 2011; Sousa et al., 2011). Time-lapse imaging in adults has beautifully demonstrated this unusual dedifferentiation process in real time (Knopf et al., 2011), with more recent work showing a similar type of regeneration in the calvarial bones of fish (Geurtzen et al., 2014). In contrast, regeneration of the lower jaw proceeds through a cartilage intermediate (S. Paul & G. D. Crump, unpublished; Wang et al., 2012; Zhang et al., 2015), much like fracture repair in humans. In the future, it will be interesting to examine the extent to which zebrafish skeletal tissues regenerate better than their mammalian counterparts, as well as the genetic and cellular basis of such regeneration. Insights gained could then be used to develop novel therapies for improving cartilage and bone healing in patients.

5. CONCLUSIONS

The zebrafish has become an immensely useful and popular genetic and developmental model for studying neural crest biology and the development of the skull. With its rapidly expanding repertoire of cutting-edge genetic and live-imaging tools, this model has opened an extraordinary window into the complexities of craniofacial morphogenesis that is unparalleled by any other model system. In addition, the more we learn about craniofacial and skeletal biology in the zebrafish, the more we can appreciate that the underlying development of many human craniofacial features (sutures, palate, jaw, middle ear, cartilage, and bone differentiation) is highly conserved in fish. Nevertheless, the amount of effort put into demonstrating the high degree of conservation in craniofacial development between zebrafish and mammals tends to downplay the important areas in which they significantly differ. The face of a fish, after all, looks quite different from that of a mammal, or even an amphibian. Far from invalidating the zebrafish as a model for studying craniofacial development, uncovering the biology that underlies the differences in facial structure between fish and tetrapods will only enrich our understanding of the developmental basis for the breathtaking diversity of vertebrate skulls.

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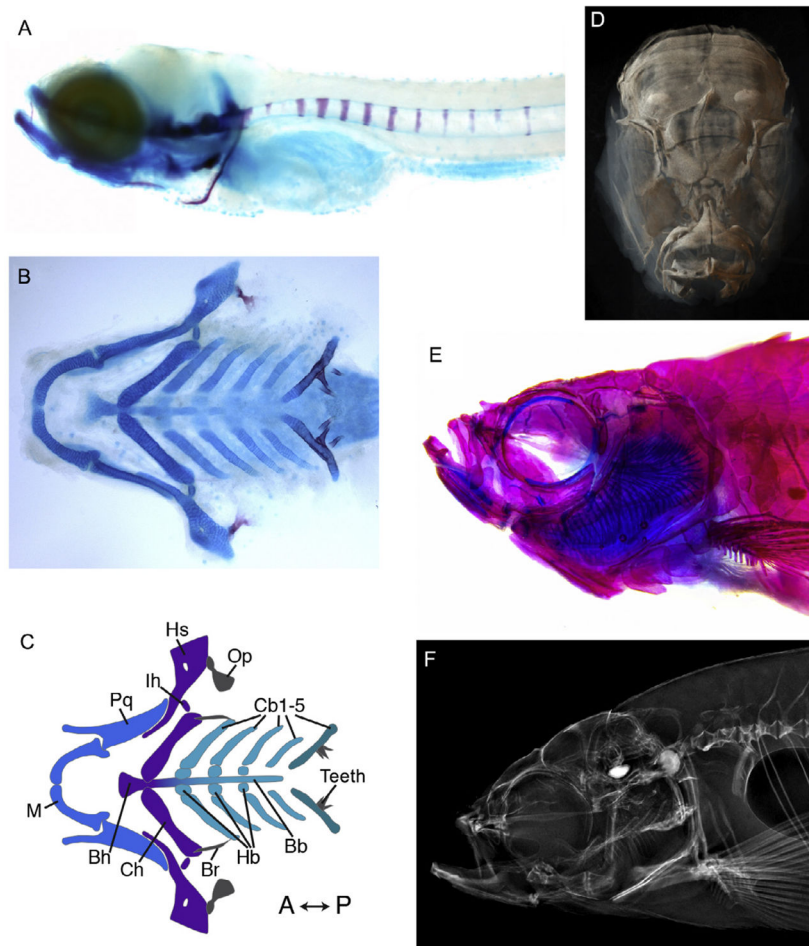


