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From Bench to Bedside and Back: Improving Diagnosis and Treatment of Craniofacial Malformations Utilizing Animal Models

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Abstract

Craniofacial anomalies are among the most common birth defects and are associated with increased mortality and, in many cases, the need for lifelong treatment. Over the past few decades, dramatic advances in the surgical and medical care of these patients have led to marked improvements in patient outcomes. However, none of the treatments currently in clinical use address the underlying molecular causes of these disorders. Fortunately, the field of craniofacial developmental biology provides a strong foundation for improved diagnosis and for therapies that target the genetic causes of birth defects. In this chapter, we discuss recent advances in our understanding of the embryology of craniofacial conditions, and we focus on the use of animal models to guide rational therapies anchored in genetics and biochemistry.

1. MODELS TO UNCOVER GENETICS OF CLEFT LIP AND PALATE

Cleft lip with or without cleft palate (CL/P) is the most common congenital anomaly of craniofacial development, affecting approximately 1 in 700 live births (Dixon, Marazita, Beaty, & Murray, 2011; Iwata, Parada, & Chai, 2011; Jugessur, Farlie, & Kilpatrick, 2009). It is estimated that 70% of CL/P cases are isolated and nonsyndromic and that 30% occur as part of one of more than 500 Mendelian syndromes (Dixon et al., 2011). Over the past few decades, mouse models have proved an invaluable tool in understanding the etiology of CL/P (Table 1; Bush & Jiang, 2012; Gritli-Linde, 2008; Jiang, Bush, & Lidral, 2006). These models have led to exponential growth in our knowledge of the molecular and cellular basis for clefts and have contributed greatly to the understanding of general developmental mechanisms. To date, the principle clinical benefit of information gained from these studies

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has been to improve diagnostics and estimates of recurrence risks (Dixon et al., 2011; Grosen et al., 2010). Looking toward the future, our improved understanding of the developmental processes underlying cleft pathogenesis increasingly suggests opportunities for developing new preventative and therapeutic approaches.

From an embryologic perspective, formation of the lip and palate has developmental similarities in that both require the coordinated growth and fusion of embryonic prominences. However, these are temporally distinct events that involve different morphogenetic programs. The development of the lip occurs during the 4th through 6th week of human development and involves fusions of the maxillary, medial nasal, and lateral nasal processes, whereas secondary palate development occurs between the 6th and 12th week and involves the fusion of the maxillary-derived secondary palatal shelves (Bush & Jiang, 2012; Jiang et al., 2006). Consistent with this temporal asynchrony, cleft lip and cleft palate can occur separately or together; cleft lip is defined as a gap between the philtrum and the lateral upper lip, and cleft palate is defined as a gap in the secondary palate (roof of the mouth). Although the lip and palate form through separate developmental programs, many genetic factors play a role in both processes.

Prior to the advent of genome-wide methodologies, candidate genes for linkage and mutation analysis were selected largely on the basis of phenotypes arising from gene-targeted mutations in mice. For example, targeted mutation of the *Msx1* homeobox gene resulted in syndromic cleft secondary palate and tooth agenesis in homozygous mutant mice and led to the evaluation of its human ortholog as a human CL/P candidate (Satokata & Maas, 1994). Linkage disequilibrium was found between *MSX1* and human CL/P, and ultimately, mutations in *MSX1* were identified in patients with syndromic cleft palate with tooth agenesis as well as in patients with non-syndromic CL/P; in total, mutations in *MSX1* may contribute to around 2% of nonsyndromic CL/P cases (Jezewski et al., 2003; Lidral et al., 1998). Integration of mouse genetics, *ex vivo*, and molecular techniques has advanced our understanding of the developmental basis for *Msx1* involvement in CL/P. *Msx1* controls cell proliferation in the anterior secondary palate by maintaining mesenchymal expression of *Bmp4* in the palatal mesenchyme (Alappat, Zhang, & Chen, 2003; Parada & Chai, 2012; Zhang et al., 2002). Whereas mutations in *MSX1* have been identified in humans with cleft lip, *Msx1*^{-/-} mice do not exhibit cleft lip, but rather cleft palate only. The related *MSX2* might partially compensate for *MSX1* loss in mice, because mice lacking *MSX1* and *MSX2* exhibit bilateral CL/P as part of dramatic craniofacial dysmorphogenesis (Ishii et al., 2005). Interestingly, *Msx1*^{-/-} and *Pax9*^{-/-} mice exhibit cleft lip with variable penetrance, possibly due to a shared role of *MSX1* and *PAX9* in the regulation of *Bmp4* or other factors critical for lip morphogenesis (Liu et al., 2005; Nakatomi et al., 2010; Zhou et al., 2013). On the other hand, compound loss of *Dlx5*^{-/-} and *Msx1*^{-/-} in mice restored cell proliferation and rescued the cleft palate phenotype (Han et al., 2009). These results exemplify the complexity of the genetic networks controlling lip and palate development and support the likely importance of genetic interaction in human CL/P. Indeed, *PAX9* has been confirmed as a contributor to human syndromic and nonsyndromic CL/P (Ichikawa et al., 2006; Schuffenhauer et al., 1999). Mouse genetics studies designed to elucidate genetic pathways have therefore been highly useful in identifying candidate genes for human genetic analysis.

The *TGF β 3* gene has been considered as a candidate human clefting gene based on the cleft palate phenotype in *Tgfb3*^{-/-} homozygous mice (Karttinen et al., 1995; Proetzel et al., 1995). Though no loss-of-function mutations have been identified in *TGF β 3* in nonsyndromic cleft patients, association with the *TGF β 3* genomic locus has been demonstrated (Lidral et al., 1998). Interestingly, treatment of mouse *Tgfb3*^{-/-} homozygous palatal shelves with exogenous TGF β 3 in explant culture resulted in a rescue of palate fusion, even when treated with a low dose for a relatively short time (Taya, O’Kane, & Ferguson, 1999). Further, exogenous TGF β 3 induced fusion of explanted lip primordia in CL/Fr, a mouse line that exhibits high susceptibility to cleft lip with unknown genetic etiology (Juriloff & Fraser, 1980; Kohama, Nonaka, Hosokawa, Shum, & Ohishi, 2002; Muraoka et al., 2005). These studies suggest the possibility of modulation of TGF β signaling as a potential therapeutic approach for nonsyndromic CL/P.

