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REVIEW

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The power of zebrafish models for understanding the co-occurrence of craniofacial and limb disorders

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Summary

Craniofacial and limb defects are two of the most common congenital anomalies in the general population. Interestingly, these defects are not mutually exclusive. Many patients with craniofacial phenotypes, such as orofacial clefting and craniosynostosis, also present with limb defects, including polydactyly, syndactyly, brachydactyly, or ectrodactyly. The gene regulatory networks governing craniofacial and limb development initially seem distinct from one another, and yet these birth defects frequently occur together. Both developmental processes are highly conserved among vertebrates, and zebrafish have emerged as an advantageous model due to their high fecundity, relative ease of genetic manipulation, and transparency during development. Here we summarize studies that have used zebrafish models to study human syndromes that present with both craniofacial and limb phenotypes. We discuss the highly conserved processes of craniofacial and limb/fin development and describe recent zebrafish studies that have explored the function of genes associated with human syndromes with phenotypes in both structures. We attempt to identify commonalities between the two to help explain why craniofacial and limb anomalies often occur together.

KEYWORDS
craniofacial, human clinical genetics, limb, zebrafish model

1 | INTRODUCTION

Structural birth defects, including craniofacial and limb anomalies, affect 3% of newborns in the United States (Update on Overall Prevalence of Major Birth Defects, 2008). Human craniofacial defects occur in 1 in every 500–1,000 live births, with orofacial clefts being the most common (1:700) (Byvaltsev, Belykh, & Belykh, 2012; Global registry and database on craniofacial anomalies, 2001), while congenital limb disorders affect 1 in 1,000–2,000 newborns (Vasluian et al., 2013; Wilcox, Coulter, & Schmitz, 2015). Interestingly, these anomalies are not mutually exclusive. Many patients with craniofacial anomalies also present with upper and/or lower limb defects, such as syndactyly (digit fusion), ectrodactyly (missing digits), polydactyly (extra digits), and/or brachydactyly (shortening of the hands/feet). Syndromes that present with defects in both structures include Apert syndrome (MIM #101200), Pfeiffer syndrome (MIM #101600), Roberts syndrome (MIM #268300), and Saethre-Chotzen syndrome (MIM #101400) among others (Table 1). This may be due to the synchronous timing of their development (Panthaki & Armstrong, 2003). Others have proposed that paired limbs/fins are evolved from gill or pharyngeal arch skeletal elements, components of which give rise to the craniofacial skeleton (Gegenbaur, 1878). These studies suggest that there is a deep homology between the two structures. Many published reviews have thoroughly discussed vertebrate craniofacial or limb development separately and described how genetic or environmental factors can lead to congenital defects (Mercader, 2007; Mork & Crump, 2015; Petit, Sears, & Ahituv, 2017; Szabo-Rogers, Smithers, Yakob, & Liu, 2010; Twigg & Wilkie, 2015; A. Zuniga, 2015); however, to our knowledge, no one has adequately explored how or why craniofacial and limb anomalies often occur together.

TABLE 1 Human congenital disorders with both craniofacial and limb defects modeled in zebrafish

Syndrome	Human gene	Inheritance pattern	Major human craniofacial/ limb phenotypes	Zebrafish ortholog	Model	Zebrafish craniofacial phenotypes	Zebrafish pectoral fin phenotypes	Experimental assays	Reference
Achondroplasia (MIM #100800)	*FGFR3*[b]	AD	Macrocephaly; prominent forehead; brachydactyly; trident hand; bowed legs	*fgfr3*	CRISPR-Cas9 LOF mutant	No phenotype	No phenotype	RT-qPCR; RNA ISH; skeletal staining	(Leerberg, Hopton, & Draper, 2019)
Acrofacial dysostosis, Cincinnati type (MIM #616462)	*POLR1A*	AD	Variable mandibulofacial dysostosis; micrognathia; upper/ lower eyelid clefts; microcephaly; brachydactyly	*polr1a*	LOF mutant	Hypoplastic/deformed: Meckel's cartilage; ceratohyal; trabeculae Absent: Ethmoid plate; hyosymplectic cartilage; ceratobranchial arches; palatoquadrate	Hypoplastic: Pectoral fin[d]	RNA ISH; immunostaining; TUNEL assay; live-cell imaging; skeletal staining	(Weaver et al., 2015)
Acromelic frontonasal dysostosis (MIM #603671)	*ZSWIM6*	AD	Frontonasal dysplasia; nasal clefting; carp-shaped mouth; preaxial polydactyly; tibial hypoplasia/aplasia	*zswim6*	N/A	N/A	N/A	RNA-FISH	(Smith et al., 2014)
Alagille syndrome 1 (MIM #118450)	*JAG1*	AD	Broad, prominent forehead; deep, wide, low-set eyes; mandibular prognathia; brachydactyly	*jag1a/ jag1b*	MO	Subtle midbrain and forebrain defects Hypoplastic: Ears	N/A	RNA ISH; skeletal staining	(Lorent et al., 2004)
					LOF mutant	Hypoplastic/deformed: Palatoquadrate; hyosymplectic cartilage; opercle bone	Hypoplastic: Pectoral fin[d]	RNA ISH; RNA-FISH; skeletal staining; tissue transplants	(E. Zuniga, Stellabotte, & Crump, 2010)
					Inducible GOF Tol2 mutant	Deformed: Meckel's cartilage; ceratohyal; hyosymplectic cartilage Ectopic cartilage near palatoquadrate and midline	N/A		
Alagille syndrome 2 (MIM #610205)	*NOTCH2*	AD	Broad, prominent forehead; deep, wide, low-set eyes; mandibular prognathia; brachydactyly	*notch2*	MO	N/A	N/A	RNA ISH	(Lorent et al., 2004)
					MO	Hypoplastic/deformed: Palatoquadrate; hyosymplectic cartilage; opercle bone Ectopic cartilage within mandibular and hyoid arches	N/A	RNA ISH; RNA-FISH; skeletal staining; tissue transplants	(E. Zuniga et al., 2010)
Andersen-Tawil syndrome (MIM #170390)	*KCNJ2*	AD	Broad forehead; micrognathia; wide-set eyes; low-set ears; syndactyly; clinodactyly	*kcnj2a/ kcnj2b*	DN mRNA OE	Deformed: Palatoquadrate; hyosymplectic cartilage; ceratohyal Wide-set eyes; protruding jaw	Hypoplastic: Pectoral fin[d]	RNA ISH; RT-qPCR; skeletal staining	(Leong, Skinner, Shelling, & Love, 2013)

TABLE 1 (Continued)

Syndrome	Human gene	Inheritance pattern	Major human craniofacial/limb phenotypes	Zebrafish ortholog	Model	Zebrafish craniofacial phenotypes	Zebrafish pectoral fin phenotypes	Experimental assays	Reference
Apert syndrome (MIM #101200)	*FGFR2*[a]	AD	Craniosynostosis; broad forehead; wide-set, bulging eyes; orofacial cleft; midface hypoplasia; syndactyly	*fgfr2*	MO	Hypoplastic/deformed: Meckel's cartilage; ceratohyal; hyosymplectic cartilage; ceratobranchial arches Absent: Ceratobranchial arches	Hypoplastic: Pectoral fin[d]	RNA ISH; RNA-FISH; skeletal staining	(Larbuisson, Dalcq, Martial, & Muller, 2013)
					CRISPR-Cas9 LOF mutant	No phenotype	No phenotype	RT-qPCR; RNA ISH; skeletal staining	(Leerberg et al., 2019)
					CRISPR-Cas9 LOF triple mutant (*fgfr1a−/−; fgfr1b−/−; fgfr2 −/−*)	Hypoplastic/deformed: Meckel's cartilage; palatoquadrate; hyosymplectic cartilage; ethmoid plate Absent: Ceratobranchial arches	Absent: Pectoral fin		
Bent bone dysplasia syndrome (MIM #614592)	*FGFR2*[a]	AD	Craniosynostosis; large, wide-set eyes; low-set ears; micrognathia; midface hypoplasia; brachydactyly	*fgfr2*	See above.	See above.	See above.	See above.	See above.
Camptodactyly, tall stature, and hearing loss syndrome (CATSHLS) (MIM# 610474)	*FGFR3*[b]	AD	Microcephaly; elevated palate; camptodactyly (bent fingers)	*fgfr3*	See above.	See above.	See above.	See above.	See above.
Carpenter syndrome 1 (MIM #201000)	*RAB23*	AR	Craniosynostosis; flat nasal bridge; low-set ears; micrognathia; downward slanting eyes; brachydactyly; syndactyly; polydactyly	*rab23*	MO; constitutively active mRNA OE	N/A	N/A	RNA ISH	(Fuller, O'Connell, Gordon, Mauti, & Eggenschwiler, 2014)
Carpenter syndrome 2 (MIM #614976)	*MEGF8*	AR	Craniosynostosis; flat nasal bridge; low-set ears; micrognathia; downward slanting eyes; brachydactyly; syndactyly or polydactyly	*megf8*	MO	N/A	N/A	N/A	(Twigg et al., 2012)
Cornelia de Lange syndrome I (MIM #122470)	*NIPBL*	AD	Microcephaly; arched eyebrows that grow toward midline; low-set ears; micrognathia; small hands/feet; ectrodactyly; syndactyly in toes	*Nipbla/ nipblb*	MO (*nipblb*)	N/A	N/A	RNA ISH; TUNEL assay; IHC; RT-qPCR	(Pistocchi et al., 2013)
					MO (*nipbla/ nipblb*)	N/A	Hypoplastic: Pectoral fin	RNA ISH	(Kawauchi et al., 2016)

