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Genetics and signaling mechanisms of orofacial clefts

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Abstract

Craniofacial development involves several complex tissue movements including several fusion processes to form the frontonasal and maxillary structures, including the upper lip and palate. Each of these movements are controlled by many different factors that are tightly regulated by several integral morphogenetic signaling pathways. Subject to both genetic and environmental influences, interruption at nearly any stage can disrupt lip, nasal, or palate fusion and result in a cleft. Here, we discuss many of the genetic risk factors that may contribute to the presentation of orofacial clefts in patients, and several of the key signaling pathways and underlying cellular mechanisms that control lip and palate formation, as identified primarily through investigating equivalent processes in animal models are examined.

Keywords

Cleft lip/palate; Syndromic/non-syndromic; Human genetics; Mouse models; Bmp/Tgfb signaling; Fgf signaling; Retinoic acid signaling; Shh signaling; Wnt signaling; Signaling crosstalk

1. Introduction

Orofacial clefts (OFCs) are among the most common birth defects worldwide and contribute a heavy burden on patients and their families as well as healthcare systems. Clefts can be observed in the context of other syndromic characteristics, but frequently occur as an isolated, nonsyndromic presentation. During embryonic development, several midfacial primordia fuse together to form orofacial structures, including the nose, upper lip, and the palate separating the oral and nasal cavities. Complex regulatory mechanisms control these

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processes and are subject to both genetic and environmental influences. When they fail, defective closure of facial tissues may manifest as a structural cleft at birth. OFCs are typically classified as either cleft lip with or without cleft palate (CL/P) or cleft palate only (CPO). As the processes that govern lip and palate formation differ along with their respective causes and risk factors, it is becoming more common for studies to group CL/P patients with cleft lip only (CLO) separately from those with cleft lip with cleft palate (CLP) to more accurately investigate the causal relationships between potential influences and specific types of resulting OFCs (L. Huang et al., 2019). This review is a part of a series discussing the underlying mechanisms that contribute to OFC formation. The developmental processes and germ layers involved in orofacial formation and their role in the presentation of clefts are reviewed in greater detail within a companion article (Ji et al., 2020 in revision). Here, we will discuss some of the known genetic influences and risk factors in oral clefts, as well as the underlying signaling pathways that govern lip and palate formation and their role in OFCs.

2. Genetics of human orofacial clefts

Prior to the genomic era, early segregation studies recognized that while many OFCs appear sporadically, they often can be attributed to heritable alleles and occur in multiple cases within the same family. Studies commonly include large multiplex or consanguineous families or smaller case-parent trios to identify inherited causes, but they may compare patients with sporadic cases and unrelated controls. Attempts to identify genetic causes of OFCs began with association of markers such as variable serum antigens and restriction site polymorphisms which segregated with OFCs within familial genetic pedigrees. These were followed by genome-wide scans to identify regions of the genome that associated with clefts and targeted genotyping and studies to pinpoint the sequence variants that were linked with OFCs. Since the completion of the human genome project, reference genome and single nucleotide polymorphism (SNP) databases have been compiled and are continuing to be improved. The availability of these tools to investigators has allowed for more efficient identification of specific variants that may be linked with traits, and evidence for the association of many important candidate genes and loci in the etiology of OFCs, both syndromic and nonsyndromic, has been amassed. The history of genetics studies of OFCs has been previously reviewed (Mary L. Marazita, 2012). Modern sequencing technologies have accelerated our ability to identify specific sequences and variants that are linked with clefts, and at least 350 potential candidate genes have been identified through association studies in human OFC patients alone. Sufficient evidence has accumulated for many genes and loci involved in craniofacial development that may contribute to OFCs, such that we can begin to describe a network of key factors that have clear implications in governing lip and/or palate formation and fusion.

2.1. Genetics of nonsyndromic OFCs

OFCs are often observed in conjunction with other abnormal morphological characteristics as part of a syndrome, and syndromic defects frequently are caused by mutations and deletions that result in a loss of function of one or more genes. However, more commonly seen are nonsyndromic OFCs, which are often not attributable to a single easily identifiable

mutation, but are likely controlled by many different risk factors and genetic variants that do not result in a loss of function and do not cause an observable phenotype on their own. As such, attempts to determine genetic causes of nonsyndromic clefts have proven in many cases to be difficult and results can be highly variable, even in studies assessing linkage of the same genomic variants with OFCs. This may be due to differences between genetic backgrounds, environmental influences across patient cohorts, or experimental techniques and analysis methods.

Genome-wide association studies (GWAS) provide an unbiased approach to identify genetic risk factors and several have been performed to determine genomic regions that segregate with clefts, most seeking linkage with nonsyndromic CL/P (NSCL/P), the most common type of OFC (Table 1). Many of the earlier genome scans provided suggestive evidence for cleft association with loci but were unable to establish genome-wide significance. However, a meta-analysis of 13 scans including data from several of these earlier studies demonstrated the strongest evidence at the time for NSCL/P association with several regions. Highly significant results were obtained at 9q21, so linkage with key candidate genes at this locus was assessed and positive associations reported with patched (*PTCH1*), receptor tyrosine kinase (RTK)-like orphan receptor 2 (*ROR2*), transforming growth factor-beta receptor 1 (*TGFBRI*), and forkhead box E1 (*FOXE1*) (Mary L. Marazita et al., 2004). A subsequent GWAS and fine-mapping in 2009 by the same group also showed evidence for NSCL/P association with *FOXE1*, which has since been strengthened by multiple other studies (Beaty et al., 2013; Elizabeth J. Leslie et al., 2017; M. L. Marazita et al., 2009; Moreno et al., 2009; Nikopensius et al., 2011), as well as several new loci including 1q32 in which interferon regulatory factor 6 (*IRF6*) lies, in important craniofacial regulator that had previously been implicated in targeted studies but not genome scans (Blanton et al., 2005; Ji Wan Park et al., 2007; Scapoli et al., 2005; Zuccherro et al., 2004). Several other GWAS around this time also identified other important loci involved in NSCL/P, including strong association with the genomic region at 8q24.21, which lies in a gene desert (Beaty et al., 2010; Birnbaum et al., 2009; Grant et al., 2009). While the role this locus plays in NSCL/P is not entirely clear, variants may affect expression of transcription factor c-MYC (*MYC*), which maps to 8q24 and is involved in craniofacial development (Uslu et al., 2014).

One 2010 GWAS found genome-wide association with markers near ventral anterior homeobox 1 (*VAX1*) and the bone morphogenetic protein (BMP) regulator noggin (*NOG*), each of which has been identified in subsequent scans (Mangold et al., 2010) (Table 1). The muscle segment homeobox (*Msx*) genes are regulated by Bmp signaling, and several targeted studies have implicated *MSX1* variants in the etiology of NSCL/P prior to being linked in GWA studies (Jezewski et al., 2003; Lidral et al., 1998; J. Suazo, Santos, Carreno, Jara, & Blanco, 2004; Y. Suzuki et al., 2004). *VAX1* is an important transcription factor with roles in craniofacial development, and targeted studies have further strengthened the evidence for association of *VAX1* SNPs with NSCL/P as well (A. Butali et al., 2013; de Araujo et al., 2016; B.-H. Zhang, Shi, Lin, Shi, & Jia, 2018). Another 2010 study reported association with 4 regions including two novel loci, V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*) and ATP-binding cassette, sub-family A, member 4 (*ABCA4*), which were confirmed in replication studies. *MAFB* has been identified in multiple genome scans since, while *ABCA4* variants may contribute to NSCL/P

by altering expression of *ARHGAP29* at the same locus (Beaty et al., 2010; Beaty et al., 2013; Azeez Butali et al., 2014; Elizabeth J. Leslie et al., 2012). One of the earlier candidate genes identified that has been thoroughly examined is transforming growth factor-alpha (*TGFA*), but inconsistent results have been unable to provide strong evidence for its role in OFCs (Vieira, 2006).

A 2017 GWAS and meta-analysis in a Chinese population linked many previously known genes as well as several novel loci with NSCL/P. These authors also performed a network analysis to describe interactions between NSCL/P-associated genes, linking several signaling pathways and transcription factors that govern lip and palate fusion (Y. Yu et al., 2017). One novel gene linked in this study, transcription factor AP-2 alpha (*TFAP2A*), regulates craniofacial *IRF6* and had previously been implicated in NSCL/P through targeted studies as a strong candidate based on its role in animal OFC models (de Araujo et al., 2016; Martinelli, Masiero, et al., 2011; M. Shi et al., 2009). Among signaling pathway genes identified are several fibroblast growth factor (FGF) pathway components with extensive network interaction, including *FGF10*, the receptor *FGFR1*, and antagonists Sprouty 1 and 2 (*SPRY1/2*), the first three of which were novel. Components of the Sonic hedgehog (SHH) and wntless-type MMTV integration site family (WNT) pathways were also identified, including *PTCH1* and *WNT9B*, and the latter is a homolog of an important CL/P mouse model gene (Juriloff, Harris, McMahon, Carroll, & Lidral, 2006; Y. Yu et al., 2017). Many genes previously linked with OFCs were also identified/confirmed in the Yu et al. study, as well as another recently performed extensive GWAS (L. Huang et al., 2019).

Tumor protein p63 (*TP63*) encodes another transcription factor associated with multiple syndromes that can include OFCs (Table 2). *TP63* is a canonical WNT signaling target, and has been recently implicated in NSCL/P in both a GWA meta-analysis and exome-sequencing study (Basha et al., 2018; Elizabeth J. Leslie et al., 2017). A recent study that identified the first OFC association with Pre-B cell leukemia homeobox 1 (*PBX1*) and *PBX2* mutations also identified gene-gene interactions between SNPs in *PBX1* and *WNT9B*, and between *IRF6* and each of *PBX1*, *PBX2*, and *TP63* in the etiology of NSCL/P (Maili et al., 2019). These findings are consistent with a prior study that linked Pbx, p63, and Irf6 with Wnt signaling in midfacial development in animal models (Ferretti et al., 2011).

The extracellular matrix (ECM) plays many important roles in the tissue movements that occur during craniofacial morphogenesis, and as such its function is intimately linked with OFCs. The mechanisms by which ECM activity contributes to OFCs is discussed in greater detail within a companion review article (Ji et al., 2020 in revision), though several genes involved in ECM regulation have been linked to NSCL/P, including the laminin-related netrin 1 (*NTN1*), implicated by both genome-wide and targeted studies (Beaty et al., 2010; Beaty et al., 2013; Q. Guo et al., 2017; S. Jiang et al., 2019; E. J. Leslie et al., 2016; Y. Yu et al., 2017). Other ECM regulatory molecule genes for which possible association with nonsyndromic OFCs have been found include those encoding the ECM remodeling proteins matrix metalloproteinase 3 and 16 (*MMP3/16*) (Kumari, Singh, & Raman, 2018; Y. Yu et al., 2017), tissue inhibitor of metalloproteinase 2 (*TIMP2*) (Nikopentius et al., 2011), and ADAM Metalloproteinase with thrombospondin type 1 motif 20 (*ADAMTS20*) (Wolf et al., 2015). The collagen genes *COL4A3* and *COL4A4* showed linkage disequilibrium with

NSCL/P in case-parent trios in 3 populations (Beaty et al., 2006), while *COL2A1* and *COL11A2* were linked with NSCPO in a case-control genotyping study (Nikopensius et al., 2010). Fgf signaling interacts with the ECM and plays key roles in lip and palate fusion, will be discussed in greater detail below. Notch signaling does so as well, and several studies have linked polymorphisms in the Notch ligand gene jagged 2 (*JAG2*) with nonsyndromic clefts (de Araujo et al., 2016; Jagomägi et al., 2010; Vieira et al., 2005).

Environmental exposures are strongly linked with OFCs, which will be discussed extensively in another companion review (Garland et al., 2020 in revision). As a result, mutations in many of the genes that code for enzymes that metabolize or detoxify chemicals associated with such exposure can also contribute to the presentation of clefts. Retinoic acid/vitamin A metabolism and signaling are especially integral to craniofacial development and deviations in cellular metabolite levels can result in birth defects including OFCs. As such, several genes associated with retinoid activity have been linked with nonsyndromic clefts, including retinoic acid receptor alpha (*RARA*) (Fan, Li, & Wu, 2007; Peanchitlertkajorn, Cooper, Liu, Field, & Marazita, 2003; Xavier et al., 2013) and possibly *ABCA4*, which acts as a flippase-type transporter for retinoids (Beaty et al., 2010; Beaty et al., 2013; Fontoura, Silva, Granjeiro, & Letra, 2012; Q. Yuan, Blanton, & Hecht, 2011).

Folates are another key vitamin required for production of the amino acid methionine and many other important cellular compounds, and insufficient dietary intake can result in OFCs. One of the most well-studied genes in NSCL/P encodes the folate metabolism enzyme methylenetetrahydrofolate reductase (*MTHFR*), and in particular it is maternal rather than fetal missense mutations that have been shown to contribute to OFC risk (Martinelli et al., 2001; Pan et al., 2015; Pezzetti et al., 2004). Genes encoding several other enzymes involved in the folate/homocysteine metabolic pathway have been associated with NSCL/P with a similar importance of maternal genotype, further demonstrating the importance of maternal folate metabolism in modulating fetal lip and palate fusion. These include methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), methylenetetrahydrofolate dehydrogenase (*MTHFD1*), reduced folate carrier 1 (*SLC19A1*), betaine-homocysteine methyltransferase (*BHMT*), and a gene-gene interaction between *BHMT* and dimethylglycine dehydrogenase (*DMGDH*), which uses folate as a cofactor, was determined to significantly contribute to risk of NSCL/P in an international cohort (Bufalino et al., 2010; Mills et al., 2008; A. Mostowska, Hozyasz, & Jagodzinski, 2006; Soghani et al., 2017; P. Wang et al., 2018; W. Wang, Jiao, Wang, Sun, & Dong, 2016).