Notably, mutations in other TGF β pathway components including *TGFB2*, *SMAD3*, *TGF β R2*, or *TGF β R2* cause syndromic cleft palate as part of autosomal dominant Loeys–Dietz syndrome (LDS; Loeys et al., 2005). The majority of LDS cases are caused by mutations in *TGF β R1* or *TGF β R2* and result in impaired kinase activity without altered expression or localization of the receptor, though the net effect is an apparent increase in TGF β signaling (Loeys et al., 2005; Van Laer, Dietz, & Loeys, 2014). Marfan syndrome (MFS) has significant clinical overlap with LDS, but does not include clefting phenotypes and is also thought to involve overactive TGF β signaling (Dietz et al., 1991; Habashi et al., 2006). In mice, TGF β -neutralizing antibodies or the angiotensin II type 1 receptor (AT1) blocker losartan was used to decrease TGF β signaling, which led to successful treatment of aortic aneurism. On this basis, several trials have been conducted in MFS patients to determine whether suppressing TGF β signaling can reduce aortic aneurism (Bowen & Connolly, 2014; Chiu et al., 2013; Groenink et al., 2013; Habashi et al., 2006; Lacro et al., 2014). It will be interesting to determine whether similar improvements can be obtained for craniofacial phenotypes in LDS mouse models. However, prenatal use of such a strategy for LDS is likely to be complicated by embryonic consequences of blockade of TGF β signaling and by timing of diagnosis, which raises an important general point: many of the genes involved in clefting have pleiotropic roles in the embryo, and it is not trivial to devise treatments that would lead to blockade of the pathway in one organ but not others.

During secondary palate fusion in mice, signaling by TgF β R2 is required for the expression of the gene encoding the IRF6 transcription factor, and this regulation is required for normal palate development (Iwata et al., 2013). Human mutations in *IRF6* cause Van der Woude syndrome (VWS), an autosomal dominant ectodermal dysplasia that causes CL/P with lip pits (Kondo et al., 2002). Linkage between *IRF6* and nonsyndromic CL/P has been shown in a number of studies, supporting its importance in human clefting (Dixon et al., 2011; Zuccherro et al., 2004). Mice harboring mutations orthologous to those causing VWS phenocopy the disease and exhibit defects in epithelial differentiation that result in oral adhesions and cleft palate (Ingraham et al., 2006; Richardson et al., 2006; Stottmann, Bjork, Doyle, & Beier, 2010). Interestingly, expression of *Irf6* is regulated by the transcription factor P63 in mice, and mutations in *P63* in humans cause ectrodactyly–ectodermal dysplasia clefting (EEC) and ankyloblepharon ectodermal dysplasia clefting (AEC). Further, mice harboring compound heterozygous mutations in *Irf6* and *p63* exhibit fully penetrant

cleft palate, connecting these ectodermal dysplasias to VWS through a molecular mechanism.

More recently, several genome-wide association studies (GWAS) have identified a number of additional loci that are likely to be involved in non-syndromic cleft lip and palate (Beaty et al., 2010, 2011; Birnbaum et al., 2009; Grant et al., 2009; Mangold et al., 2010). In addition to confirmation of *IRF6* as a key susceptibility locus, four previously unidentified loci, including candidate genes with previously unknown involvement in craniofacial development, were identified (Mangold, Ludwig, & Nothen, 2011). Interestingly, all of these studies identified a locus at 8q24 as having significant involvement in nonsyndromic CL/P. Subsequent work in mice has indicated that this region includes a remote *cis*-acting enhancer for the *Myc* transcription factor (Uslu et al., 2014). Deletion of this region resulted in a dramatic decrease in expression of *Myc* specifically in the developing lip and a low frequency of CL/P, suggesting that regulatory mutations in *MYC* may contribute to human clefting linked to the 8q24 locus (Uslu et al., 2014). Estimation of the contribution of the four loci identified in these studies (*IRF6*, 8q24, 17q22, and 10q25.3) suggests that these loci might account for approximately 55% of variation in CL/P (Marazita, 2012). These data may challenge the traditional view of CL/P as a highly multifactorial disorder, raising new hopes for designing therapeutics if mechanistic insights for these loci can be obtained.

Gene–environment interactions have long been known to be a significant contributor to orofacial clefting in humans, and maternal smoking and alcohol consumption, as well as nutritional deficiency, have been implicated (Dixon et al., 2011). The extent of this contribution is not clear, however, and few susceptibility loci have been identified consistently. Recently, a GWAS approach has been used to identify gene–environment interactions underlying nonsyndromic cleft palate. Several loci, which harbor genes with previously unrecognized roles in orofacial clefting, were shown to have significant interaction with maternal smoking or alcohol use, whereas these loci did not reach significance if considered independently of smoking or alcohol use (Beaty et al., 2011). Mouse teratogen studies have shown that environmental influences can contribute to orofacial clefting, but very few studies have brought modern molecular and cell biological approaches to bear on understanding the mechanisms of these interactions. Such studies will be critical for understanding the mechanisms of action for these genes and determining if preventative approaches can reduce the frequency of clefting in humans.

2. TREACHER COLLINS: PROOF OF CONCEPT OF A NONSURGICAL THERAPEUTIC FOR A CRANIOFACIAL SYNDROME

Treacher Collins syndrome (TCS) is a congenital disorder that affects craniofacial development. Mutations in *treacle* (*TCOF1*) were the first identified cause of TCS, and since then additional genetic causes have been identified as well (Dixon, Trainor, & Dixon, 2007). Over 120 mutations in *TCOF1*, half of which occur spontaneously, have been identified, and most result in a premature stop codon, suggesting that haploinsufficiency of *TCOF1* underlies the pathogenesis of TCS (Dixon et al., 2007).

Individuals with TCS exhibit a spectrum of craniofacial anomalies, including hypoplasia of the facial bones, particularly the mandible and zygomatic complex; downward slanting of the palpebral fissures, usually with colobomas of the lower eyelids and a paucity of lashes medial to the defect; abnormalities of the external ears and atresia of external auditory canals, resulting in hearing loss; and choanal atresia. Cleft palate and absence of the zygomatic arch may occur in severe cases (Edwards et al., 1996; Fazen, Elmore, & Nadler, 1967; Phelps, Poswillo, & Lloyd, 1981; Rovin, Dachi, Borenstein, & Cotter, 1964). There is high phenotypic variability among TCS individuals, and some individuals are retrospectively diagnosed after their more severely affected siblings or children are born.

Tcof1 expression in mouse embryos coincides with the formation and migration of neural crest cells, suggesting a role in their development (Dixon et al., 2006). Studies of neural crest cells in *Tcof1*^{+/-} embryos revealed that, although the migration pattern remained unchanged, there was a reduction in the number of migrating neural crest cells compared to wild-type embryos (Dixon et al., 2006). Consistent with this observation, there was a significant increase in apoptosis and reduction in proliferation of neuroepithelial and neural crest cells in *Tcof1*^{+/-} embryos. Interestingly, the half-life and cytoplasmic concentration of P53 were increased in *Tcof1*^{+/-} embryos, and proapoptotic genes that are transcriptional targets of P53 showed increased expression levels as well (Jones et al., 2008).