(Continues)

TABLE 1 (Continued)

Syndrome	Human gene	Inheritance pattern	Major human craniofacial/ limb phenotypes	Zebrafish ortholog	Model	Zebrafish craniofacial phenotypes	Zebrafish pectoral fin phenotypes	Experimental assays	Reference
Ectrodactyly-ectodermal dysplasia-Clefting syndrome 3 (EEC3) (MIM #604292)	*TP63*	AD	Ectodermal dysplasia; Orofacial cleft; ectrodactyly	*tp63*	MO	N/A	Absent: Fin fold; pectoral fin	RT-qPCR; RNA ISH; IHC; BrdU assay; TUNEL assay	(Lee & Kimelman, 2002)
					MO	N/A	Absent: Fin fold; pectoral fin	Transactivation assay; EMSA assay; RNA ISH	(Bakkers, Hild, Kramer, Furutani-Seiki, & Hammerschmidt, 2002)
					LOF CRISPR-Cas9 mutant	N/A	Hypoplastic: Fin fold Absent: Pectoral fin	RNA-seq; ChIPmentation; ATAC-seq	(Santos-Pereira, Gallardo-Fuentes, Neto, Acemel, & Tena, 2019)
Greig cephalopolysyndactyly syndrome (MIM #175700)	*GLI3*	AD	Macrocephaly; bulging forehead; broad nasal bridge; wide-set eyes; polydactyly; syndactyly; wide thumb or big toe	*gli3*	MO	N/A	N/A	RNA ISH; IHC	(Tyurina et al., 2005)
Hypochondroplasia (MIM #146000)	*FGFR3*[b]	AD	Macrocephaly; brachydactyly; bowed legs; shortened limbs	*fgfr3*	See above.	See above.	See above.	See above.	See above.
Jackson-Weiss syndrome (MIM #123150)	*FGFR2*[a]	AD	Craniosynostosis; wide-set eyes; bulging forehead; brachydactyly and/or syndactyly of toes only	*fgfr2*	See above.	See above.	See above.	See above.	See above.
Kabuki syndrome I (MIM #147920)	*KMT2D*	AD	Orofacial cleft; microcephaly; micrognathia; wide-set, upward slanting eyes; depressed nose; shortened pinky finger	*kmt2d*	CRISPR-Cas9 mutant	Hypoplastic/deformed: Ethmoid plate; trabeculae; palatoquadrate Absent: Meckel's cartilage, ceratobranchial arches; ceratohyal; hyosymplectic cartilage	Hypoplastic/ deformed: Pectoral fin[d]	RNA-seq; RT-qPCR; IHC; skeletal staining; drug treatment	(Serrano, Demarest, Tone-Pah-Hote, Tristani-Firouzi, & Yost, 2019)
Kabuki syndrome II (MIM #300867)	*KDM6A*	XLD	Orofacial cleft; microcephaly; micrognathia; wide-set, upward slanting eyes; depressed nose; shortened pinky finger	*kdm6a/ kdm6al*	MO	Hypoplastic/deformed: Ceratobranchial arches; Meckel's cartilage; ceratohyal (*kdm6a* only)	Hypoplastic: Pectoral fin (*kdm6a* only)[d]	Skeletal staining; H&E stain; IHC; live-cell imaging	(Van Laarhoven et al., 2015)
Lacrimo-auriculo-dento-digital syndrome (MIM #149730)	*FGFR2*[a]	AD	Hypoplasia/aplasia of lacrimal system; orofacial cleft (variable); low-set ears; polydactyly, syndactyly, and/or ectrodactyly	*fgfr2*	See above.	See above.	See above.	See above.	See above.
	FGFR3[b]	AD		*fgfr3*	See above.	See above.	See above.	See above.	See above.

TABLE 1 (Continued)

Syndrome	Human gene	Inheritance pattern	Major human craniofacial/ limb phenotypes	Zebrafish ortholog	Model	Zebrafish craniofacial phenotypes	Zebrafish pectoral fin phenotypes	Experimental assays	Reference
Muenke syndrome (MIM #602849)	*FGFR3*[b]	AD	Craniosynostosis; wide-set, bulging eyes; macrocephaly; elevated palate; brachydactyly;	*fgfr3*	See above.	See above.	See above.	See above.	See above.
Oral-facial-digital syndrome I (MIM #311200)	*OFD1*	XLD	Orofacial cleft; split tongue; broad nose; wide-set eyes; micrognathia; brachydactyly, syndactyly, clinodactyly	*ofd1*	MO	Deformed: Meckel's cartilage	N/A	RT-qPCR; RNA ISH; IHC; live-cell imaging; skeletal staining; microarray; bead & dextran injections	(Ferrante et al., 2009)
Pfeiffer syndrome (MIM #101600)	*FGFR1*	AD	Craniosynostosis; bulging, wide-set eyes; high forehead; micrognathia; beaked nose; brachydactyly; syndactyly	*fgfr1a/ fgfr1b*	MO (*fgfr1a*)	Hypoplastic: Meckel's cartilage Absent: Ceratobranchial arches	Hypoplastic: Pectoral fin[d]	RNA ISH; RNA FISH; skeletal staining	(Larbuisson et al., 2013)
					Inducible DN mutant (*fgfr1a*)	Hypoplastic/deformed: Meckel's cartilage; ceratohyal; ceratobranchial arches; hyosymplectic cartilage Absent: Meckel's cartilage; ceratohyal; ceratobranchial arches; hyosymplectic cartilage	Hypoplastic: Pectoral fin[d] Absent: Pectoral fin[d]		
					CRISPR-Cas9 LOF single mutant	No phenotype	No phenotype	RNA ISH; skeletal staining	(Leerberg et al., 2019)
					CRISPR-Cas9 LOF double mutants (*fgfr1a* −/−; *fgfr1b* −/−)	Deformed/hypoplastic: Meckel's cartilage; ceratohyal; hyosymplectic cartilage; ethmoid plate Absent: Ceratobranchial arches	Absent: Pectoral fin		
	FGFR2[a]	AD		*fgfr2*	See above.	See above.	See above.	See above.	See above.
Popliteal pterygium syndrome (MIM #119500)	*IRF6*[c]	AD	Orofacial cleft; micrognathia; thin upper lip; syndactyly	*irf6*	N/A	N/A	N/A	RNA ISH	(Ben, Jabs, & Chong, 2005)
					MO	No phenotype	No phenotype	RT-qPCR; RNA ISH; skeletal staining; TUNEL assay	(Sabel et al., 2009)
					DN mRNA OE	Hypoplastic: Meckel's cartilage; ceratohyal; palatoquadrate; ceratobranchial arches; hyosymplectic	Hypoplastic: Pectoral fin		
					DN Tol2 mutant	Hypoplastic/deformed: Ethmoid plate; Meckel's cartilage; palatoquadrate	N/A	RNA ISH; IHC; EdU assay; TUNEL assay; live-cell imaging; skeletal staining	(Dougherty et al., 2013)

(Continues)

TABLE 1 (Continued)