Lipid metabolism plays important roles during craniofacial morphogenesis, and is regulated by Tgf- β signaling during secondary palatogenesis in animal models (J. Iwata et al., 2014). Several genes involved in lipid metabolism have been implicated in nonsyndromic cleft studies, including the fatty acid hydrolase androgen-dependent TFPI-regulating protein (*ADTRP*) (J. W. Park et al., 2006), apolipoprotein C2 (*APOC2*) (Mary L. Marazita et al., 2002), and stearoyl-CoA desaturase 5 (*SCD5*) (Beiraghi et al., 2003), and a gene-gene interaction between variants in the paraoxonases 1 and 2 (*PON1/2*), which protect against damage due to low-density lipoprotein oxidation, was determined to contribute to NSCL/P risk (Machado et al., 2019). Variants in genes that encode enzymes involved in metabolizing drug and environmental toxins may contribute to formation of OFCs, including aryl

hydrocarbon receptor nuclear transporter (*ARNT*) (Kayano et al., 2004), alcohol dehydrogenase 1C (*ADH1C*) (Jugessur et al., 2009) and sulfotransferase 2A1 (*SULT2A1*) (A. Butali et al., 2019). Further, studies investigating the role toxin metabolism plays in OFC development have demonstrated gene-environment interactions contribute to NSCL/P risk, including between maternal smoking and fetal variants in glutathione S-transferase T1 (*GSTT1*), arylamine N-acetyltransferase 1 (*NAT1*), and *NAT2* (Lammer, Shaw, Iovannisci, Van Waes, & Finnell, 2004; M. Shi et al., 2007), and between maternal occupational chemical exposures and fetal *NAT2* and *GSTM1* variants (Shaw, Nelson, Iovannisci, Finnell, & Lammer, 2003).

2.2. Genetics of syndromic OFCs

While syndromic OFCs are more often attributable to one congenital cause or disrupted gene than NSOFCs, other challenges arise in determining the underlying mechanisms. Many of the syndromic diseases that can include OFCs are heterogenous in both genotype and phenotype, and often the presentation of a cleft in conjunction with other defining characteristics can be variable in penetrance. For many syndromic conditions in which clefts are mild or uncommon, the underlying etiologies associated with an OFC phenotype may be more difficult to determine. Often multiple disease-causing genes and factors can exist, and the role of specific genes in cleft-associated cases may not be described as thoroughly, especially if clefts are a minor feature and not the primary focus of studies into a particular condition. Additionally, many syndromes are caused by deletions that disrupt several genes, further complicating the connection between specific loci and lip or palate fusion. However, many instances in which a case study of a smaller group or individual patient is available with the mutation data, it is more often possible to attribute to the OFC to the role of a specific disrupted gene or locus.

Van der Woude syndrome (VWS) accounts for the most common form of syndromic clefts, which are often indistinguishable from NSCL/P but often includes lip pits. The majority of cases are caused by mutations in *IRF6*, which are also linked with dominant Popliteal pterygium syndrome (PPS), a condition that affects the skin and genitals and also frequently includes CL/P (Kondo et al., 2002). Mutations in Grainyhead-like 3 (*GRHL3*), an ectodermal *IRF6* target (Kousa et al., 2019), were identified as the cause of many of the VWS cases in which no causative variant in *IRF6* could be identified (Peyrard-Janvid et al., 2014). Cytosolic IRF6 interacts with regulators of cytoskeletal remodeling and cell adhesion, nonmetastatic expressed 1 (NME1) and NME2. *IRF6* VWS mutations interrupt this interaction, and recently a missense mutation in *NME1* was found in a patient with VWS, while a missense NME2 variant was identified in a patient with NSCL/P (Parada-Sanchez et al., 2017).

The Pierre-Robin Sequence (PRS) refers to a set of characteristic craniofacial phenotypes that are commonly observed together: glossoptosis, cleft palate, micrognathia, and upper airway obstruction. Multiple models for an underlying mechanism exist, and palate defects appear be secondary effects, resulting from altered tongue and mandible positioning, rather than intrinsic defects within the palatal shelves (PS) themselves. The dominant model proposes that mandibular hypoplasia causes a high, retrotransposed tongue to block PS

elevation and the upper airway. Alternatively, intrauterine mandible compression may restrict its growth and alter tongue development, similarly blocking the palate and airway. A third model suggests that delayed neuromuscular development reduces the tongue's ability to stimulate mandible and palate growth, causing the observed phenotypes (Giudice et al., 2018). This sequence is often observed as part of a broader syndrome, but not always. Isolated PRS has been associated with mutations in or near the SRY-related HMG box 9 (*SOX9*) gene (Benko et al., 2009; Jakobsen et al., 2007). *SOX9* mutations also cause Campomelic Dysplasia, which affects skeletal and genital development and can include PRS (Foster et al., 1994; Wagner et al., 1994). More recently, mutations in *BMPRI1B* were also reported to be the cause of PRS in two unrelated families (Y. Yang et al., 2017). Several genes that code for ECM components and ECM-interacting proteins have also been associated with syndromes that include clefts. Stickler syndrome and the similar Marshall syndrome frequently include PRS and can be caused by mutations in several collagen genes, including *COL2A1*, *COL11A1*, *COL11A2*, *COL9A1*, *COL9A2*, and the collagen crosslinking enzyme gene lysyl oxidase-like 3 (*LOXL3*). *COL2A1*, *COL11A1*, and *LOXL3* mutations have so far been identified in patients with cleft-associated forms (Alzahrani, Al Hazzaa, Tayeb, & Alkuraya, 2015; L. Guo et al., 2017). Filamins are involved in cell-ECM adhesion, and *FLNA* mutations cause Oto-palato-digital Syndrome, which is typified by cleft palate, while the glypican gene *GPC3* is associated with Simpson-Golabi-Behmel Syndrome with CL/P (Robertson et al., 2003; Tenorio et al., 2014).

Fgf signaling is integral to lip and palate development and will be discussed in further detail later, and several syndromic conditions associated with OFCs can result from mutations in Fgf pathway components. Craniosynostosis is caused by the premature fusion of cranial bones and syndromic craniosynostoses are commonly accompanied by oral clefts. These syndromes are often associated with altered Fgf signaling, especially with *FGFR2* variants. Both of Apert and Crouzon syndromes are typified by craniosynostosis and can include cleft palate, and both syndromes are linked with mutations in *FGFR2* (Reardon et al., 1994; Slaney et al., 1996; Wilkie et al., 1995). Apert syndrome is a condition marked by craniosynostosis and syndactyly that often includes CP. Clefting is most prevalent (~59%) in Apert patients with a S252W substitution near the linker region between extracellular immunoglobulin domains II and III, which eliminates ligand specificity and causes ectopic activation (Slaney et al., 1996; K. Yu, Herr, Waksman, & Ornitz, 2000). Another type of *FGFR2* gain-of-function mutation causes Crouzon syndrome, which also presents with frequent CP, and mice carrying the Crouzon mutation similarly exhibit CP (Snyder-Warwick et al., 2010). *FGFR3* mutations also can cause a form Crouzon syndrome, but patients with this form do not present with oral clefts (Meyers, Orlow, Munro, Przylepa, & Jabs, 1995). However, a missense *FGFR3* mutation was identified in a Mexican family with an atypical craniosynostosis with hypochondroplasia including CPO (Gonzalez-Del Angel et al., 2018). CLP or CPO has also been occasionally reported in patients with Muencke syndrome, another craniosynostosis caused by mutant *FGFR3* (Anderson, Snell, & Moore, 2013). Several causative mutations affecting Fgf signaling have been identified in patients with idiopathic hypogonadotropic hypogonadism (IHH) and autosomal dominant Kallmann syndrome (IHH with anosmia), which can include OFCs. *FGFR1* mutations are associated with forms of both syndromes that include CL/P, and more recently, mutations in *FGF8* have

also been linked with Kallmann syndrome (Pitteloud et al., 2006; Villanueva & de Roux, 2010).

WNT signaling is also implicated in syndromic clefts. Mutations in both of *WNT3* and the WNT modulator gene R-spondin 2 (*RSPO2*) have been identified in tetra-amelia patients with CLP (Niemann et al., 2004; Szenker-Ravi et al., 2018). Robinow syndrome is a type of skeletal dysplasia that affects limb and genital development, and characteristic craniofacial features frequently include CL/P or CPO. It is caused by mutations in several genes associated with noncanonical Wnt signaling, which is discussed further below. The more severe recessive form is associated with mutations in *ROR2* (van Bokhoven et al., 2000), while dominant forms are caused by mutations in the ligand *WNT5A* and signal transducers disheveled 1 (*DVL1*) and *DVL3* (Bunn et al., 2015; Person et al., 2010; J. White et al., 2015; J. J. White et al., 2016).

22q11.2 deletion syndrome, or velo-cardio-facial syndrome, includes a spectrum of disorders associated with a genomic deletion on chromosome 22. It affects neural crest development, resulting in a characteristic craniofacial phenotype including CP. DiGeorge Syndrome patients share phenotypic characteristics and causal mutations map to the same genomic region but patients do not have the deleted chromosome band (McDonald-McGinn et al., 2015). The affected region of these syndromes includes T-box transcription factor 1 (*TBX1*), which may be the gene responsible for associated craniofacial defects, which frequently includes palatal phenotypes ranging from arched palate or bifid uvula to overt CPO (Herman et al., 2012; Yagi et al., 2003). Opitz G/BBB syndrome, which also can include CL/P or CPO, can be classified as X-linked or autosomal dominant. However, the latter form has been determined to be similarly due to a 22q11.2 deletion, while X-Linked Opitz G/BBB is caused by mutations in *MID1* and *SPECCL1* (McDonald-McGinn et al., 1995).

Holoprosencephaly is characterized by the failure of the forebrain to develop into two hemispheres and often includes defects in the medial craniofacial structures. Alteration of genes encoding the transcriptional factor Sine oculis homeobox 3 (*SIX3*) and the Nodal/TGF- β modulator transforming growth-interacting factor (*TGIF1*) have been demonstrated to cause holoprosencephaly with CL/P (Aguilella et al., 2003; K. W. Gripp et al., 2000; Lachawan et al., 2009; Wallis et al., 1999). Additionally, hedgehog signaling is key to craniofacial patterning and mutations affecting several genes in the Hedgehog pathway are associated with holoprosencephaly with CL/P, including *SHH*, *PTCH1*, and glioma-associated oncogene 2 (*GLI2*) (Ribeiro, Murray, & Richieri-Costa, 2006; E. Roessler et al., 1996; Erich Roessler et al., 2003). *PTCH1* mutations also cause nevoid basal cell carcinoma syndrome (also known as Gorlin-Goltz Syndrome), which frequently includes OFCs (Hahn et al., 1996). Several other syndromes in which a causative gene has been identified in cases that include OFCs are listed below (Table 2).

3. Bmp/Tgf- β signaling in orofacial clefts

3.1. Bmp/Tgf- β superfamily

Tgf- β signaling is an embryonic patterning mechanism conserved across metazoa, and includes several signaling pathways that regulate a number of cellular processes to control early vertebrate development, including proliferation, apoptosis, and tissue specification (Adamska et al., 2007; Zinski, Tajer, & Mullins, 2018). The Tgf- β superfamily encompasses several classes of signaling ligand, including two which play indispensable roles in lip and palate formation and are implicated in the presentation of OFCs, the Bmp and the Tgf- β subfamilies (J. Iwata, Parada, & Chai, 2011; C. Parada & Chai, 2012) (Figure 1). The Tgf- β pathway is activated when secreted ligand dimers are recognized by multimeric serine/threonine kinase Tgf- β receptor complex consisting of two type I and two type II receptors (Budi, Duan, & Derynck, 2017; Massagué, 2012; Y. Shi & Massagué, 2003). The ligand-induced receptor complex assembly allows the phosphorylation of the type I receptors by the type II receptors. The activated type I receptor then phosphorylates and activates intracellular signaling Smad proteins (Figure 1). Two of these receptor-activated Smads (R-Smads) interact with a co-Smad, Smad4, to translocate to the nucleus and regulate transcription. Smad2/3 act as R-Smads for Tgf- β signaling, while Smad1/5/8 are R-Smads for Bmp signaling. Smad6/7 are inhibitory Smads that antagonize signaling by competitively binding to the receptor complex (Weiss & Attisano, 2013). Inhibition of Tgf- β signaling upregulates canonical Bmp activity in the posterior PS, likely because Tgf- β -specific Smad2/3 and Bmp-specific Smad1/5/8 must compete for a binding partner within the same pool of Smad4, reflecting a mechanism of mutual antagonism that occurs between the two pathways (G. Yuan, Zhan, Gou, Chen, & Yang, 2018).