Genetically ablating one or both alleles of *Trp53*, which encodes P53, in *Tcof1*^{+/-} embryos resulted in dosage-dependent rescue of the TCS-like phenotypes (Jones et al., 2008). All *Tcof1*^{+/-}; *Trp53*^{-/-} pups survived beyond weaning with a fully rescued craniofacial phenotype. However, some of the pups developed tumors, which are commonly observed in *Trp53*^{-/-} mice (reviewed in Levine, 1997). Chemical inhibition of P53 with pifithrin- α at the onset of neural crest cell migration led to a significant decrease in neuroepithelial apoptosis and subsequent alleviation of TCS-related phenotypes, suggesting the exciting possibility that small molecules administered *in utero* could treat TCS (Jones et al., 2008).

The successful rescue experiments in the TCS mouse model provide a conceptual advance in terms of medical treatment of birth defects resulting from ribosome abnormalities. This work also suggests that inhibition of P53-mediated apoptosis is a potential strategy for prenatal treatment of TCS. However, the strong correlation between P53 inhibition and tumorigenesis indicates that targeting P53 itself is not likely to be a practical therapeutic. Instead, investigating the TCOF effectors that are more specifically involved in TCS pathogenesis would be the logical next step to develop a safe and effective therapeutic for TCS.

3. RASopathies: UNDERSTANDING AND DEVELOPING TREATMENT FOR SYNDROMES OF THE RAS PATHWAY

The RASopathies are a group of syndromes characterized by dysregulation of signaling through the RAS pathway and include neurofibromatosis type 1 (NF1), Noonan syndrome (NS), NS with multiple lentigines, capillary malformation–AV malformation syndrome, Legius syndrome, Costello syndrome (CS), and cardio-facio-cutaneous syndrome (CFC; Fig. 1; Tidyman & Rauen, 2009). The RASopathies are caused by mutations in genes that encode

components of the RAS pathway, and these conditions have both unique and overlapping characteristics.

The *RAS* genes, *HRAS*, *NRAS*, and *KRAS*, encode small, monomeric GTPases that are activated by upstream regulators, including receptor tyrosine kinases, G-protein-coupled receptors, and integrins. Once activated, RAS-GTP signals through multiple effector pathways, including RAF/MEK/ERK, PI3K/AKT, TIAM1/Rac, and RALGDS/Ral. Mouse models have advanced our understanding of the *in vivo* role of the RAS signaling pathway as well as how it is regulated, and animal models have also pointed to therapeutic targets for the RASopathies. Here, we focus on three of the RASopathies, NS, CS, and CFC, all of which have important craniofacial phenotypes.

NS typically presents with proportional short stature, facial dysmorphism, including a typical “triangular facies” with dolicocephaly, prominent forehead, pointed chin and hypertelorism, as well as blood and cardiovascular abnormalities (Fig. 2A and B; Collins & Turner, 1973; Noonan, 1968; Zenker, 2009). In approximately 50% of NS cases, mutations in the *PTPN11* gene, which encodes the SHP2 phosphatase, cause pathogenesis by increasing phosphatase activity (Tartaglia et al., 2001); other genes known to cause NS include *SOS1* (~10%) (Roberts et al., 2007; Tartaglia et al., 2007), *RAF1* (3–5%) (Pandit et al., 2007; Razaque et al., 2007), *KRAS* (1–2%) (Schubbert et al., 2006; Zenker et al., 2007), *NRAS* (<1%) (Cirstea et al., 2010), and *SHOC2* (<1%) (Cordeddu et al., 2009). Several mouse models with mutations in the genes responsible for NS have revealed the different contributions of specific genes to the syndrome pathology and shed light on the complexity of the RAS pathway. Mice expressing the NS-associated mutation *Ptpn11*^{D61G} (*Ptpn11*^{D61G/+}) have decreased body weight and length, craniofacial dysmorphism including decreased skull length, increased intercanthal distance and wide, blunt snout, myeloproliferative disease (MPD), and a range of cardiac defects (Araki et al., 2004). These mice do not develop hypertrophic cardiomyopathy (HCM), reflecting the fact that only 10% of NS individuals with *PTPN11* mutations develop HCM. However, approximately 95% of patients carrying *RAF1* mutations that cause increased kinase activity develop HCM, and knock-in mice expressing the kinase-activating NS mutation *Raf1*^{L613V} have short stature, facial dysmorphism including short nose and wide-set eyes, and these mice develop HCM (Wu et al., 2011). Mice with the *Sos1*^{E846K}-activating mutation display increased embryonic and perinatal mortality, short stature, craniofacial dysmorphism with short skull length, increased intercanthal distance with blunt snout due to depression of the frontal bone, and severe cardiac hypertrophy (Chen et al., 2010). Another strain of mice carrying an endogenous *K-Ras*^{V14I} germline mutation, the most frequent *KRAS* mutation in NS individuals, display many of the phenotypic abnormalities observed in NS, including small size, craniofacial dysmorphism with increased skull width and height but decreased length, wide separation between the eyes and blunt snout, and cardiac defects (Hernandez-Porrás et al., 2014). Moreover, these mice develop fatal MPD, a disease similar to a leukemia seen in patients with NS. Although these NS mouse models share craniofacial characteristics similar to the triangular facies of NS individuals, the cardiac conditions vary depending upon the gene mutation, emphasizing the specific effects of distinct gene mutations.

CS is characterized by craniofacial malformations including macrocephaly, bitemporal narrowing, convex facial profile, full cheeks and large mouth, dermatologic anomalies, cardiac defects, musculoskeletal abnormalities, growth delay, and cognitive deficits (Fig. 2C and D; Goodwin, Oberoi, et al., 2014; Rauen, 2007). Nearly all individuals with CS have a heterozygous, *de novo* germline mutation in *HRAS* that results in a constitutively active protein (Aoki et al., 2005; Estep, Tidyman, Teitell, Cotter, & Rauen, 2006). The majority of the mutations encode a *HRAS*^{G12S} protein. There are no mouse models expressing *HRAS*^{G12S}; however, mice expressing *HRAS*^{G12V}, the mutation most commonly expressed in *HRAS* tumors (Schubbert, Bollag, & Shannon, 2007), phenocopy and provide an effective model of CS. A mouse model with a germline G12V mutation within the endogenous *H-Ras* locus (*Hras*^{G12V/geo}) had several characteristics of CS, including craniofacial dysmorphism due to depression of the anterior frontal bone, shortening and depression of the nasal bridge and premaxillary bone, and choanal atresia, and HCM including cardiac enlargement and hypertrophic cardiomyocytes (Schuhmacher et al., 2008). Another CS mouse model using a targeted conditional activating *Hras*^{G12V} mutation (*CC/FR-Hras*^{G12V}) displayed many traits associated with CS, including failure to thrive and decreased growth rate, relative acrocephaly, deviated septum, and myocardial fibrosis (Chen et al., 2009). These mice also exhibited an enamel defect characterized by hyperproliferative and disorganized ameloblasts similar to the hypoplastic enamel phenotype observed in CS individuals (Goodwin, Tidyman, et al., 2014). Although these mouse models have provided insight into the features of CS, more work is necessary to understand the underlying pathogenesis of CS.