Syndrome	Human gene	Inheritance pattern	Major human craniofacial/limb phenotypes	Zebrafish ortholog	Model	Zebrafish craniofacial phenotypes	Zebrafish pectoral fin phenotypes	Experimental assays	Reference
Roberts syndrome (MIM #268300)	*ESCO2*	AR	Orofacial cleft; micrognathia; downward slanting, wide-set eyes; beaked nose; microcephaly; hypoplastic limbs; syndactyly; ectrodactyly; joint deformities	*esco2*	MO	Hypoplastic/deformed: Ceratohyal; ceratobranchial arches Absent: Ceratobranchial arches	Hypoplastic/deformed: Pectoral fin	Microarray; RNA ISH; skeletal staining; BrdU assay; TUNEL assay; IHC; RT-qPCR; Caspase assay	(Monnich, Kuriger, Print, & Horsfield, 2011)
					MO; CRISPR-Cas9 LOF mutant	N/A	N/A	Acridine orange stain; IHC; Western blot; transgene mRNA injections	(Percival et al., 2015)
Robinow syndrome (MIM #180700)	*WNT5A*	AD	Prominent forehead; small midface; wide-set, prominent eyes; short nose; brachydactyly	*wnt5a*	mRNA OE	N/A	N/A	RNA ISH	(Person et al., 2010)
Saethre-Chotzen syndrome (MIM #101400)	*FGFR2*[a]	AD	Craniosynostosis; maxillary hypoplasia; high forehead; wide-set eyes; ptosis; broad nasal bridge; brachydactyly; syndactyly; pre-axial polydactyly	*fgfr2*	See above.	See above.	See above.	See above.	See above.
	FGFR3[b]	AD		*fgfr3*	See above.	See above.	See above.	See above.	See above.
	TWIST1	AD		*twist1a/ twist1b*	MO (*twist1a; twist1b*)	Absent: Meckel's cartilage; ceratohyal; ceratobranchial arches; palatoquadrate; hyosymplectic cartilage; ethmoid plate; trabeculae	Absent: Pectoral fin[d]	RNA ISH	(Das & Crump, 2012)
					DN Tol2 mutant (*twist1b*)	Hypoplastic: Branchial arches Absent: Meckel's cartilage; ceratohyal; hyosymplectic cartilage; palatoquadrate	No phenotype[d]		
					LOF TALENs mutants	Hypoplastic/deformed: Meckel's cartilage; hyosymplectic cartilage; palatoquadrate (*twist1a−/−; twist1b−/−*) Absent: Coronal suture (*tcf12 −/−;twist1b−/−*)	N/A	Skeletal staining; live-cell imaging; BrdU assay; RNAscope ISH	(Teng et al., 2018)
					N/A	N/A	N/A	RNA ISH; transgenic enhancer assay	(Hirsch et al., 2018)
Thanatophoric dysplasia I (MIM #187600)	*FGFR3*[b]	AD	Macrocephaly; low nasal bridge; brachydactyly; syndactyly; shortened limbs; curved thigh bones	*fgfr3*	See above.	See above.	See above.	See above.	See above.
Thanatophoric dysplasia II (MIM #187601)	*FGFR3*[b]	AD	Cloverleaf skull; macrocephaly; brachydactyly; shortened limbs	*fgfr3*	See above.	See above.	See above.	See above.	See above.

TABLE 1 (Continued)

Syndrome	Human gene	Inheritance pattern	Major human craniofacial/limb phenotypes	Zebrafish ortholog	Model	Zebrafish craniofacial phenotypes	Zebrafish pectoral fin phenotypes	Experimental assays	Reference
Van der Woude syndrome 1 (MIM #119300)	*IRF6*[c]	AD	Orofacial cleft; lower lip pits and/or sinuses; hypodontia; syndactyly	*irf6*	See above.	See above.	See above.	See above.	See above.
Van der Woude syndrome II (MIM #606713)	*GRHL3*	AD	Orofacial cleft; lower lip pits and/or sinuses; hypodontia; syndactyly	*grhl3*	MO	Hypoplastic/deformed: Ceratohyal; ceratobranchial arches; palatoquadrate; Meckel's cartilage; ethmoid plate, Absent: Ceratobranchial arches	Hypoplastic: Pectoral fin[d]	RNA ISH; skeletal staining: electron microscopy; TUNEL assay; micro-ChIP assay; EdU assay;	(Dworkin et al., 2014)
					DN mRNA OE	N/A	N/A	RNA ISH	(Peyrard-Janvid et al., 2014)
					MO; LOF CRISPR-Cas9 mutant	N/A	N/A	IHC	(Miles et al., 2017)

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; ChIP, chromatin immunoprecipitation; DN, dominant negative; EMSA, electrophoresis mobility shift assay; GOF, gain-of-function; H&E, hematoxylin and eosin; IHC, immunohistochemistry; ISH, in situ RNA hybridization; LOF, loss-of-function; MIM, Mendelian Inheritance in Man number; MO, morpholino; N/A, not applicable; OE, overexpression; RNA-FISH, RNA fluorescent in situ hybridization; RT-qPCR, real-time quantitative polymerase chain reaction; TALENs, transcription activator-like effector nucleases; XLD, X-linked dominant.

[a]*FGFR2* variants are thought to cause nonspecific craniosynostosis and are associated with multiple different craniofacial syndromes.

[b]*FGFR3* variants are associated with multiple different craniofacial syndromes.

[c]*IRF6* variants are associated with both popliteal pterygium syndrome and Van der Woude syndrome.

[d]Our interpretation of the structure of the pectoral fin based on the Alcian blue skeletal staining images provided in the article.

In this review, we analyze syndromes that present with both craniofacial and limb defects, and we focus specifically on those that have been studied using a zebrafish model. Zebrafish serve as a useful developmental model to study these syndromes because they are relatively easy to genetically modify, inexpensive to maintain, reproduce and develop quickly, and the transparency of the embryo allows for live imaging *in vivo*. The zebrafish skeleton is also simpler, with fewer structural elements and larval cartilages are only a few cell layers thick, making them easier to visualize. Its pectoral and pelvic fins are homologous to mammalian forelimbs and hindlimbs, respectively (Grandel & Schulte-Merker, 1998). Most of the gene regulatory networks involved in both craniofacial and limb/fin development are highly conserved between zebrafish and mammals. The information obtained from zebrafish studies can provide valuable insight into human development. First, we will very briefly describe the processes governing craniofacial and limb/fin development as a preface. Then we will highlight recent studies that have used zebrafish to uncover the mechanism(s) by which certain genes cause syndromes with craniofacial and limb defects. We hope to identify common themes and mechanisms that can help explain why these two defects often occur together.

2 | BRIEF OVERVIEW OF CRANIOFACIAL AND LIMB DEVELOPMENT

2.1 | Craniofacial development

Much of the facial skeleton is derived from cranial neural crest cells. Neural crest cells (NCCs) are a multipotent population of cells that originate at the dorsal most part of the neural tube. During development, NCCs undergo an epithelial-mesenchymal transition to delaminate from the neural tube, migrate to different regions of the body, and differentiate into specific cell types. Cranial NCCs are a subpopulation of NCCs that migrate from the neural tube into the pharyngeal arches and the facial prominences. They interact with head mesoderm in response to signals from the ectoderm and endoderm to differentiate into cartilage, bone, cranial neurons, glia, and connective tissues to form the frontonasal skeleton, jaw, odontoblasts of the teeth, middle ear, glia, and cranial neurons.

The skull is divided into two structures, the viscerocranium and neurocranium. The viscerocranium forms the lower part of the skull and supports the structure of the face. It is comprised entirely of NCCs from the pharyngeal arches (Kague et al., 2012; Morriss-Kay, 2001). Each pharyngeal arch gives rise to different structures. The first arch forms the lower jaw (mandibular domain) and the palate (maxillary domain); the second arch (hyoid) forms the ceratohyal and hyomandibular bones, which connect the lower jaw to the neurocranium; and in zebrafish, the third through the seventh arches give rise to the ceratobranchial cartilages, which support the gill tissues (Schilling & Kimmel, 1997) (Figure 1a,c). In mammals, these posterior arches form laryngeal cartilage. Interestingly, parts of the zebrafish jaw and hyoid skeleton are evolutionarily homologous to the mammalian middle ear bones. More specifically, the Meckel's cartilage and