3.2. Bmp signaling genes associated with OFCs

Several components of the Bmp/Tgf- β superfamily contribute to the development of oral tissues, and their dysregulation can result in clefts in patients and in animal models. The human genome contains 7 type I (*TGFBRI*, *BMPRI1A*, *BMPRI1B*, *ACVRL1*, *ACVRI*, *ACVR1B*, and *ACVR1C*) and 5 type II (*ACVR2A*, *ACVR2B*, *TGFBRI2*, *BMPRI2*, and *AMHR2*) receptors, which transduce signal from at least 30 ligands, including 11 BMPs and 3 TGF- β s (Weiss & Attisano, 2013). *BMP2* and *BMP4* are closely related genes and comprise a subclass of BMPs (McCauley & Bronner-Fraser, 2004). Both genes play a role in mediating lip and palate formation, and mutations in both have been implicated in clefts. *BMP4* SNPs have been extensively linked with NSCL/P (Antunes et al., 2013; Q. Chen et al., 2014; Y.-Y. Hu, Qin, Deng, Niu, & Long, 2015; Jianyan et al., 2010; Kempa et al., 2014; J.-Y. Lin et al., 2008; José Suazo, Santos, Jara, & Blanco, 2009), and conditional deletion of *Bmp4* by Nestin-Cre in mice causes delayed fusion between the medial and lateral nasal processes (MNP and LNP, respectively), which may result in CL/P (W. Liu et al., 2005). *Bmp2* deletion in *Wnt1*-expressing neural crest cells causes CP with failed PS elevation. *Bmp2* activity in the palate likely does not directly contribute to elevation, as mandibular osteogenesis and chondrogenesis are disrupted, resulting in altered positioning of craniofacial structures that blocks PS movement. Bmp/Smad activity is only reduced in the posterior nasal region of the PS of these embryos despite a much larger *Bmp2* expression

domain, likely reflecting a functional redundancy between different Bmp ligands in other regions of the palate (Y. Chen, Wang, Chen, & Zhang, 2019).

Msx1 is important for neural crest specification, and Bmp signaling regulates *Msx1* upstream of *Snai1/2* in the neural crest ectoderm (Tribulo, Aybar, Nguyen, Mullins, & Mayor, 2003). *Msx1* knockout mice exhibit cleft palate phenotype as well as dysregulated palatal *Bmp2* and *Shh* expression, which can be rescued by Bmp4 supplementation, evidencing a possible regulatory loop or reinforcing role for *Msx1* upstream of Bmp signaling during palatogenesis (Z. Zhang et al., 2002). *Bmp2* is a direct target of Wnt/ β -catenin in osteoblasts (R. Zhang et al., 2013), but *Bmp4* expression is not affected by Wnt signaling during lip and primary palate fusion (L. Song et al., 2009). *Bmp7* also plays a role in palatogenesis. A *BMP7* SNP has been linked with NSCL/P in a Han Chinese cohort (Q. Yu et al., 2015), while a frameshift mutation was identified in a European patient with syndromic defects that included secondary CPO (Wyatt, Osborne, Stewart, & Ragge, 2010). *Bmp7* knockout also causes cleft palate in mice, and it is expressed in both epithelium and mesenchyme, but neither epithelial nor neural crest-specific ablation alone results in CP (Kouskoura et al., 2013). Bmp7 acts downstream of Wnt, and inhibition of Wnt signaling reduces its expression domain in the converging palatal shelves (C. Lin et al., 2011).

Bmpr1a conditional knockout (cKO) by Nestin-Cre also results in cleft lip and secondary palate in mice (W. Liu et al., 2005). These embryos display expanded expression of *Pax9* and *Barx1*, but the conserved Bmp target *Msx1* is not affected (Furuta, Piston, & Hogan, 1997; J. S. Hu et al., 2008; A. Suzuki, Ueno, & Hemmati-Brivanlou, 1997; Tribulo et al., 2003). This may reflect incomplete redundancy with other Bmp receptors such as *Bmpr1b*, overexpression of which can rescue tooth (but not palate) defects in *Bmpr1a* mutants (L. Li et al., 2011). The CP phenotype in these embryos is primarily due to defective PS outgrowth, which are still able to fuse *in vitro*. However, fusion between the maxillary process (MxP) and LNP are affected, and the fusing lip displays reduced expression of *p63*, and both *Fgf8* and its target pituitary homeobox transcription factor 1 (*Pitx1*) at the edge ectoderm, along with increased apoptosis (W. Liu et al., 2005). While Nestin-Cre activates prior to neural crest migration, deletion of *Bmpr1a* later in neural crest-derived mesenchyme by *Osr2*-Cre results in delayed secondary palatogenesis, causing an anterior cleft between the primary and secondary palates, as is also observed in *Bmp4* cKO mutants (Baek et al., 2011). Deletion of another type I Bmp receptor, activin A receptor 1 (*Acvr1*), in *Wnt1*-expressing neural crest causes severe craniofacial defects including cleft secondary palate with no PS elevation (Dudas, Sridurongrit, Nagy, Okazaki, & Kaartinen, 2004). Overexpression, on the other hand, results in palatal fusion failure with a persistent medial epithelial seam (MES), leaving a submucous cleft of both hard and soft palates (Noda, Mishina, & Komatsu, 2016).

Nog, homolog of the human gene associated with NSCL/P (Table 1), encodes a pathway antagonist that preferentially binds and inhibits the three OFC-linked Bmps, Bmp2, Bmp4 and Bmp7, and is largely expressed in the palatal epithelium. Conventional knockout of *Nog* in mice causes embryos to exhibit increased epithelial Bmp/Smad activity and proliferation at E13.5, resulting in complete CP with aberrant maxillary-mandibular fusion at the posterior PS (He et al., 2010). Hypomorphic expression of the gene encoding heart-and neural crest derivatives-expressed protein 2 (*Hand2*) causes full CP with a similar posterior

PS-mandibular fusion phenotype (Xiong et al., 2009). It was previously demonstrated that *Bmp4* regulates epithelial *Shh*, which in turn regulates *Bmp2* to control PS growth (Z. Zhang et al., 2002). *Hand2* is expressed in both palatal epithelium and mesenchyme, and both sources of expression are regulated by mesenchymal *Nog*, likely via *Bmp4*. *Hand2* itself activates *Shh* in the medial edge epithelium (MEE) of the PS, linking these two critical pathways. While mesenchymal expression is dispensable for palatogenesis, epithelium-specific *Hand2* ablation phenocopies the hypomorphic allele, reflecting its importance in activating epithelial *Shh* (Xiong et al., 2009).

3.3. Tgf- β signaling and OFCs

Mutations in the common co-SMAD between *Bmp* and Tgf- β signaling, *SMAD4*, cause Myhre syndrome, and missense SNPs that cause an amino acid change at Ile500 were identified both in patients with CLO and with CPO (Caputo et al., 2012). Conventional *Smad4* knockout causes early embryonic lethality in mice (Sirard et al., 1998; X. Yang, Li, Xu, & Deng, 1998), as does conditional knockout by *Wnt1-Cre* in neural crest cells. However, these cKO embryos survive until around E12.0 and exhibit failed midline fusion of the MNPs and dysgenesis of the first branchial arch (BA1). Epithelium-specific *Smad4* deletion alone does not inhibit palatogenesis, illustrating the importance of Tgf signaling in craniofacial mesenchyme growth (Ko et al., 2007). P38 mitogen-activated protein kinase (MAPK) can transduce Tgf- β signals independent of Smads (L. Yu, Hebert, & Zhang, 2002). siRNA targeting *p38* does not affect palate fusion *in vitro*, but when *p38* is knocked down by siRNA in palatal shelves of epithelial *Smad4*-knockout embryos in culture, fusion fails, potentially implying a partial redundancy between epithelial *Smad4* and *p38* (X. Xu et al., 2008). *Smad4* deletion in *Osr2*⁺ mesenchyme does cause complete secondary palatal cleft, but altered expression of known *Bmp* targets is not detected in these embryos. However, connective tissue growth factor (*Ctgf*), is downregulated, which is also observed in *Tgfb2; Wnt1-Cre* cKO mutants. Exogenous application of *Ctgf* can rescue defective mesenchymal proliferation observed in the *Tgfb2* models, further highlighting the importance of *Smad4* in transducing Tgf- β subfamily signals upstream of *Ctgf* during palate formation (Carolina Parada, Li, Iwata, Suzuki, & Chai, 2013).

Tgf- β signaling also contributes to fusion of the MEE of converging PS. Ablation of murine *Tgfb2* by *K14-Cre* results in excessive epithelial proliferation and a submucous cleft of the hard palate due to a persistent MES resulting in a cyst at the anterior secondary palate. Fusion between the primary and secondary palate and between the palatal shelves and nasal septum fail, and the posterior soft palate is fully cleft with misdirected muscle attachments. These experiments revealed Tgf- β regulation of key epithelial transcription factor *Irf6* (X. Xu et al., 2006). Exogenous *Irf6* rescues the submucous cleft of the hard palate, demonstrating its role in epithelial apoptosis and fusion downstream of Tgf- β signaling in the MES. However, it did not rescue the soft palate cleft phenotype, suggesting that *Tgfb2* may mediate soft palate formation via a different mechanism (J.-i. Iwata et al., 2013). *Tgfb1* also appears to play different roles in each of the two major tissue lineages, as epithelial *Tgfb1* inhibition results in fully penetrant posterior CP. However, ablation in neural crest-derived mesenchyme results in a more severe phenotype including full secondary cleft palate and a midline frontonasal cleft (Dudas et al., 2006). The *Wnt*

antagonists *Dickkopf 1 (Dkk1)* and *Dkk4* are upregulated in these embryos, which is in turn accompanied by Wnt repression. Dkk inhibition is able to rescue proliferation and muscle formation defects *in vitro*, highlighting the importance of crosstalk between the Wnt and Tgf- β pathways in the developing posterior palatal mesenchyme (J.-i. Iwata et al., 2014).

FOXE1 has been strongly linked with human OFCs (Table 1), and a key facet of *Foxe1*'s role in murine craniofacial development is transcriptional activation of *Tgfb3* and *Msx1*, which regulates Bmp signaling (Venza et al., 2010). *Tgfb3*-null mice also display cleft palate with similar defective MES fusion and proliferation in the palatal mesenchyme as observed in *Tgfb2* mutants (Kaartinen et al., 1995; Proetzel et al., 1995). In addition to signaling through Smad4, epithelial Tgf- β 3 can effect a Smad-independent pathway via Tgf- β -activated kinase 1 (Tak1) (Lane et al., 2015). Recently, it was demonstrated that *Irf6* directly activates transcription of Homeodomain-interacting protein kinase 2 (*Hipk2*), and that *Hipk2* protein is activated via phosphorylation by Tak1, subsequently promoting MEE apoptosis and palate fusion (Ke, Mei, Wong, & Lo, 2019). These results imply that epithelial Tgf- β 3 signaling through *Tgfb2* contributes to palatal fusion via *Irf6-Tak1* activation of *Hipk2*.

Tgfb3 mediates both Tgf- β and Bmp signals in secondary palate, and *Tgfb3*-null embryos have fully cleft secondary palate in which PS fail to elongate and elevate, as well as showing altered vascularization and osteogenesis (Hill, Jacobs, Brown, Barnett, & Goudy, 2015). It is also required for MEE fusion, and cultured shelves treated with anti-*Tgfb3* siRNA do not fuse *in vitro* (Nakajima et al., 2007). *Tgfb3* is not thought to transduce signal but rather modulate signaling through other receptors. However, in embryos lacking mesenchymal *Tgfb2*, Tgf- β 2 is upregulated and activates a Smad-independent cascade of TNF receptor associated factor 6 (Traf6)/Tak1/p38 within the palatal mesenchyme through a receptor complex of *Tgfb1/Tgfb3* and *Tgfb2* inhibition results in partial cleft palate rescue (J.-i. Iwata, Hacia, et al., 2012). While excess activation of this pathway may contribute to the CP observed in *Tgfb2* mutants, *Tak1* knockout in mesenchyme by *Osr2-Cre* (but not in epithelium by *K14-Cre*) also causes cleft palate, suggesting mesenchymal Tgf- β may control palatogenesis through this MAPK pathway, and that appropriate signaling through both the Smad and p38/Mapk branches of the pathway are required for PS closure (Z. Song et al., 2013). Phosphorylation by Tak1 appears to also activate R-Smads and transduce Smad-dependent Tgf- β signaling, so Tak1 activity may be an important mechanism for Tgf- β target cells to mediate the balance between Smad and p38/Mapk signaling (Yumoto et al., 2013).

4. Fgf signaling in orofacial clefts

4.1. Fgf signaling pathway

Fgf signaling is another morphogenetic pathway that is intimately linked with OFCs (Figure 2). Fgf ligands are recognized by their cognate receptors (Fgfrs), class V RTKs that function as hetero- or homodimers. Binding of a ligand facilitates receptor dimerization and allows their cross-phosphorylation and activation. Phosphorylated receptors are bound by intracellular transducers of several key pathways that regulate fundamental cellular processes, such as proliferation, survival, migration, differentiation, and metabolism, including signal transducer and activator of transcription (STAT), Fgfr substrate 2 α (FS2 α),

which activates RAS/MAPK and PI3K/AKT pathways, and phospholipase C (PLC) pathways (Ornitz & Itoh, 2015). There are 22 mammalian Fgf genes, including 4 that code for intracellular proteins and 3 endocrine Fgfs, in addition to the canonical secreted paracrine Fgfs that facilitate communication between proximal cells in developing tissue. Canonical Fgfs associate with heparin/heparin sulfate, which acts as a cofactor for the Fgf ligand, so Fgf signaling is intricately linked with extracellular matrix assembly and function (Lonai, 2005).