CFC is a multiple congenital anomaly disorder characterized by craniofacial dysmorphism including macrocephaly, bitemporal narrowing, convex facial profile and hypoplastic supraorbital ridges, ectodermal abnormalities, congenital heart defects, growth delays, and neurocognitive deficits (Fig. 2E and F; Goodwin et al., 2013; Pierpont et al., 2014). CFC is caused by heterozygous, activating germline mutations in *KRAS*, *BRAF*, *MAP2K1* (*MEK1*), or *MAP2K2* (*MEK2*), all components of the RAS/MAPK pathway (Niihori et al., 2006; Rodriguez-Viciana et al., 2006). *BRAF* mutations have been found in human cancers, and the *BRAF*^{V600E} mutation is the most commonly detected (Davies et al., 2002); the kinase activity of this mutation is 10- to 50-fold higher than that of other *BRAF* mutations responsible for CFC (Wan et al., 2004). The *B-Raf*^{+/LSLV600E} mouse model expresses the constitutively active *B-Raf*^{V600E} allele but at 5–10% the level of the wild-type allele to model the *BRAF* activity in CFC individuals more closely. This model phenocopies features of CFC, including reduced size and body weight, more rounded and shorter heads due to changes in the shape of the frontal and parietal bones, cataracts, neurological defects including hyperactivity and seizures, and cardiomegaly due to an increase in the number of cardiomyocytes (Urosevic et al., 2011).

Additional mouse models of high- or intermediate-activity *BRAF* mutations have been developed. A conditional knock-in mouse for the intermediate-activity mutant *Braf*^{L597V} phenocopies features of CFC including short stature, blunt nose, cardiac hypertrophy, and predisposition to develop benign tumors including papillomas and intestinal polyps (Andreadi et al., 2012). Another model expressing the *Braf*^{Q241R} mutation, which is the most frequent mutation in the CFC population, manifests several features of CFC, including heart defects and craniofacial anomalies such as mandibular hypoplasia, before dying

embryonically (Inoue et al., 2014). Also, CFC zebrafish models expressing a kinase-activating *BRAF*^{Q257R} allele or kinase-inactivating *BRAF*^{G596V} allele develop craniofacial anomalies (Anastasaki, Estep, Marais, Rauen, & Patton, 2009). These CFC models have elucidated the biological effects of specific BRAF mutations and revealed the importance of BRAF activity levels in CFC.

Drugs developed to target the RAS pathway in cancer have enormous potential for treatment of developmental syndromes caused by its dysregulation (Rauen et al., 2011, 2015). Utilizing animal models, researchers have begun testing the potential of RAS pathway inhibitors in treatment of the RASopathies. Treatment of NS mice with the MEK1/2 inhibitor PD0325901 restored their weight and length with partial improvement of the heart defects (Chen et al., 2010; Wu et al., 2011). Additionally, when NS mice were treated *in utero* with PD0325901, the craniofacial defects in the mice improved (Wu et al., 2011), and *in utero* treatment with U0126, an inhibitor of MEK1/2, resulted in complete rescue of the development and growth of the neural crest-derived bones of the face (Nakamura et al., 2007). In CS mice, the enamel defect was rescued in adult mice treated with PD0325901; however, treatment with the PI3K inhibitor GDC0941 had no effect on the disrupted ameloblasts or enamel defect, indicating that signaling through MEK is critical in enamel development (Goodwin, Tidyman, et al., 2014). Treatment of the CFC zebrafish model with low doses of PD0325901 at early stages of development ameliorated craniofacial defects (Anastasaki, Rauen, & Patton, 2012). Treatment of the *Braf*^{Q241R/+} embryos with PD0325901 rescued embryonic lethality and ameliorated edema and craniofacial defects, and furthermore, treatment in combination with a histone 3 demethylase inhibitor, GSK-J4, additionally rescued enlarged cardiac valves, suggesting the potential of combined therapies in treating the RASopathies (Inoue et al., 2014). Thus, drugs targeting the RAS pathway have potential in the treatment of RASopathies, but the complexity of RAS dysregulation in these syndromes means that further work will be required to determine optimal timing and dosage of drugs with specific targets.

4. CRANIOSYNOSTOSIS: PURSUING GENETIC AND PHARMACEUTICAL ALTERNATIVES TO SURGICAL TREATMENT

Craniosynostosis is the premature fusion of calvarial sutures, and multiple craniosynostosis syndromes, including Apert, Pfeiffer, and Crouzon, are caused by mutations in fibroblast growth factor receptors (FGFRs). Several mutations in FGFR1 and FGFR2 result in phenotypes that vary in severity yet share some characteristics. These mutations all result in gain of function of FGFR signaling, and a variety of specific molecular mechanisms have been shown to achieve this net effect. Mouse models have been invaluable in understanding how altered FGFR signaling contributes to the pathogenesis of these syndromes.

Apert syndrome is defined by craniosynostosis involving coronal sutures, midface hypoplasia, and bony and/or cutaneous syndactyly of the hands and feet (Bonaventure & El Ghouzzi, 2003; Wilkie, Oldridge, Tang, & Maxson, 2001). Individuals with Apert syndrome also have varying degrees of malformations of the nervous, respiratory, cardiovascular, and genitourinary systems. Furthermore, they have distinct craniofacial characteristics including

increased incidence of cleft palate, narrow, high-arched palate and hypoplastic maxilla, hypodontia, delayed eruption, and dental crowding (Kreiborg & Cohen, 1992; Letra et al., 2007). Apert syndrome is caused by heterozygous, gain-of-function mutations affecting FGFR2, and more than 98% of individuals diagnosed with Apert syndrome carry a Ser252Trp (FGFR2 S252W; 66%) or Pro253Arg (FGFR2 P253R; 32%) mutation (Park et al., 1995; Wilkie et al., 1995). The altered amino acids lie in the linker region between the second and third extracellular immunoglobulin-like domains (IgII and IgIII) that mediate FGF ligand binding. The region encoding IgIII undergoes alternative splicing to form two well-described isoforms, FGFR2b and FGFR2c, which are expressed in the epithelium and mesenchyme, respectively, and have differing affinities for specific FGF ligands. Both mutations have been shown to increase FGFR2 ligand-binding affinity (Anderson, Burns, Enriquez-Harris, Wilkie, & Heath, 1998; Yu, Herr, Waksman, & Ornitz, 2000); the Ser252Trp mutation increases the affinity of FGFR2 for a subset of FGFs, whereas the Pro253Arg mutation increases affinity of FGFR2 for FGF indiscriminately (Ibrahimi et al., 2001).