Figure 1. Zebrafish larval and adult craniofacial skeletons. (A) Larval zebrafish (6 dpf) stained with Alcian blue for cartilage and Alizarin red for mineralized bone reveals the early formation of the craniofacial skeleton. (B) Ventral view of an Alcian blue/Alizarin red-stained larval viscerocranium dissected away from the rest of the head, showing the skeletal derivatives of the seven pharyngeal arches. (C) Schematic of the larval viscerocranium. First-arch structures (blue): Pq, palatoquadrate; M, Meckel's; second-arch structures (purple): Hs, hyosymplectic; Ih, interhyal; Ch, ceratohyal; Bh, basihyal; Op, opercle (bone); Br, branchiostegal ray (bone); arches 3–7 (teal): Cb, ceratobranchial; Hb, hypobranchial; Bb, basibranchial. (D–F) The complex adult zebrafish craniofacial skeleton can be visualized in multiple modalities. (D) Micro-CT projection of an adult zebrafish head (frontal view). (F) Adult zebrafish head stained with Alcian blue and Alizarin red (lateral view). (E) X-ray of an adult zebrafish head (lateral view). Images not to scale. Anterior is to the left in A–C, E, and F. Panel (D) Image courtesy of S. Ruffins and Panel (E) photo courtesy of S. Paul.

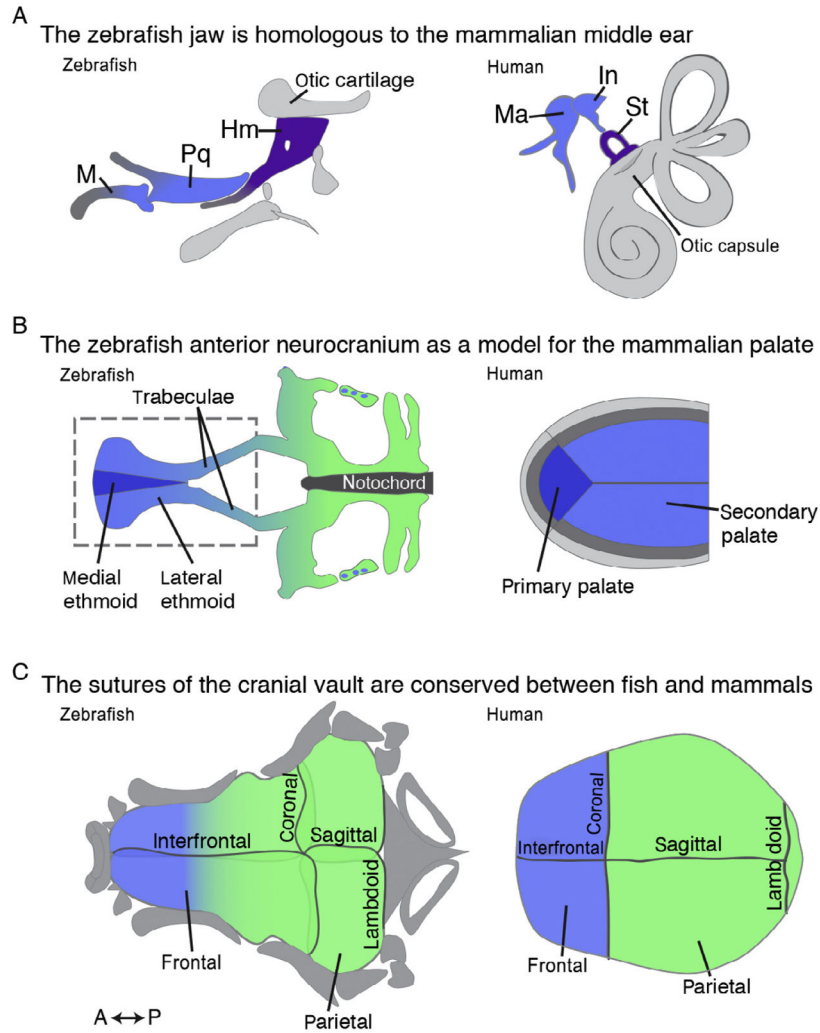


Figure 2.