4.2. FGF signals involved in midfacial patterning

Many *Fgf* and *Fgfr* genes are expressed in the orofacial primordia, and mutations in several are linked with OFCs in human patients or animal models. During midfacial morphogenesis, *Fgf3*, *Fgf8*, *Fgf9*, *Fgf10*, and *Fgf17* are broadly expressed within the facial ectoderm of E9.5 mouse embryos (Bachler & Neubuser, 2001). By E10.5, the MNP and LNP have formed, and their expression domains have become restricted. *Fgf9*, *Fgf10*, and the particularly strong *Fgf8* are expressed around the ectodermal ridge of the nasal pits, while *Fgf3*, *Fgf17*, and a new expression domain of *Fgf15* are restricted to the medial side of the nasal pit. At E10.5, *Fgf8*, *Fgf9*, and *Fgf17* are also expressed at the oral side of the MNP, along with *Fgf18*, which is at the oral MNP but not the nasal pit. *Fgf8*, *Fgf9*, and *Fgf17* are also expressed in the oral ectoderm of the MxP at E10.5 as it fuses with the LNP, and by E11.5, there are continuous expression domains of *Fgf8*, *Fgf9*, and *Fgf17* across the dorsal side of the oral ectoderm derived from both progenitor structures (Bachler & Neubuser, 2001). These Fgf signals are likely transduced through Fgfr1 and Fgfr2, which are expressed in both ectoderm and mesenchyme of the facial prominences, while Fgfr3 and Fgfr4 are not detected (Bachler & Neubuser, 2001). The roles of many of these genes are likely conserved, as possible associations have been found between NSCL/P and SNPs in the human homologs of *FGF7*, *FGF10*, *FGF18*, *FGFR1*, and *FGFR2* (M. Hu et al., 2013; E. J. Leslie et al., 2015; Riley et al., 2007; Y. Yu et al., 2017).

4.3. Fgf signaling in secondary palatogenesis

Fgf7 and Fgf10 are involved in patterning along the oronasal axis of the PS, with *Fgf7* primarily expressed at the nasal side and *Fgf10* at the oral side of the palatal mesenchyme (which correspond with and are sometimes described as the medial and lateral MxP, respectively, prior to elevation) (Alappat et al., 2005; J. Han et al., 2009). *Fgfr1* and *Fgfr2* are also expressed in the palatal epithelium and mesenchyme from E13 through midline fusion around E15. *Fgfr1* is expressed more broadly throughout the mesenchyme, while *Fgfr2* expression is more focused at the lateral/maxillary region. Both are more highly expressed at the nasal side, and epithelial expression is especially strong at the MES during its dissolution (S. Lee et al., 2001; Kai Yu, Karuppaiah, & Ornitz, 2015). Fgf signaling mediates epithelial-mesenchymal interactions (EMI) during palate development, and differential receptor-ligand specificity is integral to maintaining differential signals. *Fgfr2* encodes two main isoforms that differ in their immunoglobulin-like domain III, Fgfr2IIIb and Fgfr2IIIc (*Fgfr2b/c*) (De Moerloose et al., 2000). Epithelial proliferation is normally activated by mesenchyme-derived Fgf10, which preferentially signals through Fgfr2IIIb, while Fgf8 preferentially activates Fgfr2IIIc, which is distributed throughout the mesenchyme (Rice et al., 2004; X. Zhang et al., 2006).

Fgfr1 is thought to have similar spliceforms and ligand preferences as *Fgfr2*. Conditional neural crest (by *Wnt1-Cre*) deletion of *Fgfr1* in mice causes midline clefts including nasal and lip, as well as cleft primary and secondary palate with delayed maxillary growth and no PS elevation (C. Wang et al., 2013). When *Fgfr1* is targeted using a Cre driver that activates later (*Twist2*), penetrance of CP is only 16%, and palatal shelf defects are described only after E13.5, while the *Wnt1^{Cre/+};Fgfr1^{flox/flox}* palate exhibits reduced proliferation at least a full day earlier. While mesenchymal *Fgfr2* cKO in *Twist2⁺* cells does not cause cleft palate on its own, double cKO of *Fgfr1* and *Fgfr2* results in completely penetrant CP, reflecting the potential for compensation between the two receptors (K. Yu et al., 2015). Unlike mesenchymal *Fgfr2* cKO, a substitution mutation that causes ectopic Fgfr2IIIc activation does result in cleft palate with increased posterior proliferation as a likely mechanism (Snyder-Warwick et al., 2010). Both *Fgf10*-null and *Fgfr2b*-deficient mice display cleft palate, highlighting the importance of this ligand-receptor interaction in mediating communication between the palatal epithelium and mesenchyme (Rice et al., 2004).

Epithelial Fgf8 is important for mesenchymal patterning and growth within BA1-derived structures, including the MxPs as they grow to form the PS (Trumpp, Depew, Rubenstein, Bishop, & Martin, 1999). However, when *Fgf8* is constitutively expressed in *Osr2⁺* mesenchyme, *Fgf7* and *Fgf10* expression is diminished, and palatal shelves fail to elevate, demonstrating the ligand specificity of mesenchyme-expressed receptors, as well as a feedback mechanism modulating Fgf expression. Proliferation is increased in the mesenchyme but not the epithelium in response to ectopic Fgf8, consistent with the model that it targets mesenchymal cells from the epithelium to help coordinate EMI during palatogenesis (Wu et al., 2015). Additionally, activation of Fgf effector pathways are altered in the palatal shelves of these mutants, including increased phosphorylated extracellular signal-regulated kinase (p-Erk) within the nasal/medial mesenchyme, and Janus kinase 1 (p-Jak1) throughout the mesenchyme, while p-Jak2 is increased in the epithelium but decreased in the mesenchyme (Wu et al., 2015).

4.4. Fgf regulation and target pathway interactions during lip and palate development

Fgf signaling is a key mediator of several other signaling pathways and factors involved in craniofacial development, and a number of important cleft-associated genes are regulated by craniofacial Fgf signaling (Figure 2). Fgf signaling is activated downstream of canonical Wnt pathway in the MNP and LNP. *Fgf8* is a direct transcriptional target of Wnt/ β -catenin signaling during lip and primary palate development, and is dysregulated in the facial ectoderm and neural ridge of *Ctnnb1* (β -catenin)-mutant mouse embryos, accompanied by increased apoptosis (Y. Wang, Song, & Zhou, 2011). Not surprisingly, *Fgf8* expression is also reduced in the BA1 ectoderm of embryos lacking the Wnt signaling amplifier *Rspo2* (Jin, Turcotte, Crocker, Han, & Yoon, 2011). *Fgf8*, *Fgf10*, and *Fgf17* are all downregulated in MNP and LNP ectoderm of *Wnt9b*-null mouse embryos, while all three are in turn upregulated in embryos with a *Ctnnb1* allele that is constitutively activated in *Foxg1*-expressing ectoderm (Jin, Han, Taketo, & Yoon, 2012). This epithelial *Wnt9b*-Fgf cascade is further demonstrated to regulate mesenchymal proliferation via Fgfr-dependent Erk1/2 phosphorylation (Jin et al., 2012).

The zinc finger transcription factor Sp8 is integral to craniofacial morphogenesis and establishes signaling centers in the olfactory pit and anterior neural ridge of the developing mouse embryos (Kasberg, Brunskill, & Steven Potter, 2013). Loss of Sp8 function results in CLP, due to its role in activating *Fgf8* and *Fgf17* to regulate Shh, but not Wnt signaling in the developing maxillary and nasal processes, consistent with Wnt acting upstream of Fgf (Kasberg et al., 2013). Fgf signaling may also be regulated by Bmp, though their relationship in midfacial development is less clear. Liu et al. demonstrated that ectodermal *Fgf8* expression is decreased where the LNP and MNP fuse during lip formation in *Nestin-Cre;Bmpr1a* cKO mouse mutants (W. Liu et al., 2005). However, *Fgf8* expression is expanded in chick embryos in which mesenchymal Bmp is inhibited via ectopic Noggin application (Ashique, Fu, & Richman, 2002). Additionally, midfacial *Fgf8* is a target of the micro RNA *miR-17-92*, which is activated by the transcription factor AP-2 α (*Tfap2a*). *Tfap2a* is critical for craniofacial development and represses Fgf signaling in the MNP and LNP. Loss of function results in bilateral CLP, which can be partially rescued by reduction of *Fgf8* (Green et al., 2015; J. Wang et al., 2013). The *miR-17-92* promoter also contains a SMAD-binding element, and is directly activated by canonical Bmp signaling (J. Wang et al., 2010).

When *Bmpr1a* is ablated in *Osr2*-expressing mesenchyme, *Fgf10* is reduced in the palatal shelves of E13.5 and E14.5 mouse embryos (Baek et al., 2011). In *Fgf10* mutants, the Notch ligand *Jag2* is lost in the palatal epithelium, while *Tgfb3* is expanded into the oral PS epithelium where Fgf10 normally signals through Fgfr2IIIb, reflecting their antagonistic relationship (Alappat et al., 2005). *Jag2* may be an important negative regulator of oral fusion processes, and *Jag2*-null mice also have CP associated with aberrant epithelial adhesion between the palatal shelves and tongue (R. Jiang et al., 1998). P63 is a cleft-associated transcription factor activated in the oral ectoderm that may be regulated by both Wnt and Tgf- β signaling (Ferretti et al., 2011; R. Richardson et al., 2017). P63 directly activates expression of *Fgfr2* and *Fgfr3* in the palatal epithelium and positively regulates *Fgf8*, as well as both Shh and Bmp signaling, further linking these several key pathways in the developing palatal shelves (Ferone et al., 2012; R. Richardson et al., 2017; H. A. Thomason, M. J. Dixon, & J. Dixon, 2008). Mesenchymal *Fgf10* and epithelial *Shh* reciprocally activate each other at the oral side of the PS to mediate communication between the two tissue layers (Lan & Jiang, 2009; Rice et al., 2004). An important orofacial patterning transcription factor that plays important roles in osteoblast differentiation, distal-less homeobox 5 (*Dlx5*), activates mesenchymal *Fgf7* at the nasal side while restricting *Shh* expression, establishing the Fgf expression domains across the PS epithelial surface. This axis is interrupted by ectopic activation of *Fgf8* in neural crest due to its antagonistic relationship with *Fgf7*, and both *Fgf7* and *Shh* are diminished (J. Han et al., 2009; Wu et al., 2015).

Runt-related transcription factor 2 (*Runx2*) and its target Osterix (*Sp7*) are also key transcription factors that are also involved in promoting osteogenesis. They are activated downstream of Fgf signaling in certain contexts, and are thought to play a role in craniosynostosis associated with excess Fgf signaling (Komori, 2011). When ectopic *Fgf8* is activated in cells expressing the anterior palatal mesenchyme transcription factor Short stature homeobox 2 (*Shox2*), however, *Runx2* and *Sp7* are inhibited in that region. This may

be due to an antagonistic relationship between *Fgf8* and a different Fgf that promotes osteoblast differentiation, such as *Fgf18* (J. Xu et al., 2018). While these embryos do not display secondary CP, osteogenesis is interrupted and the palatine process of the maxilla is absent, with excess cartilaginous tissue formed in its place (J. Xu et al., 2018).

Fgf18 is expressed in the palatal mesenchyme just under the MEE where it activates epithelial *Runx1*, which is required for completing palate fusion (Charoenchaikorn et al., 2009). *Fgf18* also helps regulate craniofacial chondrogenesis and osteogenesis, and *Fgf18*-null mice exhibit reduced cranial ossification and cleft secondary palate with PS elevation failure (Z. Liu, Xu, Colvin, & Ornitz, 2002). *Foxf1* and *Foxf2* activity in the palatal mesenchyme inhibits *Fgf18* to maintain expression of its repressive target *Shh*. *Foxf1/2* are partially redundant, and conditional *Foxf2* deletion in neural crest lineage results in palate elevation failure with expanded *Fgf18* expression domains (J. Xu et al., 2016). CP also presents in *Fgf9*^{-/-} mice with 40% penetrance, but few details on the cause are available (J.-i. Iwata, Tung, et al., 2012). *Fgf9* is expressed in the maxillary epithelium at E13.5 and both mesenchyme and epithelium at E14.5 and more recently, Tgf- β signaling has been demonstrated to regulate PS proliferation through a *Fgf9-Pitx2* axis (Colvin, White, Pratt, & Ornitz, 2001; J.-i. Iwata, Tung, et al., 2012). *Sox11* also regulates proliferation in the palatal shelves through *Fgf9*, which can be activated to rescue growth defects in *Sox11*^{-/-} palate *in vitro* (H. Huang et al., 2016).