Mouse models of the two most common Apert syndrome mutations in FGFR2 have shed light on the cellular mechanisms by which these mutations lead to syndromic phenotypes. The *Fgfr2*-Ser250Trp mutation resulted in mice that exhibit craniosynostosis, which is most pronounced in the coronal sutures, and skull malformations (Chen, Li, Li, Engel, & Deng, 2003; Wang et al., 2005). Increased *Bax* expression and apoptosis in the suture mesenchyme (Chen et al., 2003), as well as increased proliferation of chondrocytes and osteoblasts (Wang et al., 2005), have been suggested to contribute to craniosynostosis pathology in other mouse models of Apert syndrome.

When the P253R mutation was inserted in the *Fgfr2* gene using a knock-in approach, the mice had premature closure of the coronal suture, as well as retarded growth of the synchondroses of the cranial base and growth plates of long bones, due to abnormalities in both osteogenesis and chondrogenesis (Wang et al., 2010; Yin et al., 2008). Of note, *Fgfr2*S250T mice did not develop syndactyly, whereas in rare instances *Fgfr2*P253R mice did. These data correlate with genotype–phenotype studies showing that the Pro253Arg mutation causes more severe syndactyly, whereas cleft palate is more common in Ser252Trp individuals (Slaney et al., 1996).

Furthermore, mutations causing ectopic expression of FGFR2 splice variants can result in increased ligand-dependent activation of the receptor. *De novo Alu* insertions within or upstream of alternatively spliced exon 9 (IIIc) of *FGFR2* (Oldridge et al., 1999) resulted in ectopic expression of FGFR2b in mesenchymal tissues of the limb bud that normally express FGFR2c, suggesting a possible mechanism for the syndactyly phenotype in Apert syndrome. Similarly, excision of a single copy of *Fgfr2-IIIc* in mice resulted in a gain-of-function mutation causing precocious ossification of the coronal sutures, zygomatic arch joints, and the sternbrae, as well as major defects in the kidney, lung, and lacrimal glands (Hajihosseini, Wilson, De Moerloose, & Dickson, 2001). In this model, *Fgfr2-IIIb* expression is substantially elevated in calvarial sutures and zygomatic joints, suggesting that cells in the sutures would respond to a broader array of FGF ligands and undergo differentiation prematurely, resulting in premature suture ossification. Thus, Apert syndrome

appears to circumvent the biochemical and developmental regulatory mechanisms that are normally imposed by tissue-specific alternative splicing of *Fgfr2*, resulting in ectopic ligand-dependent receptor activation (Yu & Ornitz, 2001).

Similar to Apert syndrome, Pfeiffer syndrome is characterized by premature fusion of several cranial sutures, widely spaced eyes, a small nose, and midface hypoplasia (Pfeiffer, 1964; Vogels & Fryns, 2006). Additionally, individuals with Pfeiffer syndrome have hand and foot anomalies, including broad thumbs, cutaneous syndactyly, shortened fingers, and medially deviated broad toes; however, unlike Apert syndrome, syndactyly is not a feature of Pfeiffer syndrome. Pfeiffer syndrome is caused by mutations in both FGFR1 and FGFR2, including a cytosine to guanine conversion in exon 5 of FGFR1 that results in a proline to arginine substitution in the extracellular domain (Muenke et al., 1994; Schell et al., 1995). When the Pro250Arg mutation was introduced into *Fgfr1*, mimicking the human mutation, the mice exhibited craniosynostosis, a dome-shaped skull, and a shortened snout (Zhou et al., 2000).

The phenotype of Crouzon syndrome is similar to that of Pfeiffer syndrome and includes craniosynostosis with abnormal skull shape and prominent eyes secondary to early fusion of the cranial sutures, without digital malformations like syndactyly (Crouzon, 1912). More than 30 different point mutations in the extracellular domain of FGFR2, most destroying or creating a cysteine residue, have been identified in individuals with Crouzon syndrome (Reardon et al., 1994; Wilkie et al., 1995). When FGFR2/Neu chimeras were generated with these mutations and expressed in NIH 3T3 cell cultures, FGFR2 was constitutively active due to aberrant intermolecular disulfide bonds (Galvin, Hart, Meyer, Webster, & Donoghue, 1996). These data suggest that the underlying pathology of Crouzon syndrome is the creation of unpaired cysteine residues, which facilitate the formation of intermolecular disulfide bonds, causing ligand-independent dimerization, phosphorylation, and signaling. The common Pfeiffer/Crouzon gain-of-function mutation Cys342Tyr was introduced into *Fgfr2c*, and the *Fgfr2c*^{C342Y/+} heterozygote mice were characterized by a shortened face, protruding eyes, and premature fusion of cranial sutures (Eswarakumar et al., 2004), due to ligand-independent activation of FGFR2 via disulfide bond dimerization (Eswarakumar et al., 2006). Notably, when a mutation inhibiting FRS2 binding was introduced together with the C342Y mutation, the phenotype was rescued, indicating that the FRS2 adaptor is a key mediator of FGFR2 signaling in craniosynostosis pathogenesis and suggesting a potential point of therapeutic intervention (Eswarakumar et al., 2006).

Currently, treatment of craniosynostosis is almost exclusively surgical and consists of removing fused sutures with osteotomies and reconstructing the skull (Panchal & Uttchin, 2003). More recently, surgeons have utilized distraction osteogenesis, in which osteotomies are performed and force is applied by an external or internal device to separate the skull fragments (Gasparini, Di Rocco, Tamburrini, & Pelo, 2012). Additionally, specific synostoses may be treated with minimally invasive endoscopic repairs that result in reduced blood loss and earlier hospital discharge (Jimenez & Barone, 1998). Due to the morbidity associated with multiple surgeries to treat craniosynostosis, pharmacologic approaches are an attractive option to consider. Utilizing mouse models to understand the underlying pathogenesis of craniosynostosis associated with FGFR mutations has pinpointed

downstream effectors that may serve as targets in treatment of FGFR-related craniosynostosis syndromes. Treatment of calvarial explants from Crouzon-like *Fgfr2^{c342Y/+}* mice with the small-molecule FGFR inhibitors PLX052 or PD173074 prevented premature suture fusion (Eswarakumar et al., 2006; Perlyn, Morriss-Kay, Darvann, Tenenbaum, & Ornitz, 2006). Similarly, treatment of cultured calvaria from Apert-like P253R mice with the ERK1/2 inhibitor PD98059 partially alleviated the coronal suture fusion (Yin et al., 2008). *In vivo*, mice with the *Fgfr2^{S252W}* mutation expressing a small hairpin RNA targeting *Fgfr2^{S252W}* had patent cranial sutures and normal skull development (Shukla, Coumoul, Wang, Kim, & Deng, 2007). Furthermore, increased phosphorylation of ERK1/2 was observed in *Fgfr2^{S252W}* mice, and treatment of these mice *in utero* at E18.5 and postnatally with U0126, an inhibitor of MEK1/2, resulted in rescue of the craniosynostosis phenotype (Shukla et al., 2007). Thus, utilization of mouse models has elucidated potential downstream targets of FGFR signaling for pharmacological treatment of craniosynostosis. However, resynostosis has been noted after withdrawal of inhibitors, and further investigation is necessary to develop drugs with specific targets to maximize efficacy and minimize side effects.