Zebrafish models of mammalian craniofacial development. (A) The larval zebrafish jaw is evolutionarily homologous to the mammalian middle ear ossicles. The posterior parts of the first arch-derived Meckel's (M) and palatoquadrate (Pq) cartilages (blue), which articulate the jaw in fish, correspond to the malleus (Ma) and incus (In) of mammals. The second arch-derived hyomandibula (purple) (Hm; the dorsal component of the hyosymplectic), which affixes the viscerocranium to the neurocranium via the otic cartilage, is the homolog of the mammalian stapes (St). (B) The anterior end of the larval fish neurocranium is a model for the mammalian hard palate. The medial ethmoid plate (dark blue) derives from frontonasal NCCs, with the lateral ethmoid and trabeculae (light blue) formed by medially migrating anterior maxillary NCCs (left panel), akin to the origins of the primary and secondary hard palate, respectively, in mammals (right panel). The posterior zebrafish neurocranium derives from mesoderm (green). (C) The overlapping calvarial bones of the adult zebrafish form the same suture pattern as observed in mammals. The two frontal bones are separated from each other by the interfrontal (or metopic) suture and from the parietal bones by the bilateral coronal sutures. The parietals are divided along the midline by the sagittal suture and

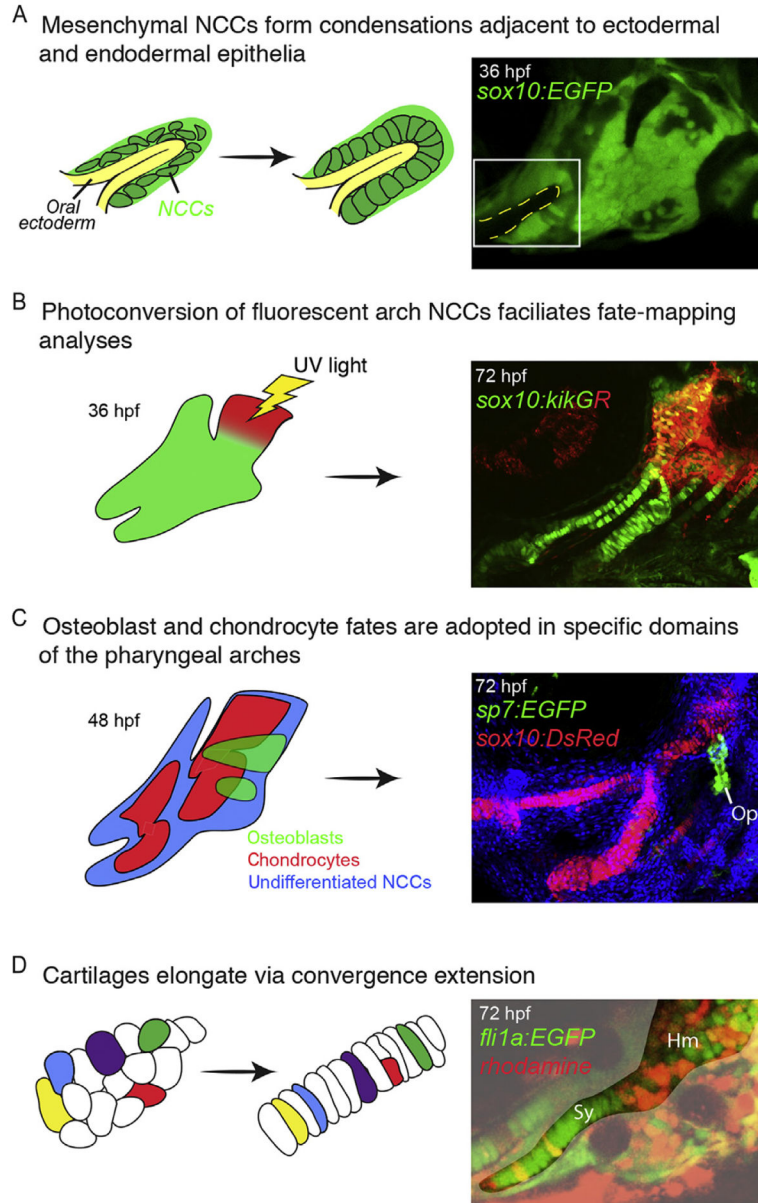
posteriorly bounded by the lambdoid suture. The boundary between neural crest (blue) and mesoderm (green) falls within the frontal bones in fish and along the coronal suture in mammals. Anterior is to the left.