4.5. Fgf signaling inhibitors associated with OFCs

Spry family members are antagonists of Fgf signaling and specifically inhibit Ras-Erk pathway activation by Fgf receptors, and Fgf signaling itself regulates Spry gene expression, which can then act as negative feedback mechanism to modulate signaling activity (Mason, Morrison, Basson, & Licht, 2006). Spry genes are involved in facial morphogenesis and *SPRY1*, *SPRY2*, and *SPRY4* variants have been implicated in NSCL/P in human patients (K. U. Ludwig et al., 2012; Vieira et al., 2005; Y. Yu et al., 2017; R. Zhou et al., 2019). Ectopic activation of *Spry1* in mouse neural crest by Wnt1-Cre causes severe craniofacial defects including CLP, with decreased proliferation and increased apoptosis (X. Yang et al., 2010). *Spry2* and *Spry4* are integral to craniofacial morphogenesis and exhibit partial redundancy, evidenced by an increased severity of defects in compound mutant mouse embryos, though embryos lacking only *Spry4* do not present with clefts (Taniguchi et al., 2007). A >1 Mb genomic deletion including *Spry2* among multiple genes in mouse causes cleft palate with occasional cleft lip, with altered expression of *Etv5*, *Msx1*, *Barx1*, and *Shh* signaling (Welsh, Hagge-Greenberg, & O'Brien, 2007). Transgenic *Spry2* expression is able to rescue the cleft palate phenotype from 83% to 8% penetrance, demonstrating *Spry2* is the gene likely responsible for the oral clefts observed in this strain (Welsh et al., 2007). Targeted *Spry2* deletion further reveals its role, as ossification or MES fusion potential are unaffected, while palatal mesenchyme exhibits excess proliferation due to enhanced Fgf signaling, leading to PS elevation failure (Matsumura et al., 2011). Similarly, *Spry2* overexpression results in defective outgrowth of facial prominences but does not affect the chondrogenesis or osteogenesis of mesenchymal cells (Goodnough, Brugmann, Hu, & Helms, 2007).

5. Retinoic acid signaling in orofacial clefts

5.1. RA signaling pathways

Retinoic acid (RA) is a metabolic derivative produced through the retinol (Vitamin A) metabolic pathway (Figure 3), and it is an important signaling molecule in morphogenesis and differentiation during embryonic development. Retinoids cannot be synthesized by animal cells and dietary intake of retinoids or their carotenoid precursors is necessary for RA production. Circulating dietary retinol is bound by a retinol binding protein (RBP) family carrier, and this complex is recognized by the RBP receptor STRA6 and taken up by target cells (Kawaguchi et al., 2007; Quadro et al., 1999). Cellular retinol binding protein (CRBP) recognizes free cytosolic retinol and facilitates its interaction with alcohol dehydrogenase (ADH) or retinol dehydrogenase family (RDH) enzymes that catalyze its oxidization into retinaldehyde (Ang, Deltour, Hayamizu, Žgombic-Knight, & Duester, 1996; P. N. MacDonald & Ong, 1987; Sandell et al., 2007; M. Zhang, Chen, Smith, & Napoli, 2001). Retinaldehyde is subsequently oxidized to RA by one of three tissue-specific retinaldehyde dehydrogenase enzymes, RALDH1, RALDH2, and RALDH3 (encoded by *ALDH1A1–3*) (Niederreither & Dollé, 2008) (Figure 3). RA is in turn metabolized into 4-hydroxy-RA by one of three members of the Cytochrome P450 26 subfamily (CYP26) (J. A. White et al., 1997). Cytosolic RA signals through the nuclear receptor family retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are imported to the nucleus upon binding RA. Activated RAR and RXR proteins function as heterodimers to recognize Retinoic Acid Response Element (RARE) genomic sequences and recruit co-regulator proteins to activate transcription of target genes (Pogenberg et al., 2005). Several isoforms of RA exist, including notably all-trans, 9-cis, and 13-cis forms. The RAR and RXR receptor families each contain an α , β , and γ form, of which each has three subtypes (Okano, Udagawa, & Shiota, 2014). While RXR is specifically activated by all-trans retinoic acid (atRA), RAR receptors are activated by both cis- and trans- RA (Leid, Kastner, & Chambon, 1992). Cellular retinoic acid-binding proteins I and II (CRABP-I and CRABP-II) facilitate RA interaction with CYP26 enzymes or RAR/RXR receptors, with CRABP-II associated with receptor activation and CRABP-I favoring interaction with CYP26 (Dong, Ruuska, Levinthal, & Noy, 1999; Rhinn & Dollé, 2012). The metabolic and signaling pathways of RA have previously been reviewed in greater detail (Das et al., 2014; Duester, 2008; Ghyselinck & Duester, 2019; Rhinn & Dollé, 2012). Retinoid metabolism is strongly implicated in OFCs, which is discussed at greater length in a companion article (Garland et al., 2020 in revision), while the following section will discuss some of the underlying genetics involved in RA signaling and its interaction with other signaling pathways during craniofacial development.

5.2. Abnormal RA signaling in the cause of orofacial clefts

RA signaling regulates developmental processes of many embryonic tissues/organs, including the neural tube, somite, foregut, limb, brain, and eye. RA is also required for appropriate craniofacial morphogenesis. Deficiencies of vitamin A and its metabolites have long been known to cause congenital craniofacial defects (J. G. Wilson, Roth, & Warkany, 1953), and excess retinoids similarly induce syndromic defects in rodents, including cleft palate (Harold Kalter, 1960; H. Kalter & Warkany, 1961; Walker & Crain Jr, 1960) (Figure

3). The balance between RA production and degradation by RALDH and CYP26 enzymes mediates cellular RA levels and signaling (Cunningham & Duester, 2015), while RARE-inducible transcriptional activation of *Cyp26a1* acts as a negative feedback mechanism to regulate excess RA activity (Loudig, Maclean, Dore, Luu, & Petkovich, 2005).

Excess endogenous RA also can result in cleft palate and palatal shelves fail to elevate in *Cyp26b1*-null mice, as a reduced ability to metabolize RA results in its accumulation and overactivation of its receptors (Okano et al., 2012). Retinoid signaling deficiency is also detrimental and knockout is lethal, either embryonically or within 12 hours postnatally, for 8 out of the 10 RAR isoforms encoded by *Rara*, *Rarb*, and *Rarg*. Two types of compound RAR mutants ($\alpha I^{-/-}; \alpha^{-/+}; \gamma^{-/-}$, and $\alpha^{-/-}; \gamma^{-/-}$) display midfacial and secondary palatal clefts, as RAR β is unable to significantly compensate for loss of activity by RAR α and RAR γ (Lohnes et al., 1994). *Aldh1a3*-deficient neonatal mice also exhibit reduced RA activity due to deficient retinaldehyde metabolism, and exhibit lethal choanal atresia, associated with over-fusion of the nasal primordia (Dupé et al., 2003). Compound *Aldh1a2/Aldh1a3* mutants display a midline facial cleft and bilateral cleft lip, and the midline cleft can be rescued by maternal RA supplementation from E7.5 to E10.5. However, the bilateral lip defect is persistent and more precise modulation of cellular RA levels may be required for the frontonasal structures to fully form (Halilagic et al., 2007).

5.3. RA signaling interactions in orofacial clefts

A reciprocal regulation between the Cyp26 RA degradation enzymes and Tbx1 has been revealed in palatogenesis (Figure 3). Tbx1 regulates oral epithelial differentiation and adhesion, and *Tbx1*-deficient mice exhibit cleft palate (Funato, Nakamura, Richardson, Srivastava, & Yanagisawa, 2012). All three Cyp26 genes (*Cyp26a1*, *Cyp26b1* and *Cyp26c1*) are expressed in the pharyngeal regions which give rise to maxillary and palatal tissues and are dysregulated in *Tbx1* mutants (Roberts, Ivins, Cook, Baldini, & Scambler, 2006). During palatal development, *Cyp26b1* is expressed primarily in mesenchymal cells, while *Cyp26a1* is localized to the epithelium (Okano et al., 2012). *Tbx1* is downregulated in the maxillary tissue in *Cyp26b1*-null mice (which also have CP) which accumulate RA in mesenchymal cells, also resulting in reduced expression of key palatal regulators *Bmp2* and *Fgf10* (Okano et al., 2012), and a reduction in *Tbx1* was also reported in the embryonic tongue in response to exogenous RA treatment (Okano, Sakai, & Shiota, 2008).

RA has a mutually inhibitory relationship with Tgf- β signaling in palatogenesis, and atRA treatment of cultured palatal shelves prevents fusion, upregulating expression of the inhibitory *Smad7*, and reducing Smad2 phosphorylation (Yuming Wang et al., 2011). In mouse embryonic palatal mesenchyme cells (MEPM), atRA similarly antagonizes Tgf- β signaling, reducing Smad2 activation and increasing expression of both *Rarb* and *Smad7*, resulting in reduced proliferation. Tgf- β 3 treatment in turn antagonizes RA signaling, reducing *Rarb* and *Smad7* expression. TGIF1 interacts with RAR- α in addition to Smad2/3, and is a co-repressor of both signaling pathways. Treatment with either atRA or Tgf- β 3 causes a significant increase in *Tgif1* expression, which was shown to be a key mechanism of each pathway's antagonism of the other (X. Liu et al., 2014).

Mutual repression between RA and Wnt signaling may also play a critical role in orofacial development (Figure 3). In Wnt-deficient *Lrp6*-knockout mice, which present with fully penetrant CLP, the expression domain of *Aldh1a3* in the nasal pits is expanded, resulting in increased RA activity (L. Song et al., 2009). In contrast, RA treatment has been shown to repress canonical Wnt/ β -catenin signaling, along with alterations in proliferation and apoptosis in mouse embryonic palatal shelves through dysregulation of several cell cycle regulators (including *cyclinD1*, *p21*, and *p27*), and of p38 MAPK signaling, respectively (X. Hu, Gao, Liao, Tang, & Lu, 2013).

RA signaling has been shown to maintain both *Shh* and *Fgf8* expression in the frontonasal process of chick embryos, and chemical disruption of RA signaling results in reduced proliferation and increased apoptosis (Schneider, Hu, Rubenstein, Maden, & Helms, 2001). Similar results are observed in embryos treated with a RALDH antagonist, and exogenous Fgf8 is able to partially rescue many of the craniofacial defects from insufficient RA signaling (Y. Song, Hui, Fu, & Richman, 2004). However, more recently, addition of exogenous RA in mouse embryos also resulted in increased apoptosis in maxillary tissue and downregulation of hedgehog pathway components *Shh*, *Ptch1*, and *Gli1*, while addition of a hedgehog agonist partially rescues the penetrance of RA-induced OFC (Q. Wang et al., 2019). This is consistent with previous studies demonstrating RA treatment repressed Shh signaling, further evidencing the importance of Shh inhibition as a function RA signaling (Helms et al., 1997). Shh in turn antagonizes RA signaling by activating *Cyp26a1* and *Cyp26b1* to reduce cellular RA levels. Shh also regulates the RA receptors, and cKO induced at E10.5 (*Shh^{flox/Cre-ERT}*) results in significantly increased *RARB* and *RARG* expression (El Shahawy et al., 2019).

6. Shh signaling in orofacial clefts

6.1. Shh signaling

Hedgehog signaling is fundamental to many aspects of animal development, and is implicated in the pathology of numerous congenital disorders and various cancers, which has previously been reviewed by Briscoe and Thérond (Briscoe & Thérond, 2013), and in craniofacial development, specifically reviewed by Abramyan (Abramyan, 2019). Of three mammalian hedgehog ligands (Indian hedgehog, Desert hedgehog, and Sonic hedgehog), *Shh* is the best studied and the hedgehog gene most commonly associated with congenital defects and disease. Upon synthesis, Shh is post-translationally modified via cleavage and lipidation, by which a cholesterol and palmitoyl group are added to the N-terminal fragment. Lipid modification is required for association with the membrane and secretion, which occurs through activity of dispatched (Disp) and signal peptide CUB EGF-like domain-containing protein (Scube2), allowing secreted ligand to then signal both short- and long-distance to target cells. In the absence of signal, the 12-pass transmembrane receptor patched (Ptch) represses signaling by inhibiting the GPCR family member smoothened (Smo), while the downstream effector glioma-associated oncogene (Gli) is sequestered and inhibited by Suppressor of Fused (Sufu). Upon binding of the Hh ligand to the Ptch receptor, Smo inhibition is relieved, which allows it to accumulate in the distal tip of the primary cilium. There it dismantles the Sufu-Gli complex, allowing Gli to freely enter the nucleus and act as

a transcription factor to alter target gene expression (Abramyan, 2019). Several components of the Hedgehog pathway have been associated with OFCs in both human patients and animal models (Figure 4). Hedgehog signaling is intimately linked with primary cilia activity, and many genes that encode ciliary proteins are similarly associated with clefts, which is extensively discussed in an associated review article (Ji et al., 2020 in revision).

6.2. Shh signaling in craniofacial morphogenesis and OFCs

Shh is expressed in the craniofacial ectoderm and is key for regulating development of the cranial neural crest, and dysregulated signaling causes severe defects of BA1-derived structures and midline patterning (Ahlgren & Bronner-Fraser, 1999; Chiang et al., 1996). Holoprosencephaly occurs when the forebrain fails to develop into two hemispheres, and often involves narrowing of craniofacial midline structures. It is often associated with CL/P, and mutations in several components of the Hh pathway, including *SHH*, *PTCH1*, and *GLI2*, can result in holoprosencephaly in human patients (Table 2). Cyclopamine was identified as a teratogen in sheep who gave birth to holoprosencephalic offspring after exposure, which was later determined to confer this effect via inhibition of hedgehog signaling (Chiang et al., 1996; Keeler, 1978). Both cyclopamine and vismodegib, another hedgehog antagonist, cause cleft lip and palate in mouse embryos when introduced between embryonic days 7 and 10 (Heyne et al., 2015; Lipinski et al., 2010). Hedgehog acyltransferase (Hhat) palmitoylates Shh, a modification that is essential to its long-range signaling capability (Buglino & Resh, 2008; M.-H. Chen, Li, Kawakami, Xu, & Chuang, 2004). Mouse embryos lacking *Hhat* exhibit severe craniofacial defects including holoprosencephaly, and vertical PS growth from the MxP is disrupted, highlighting the key role hedgehog signaling plays in secondary palatogenesis and cleft palate (Dennis et al., 2012).