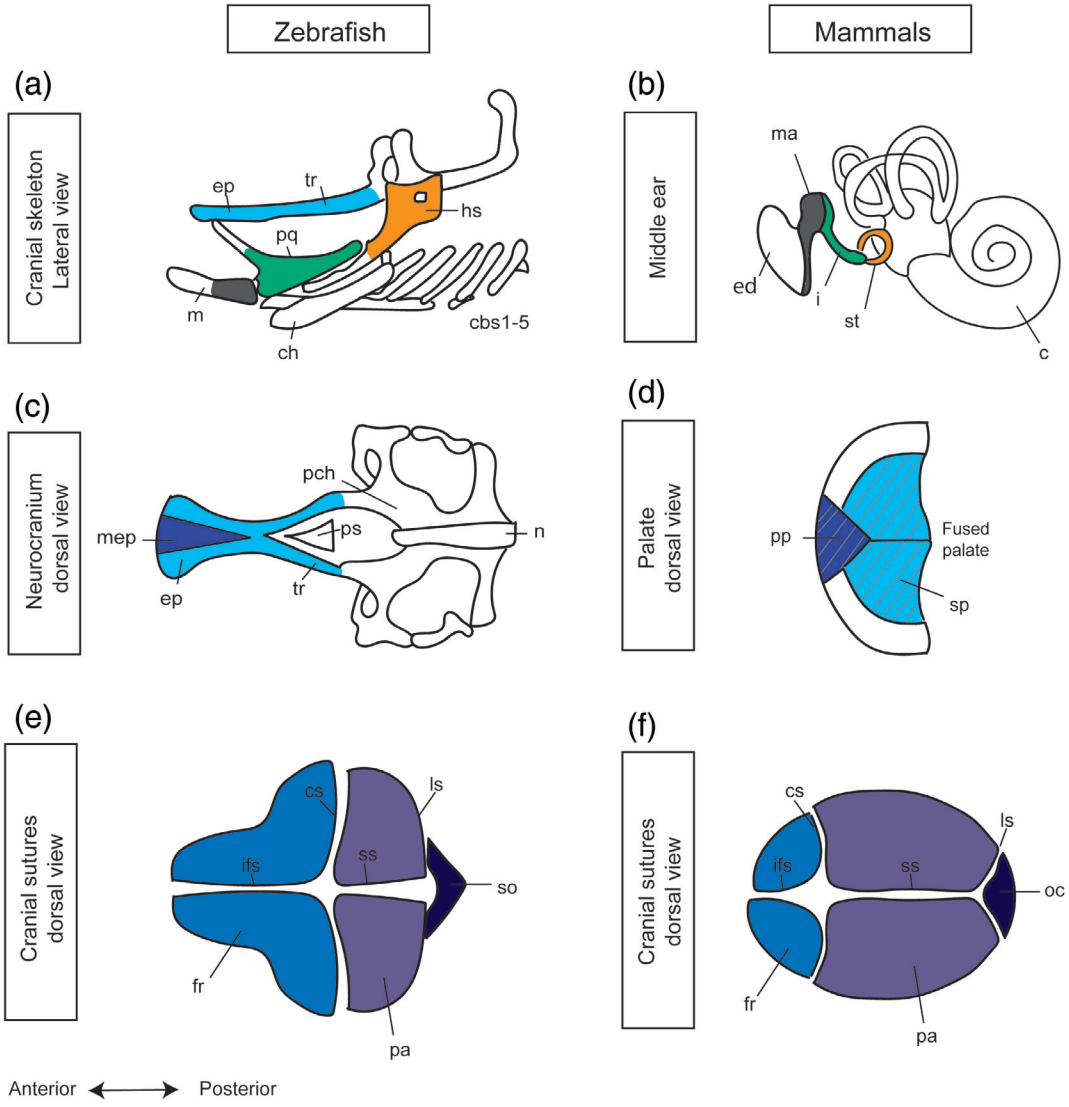

FIGURE 1 Homologous craniofacial structures between zebrafish and mammals. (a) Lateral view of zebrafish head skeleton. The posterior end of the Meckel's cartilage, the palatoquadrate and hyosymplectic cartilage are homologous to structures in the mammalian middle ear, particularly the malleus, incus, and stapes bone (b). (c) Dorsal view of the zebrafish neurocranium, which is analogous to the mammalian palate (d). (e,f) Dorsal view of the zebrafish and mammalian cranial sutures. Homologous structures have the same color, and analogous structures have added hashmarks. Adapted from Mork and Crump (2015). c, cochlea; cbs, ceratobranchial arches #1–5; ch, ceratohyal; cs, coronal sutures; ed, ear drum; ep, ethmoid plate; fr, frontal bones; hs, hyosymplectic cartilage; i, incus; ifs, interfrontal sutures; ls, lambdoid sutures; m, Meckel's cartilage; ma, malleus; mep, medial ethmoid plate; n, notochord; oc, occipital bone; pa, parietal bones; pch, parachordal; pp, primary palate; pq, palatoquadrate; ps, parasphenoid; so, supraoccipital bone; sp, secondary palate; ss, sagittal sutures; st, stapes bone; tr, trabecula

part of the palatoquadrate are homologous to the malleus and incus of the middle ear, while the hyomandibula portion of the hyosymplectic cartilage is homologous to the stapes bone (Figure 1a, b) [reviewed in Anthwal, Joshi, & Tucker, 2012, Mork & Crump, 2015]. Molecular mechanisms governing lower jaw development have been well-studied in both mice and zebrafish. For a more detailed review, we refer to Clouthier, Garcia, and Schilling (2010) and Santagati and Rijli (2003). One of the most critical factors in lower jaw development is endothelin-1 (Edn1) signaling through the endothelin-A receptor (Ednra) (schematic in Figure 2a). In zebrafish, loss of *edn1* leads to a severe truncation of the Meckel's cartilage, a loss of the ceratohyal bone, and a homeotic transformation of the lower jaw to an upper jaw (Kimmel, Ullmann, Walker, Miller, & Crump, 2003; Miller, Schilling, Lee, Parker, & Kimmel, 2000). Similar phenotypes are observed in *Edn1*−/− and *Ednra*−/− mice, suggesting that their roles in craniofacial development are conserved (Clouthier et al., 1998; Kurihara et al., 1994). Endothelin signaling is required for downstream expression of Dlx5/Dlx6, as well as Dlx3 and Hand2 in the ventral most part of the pharyngeal arch. The *dlx* homeobox genes are important for establishing the dorsal/ventral axis in craniofacial development and are further regulated by Mef2c (Miller et al., 2007). *nkx3.2* (*bapx1*) is an additional homeobox gene that regulates genes involved in jaw joint formation. Its expression is limited to the intermediate region of the pharyngeal arch, and it is repressed by Hand2 in

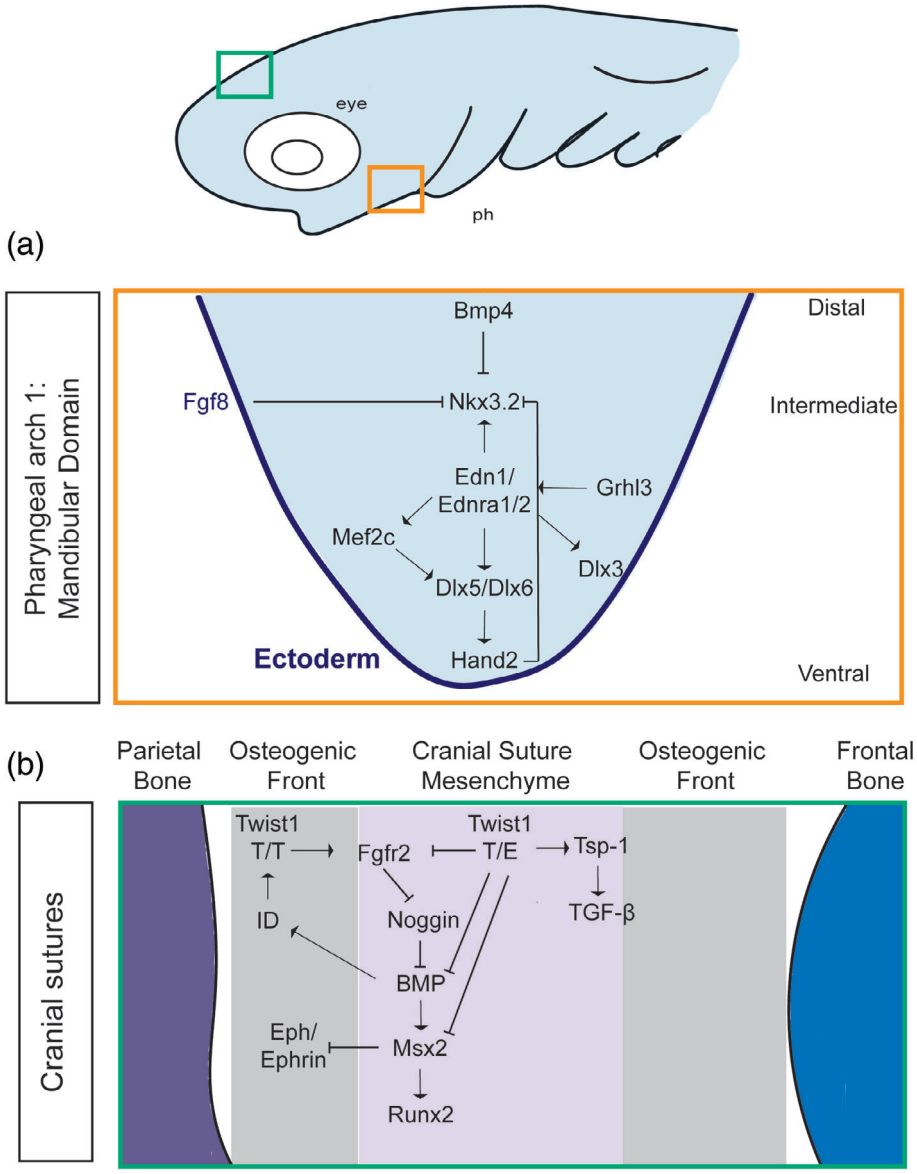

FIGURE 2 Molecular mechanisms governing lower jaw and cranial suture development. (a) Simplified schematic of endothelin-1 signaling in the first pharyngeal arch during lower jaw development. Endothelin signaling is required for downstream expression of Dlx homeobox genes, which are required for dorsal/ventral patterning, and Hand2, which represses Nkx3.2 in the ventral region of the pharyngeal arch. Nkx3.2 is also regulated by Bmp4 in the most dorsal part of the arch and Fgf8 in the ectoderm. (b) Schematic of genes and pathways involved in cranial suture development. Twist1 is capable of forming homodimers (T/T) and heterodimers with E proteins (T/E). These two forms act antagonistically with one another to regulate FGFR2 activity, downstream BMP signaling, osteogenic differentiation, and suture closure. ID, inhibitor of DNA binding; ph, pharyngeal arch; T/E, Twist1 heterodimer; T/T, Twist1 homodimer

the ventral region, Fgf8 in the oral epithelium, and Bmp4 in the distal pharyngeal arch (Miller, Yelon, Stainier, & Kimmel, 2003; Miyashita et al., 2020; Wilson & Tucker, 2004). *DLX5/DLX6* and *NKX3.2* are also involved in human limb development. Variants are associated with split hand/foot malformation (MIM #183600) and spondylo-megaepiphyseal-metaphyseal dysplasia (MIM #613330), respectively.