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**Figure 3.**

Morphogenetic processes that shape the facial skeleton. (A) Mesenchymal NCCs form tight condensations next to ectodermal or endodermal epithelia, including the stomodeum (i.e., oral ectoderm). The *sox10:EGFP* transgene (green) is expressed by all NCCs. Boxed area shows NCCs condensed around the oral ectoderm (dashed line). (B) Fate-mapping analyses demonstrated that NCCs at certain locations within the arches reproducibly give rise to specific parts of facial skeletal elements. For example, arch NCCs in the dorsal half of the second arch make the hyomandibular portion of the hyosymplectic. One way to track cells is to use focused applications of ultraviolet light to convert fluorescent proteins such as *kikGR* from green to red in specific cell populations at early stages. The labeled cells are then reimaged at a later time point. (C) Following mesenchymal condensation, chondrocyte and osteoblast fates are adopted within specific domains of the pharyngeal arches around 48 hpf.

At 72 hpf, the *sp7:EGFP* transgene (green) is expressed in osteoblasts, while *sox10:DsRed* (red) is specifically expressed by chondrocytes. Op, opercle. Nuclei are labeled with Hoechst (blue). (D) Convergence-extension movements of differentiating prechondrocytes, rather than cell proliferation, drive the elongation of cartilages from bulky precartilaginous condensations into stacks of disc-shaped chondrocytes. The symplectic rod (Sy) lengthens anteriorly from the plate-like hyomandibula (Hm) in this manner. The right panel shows a *fli1a:EGFP* (green) transgenic embryo with mosaic rhodamine-dextran injection (red), illustrating the intercalation of dye-labeled cells into the elongating Sy.

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Table 1

Human Genetic Diseases with Craniofacial Abnormalities Modeled in Zebrafish

Human Disease	Zebrafish Homolog	Type of Model	References
Alagille syndrome	<i>jag1b</i>	Mutant	Zuniga et al. (2010)
Axenfeld–Rieger syndrome	<i>pitx2</i>	Morpholino	Bohnsack, Kasprick, Kish, Goldman, and Kahana (2012) and Liu and Semina (2012)
Auriculocondylar syndrome	<i>edn1</i>	Mutant	Miller et al. (2000)
	<i>furinA</i>	Mutant	Walker et al. (2006)
	<i>mef2ca</i>	Mutant	Miller et al. (2007)
	<i>plcb3</i>	Mutant	Walker et al. (2007)
Bamforth–Lazarus syndrome	<i>foxe1</i>	Morpholino	Nakada, Iida, Tabata, and Watanabe (2009)
Branchio-oculo-facial syndrome	<i>tfap2a</i>	Mutant, morpholino	Gestri et al. (2009)
Campomelic dysplasia	<i>sox9a, sox9b</i>	Mutants	Yan et al. (2002, 2005)
Cardiofaciocutaneous syndrome	<i>braf</i>	Injection of mutant mRNA	Anastasaki, Rauen, and Patton (2012)
CHARGE syndrome	<i>chd7</i>	Morpholino	Patten et al. (2012)
Cleft palate	<i>faj1</i>	Morpholino	Ghassibe-Sabbagh et al. (2011)
	<i>mir140, pdgfra</i>	miRNA injection; mutant	Eberhart et al. (2008)
	<i>tgfb3</i>	Morpholino	Cheah, Winkler, Jabs, and Chong (2010)
	<i>smad5</i>	Mutant	Swartz et al. (2011)
	<i>tgfb2, fgf10a</i>	Morpholino	Swartz et al. (2011)
	<i>wwp2, med25</i>	Morpholino	Nakamura et al. (2011)
	<i>crispld2</i>	Morpholino	Yuan et al. (2012)
	<i>irf6</i>	Dominant-negative transgene	Dougherty et al. (2013)
Cleidocranial dysplasia	<i>runx2b</i>	Morpholino	Flores, Lam, Crosier, and Crosier (2006)
Cornelia de Lange syndrome	<i>nipbla/nipblb</i>	Morpholino	Muto, Calof, Lander, and Schilling (2011)
Coronal craniosynostosis	<i>cyp26b1</i>	Mutant	Laue et al. (2011)
Cranio-lenticulo-sutural dysplasia	<i>sec23a</i>	Mutant	Lang, Lapierre, Frotscher, Goldenring, and Knapik (2006)
Diamond–Blackfan anemia	<i>rpl11</i>	Mutant	Danilova, Sakamoto, and Lin (2011) and Zhang et al. (2014)
	<i>rps19</i>	Mutant	Zhang et al. (2014)
	<i>rps24</i>	Morpholino	Song et al. (2014)
DiGeorge syndrome	<i>tbx1</i>	Mutant	Piotrowski et al. (2003) and Zhang, Gui, Wang, Jiang, and Song (2010)
			Choudhry and Trede (2013) and Choe and Crump (2014)
Frontonasal dysplasia	<i>alx1</i>	Morpholino	Dee, Szymoniuk, Mills, and Takahashi (2013)
Hereditary multiple exocytoses	<i>ext2, slc35b2</i>	Mutants	Clement et al. (2008)
Holoprosencephaly	<i>shha, smo, disp1</i>	Mutants	Brand et al. (1996), Kimmel et al. (2001), and Wada et al. (2005)
	<i>dzip1, gli1, gli2</i>		Eberhart et al. (2006) and Schwend and Ahlgren (2009)