5. XLHED: DEVELOPING TREATMENT BASED ON KNOWLEDGE GAINED FROM MOUSE AND CANINE MODELS

In 1848, an early case study reported a male subject with an “almost complete absence of hair; the teeth being not more than four in number; the delicate structure of the skin; and the absence of sensible perspiration and tears” (Thurnam, 1848). In 1875, Charles Darwin made similar observations on a family of males in India (Darwin, 1875). These men suffered from a hereditary condition that disrupts the development of ectodermal structures and their appendages, termed ectodermal dysplasia (ED; Smith, 1929).

Over 150 syndromes have been categorized as EDs, and the most prevalent subgroup is comprised of the hypohidrotic ectodermal dysplasias (HEDs), which affect more than 1 in 17,000 live births (Itin & Fistarol, 2004; Pinheiro & Freire-Maia, 1994). Clinical features of HED include sparseness of scalp and body hair (hypotrichosis); reduced sweating (hypohidrosis) and tear production; and malformation and reduced number of teeth (hypodontia), with an average of nine permanent teeth (Fig. 3; Clarke, 1987; Kobiela et al., 2001; Lexner, Bardow, Hertz, Nielsen, & Kreiborg, 2007; Solomon & Keuer, 1980). In addition, many HED patients exhibit abnormalities in facial appearance, such as prominent forehead, depressed nasal bridge, hypoplastic nose, prominent supraorbital ridges, and thick lips (Goodwin, Larson, et al., 2014; Gunduz Arslan, Devocioglu Kama, Ozer, & Yavuz, 2007; Johnson et al., 2002; Lexner et al., 2007). These clinical phenotypes serve as the initial diagnostic features for most affected individuals with HED. Early diagnosis is crucial because affected children can develop recurrent hyperpyrexia due to impaired sweating and temperature control ability, which can result in febrile seizures; some authors have suggested that neurologic damage can result and have proposed a mortality rate of about 30% during the first 2 years of life (Clarke, 1987; Salisbury & Stothers, 1981).

HED individuals carry mutations in one or more of the core genes in the ectodysplasin pathway: ectodysplasin ligand (*EDA*), ectodysplasin receptor (*EDAR*), or EDAR-associated death domain adaptor protein (*EDARADD*; reviewed in Sadier, Viriot, Pantalacci, & Laudet 2014). *EDA* is a type II transmembrane tumor necrosis factor superfamily member that is proteolytically processed into a soluble trimeric form to bind the receptor, *EDAR*. Upon activation, *EDAR* recruits *EDARADD* via its intracellular death domain region. In turn, *EDARADD* transduces the signal to activate the NF- κ B pathway, which mediates the transcriptional activation of the target genes involved in hair, sweat gland, and tooth development (Lefebvre, Fliniaux, Schneider, & Mikkola, 2012). In rare cases, HED can be associated with immune deficiency caused by dysregulation of NF- κ B (Doffinger et al., 2001; Jain et al., 2001; Zonana et al., 2000). *EDA* has several splicing isoforms; *EDA-A1* and *-A2* interact with *EDAR* and *EDA2R* (formerly known as *XEDAR*), respectively.

Mutations in *EDAR* and *EDARADD* are inherited in an autosomal dominant or recessive fashion, whereas *EDA* mutations are inherited in an X-linked manner. Phenotypes caused by mutations at different points along the *EDA* pathway are clinically similar, with autosomal dominant forms of *EDAR* and *EDARADD* exhibiting milder features. Here, we will focus on X-linked HED (XLHED), which is caused by mutations in *EDA* and is the most common form of HED.

Tabby mice that exhibit clinical features of XLHED were described in the mid-1900s, but it was not until three decades later that the *Tabby* gene was confirmed to be orthologous to human *EDA* through synteny mapping and comparison of predicted amino sequence (Bayes et al., 1998; Falconer, 1952; Ferguson et al., 1997; Srivastava et al., 1997). *Tabby* mice carry spontaneous loss-of-function mutations in *Eda*, resulting in phenotypes comparable to those observed in XLHED individuals: hypodontia with incomplete penetrance; reduced size and cusps of the molars; reduction in four pelage hair types (awl, auchene, guard, zigzag) to a single awl-like hair type; missing hair on tail and retroauricular regions; missing sweat glands on footpads; and reduction in size of various glands, including lacrimal and salivary glands (Cui et al., 2003; Gruneberg, 1965, 1971; Pispá et al., 1999; Srivastava et al., 1997). In contrast, overexpression of the *Eda-A1* transgene in developing murine ectoderm resulted in supernumerary mammary glands and teeth, hypertrophy of sebaceous glands, and abnormal composition and structure of pelage hairs (Mustonen et al., 2003; Srivastava et al., 2001). These mouse models with dysregulation of the *EDA* pathway suggest the importance of the *EDA* pathway in fine-tuning the size, number, and morphology of ectodermal organs.

Controlled expression of a tetracycline-regulated *Eda-A1* transgene during *Tabby* embryogenesis revealed that ectodysplasin signaling is required for hair development within defined spatiotemporal windows (Cui, Kunisada, Esibizione, Douglass, & Schlessinger, 2009). *Tabby* phenotypes can also be ameliorated by administration of recombinant Fc:EDA1, which contains the EDAR-binding domain linked to the IgG1 Fc domain to allow trafficking across the placental barrier and trimerization of the ligand (Fig. 4; Gaide & Schneider, 2003). As with transgenic rescue, Fc:EDA1 efficacy was timing dependent; fetal, but not neonatal, administration rescued tooth morphology, as well as some hair and gland phenotypes. Interestingly, whereas transgenic expression of *Eda-A1* was able to rescue the

number but not the morphology of molars, Fc:EDA1 treatment restored morphology but not molar tooth number (Gaide & Schneider, 2003).