The neurocranium is composed of both cranial NCCs and mesoderm and protects and supports the brain (Wada et al., 2005). Cranial NCCs in the maxillary domain of the first pharyngeal arch give rise to the palate after cells migrate medially to converge at the midline of the roof of the mouth. In mammals, palatal shelves composed of cranial NCCs grow and converge at the midline to form an epithelial seam, while the primary and secondary palates fuse to create the palatal skeleton (Figure 1d). The zebrafish palate is located in the anterior part of the neurocranium and consists of the ethmoid plate, trabeculae, and parasphenoid bone (Figure 1c). Orofacial clefting occurs when the palatal shelves fail to come together. Clefting, truncation, hypoplasia, or the absence of these structures is indicative of orofacial clefting in zebrafish (Dougherty et al., 2013; Eberhart et al., 2008; Swartz, Sheehan-Rooney, Dixon, & Eberhart, 2011; Wada et al., 2005). It is still unclear whether cranial NCCs that form the palate fuse in zebrafish as well or if they are differentiating in a posterior to anterior pattern (Dougherty et al., 2013; Swartz et al., 2011). Early palatogenesis is conserved among vertebrates. Mice and zebrafish share similar expression patterns of critical genes involved in palate formation in the anterior maxillary domain (*msxe*, *bmp4*, *bmp2b*, and *fgf10a*) as well as the posterior maxillary domain (*tbx22*, *osr1*, *osr2*, *pax9a*) (Braybrook et al., 2002; Peters, Neubuser, Kratochwil, & Balling, 1998; Swartz et al., 2011) [reviewed in (Hilliard, Yu, Gu, Zhang, & Chen, 2005). They also utilize the same signaling pathways, such as Fgf, Pdgfr, Bmp, Tgfb, Wnt, and Shh, and disruptions to any of these pathways result in craniofacial defects, particularly in the anterior neurocranium (Dougherty et al., 2013; Eberhart et al., 2008; Swartz et al., 2011; Wada et al., 2005). For example, *fgf10a* is

expressed throughout the oral ectoderm and knocking down this gene produces a shortened trabeculae and parasphenoid bone and a misshapen Meckel's cartilage and palatoquadrate (Swartz et al., 2011). Fgf10a regulates Shh signaling, which is critical for cranial NCC migration to the midline and induction of chondrogenesis. Loss of Shh in zebrafish causes inappropriate fusion of the trabeculae (Wada et al., 2005). Interestingly, Fgf10a and Shh are both critical for limb development as well (see below). Loss of either gene leads to defects in the pectoral fin, which is homologous to mammalian forelimbs (Swartz et al., 2011; van Eeden et al., 1996). Although early development of the mammalian and zebrafish palates appear genetically similar with similar patterning formation, there are distinct morphogenic differences at later stages, warranting caution when comparing zebrafish and mammalian palatogenesis [reviewed in (Bush & Jiang, 2012)].

The skull vault of the neurocranium has five bones that are connected by cranial sutures, or fibrous tissues. The anatomical structure of the skull vault and its cranial sutures is conserved between humans and zebrafish (Figure 1e,f). The sutures are patent in early development, providing room for the skull and brain to grow. The timing of suture closure differs as human sutures are only patent during early childhood whereas zebrafish sutures remain patent throughout the life of the fish (Quarto & Longaker, 2005). The molecular mechanisms dictating cranial suture formation and closure are conserved among vertebrates, rendering zebrafish a useful model for studying craniosynostosis, a common skeletal defect that occurs when cranial sutures prematurely fuse (Quarto & Longaker, 2005; Topczewska, Shoela, Tomaszewski, Mirmira, & Gosain, 2016) [reviewed in (Holmes, 2012)]. The skull stops growing perpendicular to the fused suture and compensates by growing in a parallel fashion. Therefore, patients with craniosynostosis have abnormally shaped skulls. Most cases of craniosynostosis are caused by genetic variants in *TWIST1*, FGFRs, and *EFNB1* among other genes [reviewed in Wu & Gu, 2019]. Twist1 is a transcription factor that is expressed in either the osteogenic front as a homodimer (T/T) or as a heterodimer with E proteins (T/E), such as Tcf12, in the mesenchymal cells of cranial sutures (schematic in Figure 2b). These two forms of Twist1 act in opposition to one another. The T/T homodimers activate FGFR2 and promote osteogenic differentiation by increasing BMP signaling as well as *Msx2* and *Runx2* expression. This leads to suture closure. In contrast, the T/E heterodimer represses FGFR2, preventing osteogenesis and suture closure. The ratio of Twist1 homodimer to heterodimer changes over time as an organism grows and develops. An untimely excess of T/T homodimer leads to increased FGFR2 expression, decreased Eph/Ephrin signaling, an inappropriate influx of neural crest cells to the paraxial mesoderm, and craniosynostosis (Connerney et al., 2008; Merrill et al., 2006).

Later in this review, we summarize studies that have used zebrafish to study syndromes with craniofacial anomalies, including orofacial clefting and craniosynostosis.

2.2 | Limb development

The zebrafish pectoral and pelvic fins are homologous to mammalian forelimbs and hindlimbs, respectively. Limb growth begins at the lateral plate mesoderm, where mesenchyme precursors form a small bud surrounded by an ectodermal layer (schematic in Figure 3d). Retinoic acid is synthesized in the surrounding somites and, along with Wnt signaling, establishes the limb field and initiates limb induction (Grandel et al., 2002; Ng et al., 2002). Fgf signaling is required for the formation and function of the apical ectodermal ridge (AER) (Niswander, Tickle, Vogel, Booth & Martin 1993; Sekine et al., 1999; Sun, Mariani, & Martin, 2002), which regulates limb outgrowth and establishes the proximodistal axis (shoulder to digits) (Saunders, 1948). Transcription factors Tbx5 (Agarwal et al., 2003; Ng et al., 2002) and p63 (Bakkers et al., 2002) induce Fgf signaling in the mesenchyme and AER. This establishes a complex epithelial-mesenchymal feedback loop that activates proliferation and differentiation of mesenchymal cells resulting in limb outgrowth (Ohuchi et al., 1997). Fgf signaling in the AER is negatively regulated by BMP signaling (Niswander & Martin, 1993; Pajni-Underwood, Wilson, Elder, Mishina, & Lewandoski, 2007; Pizette & Niswander, 1999). Moreover, BMP signaling is required for interdigital programmed cell death and preventing finger webbing as well as polydactyly (Pajni-Underwood et al., 2007; Selever, Liu, Lu, Behringer, & Martin, 2004). The anterior/posterior axis (digits 1–5) is established by Shh signaling in the zone of polarizing activity (ZPA) (Riddle, Johnson, Laufer, & Tabin, 1993; Saunders & Gasseling, 1968). Shh also regulates and is regulated by Fgf signaling in the AER (Laufer, Nelson, Johnson, Morgan, & Tabin, 1994; Niswander, Jeffrey, Martin, & Tickle, 1994). The dorsal/ventral axis (back of hand to palm) is dictated by expression of Wnt7a (dorsal) and En1(ventral) (Davis, Holmyard, Millen, & Joyner, 1991; Gardner & Barald, 1992; Loomis, Kimmel, Tong, Michaud, & Joyner, 1998; Parr & McMahon, 1995). Each gene and pathway are interconnected, and dysregulation at any point can cause abnormal limb growth [reviewed in (Kantaputra & Carlson, 2019)] (Figure 3d).