Shh contributes to midfacial expansion and excess signaling can result in hypertelorism, which can range from wider spacing between the eyes to complete facial duplication and may also contribute to OFC formation. Conditional targeting of hedgehog repressor *Ptch1* in mouse neural crest, which effectively results in constitutive hedgehog activation, results in failed fusion of the MNP and LNP. These embryos exhibit reduced Fgf signaling within the nasal pit and prominences with lower mesenchymal cell density and increased epithelial cell death, which would result in bilateral CLP if embryos survived to term (Metzis et al., 2013). While this model is embryonic lethal prior to secondary palatogenesis, transgenic mice with ectopic *Shh* expression in *Krt14*⁺ basal epithelium survive longer and similarly present with severe craniofacial defects, including fully penetrant cleft secondary palate (Cobourne et al., 2009). Constitutive *Smo* expression in *K14*⁺ cells causes submucous CP due to a persistent MES that is resistant to apoptosis and maintains a proliferative state beyond that which is observed during normal palatogenesis (J. Li et al., 2018). However, constitutive *Smo* activation in *Osr2*⁺ mesenchyme causes reduced vertical growth of palatal shelves with a rounded appearance that fail to elevate, resulting in a wide cleft with maxillary skeletal defects (Hammond, Brookes, & Dixon, 2018). Among upregulated target genes in the palatal tissue of these embryos is Sclerostin domain-containing 1 (*Sostdc1*), which directly antagonizes both Bmp and Wnt signaling (Ahn, Sanderson, Klein, & Krumlauf, 2010; Hammond et al., 2018; Laurikkala, Kassai, Pakkasjärvi, Thesleff, & Itoh, 2003; Lintern, Guidato, Rowe, Saldanha, & Itasaki, 2009).

Shh is expressed in periodically spaced domains along the anteroposterior axis of palatal epithelium and contributes to the formation of palatal rugae, a series of palatal ridges involved in sensing and manipulating food that are conserved throughout mammals (Economou et al., 2012). New expression domains of the rugae patterning genes are sequentially established as the palatal shelves grow. Each new rugal domain is formed immediately anterior to ruga 8, which is formed first and corresponds with the boundary between anterior *Shox2* and posterior *Meox2* expression (Pantalacci et al., 2008). This expression pattern is regulated by a reaction-diffusion mechanism involving antagonistic activities between *Lrp4* and *Sostdc1*, which are complementarily expressed in the prospective rugal and inter-rugal domains, respectively (Kawasaki et al., 2018). *Lrp4* and *Sostdc1* may modulate Wnt signaling and Wnt is required to establish Shh signaling centers during rugae induction. (C. Lin et al., 2011). When either of *Lrp4* or *Sostdc1* is knocked out, *Shh* expression domains become disordered, resulting in erratic ridge patterning (Kawasaki et al., 2018).

6.3. Shh signaling interactions during lip and palate formation

Shh acts downstream of Fgf signaling in the oral epithelium during secondary palate formation, activated by signaling through Fgfr2IIIb by mesenchyme-derived Fgf10. This axis is disrupted in either *Fgf10* or *Fgfr2b* mouse mutants, which present with dysregulated *Shh* and CP. This is likely due to proliferation and growth defects observed in the palatal shelves, though the potential for medial fusion remains *in vitro* (Rice et al., 2004). *Shh* is also repressed by retinoic acid signaling in the craniofacial primordium, and the role excess RA plays in causing clefts is due in part to its modulation of *Shh* polarizing activity (Helms et al., 1997).

Shh mediates EMI in the developing palate, and epithelial Shh activates several downstream palatal regulators within the mesenchyme, including *Foxf2*, *Osr2*, *Bmp2*, and *Fgf10*. It also controls mesenchymal proliferation by maintaining expression of *Ccnd1* and *Ccnd2* (Lan & Jiang, 2009). *Foxf2* is a direct target and key effector of Shh signaling in lip and primary palate formation. Transient cyclopamine-induced hedgehog inhibition at E9.0 causes a reduction of *Foxf2* expression, and *Foxf2* was shown to promote Shh-dependent mesenchymal proliferation (Everson et al., 2017). In addition to *Foxf2*, expression of several other Fox genes in neural crest-derived mesenchyme of facial primordia are dependent on Shh signaling, including *Foxc2*, *Foxd1*, *Foxd2*, and *Foxf1* (Jeong, Mao, Tenzen, Kottmann, & McMahon, 2004). *Foxf1* and *Foxf2* are an important facet of a regulatory feedback loop in the secondary palate between Shh and Fgf, in which *Shh*-dependent *Foxf1/2* activates *Fgf18*, which feeds back to regulate *Shh* expression (J. Xu et al., 2016).

In another important EMI loop, *Msx1* activates *Bmp4* in the palatal mesenchyme to signal to the epithelium and activate *Shh*. Shh in turn signals back to the mesenchyme to activate *Bmp2* and control proliferation (Z. Zhang et al., 2002), as well as positively regulate both *Msx1* and *Bmp4* (Lan & Jiang, 2009). Ablation of epithelial *Shh*, but not *Smo*, causes cleft palate, demonstrating that specifically epithelium-to-mesenchyme hedgehog signaling is required for PS growth (Lan & Jiang, 2009). *Tp63*-null embryos display bilateral cleft lip and palate, and reduced proliferation in the MxP mesenchyme prevents formation of the

region that would otherwise develop into palatal shelves. While *Bmp4* positively regulates *Shh* in the developing craniofacial processes, both of *Shh* and *Fgf8* are diminished in the nasal and lip primordia in these embryos, with *Bmp4* upregulated in the posterior LNP and anterior MxP between E10.5 and E11.5. These differential responses may be independent of each other and Bmp and Shh likely act in parallel downstream of *Tp63* (Helen A. Thomason, Michael J. Dixon, & Jill Dixon, 2008). Studies in chick demonstrated that blocking Bmp signaling in the frontonasal process with ectopic noggin results in diminished *Shh* expression (Foppiano, Hu, & Marcucio, 2007). Shh activates all three of *Bmp2*, *Bmp4*, and *Bmp7* in the frontonasal process, and this positive reinforcement loop between Bmp and Shh signaling contributes to growth of the facial prominences during fusion of the primary palate (D. Hu et al., 2015).

7. Wnt signaling in orofacial clefts

7.1. Wnt lipidation and secretion molecules

Wnt signaling is integral to many aspects of development and is often associated with disease, which has been more extensively reviewed elsewhere (Clevers & Nusse, 2012; Komiya & Habas, 2008; B. T. MacDonald, Tamai, & He, 2009; Nusse & Clevers, 2017; van Amerongen, 2012). Wnt can activate a number of downstream pathways, traditionally classified as canonical or non-canonical depending on the primary response by a target cell, but there are many feedback mechanisms and points of interaction between the pathways that complicate this division. In brief, in the absence of Wnt, cytoplasmic β -catenin is continually targeted for proteasomal degradation. A canonical Wnt signal results in inhibition of the β -catenin destruction complex, allowing accumulation of free β -catenin to act as a transcription factor and alter target gene expression. Pathways that Wnt can activate independent of β -catenin include calcium/calmodulin signaling and the planar cell polarity (PCP) signaling cascades. Wnt signaling interacts with many other signaling axes and regulators during craniofacial development, and several components of the Wnt pathways are implicated in the etiology of OFCs (Reynolds et al., 2019).

In a Wnt-secreting cell, nascent Wnt ligands are post-translationally glycosylated, and then subsequently palmitoylated by the O-acyltransferase Porcupine (Porcn) within the endoplasmic reticulum (Galli, Barnes, Secrest, Kadowaki, & Burrus, 2007; Komekado, Yamamoto, Chiba, & Kikuchi, 2007) (Figure 5). Mutations within or near *PORCN* cause Goltz-Gorlin syndrome/focal dermal hypoplasia, which can include CL/P (Bornholdt et al., 2009; Lombardi et al., 2011; X. Wang et al., 2007). Wntless (*Wls*, homologous to the human *GPR177*) promotes secretion of Wnt ligands and is required for many of the patterning processes established by Wnt signaling during embryonic development (Bänziger et al., 2006). *Wls* recognition of a nascent Wnt ligand is dependent on Porcn-mediated acylation, and disrupted signaling in its absence reflects a requirement for lipid linkage in secretion and targeting to the membrane of a recipient cell (Herr & Basler, 2012). *Wls* cKO in *Wnt1*-expressing neural crest cells results in complete secondary CP. Both canonical β -catenin-dependent and Wnt5a-mediated noncanonical signaling are disrupted, causing altered patterns of proliferation and excessive cell death in palatal mesenchyme and epithelium. (Y. Liu et al., 2015). On the other hand, *Wls* deletion in the ectoderm using *Foxg1*-Cre inhibited

the cell adhesion and motility of epithelial cells during nasal pit invagination. This was accompanied by increased cell death and reduced proliferation, leading to severe frontonasal defects that were not observed in *Wnt1*-Cre-mediated *Wls* mutants (Zhu et al., 2016). Bmp, Fgf, and Shh signaling pathways, as well as JNK and ERK signaling, were all diminished in the facial prominence of these embryos (Zhu et al., 2016), suggesting multiple key roles for Wnt in modulating a complex signaling network to regulate frontonasal development.

7.2. WNT mutations associated with orofacial clefts

There are 19 Wnt ligands in the mammalian genome, and several have been linked with oral clefts in human patients, in both syndromic (Table 2) and nonsyndromic cases. One NSCL/P association study found evidence of linkage with SNPs in *WNT5A*, *WNT7A*, *WNT8A*, and *WNT11* across multiple populations (Brett T. Chiquet et al., 2008). Human *WNT6* and *WNT10A* are clustered together at 2q35 (Kirikoshi, Sekihara, & Katoh, 2001), and an analysis of 3 populations found significant association with SNPs at this cluster for both CPO and CL/P (Beaty et al., 2006). Additionally, suppressing the mouse homolog of either of these genes has been demonstrated to perturb growth of MEPM cells *in vitro* (Feng et al., 2013; Z. Jiang, Pan, Chen, Chen, & Xu, 2017). Several studies have linked *WNT3* mutations with NSCL/P, including haplotype association with *WNT3A* and *COL2A1* (B. T. Chiquet et al., 2008; Y. P. Lu et al., 2015; Adrianna Mostowska et al., 2012; Nikopensius et al., 2010; Nikopensius et al., 2011). *WNT3* and *WNT9B* are clustered at 17q21, and an association with *WNT9B*, but not *WNT3*, was identified in a Brazilian NSCL/P cohort (Fontoura, Silva, Granjeiro, & Letra, 2015), while another study found haplotype association with two *WNT9B* SNPs and epistasis between *WNT9B* and *MSX1* ($P = 2.564 \times 10^{-4}$), a conserved craniofacial BMP and WNT target (Medio et al., 2012; Nikopensius et al., 2011).

Wnt3 is expressed in the maxillary and medial nasal ectoderm during midfacial morphogenesis in mice (Lan et al., 2006), but *Wnt3*-null mice die early in development (P. Liu et al., 1999), prior to emergence of the palatal shelves from the MxP, and currently no models have yet been generated to describe a role *Wnt3* plays in lip and palate formation. *Wnt9b* is one of two Wnt ligands whose role in the formation of OFCs has been clarified in mouse models, however, along with *Wnt5a*. The A/WySn mouse strain exhibits spontaneous CL/P due to a mutation that maps to a region previously called the *Clf1* locus associated with CL/P. It was subsequently determined that *Wnt9b* is the affected gene in A/WySn mice and that *Clf1* is a mutant allele of *Wnt9b* (Juriloff et al., 2006). Targeted *Wnt9b* mutation also causes partially penetrant CL/P in which palatal shelves fail or delay to elevate and converge, but do not show altered proliferation or apoptosis (Carroll, Park, Hayashi, Majumdar, & McMahon, 2005; Jin et al., 2012). While *Wnt9b* is particularly important for lip and primary palate formation, *Wnt5a* is integral to secondary palate development. *Wnt5a*-null mice present with fully penetrant CPO due to directional cell migration defects in the palatal mesenchyme which result in failed PS elevation (He et al., 2008).

7.3. Canonical Wnt signaling pathway and orofacial clefts

Canonical Wnt signals are recognized at a target cell membrane by a Frz (Frizzled) GPCR family receptor and a single pass Lrp (lipoprotein receptor-related protein) 5 or 6 co-receptor. Recognition of a Wnt ligand facilitates binding to the Wnt-Fzd-Lrp complex by

disheveled (Dvl), which contains a central PDZ domain that physically interacts with the C terminal tail of Fzd (Wong et al., 2003). Several studies have linked various Wnt receptors to OFCs (Figure 5). A *FZD2* nonsense mutation was linked to omodysplasia with CLP (Saal et al., 2015), and a rare intronic SNP in *FZD6* was identified in an African American family with NSCLP (Cyjetkovic et al., 2015). In mouse models, *Fzd2* knockout causes 50% penetrant CPO in mice, and *Fzd1/Fzd2* compound mutants exhibit fully penetrant CPO (Huimin Yu et al., 2010). Mutations in *LRP6* have been identified in both sporadic and familial NSCL/P cases (Basha et al., 2018; Ockeloen et al., 2016), while *Lrp6*-knockout mice have fully penetrant CLP (L. Song et al., 2009).