Subsequently, a colony of dogs with XLHED-like features was characterized and found to carry a spontaneous mutation in *Eda* that resulted in truncated EDA-A1 and -A2 (Casal, Jezyk, Greek, Goldschmidt, & Patterson, 1997). EDA replacement experiments were then performed in the canine model, but because immunoglobulins are not transferred transplacentally in dogs, Fc:EDA1 was administered postnatally via intravenous injection. The treatment effectively rescued the sweat glands, as observed in *Tabby* mice. Although hair growth was not improved, the number and shape of permanent teeth were restored in most of the treated dogs (Casal et al., 2007). Furthermore, susceptibility to eye and respiratory infections was also reduced through restoration of lacrimal as well as tracheal and bronchial glands. These results collectively demonstrate that short-term treatment with recombinant EDA during a therapeutic window can dramatically improve the XLHED phenotype in murine and canine models. Importantly, the treatment did not cause any noticeable side effects during or after the injections, showing great promise for the potential utility of such a therapeutic intervention in patients with XLHED. Currently, clinical trials are underway to administer recombinant EDA to XLHED-affected males in the immediate postnatal period (Fig. 4), constituting the first attempt to treat a structural birth defect in humans with a targeted drug therapy.

6. CONCLUDING THOUGHTS

The conditions that we have reviewed in this chapter, including orofacial clefting, the RASopathies, craniosynostosis syndromes, and XLHED, illustrate the exciting recent advances in our understanding of the molecular underpinnings of craniofacial conditions. These disorders lie at various points along a spectrum that has at one end basic discovery science and at the other end clinical trials of targeted therapies. The studies described above have led to an increased understanding of the etiology of these conditions and have revealed the pathways and processes underlying craniofacial development as well as general principles that are applicable to our broader understanding of organogenesis. These examples provide hope that, as the field of craniofacial biology continues to move forward, a shift will ensue from surgical correction toward medical treatment and even prevention. The barriers to such a goal include the timing of diagnosis, developmental pleiotropy of signaling pathways, and the unpredictable consequences of specific gene mutations in a given individual. Overcoming these barriers will require continued intensive study of the basic mechanisms that underlie these diseases.

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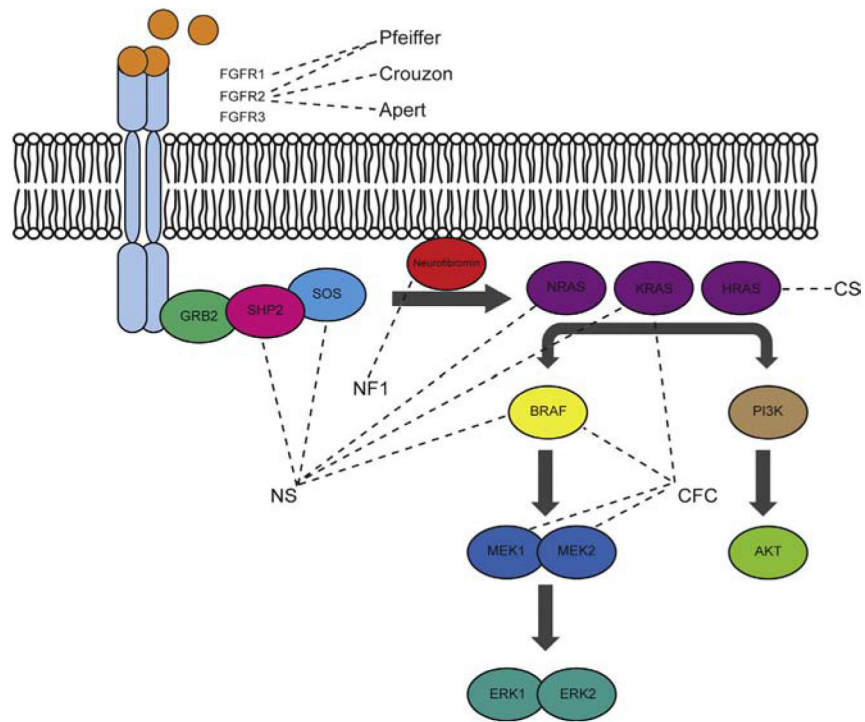


Figure 1. Syndromes of the RAS pathway. Schematic of the RAS signaling pathway with dashed lines connecting the syndrome with the protein in the pathway encoded by the causative mutated gene. Syndromes described in the text. NS, Noonan syndrome; NF1, neurofibromatosis 1; CFC, cardio-facio-cutaneous syndrome; CS, Costello syndrome.



Figure 2. Craniofacial features of the RASopathies. The RASopathies are caused by mutations in genes encoding different proteins in the RAS pathway and have both unique and overlapping craniofacial characteristics. (A) Frontal and profile photographs of a 7-year-old male and (B) 21-year-old female with Noonan syndrome (NS), (C) a 20-year-old male and (D) 23-year-old female with Costello syndrome (CS), and (E) a 15-year-old male and (F) 15-year-old female with cardio-facio-cutaneous syndrome (CFC).



Figure 3. Craniofacial and dental characteristics of XLHED. (A) 13-year-old male with facial characteristics of XLHED including short face with proportionally longer chin and midface, narrow and pointed nose, narrow mouth with full, rounded lower lip and (B) dental features such as missing teeth and conical-shaped incisors and molars with abnormal cuspal morphology. (C) A 21-year-old male with midface hypoplasia and prognathic mandible and chin and short philtrum, and (D) missing teeth, typical of XLHED.

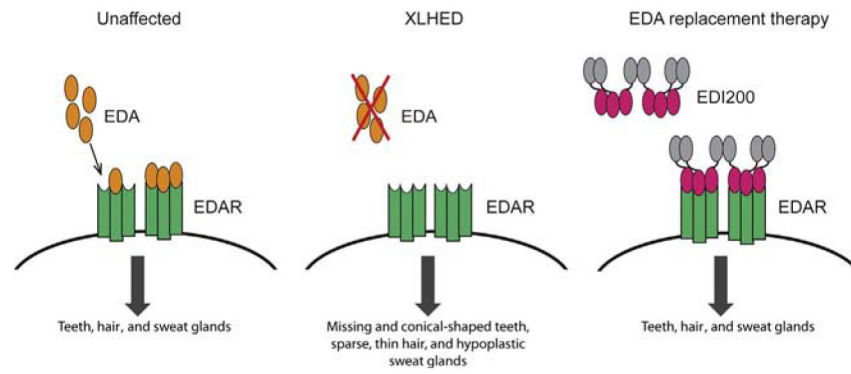


Figure 4. Schematic of recombinant EDA therapy. In unaffected individuals, EDA binds EDAR, activating downstream signaling that results in tooth, hair, and sweat gland development. XLHED individuals are missing EDA, which results in missing and conical-shaped teeth, sparse, thin hair, and hypoplastic sweat glands. EDI200 therapy replaces missing EDA in XLHED and rescues tooth, hair, and sweat gland development.