The first skeletal elements to form in the zebrafish pectoral fin bud are the scapulocoracoid, postcoracoid, and endoskeletal disk, which are all derived from fin mesenchymal cells. By 4 dpf (days post fertilization), collagenous rays called actinotrichia form and act as supportive elements for the fin fold (Figure 3a). This larval endoskeleton structure persists for several weeks until cells in the endoskeletal disk proliferate and expand the fin. The cartilage matrix of the intermediate larval structure decomposes, and at 23 dpf (7.1 mm standard length), rod-like dermal bones called lepidotrichia, or fin rays, form. By 28 dpf (9.5 mm), the endoskeletal disk splits to form four proximal radial bones. There are also six to eight distal radial bones located above and distal to the proximal radials that later fuse to the lepidotrichia (Grandel & Schulte-Merker, 1998) (Figure 3b). The pelvic fins do not begin formation until 18 dpf (6 mm), and their development is similar to that of pectoral fins. The main difference is that the pelvic fin bud develops more quickly and does not require an intermediate larval endoskeleton before the terminal adult fin structure (29 dpf, 10.1 mm). For a more detailed description of the anatomy of zebrafish fin development, we refer to Grandel and Schulte-Merker (1998).

Morphologically, zebrafish fins and mammalian limbs are distinct from one another, and it is difficult to assign which elements are truly

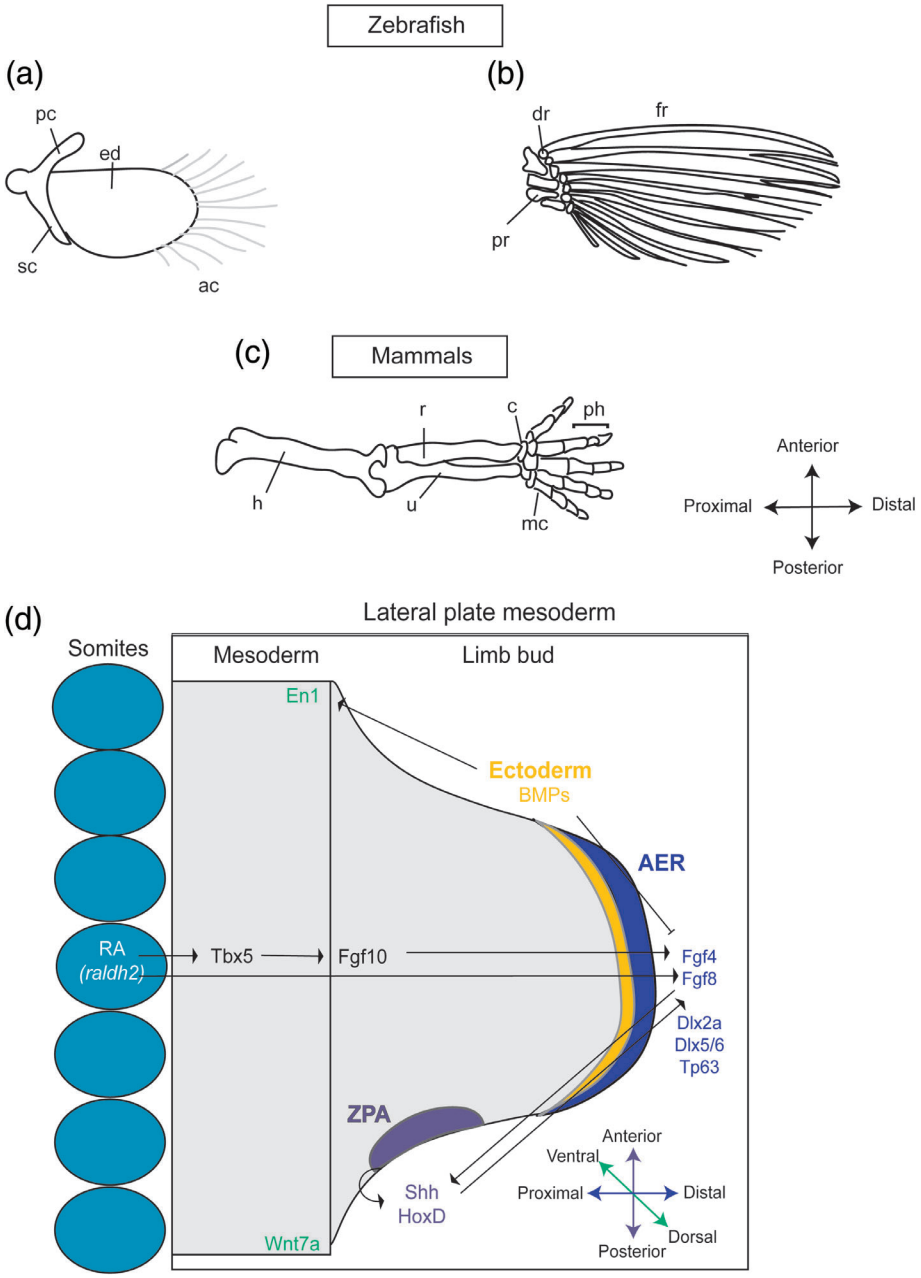

FIGURE 3 The gene regulatory networks involved in zebrafish and mammalian limb development are highly conserved. The zebrafish pectoral fin, shown at 4 days post fertilization (a) and adulthood (b), is homologous to the mammalian forelimb (c). (d) Schematic of genes and pathways involved in early limb development. Limb bud initiation begins with retinoic acid (RA) signaling in the surrounding somites (teal). This then leads to the initiation of Fgf signaling in the mesoderm and ectoderm and limb bud growth at the lateral plate mesoderm. The AER (navy) dictates outgrowth and the proximal/distal axis; the ZPA (purple) determines the anterior/posterior axis; and WNT7A/EN1 (green) establish the dorsal/ventral axis. As such, each gene and pathway are interconnected and rely on one another for proper limb growth. Abbreviations: ac, actinotrichia; AER, apical ectodermal ridge; c, carpal bones; dr, distal radials; ed, endoskeletal disk; fr, fin rays; h, humerus; mc, metacarpals; pc, postcoracoid; ph, phalanges; pr, proximal radials; r, radius; RA, retinoic acid; sc, scapulocoracoid; u, ulna; ZPA, zone of polarizing activity

homologous [reviewed in (Yano & Tamura, 2013)] (Figure 3a–c). Nevertheless, the conservation of the gene regulatory networks indicates that they are still a useful model for limb development. Researchers can examine the larval pectoral fin (4–5 dpf) and look for deformities in the shape and length of cartilage structures. If a mutant survives to adulthood, researchers can look more closely at the bones that develop later and use them as a model for human limb bones. Differences in the number of proximal radial bones, number of distal radial bones, or the length of lepidotrichia can be indicators of human polydactyly (more bones), ectrodactyly (fewer bones), or brachydactyly (shortening of bones). Medaka fish (*Oryzias latipes*) has been used by Letelier et al. (2018) to model human ectrodactyly caused by decreased *SHH* signaling by partially deleting an upstream *shh* limb enhancer known as ZRS. This deletion leads to a decrease in proximal radial bones from four to two in adult fish. Below, we discuss ways in which zebrafish have been used to study human craniofacial and limb defects.

3 | ZEBRAFISH MODELS OF CRANIOFACIAL ANOMALIES WITH ACCOMPANYING LIMB DEFECTS

Structures of the craniofacial and limb skeleton are clearly distinct from one another, but they utilize many of the same gene regulatory networks and mechanisms for their development, such as Fgf, Bmp, Wnt, and Shh signaling. It follows then that several human congenital syndromes caused by single gene variants present with defects in both structures (Panthaki & Armstrong, 2003). Here we will describe a few examples in which zebrafish were used to study specific

syndromes characterized by orofacial clefting, craniosynostosis, and limb defects. Orofacial clefting and craniosynostosis are the two most common craniofacial phenotypes, so our primary focus is here. A full list of craniofacial and limb human syndromes that have been modeled with zebrafish can be found in Table 1. Some of these studies explored different phenotypes associated with the syndrome. For example, Fuller et al. (2014) use zebrafish to model the left/right patterning defects observed in patients with Carpenter syndrome I (MIM #201000). We have chosen to highlight studies that specifically studied genes in the context of craniofacial and/or limb development.

3.1 | Orofacial clefting and missing/fused digits

Orofacial clefting, namely cleft lip with or without cleft palate, is the most common craniofacial anomaly occurring in 1 in 700 newborn babies (Global registry and database on craniofacial anomalies, 2001). Cleft palate occurs in humans when the palatal shelves fail to elevate or properly fuse at the midline. As mentioned above, the zebrafish ethmoid plate is often used to model the mammalian palate. For a comprehensive review on zebrafish models of orofacial clefting we refer to Duncan, Mukherjee, Cornell, and Liao (2017). Here, we will discuss recent zebrafish studies of syndromes that present with both orofacial clefting and limb defects, primarily ectrodactyly (missing digits) and syndactyly (fused digits).