Human Disease	Zebrafish Homolog	Type of Model	References
	<i>six3</i>	Injection of mutant mRNA	Geng et al. (2008)
Jeune and Mainzer-Saldino syndromes	<i>ift172</i>	Morpholino	Halbritter et al. (2013)
Leri–Weill dyschondrosteosis	<i>shox</i>	Morpholino	Sawada, Kamei, Hakuno, Takahashi, and Shimizu (2015)
Meier–Gorlin syndrome	<i>orc1</i>	Morpholino	Bicknell et al. (2011)
Noonan syndrome	<i>raf1</i>	Morpholino	Razzaque et al. (2007)
	<i>kras</i>	Injection of mutant mRNA	Razzaque et al. (2012)
	<i>ptpn11a, ptpn11b</i>	Mutant	Bonetti et al. (2014)
Oblique facial clefts	<i>specc11</i>	Morpholino	Saadi et al. (2011) and Gfrerer et al. (2014)
Oculofaciocardiodental syndrome	<i>bcor</i>	Morpholino	Ng et al. (2004)
Osteogenesis imperfecta	<i>sec24d</i>	Mutant	Sarmah et al. (2010) and Garbes et al. (2015)
Oro-facial-digital type 1 syndrome	<i>ofdl</i>	Morpholino	Ferrante et al. (2009)
Pierre Robin Sequence	<i>satb2 cis-regulatory</i>	Transgenic enhancer lines	Rainger et al. (2014)
	<i>sox9a cis-regulatory</i>	Transgenic enhancer lines	Gordon et al. (2014)
Richieri–Costa–Pereira syndrome	<i>eif4a3</i>	Morpholino	Favaro et al. (2014)
Roberts syndrome	<i>esco2</i>	Morpholino	Monnich, Kuriger, Print, and Horsfield (2011)
Robinow syndrome	<i>wnt5a</i>	Morpholino	Huang et al. (2014)
Shprintzen–Goldberg syndrome	<i>skia, skib</i>	Morpholinos	Doyle et al. (2012)
Simpson–Golabi–Behmel syndrome	<i>gpc4</i>	Mutant	Wiweger et al. (2011)
Stickler/Marshall syndrome	<i>coll11a1a/b</i>	Morpholino	Baas, Malbouyres, Haftek-Terreau, Le Guellec, and Ruggiero (2009)
Treacher–Collins syndrome	<i>tcofl</i>	Morpholino	Weiner, Scampoli, and Calcaterra (2012)
X-linked mental retardation	<i>phf8</i>	Morpholino	Qi et al. (2010)