Table 1

Cleaving Genes and Candidate Loci with Associated Mouse Models of Orofacial Clefting

Human Disease	Gene/Locus	Mouse Phenotype
Nonsyndromic CL/P	<i>MSX1</i>	Cleft palate (Satokata & Maas, 1994)
Nonsyndromic CL/P	<i>TGFβ3</i>	Cleft palate (Kaartinen et al., 1995; Proetzel et al., 1995)
Nonsyndromic CL/P	<i>IRF6</i>	CP (Ingraham et al., 2006; Richardson et al., 2006)
Nonsyndromic CL/P	<i>8q24 locus</i>	Deletion of 8q24 region in mice results in CL/P possibly by loss of <i>Myc</i> regulation (Uslu et al., 2014)
Nonsyndromic CL/P	<i>PDGFC</i>	CP (Ding et al., 2004)
Nonsyndromic CL/P	<i>VAX1</i>	CP (Bertuzzi, Hindges, Mui, O'Leary, & Lemke, 1999)
Nonsyndromic CL/P	<i>ARHGAP29</i> (<i>ABCA4 locus</i>)	CP (Leslie et al., 2012)
Nonsyndromic CL/P	<i>BMP4</i>	Conditional ablation in the epithelium results in CL (Liu et al., 2005)
Nonsyndromic CL/P	<i>FGFR2</i>	Conditional ablation in the epithelium results in CP (Hosokawa et al., 2009; Rice et al., 2004)
Nonsyndromic CL/P	<i>MYH9</i>	Conditional ablation in the epithelium results in fusion defects; compound homozygous loss of <i>Myh9</i> ; <i>Myh10</i> results in CP (Kim et al., 2015)
Syndromic CL/P	Gene/Locus	Mouse Phenotype
Van Der Woude syndrome (OMIM#119300)	<i>IRF6</i>	CP (Ingraham et al., 2006; Richardson et al., 2006)
Ankyloblepharon–ectodermal defects–cleft lip/palate (AEC) (OMIM#106260); Ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) (OMIM#129900)	<i>P63</i>	Knock-in of human allele results in autosomal dominant CP and phenocopies AEC (Ferone et al., 2012), and homozygous null displays CL/P and phenocopies EEC (Moretti et al., 2010; Thomason, Dixon, & Dixon, 2008)
Loeys–Dietz syndrome (OMIM#609192)	<i>TGFBR1</i> , <i>TGFBR2</i> , <i>SMAD3</i> , <i>TGFB2</i>	Conditional ablation of <i>TgfBR1</i> or <i>TgfBR2</i> in palate epithelium or NCC results in CP (Dudas et al., 2006; Ito et al., 2003; Iwata et al., 2012; Xu et al., 2006)
Stickler type 1 (OMIM#108300)	<i>COL2A1</i>	CP (Li, Prockop, et al., 1995)
Stickler type 2 (OMIM#604841)	<i>COL11A1</i>	CP (Li, Lacerda, et al., 1995; Seegmiller, Fraser, & Sheldon, 1971)
Smith–Lemli–Opitz syndrome (OMIM#270400)	<i>DHCR7</i>	CP phenocopies SLOS (Wassif et al., 2001)
Treacher Collins (OMIM#154500)	<i>TCOF1</i>	CP phenocopies Treacher Collins (Dixon et al., 2006; Jones et al., 2008)
Craniofrontonasal syndrome (CFNS) (OMIM#304110)	<i>EFNB1</i>	CP phenocopies CFNS (Bush & Soriano, 2010; Compagni, Logan, Klein, & Adams, 2003)
Pierre Robin syndrome (OMIM#261800)	<i>SOX9</i>	CP (Bi et al., 2001)
Andersen Syndrome (OMIM#170390)	<i>KCNJ2</i>	CP (Zaritsky, Eckman, Wellman, Nelson, & Schwarz, 2000)
Cleft palate with or without ankyloglossia, X-linked (CPX) (OMIM#303400)	<i>TBX22</i>	CP phenocopies CPX (Pauws et al., 2009)
DiGeorge syndrome (OMIM#188400)	<i>TBX1</i>	CP phenocopies DiGeorge (Jerome & Papaioannou, 2001)
Branchiooculofacial syndrome (OMIM#113620)	<i>TFAP2a</i>	Chimeras and hypomorphic embryos have CL/P (Green et al., 2015; Nottoli, Hagopian-Donaldson, Zhang, Perkins, & Williams, 1998), ablation from NCC results in CP (Brewer, Feng, Huang, Sullivan, & Williams, 2004), deletion from midface results in midline cleft (Nelson & Williams, 2004), hypomorphic allele results in CL/P
Greig cephalopolysyndactyly syndrome (OMIM#175700)	<i>GLI3</i>	CP (Huang, Goudy, Ketova, Litingtung, & Chiang, 2008)
Holoprosencephaly 9 (OMIM#610829)	<i>GLI2</i>	CP (Mo et al., 1997)

Human Disease	Gene/Locus	Mouse Phenotype
Coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies (CHARGE) (OMIM#214800)	CHD7	CP phenocopies CHARGE (Sperry et al., 2014)
Hypogonadotropic hypogonadism with or without anosmia (Kallman syndrome) (OMIM#147950)	FGFR1	CP (Trokovic, Trokovic, Mai, & Partanen, 2003) or CP with midline cleft (Wang et al., 2013)
Crouzon syndrome(OMIM#101200)	FGFR2	CP, phenocopies Crouzon (Eswarakumar, Horowitz, Locklin, Morriss-Kay, & Lonai, 2004)
Apert syndrome (OMIM#101200)	FGFR2	CP, phenocopies Apert (Martinez-Abadias et al., 2013)
Otopalatodigital syndrome (OMIM#311300)	FLNA	CP (Hart et al., 2006)
Lymphedema-distichiasis syndrome (OMIM#153400)	FOXC2	CP (Iida et al., 1997; Winnier, Hargett, & Hogan, 1997)
Hypothyroidism, athyroidal, with spiky hair and cleft Palate (OMIM#241850)	FOXE1	CP, models hereditary thyroid dysgenesis and cleft palate (De Felice et al., 1998)
Glass syndrome (OMIM#612313)	<i>SATB2</i>	CP phenocopies 2q32-q33 deletion syndrome (Britanova et al., 2006)
Saethre–Chotzen syndrome (OMIM#101400)	TWIST1	Ablation in mandibular NCCs results in posterior CP (Zhang et al., 2012)

Complete tables of human clefting genes and loci, including those without mouse models can be found in Dixon et al. (2011).