3.1.1 | Roberts syndrome

Roberts syndrome (MIM #268300) is an autosomal recessive disorder characterized by orofacial clefting, micrognathia (hypoplastic jaw), downward slanting, wide-set eyes, a beaked nose, microcephaly, hypoplastic limbs, syndactyly, and joint deformities (Roberts, 1919). Using multipoint linkage analysis, Vega et al. (2005) found eight different homozygous variants in *ESCO2* in affected individuals. *ESCO2* is a highly conserved gene that encodes an N-acetyltransferase required for holding sister chromatids together after DNA replication and before mitosis (Rolef Ben-Shahar et al., 2008). In zebrafish, *esco2* is expressed in the branchial arches and pectoral fins, as well as brain ventricles, optic vesicles and retinal cells during early development (Monnich et al., 2011). Partial knockdown of *esco2* in zebrafish morphants (organisms treated with morpholino antisense oligonucleotides to knockdown gene expression) leads to disorganized craniofacial cartilage and hypoplastic jaw elements as well as shortened, abnormally shaped pectoral fins. This is due to cells being blocked at the onset of mitosis, leading to increased cell death. *esco2* morphant cells in mitosis have distorted, disorganized mitotic spindles, likely from the instability of the sister chromatids (Vega et al., 2005). Consistent with this study, stable *esco2*−/− mutants created with CRISPR-Cas9 also have smaller heads, missing pectoral fins, and increased apoptosis, particularly at the neural tube (Percival et al., 2015). This group observed that cells are trapped in mitosis. Indeed, after the nuclear envelope breaks down, chromosomes in *esco2*−/− mutants scatter and are not captured on the metaphase plate, blocking mitosis from proceeding. This leads to aneuploidy and/or micronuclei and the cells are forced to undergo apoptosis. The craniofacial and limb defects, particularly the hypoplastic jaw and limbs, observed in patients from loss of *ESCO2* are hypothesized to result from increased cell death, based on these zebrafish studies.

3.1.2 | Van der Woude syndrome

Van der Woude syndrome is an autosomal dominant disorder caused by variants in either *IRF6* (MIM #119300) (Burdick, Bixler, & Puckett, 1985; Kondo et al., 2002) or *GRHL3* (MIM #606713) (Peyrard-Janvid et al., 2014). Patients present with orofacial clefting, lower lip pits and/or sinuses, hypodontia, and syndactyly.

IRF6 is a member of the interferon regulatory transcription factor family. In zebrafish larvae, it is expressed in the pharyngeal arches and in the epithelial cells of the mouth, esophagus, and pharynx, as well as the olfactory and otic placodes (2–72 hpf) (Ben et al., 2005). Expression of *irf6* in the pectoral fin has not yet been shown; however, injection of a dominant-negative form of *irf6* RNA into zebrafish embryos at the single-cell stage leads to shortened/loss of the pectoral fin as well as hypoplastic, disorganized craniofacial elements and a clefted ethmoid plate (Sabel et al., 2009). Dougherty et al. (2013) created stable, dominant-negative *irf6* mutants driven by a *sox10* promoter to limit mutant expression to NCCs. With time-lapse imaging, they show that chondrocytes at the median and lateral ethmoid plate fail to come together in mutants, creating a cleft (Dougherty et al., 2013). Similarly, *Irf6*−/− mutant mice have clefting in the secondary palate and a hypoplastic snout and jaw (Ingraham et al., 2006). Irf6 is thought to be involved in endothelin signaling during palate formation (Fakhouri et al., 2017). The clefting phenotypes observed in zebrafish and mice are consistent with what is seen in Van der Woude patients and demonstrate an important role for *IRF6* in palatogenesis.

grhl3 is a transcription factor selectively expressed in the non-neural ectoderm as well as endoderm pouches surrounding the pharynx of developing zebrafish embryos. Knockdown of *grhl3* causes severe hypoplasia of the palatoquadrate, ceratohyal, and Meckel's cartilage and loss of the ceratobranchial arches and pectoral fins (Dworkin et al., 2014). There is increased cell apoptosis in the pharyngeal arches with loss of *grhl3*. Using a micro-ChIP assay, Dworkin et al. (2014) found that Grhl3 directly binds to the promoter of *edn1*, a highly conserved gene required for lower jaw development (Clouthier et al., 1998; Miller et al., 2000). Expression of *edn1* and its known downstream targets, *hand2* and *dlx3*, are significantly reduced in the endoderm of *grhl3* morphants. Importantly, injection of *edn1* mRNA into *grhl3* morphants rescues *hand2* and *dlx3* expression as well as the craniofacial and limb skeletons (Dworkin et al., 2014). It is thought that *grhl3* is required for *edn1* expression in the pharyngeal endoderm, which is then important for NCC growth and proliferation and palatogenesis.

irf6 and *grhl3* are likely required for limb development in zebrafish, as shown by the loss of pectoral fins in different models,

but their roles remain unclear. During mammalian development, loss of either *Irf6* or *Grhl3* results in shortened forelimbs, ectrodactyly, or syndactyly (Ingraham et al., 2006; Kashgari et al., 2020). Irf6 is thought to be required for formation of the periderm, a single layer of epithelial cells surrounding the embryonic epidermis. In mice, Irf6 directly regulates transcription of *Grhl3* (de la Garza et al., 2013), which was recently shown to be required for digit separation (Kashgari et al., 2020). It is unknown whether the interaction between *irf6* and *grhl3* during limb formation is conserved and if this accounts for the loss of pectoral fins seen in zebrafish.

3.1.3 | Ectrodactyly-ectodermal dysplasia-clefting syndrome 3 (EEC3)

Ectrodactyly-ectodermal dysplasia-clefting syndrome 3 (EEC3) (MIM #604292) is an autosomal dominant disorder caused by mutations in *TP63*, a transcription factor and member of the *TP53* family (Celli et al., 1999). EEC3 is characterized by orofacial clefting, ectrodactyly, and ectodermal dysplasia such as hypopigmented, scaly skin and malformed/decayed teeth (Penchaszadeh & de Negrotti, 1976). *TP63* is transcribed from two different promoters, creating two isoforms of the gene, Tap63 and ΔNp63, which then act as an activator or repressor, respectively (Yang et al., 1998). Zebrafish studies have shown that knockdown of the dominant negative repressive isoform, ΔNp63, results in loss of the pectoral fin and a decrease in proliferation in the epidermis (Bakkers et al., 2002; Lee & Kimelman, 2002). p63 loss-of-function mutants (*tp63−/−*) created with CRISPR-Cas9 recapitulate this phenotype (Santos-Pereira et al., 2019). Unfortunately, the authors did not note any craniofacial defects in the *tp63−/−* mutants, and this is likely due to the larvae dying between 40–50 hours post fertilization (hpf). *tp63−/−* mutants have a significant decrease in expression of epidermal genes as well as genes relating to fin development. Additionally, Gene Ontology (GO) term enrichment analyses show a downregulation in genes relating to cell-matrix adhesion, cell adhesion, and skeletal muscle fiber development, genes which are likely to contribute to craniofacial and limb development. However, further studies exploring these functions have not yet been explored. *Tp63−/−* mutant mice display severe craniofacial and limb defects, including cleft lip and palate, hypoplastic upper and lower jaw, and limb truncations (Yang et al., 1999). Moreover, it has been shown in mice that ΔNp63 directly regulates the transcription of *Dlx5/Dlx6* (Lo Iacono et al., 2008) and *Fgf8/Fgf4* (Kawata et al., 2017), which are all important for both limb and craniofacial development (Figures 2a and 3d). Therefore, it may be worth revisiting the zebrafish model to determine whether craniofacial defects are present in *tp63−/−* mutants and if Tp63 is regulating these key developmental genes.

3.1.4 | Craniosynostosis and variable limb defects

Craniosynostosis is the second most common craniofacial anomaly with a prevalence of 1 in 2,500 live births (Boulet, Rasmussen, & Honein, 2008). Craniosynostosis is the premature fusion of skull bones at the sutures, or fibrous structures that join the bones, before the brain has fully formed. As a result, the skull is misshapen. During normal development, the sutures hold the skull in place while remaining flexible to allow proper brain growth. The anatomical structure and molecular mechanisms dictating formation of cranial sutures is conserved between humans and zebrafish, making them a useful model for studying craniosynostosis (Quarto & Longaker, 2005). Here, we will discuss Saethre-Chotzen syndrome and syndromes associated with mutations in *FGFR2*.