The β -catenin destruction complex continually targets cytoplasmic β -catenin for proteasomal degradation, reviewed in more detail elsewhere (Stamos & Weis, 2013). The destruction complex contains two scaffold proteins, adenomatous polyposis coli (Apc) and axis inhibition (Axin) (E. Lee, Salic, Krüger, Heinrich, & Kirschner, 2003). Association with Fzd-Wnt allows Dvl to recruit Axin, promoting assembly of a “signalosome” complex at the cell membrane. This assembly is dependent on phosphorylation of the Lrp coreceptor by another destruction complex protein, casein kinase 1 (Ck1) (Cliffe, Hamada, & Bienz, 2003; Kishida et al., 1999). Mammals possess two Axin genes. Mouse models with loss of *Axin1* function are early embryonic lethal, due to excess β -catenin activation. Reducing *Ctnnb1* gene dosage can partially rescue the severity of defects, allowing further progression of craniofacial development, and these mutants exhibit median cleft lip (Chia, Kim, Itoh, Sokol, & Costantini, 2009). While *Axin2* mutant mice also exhibit craniofacial defects, no oral clefts have been reported (H.-M. I. Yu et al., 2005). However, *AXIN2* variants may play a role in the etiology of NSCL/P or NSCPO in human patients (Y. Han et al., 2014; A. Letra et al., 2012; Ariadne Letra, Menezes, Granjeiro, & Vieira, 2009). Axin also interacts with glycogen synthase kinase 3 (Gsk-3) and β -transducin repeat-containing protein (β -TrCP), which phosphorylate and ubiquitinate β -catenin, respectively. A significant association between a *GSK3B* variant and NSCL/P was identified in a Brazilian population (Vijayan, Ummer, Weber, Silva, & Letra, 2017), while *Gsk3b* knockout in mice causes complete secondary CP in which the shelves elevate, but do not converge toward the midline (K. J. Liu, Arron, Stankunas, Crabtree, & Longaker, 2007), further illustrating the requirement for appropriate β -catenin levels during palatogenesis.

Recruitment of Axin to the membrane facilitates its dephosphorylation, resulting in reduced binding affinity for β -catenin and inhibition of the destruction complex (Willert, Shibamoto, & Nusse, 1999). This allows accumulation of β -catenin, which is imported into the nucleus and can heterodimerize with a T-cell factor/Lymphoid enhancer factor TCF/LEF family transcription factor. TCF/LEF recognizes Wnt Responsive Element (WRE) genomic sequences, and normally represses transcription of target genes. However, nuclear β -catenin converts TCF/LEF into an activator and recruits co-activators to transcribe target genes (Cadigan & Waterman, 2012). Mice with epithelial *Ctnnb1* cKO display CP in which shelves elevate but do not fuse, with suppressed apoptosis and downregulated Tgf- β activity at the MEE (He et al., 2011). Conversely, epithelial *Ctnnb1* overexpression also causes CP with aberrant fusion events between the PS and mandibular epithelium (He et al., 2011). β -catenin also participates in cell adhesion, though it hasn't been demonstrated whether this

role uniquely contributes to palatogenesis independent of its Wnt-induced function (Yamada, Pokutta, Drees, Weis, & Nelson, 2005).

Tcf/Lef1- β -catenin-dependent transcription is activated in the facial ectoderm in response to Wnt signaling during midfacial development, and *Lef1* may provide a mechanism for modulating the Wnt and Tgf- β pathways and can participate in Smad-dependent transcriptional activation in response to Tgf- β signaling in the palatal epithelium as well (Nawshad, Medici, Liu, & Hay, 2007). *LEF1* variants have been linked with CL/P and CPO in humans (Martinelli, Carinci, et al., 2011), though CP is not among craniofacial defects seen in *Lef1*-null mice, possibly reflecting partial redundancy with other Tcf/Lef1 family members (van Genderen et al., 1994).

7.4. Non-canonical Wnt signaling in orofacial clefts

Non-canonical Wnt signaling includes several distinct cellular responses that are independent of β -catenin, and can be transduced through Frz and the RTK family paralogues Ror1/2 and Ryk, and may involve Dvl. The PCP pathway regulates how cells in a tissue align themselves in a uniform orientation and undergo convergent extension, polarized cellular movements during which cells narrow along one axis and extend along the perpendicular axis to change the structure of a tissue (Butler & Wallingford, 2017; Dabdoub et al., 2003; Heisenberg et al., 2000; Qian et al., 2007; Tada & Smith, 2000; Jianbo Wang et al., 2006). Wnt can also activate several Rho family GTPases to regulate cytoarchitecture and the Jun N-terminal kinase (JNK) signaling cascade. The other major noncanonical Wnt pathway, Wnt/ Ca^{2+} signaling, triggers cleavage of phosphatidyl inositol 4,5-bisphosphate (PIP_2) by Phospholipase C (PLC) to generate secondary messengers 1,2-diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP_3), which then activate Protein Kinase C (PKC) and calcium channels, which in turn activates calmodulin and Ca^{2+} /calmodulin-dependent protein kinase II (CamKII), with a number of downstream functions including activation of nuclear factor kappa-light-chain-enhancer of activated B cells ($\text{NF}\kappa\text{B}$) and nuclear factor of activated T-cells (NFAT) transcription factors (De, 2011)..

Multiple *ROR2* SNPs were linked with NSCPO in an Asian population (H. Wang et al., 2012), and *Ror2*-null mice exhibit craniofacial defects including CPO (Schwabe et al., 2004). *Ror2* has been demonstrated to transduce Wnt5a signaling through Dvl (Ho et al., 2012), and *WNT5A*, *ROR2*, and *DVL1/3* contribute to Robinow syndrome (Table 2). While neither of *Ror2* or *Wnt5a* heterozygous mutations cause CPO alone, palatal shelves in compound heterozygous mouse embryos fail to elevate due to mesenchymal migration defects, similar to *Wnt5a* mutants, demonstrating an epistatic relationship between the two genes and evidencing *Ror2* as the primary receptor for Wnt5a during PS elevation (He et al., 2008). *Wnt5a* and *Ror2* are both dysregulated in mice with RA-induced tongue malformation and CPO, suggesting not only that the teratogenic effects on palate development from RA may be at least in part due to an interaction with Wnt signaling, but also strengthening the evidence of *Ror2* as a key receptor for Wnt5a during palatogenesis (Cong et al., 2014). In mice with neural crest-specific *Wls* deletion, both canonical Wnt signaling and activation of noncanonical effectors c-Jun and JNK are dysregulated in the palatal mesenchyme. Addition of exogenous Wnt5a is able to rescue phosphorylation of c-

Jun and JNK, evidencing Wnt5a as a primary signal responsible for their activity (Y. Liu et al., 2015). Ryk is an important receptor involved in axon guidance and may transduce Wnt5a signal independent of Frz/Dvl (Keeble et al., 2006), but can also activate Tcf/Lef in response to Wnt1 or Wnt3a (W. Lu, Yamamoto, Ortega, & Baltimore, 2004). *RYK* mutations have been identified in Asian patients with NSCL/P (Watanabe et al., 2006), and *Ryk* knockout in mouse results in complete secondary cleft palate, though a mechanism has not been described (Halford et al., 2000).

In addition to Fzd and Dvl, the “core” vertebrate PCP proteins include Vangl, Prickle, Celsr, Ankrd6/Dvsn, Scrib, and Ptk7 (Butler & Wallingford, 2017; Jones & Chen, 2007; Strutt, 2008). Variants in both coding and noncoding regions of *PRICKLE1* were linked with NSCL/P in a Filipino cohort, and *Prickle1*-null and -hypomorphic mouse mutants present with Robinow-like traits including CPO (C. Liu et al., 2014; T. Yang et al., 2014), supporting its role as a possible downstream effector of Wnt5a-Ror2-Dvl in mediating secondary palate formation (Figure 5). *Fzd2* knockout mice present with CPO at around 50% penetrance, as do compound *Fzd2^{+/-};Fzd7^{-/-}*; *Vangl2^{+Lp}* (The *Vangl2* Lp allele is hypomorphic), which not observed in *Fzd2^{+/-};Fzd7^{-/-}* or *Fzd7^{-/-};Vangl2^{+Lp}*, suggesting *Vangl2*-dependent PCP signaling may be activated through Fzd2 and possibly Fzd7 in secondary palatogenesis (H. Yu, Ye, Guo, & Nathans, 2012). Ca^{2+} /calmodulin-associated serine/threonine kinase (CASK) associates with activated calmodulin and Syndecan-4, which itself interacts with *Vangl2* to regulate PCP. *Cask* mutant mice exhibit highly penetrant CPO, though it was not demonstrated whether this was specifically related to its activity downstream of noncanonical Wnt signaling (Atasoy et al., 2007; Escobedo et al., 2013).

7.5. Wnt signaling effectors and pathway interactions in orofacial clefts

Several Wnt targets are also associated with orofacial clefts, and Wnt regulates important pathways that provide insight into the mechanisms by which Wnt/ β -catenin signaling regulates lip and palate formation. Ectodermal *Wls* cKO results in reduced proliferation and increased apoptosis in the frontal MNP and LNP, with cell adhesion and elongation defects. These embryos showed drastically reduced expression of several Fgfs, including *Fgf3*, *Fgf4*, *Fgf7*, *Fgf8*, *Fgf9*, *Fgf10* (Zhu et al., 2016). *Fgf8* is an important patterning gene in the frontonasal ectoderm and is directly activated by Wnt/ β -catenin signaling. *Ctnnb1* mutant mice exhibit reduced *Fgf8* expression with excessive apoptosis in both the facial epithelium and mesenchyme (Yongping Wang et al., 2011). In *Wnt9b* mutants, *Fgf8*, *Fgf10*, and *Fgf17* expression are reduced in the MNP/LNP at E10.5, with *Fgf8* particularly affected and diminished at the nasal pit and lamboidal junction at E11.0 (Jin et al., 2012). In turn, *Wnt11* expression is modulated by Fgf signaling during secondary palatogenesis and this interaction is required for PS fusion *in vitro* (J.-M. Lee et al., 2008).

In addition to Fgf, the ectodermal *Wls* mutants showed reduced expression of *Bmp4* and its regulators *Msx1/2* (Zhu et al., 2016). While *Msx1* is regulated by canonical Wnt signaling, it also directly activates *Wnt5a* via an enhancer specific to the frontonasal region and palatal shelves, linking the canonical and noncanonical pathways (Nishihara et al., 2016). β -catenin can activate the *Bmp4* promoter (Shu et al., 2005), and when Wnt signaling is blocked by

ectopic Dkk in the chick MxP, *Bmp4* transcript levels are significantly reduced (Shimomura et al., 2019). However, in *Lrp6*-knockout mouse embryos, *Msx1/2* are significantly reduced in the MNP and LNP during lip formation, while *Bmp4* is only mildly affected (L. Song et al., 2009). This may be due to differences between the MxP and MNP/LNP or across species, or could reflect an incomplete share in the role of β -catenin activation by *Lrp6*.

Loss of *Tbx1* function in mouse causes excess maxillary-mandibular fusion, resulting in CP with variable severity, while epithelium-specific cKO causes an anterior cleft at the region where the two shelves meet the primary palate (Funato et al., 2012). *Tbx1* is repressed by canonical Wnt/ β -catenin signaling (Freyer & Morrow, 2010; Huh & Ornitz, 2010) and by RA signaling (Okano et al., 2008) and may represent a point of convergence between the two pathways during facial development. While both pathways inhibit *Tbx1*, Wnt also represses RA signaling, and in *Lrp6*-deficient embryos, the RA-synthesizing gene *Aldh1a3* is upregulated in the developing lip (L. Song et al., 2009). Though it has not been demonstrated in palate, *Ctnnb1* expression is increased in *Tbx1*-deficient anterior heart field, suggesting a negative feedback loop between canonical Wnt signaling and *Tbx1* may be possible (Racedo et al., 2017).

Several other transcription factors associated with CL/P may be directly activated by canonical Wnt signaling. *Gbx2* is a direct Wnt target involved in neural crest induction, and *Gbx2*-null mice sometimes have CP (Byrd & Meyers, 2005; B. Li, Kuriyama, Moreno, & Mayor, 2009). *Pitx2*-deficient embryos also display CP, and *Pitx2* expression is diminished in *Ctnnb1*;*Shh-Cre* cKO mutants. The *Pitx2* promoter contains WREs and Wnt-activated *Pitx2* controls proliferation in palatal mesenchyme through Tgf- β signaling (J.-i. Iwata, Tung, et al., 2012; Kiousi et al., 2002; C. Lin et al., 2011; M. F. Lu, Pressman, Dyer, Johnson, & Martin, 1999). *Snai1* and *Snai2* (Slug) promote EMT in part by repressing E-cadherin (*Cdh1*), a binding partner for β -catenin. *Snai2*-null and compound *Snai1*^{+/-};*Snai2*^{-/-} mutant mice have CP with elevated shelves that fail to adhere and form a MES (Murray, Oram, & Gridley, 2007). *Snai1* is targeted by Gsk3- β and β -TrCP, and was shown to be stabilized by canonical Wnt signaling in a manner similar to β -catenin (Yook, Li, Ota, Fearon, & Weiss, 2005), while *Snai1/2* in turn reinforce Wnt signaling by promoting β -catenin/Lef1 complex assembly to activate target transcription (Medici, Hay, & Olsen, 2008).