3.1.5 | Saethre-Chotzen syndrome

Saethre-Chotzen syndrome is an autosomal dominant disorder featuring craniosynostosis, due to loss of the coronal suture, maxillary hypoplasia, a high forehead, wide-set eyes, ptosis (droopy eyelids), a broad nasal bridge, brachydactyly, syndactyly, and polydactyly in the feet (Chotzen, 1932; Saethre, 1931). It is associated with variants in *FGFR2* (see below), *FGFR3*, and *TWIST1* (MIM #101400) (el Ghouzzi et al., 1997; Howard et al., 1997; Paznekas et al., 1998). Transcription factors Twist1a and Twist1b, the zebrafish orthologs of TWIST1, are both required for ectomesenchyme specification from NCCs (Das & Crump, 2012). Knockdown of either gene with morpholinos results in minor skeletal defects, such as minor hypoplasia of the ventral mandibular and hyoid cartilages, but a double knockdown leads to an almost complete loss of the viscerocranium and loss of pectoral fins (Das & Crump, 2012). Stable double *twist1a−/−;twist1b−/−* knockout mutants created using TALENs (transcription activator-like effector nucleases) have a milder phenotype than the morphants and present with hypoplasia in the Meckel's cartilage, palatoquadrate, and hyosymplectic cartilage (Teng et al., 2018). Using transgenic models and the GAL4:UAS system, Das and Crump (2012) show that Twist1 promotes ectomesenchyme cell fates (i.e., cartilage, bones, etc.) in cranial NCCs. These cells primarily make up the craniofacial skeleton.

One of the most distinct phenotypes of Saethre-Chotzen syndrome is the selective loss of the coronal suture, which separates the two parietal bones from the frontal bone in the skull. Teng et al. (2018) created viable, triple *tcf12−/−;twist1a−/−;twist1b−/−* mutants that show mild craniofacial phenotypes at 5 dpf but adult mutants develop severe unilateral or bilateral coronal synostosis. Juvenile *tcf12−/−;twist1b−/−* double mutants assessed for mineralization (Calcein green staining), bone (Alizarin red staining), and live-cell imaging of osteoblasts (*sp7:EGFP* transgenic line) all show accelerated growth of the frontal and parietal bones in mutants compared to wildtype. These bones grow diagonally toward one another, becoming aberrantly shaped and leading to premature fusion, which is consistent with the human phenotype. Premature fusion of the suture prevents bone enlargement and growth of the skull along the anterior/posterior axis. Finally, using RNAscope in situ hybridization, a recently developed technique that uses probes to amplify target RNA in intact cells and tissues, they show that *tcf12−/−;twist1b−/−* mutants have reduced expression of skeletal stem cell markers, *gli1*, *grem1a*, and

prrx1a in the coronal sutures as well as a decrease in osteoprogenitors compared to the wildtype. This is true only in the coronal sutures, suggesting that there is a selective exhaustion of osteoprogenitors in this region and bone growth has ceased in the mutants (Teng et al., 2018). These studies demonstrate the importance of *twist1* in craniofacial development.

twist1 also appears to be required for pectoral fin development, as suggested by the skeletal preparations for *twist1a* and *twist1b* morphants (Das & Crump, 2012). However, the mechanism is not well understood. In mice, loss of Twist1 blocks forelimb growth. It is required for AER and ZPA maintenance by regulating Fgf and Shh signaling, respectively (O'Rourke, Soo, Behringer, Hui, & Tam, 2002). Further studies in zebrafish are needed to determine if these interactions are conserved in the growing pectoral fin bud.

3.1.6 | *FGFR2* syndromes

It is well-known that fibroblast growth factor (FGF) signaling is critical in many aspects of development. Humans have four tyrosine kinase FGF receptors (FGFR) than can bind to eighteen different secreted FGF proteins. Variants in *FGFR2* specifically are known to be associated with several different craniofacial and limb syndromes, including Apert syndrome (MIM #101200), bent bone dysplasia (MIM #614592), Jackson Weiss syndrome (MIM #123150), Pfeiffer syndrome (MIM #101600), and Saethre-Chotzen syndrome (MIM #101400) [reviewed in Wenger, Miller, & Evans, 1998–(2020)]. Interestingly, each of these syndromes presents with craniosynostosis and either brachydactyly or syndactyly. A more detailed description of phenotypes for each syndrome can be found in Table 1. Mutations in other FGFRs and secreted FGF proteins are also known to lead to developmental disorders [reviewed in Wenger et al., 1998–(2020)].

In zebrafish embryos, *fgfr2* is expressed in the pharyngeal endoderm and hindbrain between 24–72 hpf (Larbuisson et al., 2013) and in the cranial sutures at 6 weeks post fertilization (Topczewska et al., 2016). Treatment of embryos between 18–24 hpf with SU5402, an inhibitor of FGFRs, leads to loss of the pharyngeal arches (Walshe & Mason, 2003). Injection of morpholinos against *fgfr2* leads to hypoplastic skeletal structures, including the Meckel's cartilage and palatoquadrate, as well as shortened pectoral fins. Additionally, knockdown of both *fgfr1a* and *fgfr2* produces an even more severe phenotype with loss of the ceratohyal, ceratobranchial arches, neurocranium, and pectoral fins (Larbuisson et al., 2013). In situ RNA hybridization experiments in single morphants show no changes in expression of *sox9*, *dlx2a*, *or fli1*, suggesting that cranial NCC migration into the pharyngeal arches is not affected by loss of *fgfr2*. However, there is decreased expression of *barx1* and *runx2b*, suggesting Fgfr2 required for chondrocyte condensation and maturation (Larbuisson et al., 2013).

Leerberg et al. (2019) generated knockout zebrafish mutants for single *fgfr* genes using CRISPR-Cas9. Surprisingly, single homozygous mutants are viable and mRNA expression levels of non-mutated *fgfr* genes are not elevated, suggesting genetic redundancy. *fgfr1a−/−;fgfr1b−/−;fgfr2−/−* triple mutants had the most severe phenotypes in the limb and craniofacial skeleton. Consistent with the morphant studies, these mutants had shortened or loss of pectoral fins. By in situ RNA hybridization, there was a significant decrease in *fgf24*, *fgf8a*, and *dlx2a*, which are markers of limb outgrowth. The triple mutants also exhibited severe craniofacial phenotypes, including a loss of the ceratobranchial arches, ceratohyal, and hyosymplectic cartilage; a misshapen palatoquadrate; a downward facing Meckel's cartilage; and a clefted ethmoid plate. This is likely not due to issues with cranial NCC migration, but rather maintenance of the NCCs (Leerberg et al., 2019).

Fgf signaling is critical in both craniofacial and limb development. As shown in these studies and in mouse studies, Fgf signaling is required in the facial skeleton for early patterning, growth regulation, tooth growth, palatogenesis, and suture formation [reviewed in Nie, Luukko, and Kettunen (2006)]. In the limb, it is necessary for proximodistal outgrowth and regulating the anterior/posterior axis [reviewed in Mercader (2007). Therefore, mutations in *FGFR* genes can lead to syndromes that present with defects in either or both craniofacial and limb structures.

4 | SUMMARY

In this review, we have provided a brief overview of both craniofacial and limb development and discussed the relevance of using zebrafish as a model for studying both developmental mechanisms. We then reviewed studies that have used zebrafish to study human syndromes that present with both craniofacial and limb defects and attempted to understand why these defects often occur together. We speculate that there is a pleiotropic effect, in which a single gene affects more than one developmental process. Indeed, we have discussed how many signaling pathways, such as Fgf, Shh, Bmp, and Wnt signaling, are involved in both craniofacial and limb development. Disruptions to components of these pathways results in both craniofacial and limb defects. Moreover, some transcription factors, such as Twist1, Irf6, and Tp63 have been shown in mice to bind to and regulate genes that are critical for craniofacial and limb development. Additional studies are required to show that this is conserved in zebrafish as well. NCCs and their derivatives, namely melanoblasts and neurons, can be found in the limb mesenchyme, which may contribute to the similar gene regulatory networks (Erickson, 1985; Grim & Christ, 1993). It has also been proposed that there is a deep homology between the craniofacial and limb skeletons. In cartilaginous fishes (Chondrichthyes), appendages grow out of the gill arches, a structure that later gives rise to part of the craniofacial skeleton. This led Carl Gegenbaur in 1878 to hypothesize that paired limbs/fins are evolved from the gill arches (Gegenbaur, 1878). This idea was recently supported by the work of Gillis and colleagues. In the little skate (*L. erinacea*), they demonstrated that chondrichthyan branchial rays and appendages both rely on a complex interplay between retinoic acid, *Shh* and *Fgf8* signaling to drive endoskeleton outgrowth and patterning of both the gill arches and appendages (Gillis, Dahn, & Shubin, 2009; Gillis & Hall, 2016).

They argue that this is due to a deep homology of the structures. More recently, they have shown in the little skate that the first gill arch is composed of NCCs and the fins are made of mesoderm cells, as expected. Interestingly, the more posterior gill arches are composed of both NCCs and lateral mesoderm cells, suggesting that gills and fins develop from a common pool of cells that have the potential to develop into either structure (Sleight & Gillis, 2020). This may explain why craniofacial and limb patterning are so similar. Despite the seemingly stark differences between humans and zebrafish, we have shown that zebrafish have emerged as a powerful tool to study human craniofacial and limb development.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were generated in this study.

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