Hoxa2 is required for palate development in mice (T. M. Smith, Wang, Zhang, Kulyk, & Nazarali, 2009), and regulates many targets involved in Wnt signaling (Donaldson et al., 2012). *Barx1* is a key palatogenetic regulator that is inhibited by *Hoxa2* (T. M. Smith et al., 2009) and was shown to act downstream of Wnt in chick MxP, along with *Sox9* and *Tbx22* (Shimomura et al., 2019). *TBX22* mutations have been linked with NSCL/P and *Tbx22* deletion in mice results in a submucous CP with reduced ossification of the palatal mesenchyme (Marçano et al., 2004; Pauws et al., 2009). Tcf/ β -catenin can directly activate *Tbx3*, and while the mechanism is less well defined, *Tbx3* mutant mice display full secondary CP (López et al., 2018; Renard et al., 2007). Wnt signaling also directly activates the osteogenic factor *Runx2*, and *Runx2* deletion similarly causes CPO with reduced ossification in oral tissues (Åberg et al., 2004; Gaur et al., 2005). *SOX9* is associated with syndromic clefts (Table 2), and *Sox9* mutant mice have altered chondrocyte differentiation

and ectopic osteogenesis in neural crest lineage cells, resulting in full secondary CPO, reflecting its role as a chondrogenic specifier (Mori-Akiyama, Akiyama, Rowitch, & de Crombrughe, 2003). Wnt signaling induces *Hand2*, which represses *Sox9* and a chondrogenic fate in favor of an osteogenic one (Abe et al., 2010). Epithelial *Hand2* regulates *Shh* at the MEE independent of *Fgf10* and *Bmp4*, and hypomorphic *Hand2* expression results in complete CPO (Xiong et al., 2009).

Compound *Pbx1/2* mutants present with fully penetrant CL/P, and Pbx-Prep binding sites may facilitate transactivation at the *Wnt3-Wnt9b* locus and activation of Wnt/ β -catenin activity in the developing maxillary and nasal processes (Ferretti et al., 2011). *Wnt9b* subsequently regulates Fgf signaling to control mesenchymal proliferation during lip and primary palate formation (Jin et al., 2012). Both *Tp63* and *Irf6* are important effectors in the developing lamboid junction between the MNP, LNP, and MxP. *Pbx*-induced Wnt signaling activates *Tp63*, which in turn activates *Irf6* to promote epithelial apoptosis, and disrupting these interactions results in CL/P (Ferretti et al., 2011). While this sequence was initially identified in mice, a recent study reported gene-gene interactions between several of these factors in association with NSCL/P in human patients, likely demonstrating a conserved mechanism of regulating lip fusion (Maili et al., 2019). *Tp63* also represses *Bmp* and promotes *Shh* and *Fgf* signaling, and *Tp63*-null mouse embryos have bilateral CPO (H. A. Thomason et al., 2008). *p63* directly activates *Irf6* during palate development, and opposing activities of *p63* and *Twist* activate and repress, respectively, craniofacial *Irf6* expression (Fakhouri et al., 2017; Thomason et al., 2010). *Irf6* is required for keratinocyte differentiation, and failed PS elevation is among the severe defects exhibited by *Irf6*-null embryos (Ingraham et al., 2006; R. J. Richardson et al., 2006). *Grhl3* is a key target of *Irf6* in oral peridermal development (de la Garza et al., 2013), and the human orthologs of both genes are associated with OFCs (Tables 1 & 2).

7.6. Wnt signaling modulators in orofacial clefts

Several classes of protein also modulate Wnt signaling, including the antagonistic *Sostdc1*, *Dkk*, and Secreted frizzled-related protein (*Sfrp*) families and the R-spondins that amplify Wnt signals. *RSPO2* mutations are identified in multiple families with tetra-amelia including CL/P, as with *WNT3*, though it has not been demonstrated whether the characteristics observed in these two sets of patients are caused the same mechanism (Szenker-Ravi et al., 2018). *Rspo2*-null mice display several craniofacial defects including 75% penetrant cleft palate, likely an indirect effect from abnormal facial structure due to altered patterning of the BA1, rather than defective activity within the palatal shelves themselves (Jin et al., 2011). R-spondins interact with receptors *Lgr4*, *Lgr5*, and *Lgr6* to inhibit Wnt receptor degradation by *Znrf3/Rnf43* ubiquitin ligases (Zebisch et al., 2013). *Lgr5/Lgr6* and *Lgr4/Lgr5/Lgr6* compound knockout mutants exhibit CPO in which the palatal shelves fail to elevate, which is not observed in *Lgr4/Lgr6* knockouts, implying a particular requirement for at least *Lgr5* and possibly *Lgr6* in secondary palatogenesis (Szenker-Ravi et al., 2018).

While epithelial *Ctnnb1* cKO revealed Wnt regulates *Tgfb3* in palatogenesis, mouse embryos with epithelial *Tgfb2* cKO have upregulated *Dkk1* and *Dkk4*. *Dkk* represses Wnt signaling by binding and inhibiting the Lrp co-receptor. These embryos have dysregulated

Wnt activity and cleft soft palate with muscular defects, evidencing a reciprocal positive reinforcement between Wnt signaling and Tgf- β in palatal epithelium via Dkk (J.-i. Iwata et al., 2014). *Pax9* regulates a network of several important craniofacial regulators, including *Osr2*, *Msx1*, *Shh*, *Fgf10*, and *Bmp4* (J. Zhou, Gao, Lan, Jia, & Jiang, 2013). *Pax9* also functions upstream of Wnt and maintains signaling activity in the developing palatal shelves by repressing Dkk. *Pax9*-deficient mice have secondary cleft palate due to upregulated *Dkk1* and *Dkk2* expression, which can be rescued with a small-molecule Dkk inhibitor (Jia et al., 2017). *Sostdc1* is a target of Shh signaling, and ectopic Smo activation causes cleft palate, with upregulation of *Sostdc1* and downregulation of *Sox9* and *Runx2* (Hammond et al., 2018). Loss of *Sostdc1* (which also suppresses Bmp) function similarly rescues the secondary CP, further illustrating a key role for *Pax9* in secondary palate is to modulate Wnt (C. Li, Lan, Krumlauf, & Jiang, 2017).

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Frequently used lip/palate abbreviations

BA1	First Brachial Arch
BCL	Bilateral Cleft Lip
CLP	Cleft Lip and Cleft Palate
CL/P	Cleft Lip with or without Cleft Palate
CLO	Cleft Lip Only
CP	Cleft Palate
CPO	Cleft Palate Only
EMI	Epithelial-Mesenchymal Interaction
LNP	Lateral Nasal Prominence
MCL	Median Cleft Lip
MEE	Medial Edge Epithelium
MEPM	Mouse Embryonic Palatal Mesenchyme
MES	Medial Epithelial Seam
MNP	Medial Nasal Prominence
MxP	Maxillary Prominence
NS	Nonsyndromic [CL/P, CPO]

OFC	Orofacial Cleft
PS	Palatal Shelf
UCL	Unilateral Cleft Lip

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Figure 1. Bmp/Tgf-β signaling and OFCs.

The canonical Tgf-β and Bmp pathways with OFC phenotypes of the signaling molecules demonstrated in mutant mouse models in red. clp, cleft lip/palate; cp, cleft palate.

Figure 2. Fgf signaling and OFCs.

The Fgf signaling and regulatory molecules associated with OFCs in mutant mouse models are listed in red.

Figure 3. RA signaling and OFCs.

The retinoid signaling and metabolic pathway components associated with OFCs demonstrated in mouse models are listed in red. cp, cleft palate.

Figure 4. Shh signaling and OFCs.

The OFC phenotypes of the Shh signaling molecules (in red font) which have been demonstrated in mutant mouse models. Cyclopamine and Vismodegib are teratogens that inhibit Shh signaling and can induce OFCs in mouse embryos. clp, cleft lip/palate; cp, cleft palate.

Figure 5. Wnt signaling and OFCs.

Wnt signaling pathways with components in which OFC phenotypes have been demonstrated in mutant mouse models listed in red. clp, cleft lip/palate; cp, cleft palate; mcl, median cleft lip.

Table 1.

Major GWAS to identify genomic regions associated with nonsyndromic OFC risk

Study Details	Associated Loci or Candidate Genes if Identified	Reference
GWAS for NSCL/P; UK sibling pairs	Suggestive evidence for association at 1p36; 2p13; 2q37; 6p23; 6q25; 8q23–24; 11p12–q14; 12q13; 16p13; 16q24; Xcen-q21	(Prescott, Lees, Winter, & Malcolm, 2000)
GWAS for NSCL/P in Chinese families	Positive results for potential NSCL/P linkage with markers on chromosomes 1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 16, 20, and 21.	(Mary L. Marazita et al., 2002)
NSCL/P GWAS Meta-Analysis using data from 13 prior scans	1p12–13; 1q32; 2q32–35; 3p25; 6p23; 6q23–25; 7p12; 8q21; 8q23; 9q21; 12p11; 14q21–24; 15q15; 17q21; 18q21; 20q13	(Mary L. Marazita et al., 2004)
GWAS, fine-mapping and candidate analysis in multi-ethnic NSCL/P cohort	<i>IRF6</i> ; 2p13; 3q27–28; <i>FOXE1</i> ; 12p11; 14q21–24; 16q24	(M. L. Marazita et al., 2009)
GWAS for NSCL/P; German cohort	8q24.21	(Birnbbaum et al., 2009)
GWAS for NSCL/P; US patients of European ancestry	8q24.21	(Grant et al., 2009)
European and Asian Ancestry groups	8q24; <i>IRF6</i> ; <i>MAFB</i> ; 1p22 (Reported as <i>ABCA4</i> ; Variants may affect <i>ARHGAP29</i>)	(Beaty et al., 2010)
GWAS for NSCL/P; Central European ancestry, enlarged dataset from (Birnbbaum et al., 2009)	<i>VAX1</i> ; <i>NOG</i>	(Mangold et al., 2010)
GWAS for NSCPO and gene-environment interaction; multi-ethnic group	<i>MLLT3</i> , <i>SMC2</i> × Maternal Alcohol; <i>TBK1</i> , <i>ZNF236</i> × Maternal Smoking; <i>BAALC</i> × Maternal Multivitamin Supplementation (decreased risk) [No Loci Yielded Significant Association Alone]	(Beaty et al., 2011)
GWAS meta-analysis; multi-ethnic ancestry	<i>ARHGAP29</i> ; <i>PAX7</i> ; <i>IRF6</i> ; <i>THADA</i> ; <i>EPHA3</i> ; 8q21.3; 8q24; <i>VAX1</i> ; <i>SPRY2</i> ; <i>TPM1</i> ; <i>NOG</i> ; <i>MAFB</i>	(Kerstin U. Ludwig et al., 2012)
NSCL/P GWAS in Chinese population	<i>IRF6</i> ; <i>VAX1</i> ; 16p13.3 (<i>CREBBP</i> / <i>ADCY9</i>); <i>NTN1</i> ; <i>MAFB</i>	(Sun et al., 2015)
NSCL/P GWAS meta-analysis	<i>GREM1</i>	(Ludwig et al., 2016)
NSCPO GWAS; patients of European ancestry	<i>GRHL3</i>	(Elizabeth J. Leslie et al., 2016)
NSCL/P GWAS in multi-ethnic group	<i>ARHGAP29</i> ; <i>PAX7</i> ; <i>IRF6</i> ; <i>FAM49A</i> ; 8q24; <i>NTN1</i> ; 17q23 (<i>TANC2</i> / <i>DCAF7</i>); <i>RHPN2</i>	(E. J. Leslie et al., 2016)
GWAS and meta-analysis, Han Chinese cohort	<i>ARHGAP29</i> ; <i>IRF6</i> ; <i>FAM49A</i> ; <i>MYC</i> ; <i>MMP16</i> ; <i>GADD45G</i> ; <i>VAX1</i> ; <i>SPRY2</i> ; <i>SPRY1</i> ; <i>CREBBP</i> ; <i>NTN1</i> ; <i>NOG</i> ; <i>MAFB</i> ; <i>TAF1B</i> ; <i>MSX1</i> ; <i>FGF10</i> ; <i>TFAP2A</i> ; <i>FGFR1</i> ; <i>RAD54B</i> ; <i>PTCH1</i> ; <i>KRT18</i> ; <i>RPS26</i> ; <i>TMEM19</i> ; <i>LINC00640</i> ; <i>GSC</i> / <i>DICER1</i> ; <i>WNT9B</i>	(Y. Yu et al., 2017)
GWAS meta-analysis for NSCPO and NSCL/P; multi-ethnic groups	NSCPO: <i>GRHL3</i> NSCL/P: <i>ARHGAP29</i> ; <i>IRF6</i> ; <i>PAX7</i> ; 2p24; <i>TP63</i> ; 8q21; 8q24; <i>VAX1</i> ; 13q31; <i>ARID3B</i> ; <i>NTN1</i> ; <i>TANC2</i> ; <i>MAFB</i> All clefts combined: <i>IRF6</i> ; <i>ARHGAP29</i> ; <i>PAX7</i> ; 2p24; 8q21; 8q24; <i>FOXE1</i> ; <i>VAX1</i> ; 13q31; <i>NTN1</i> ; <i>MAFB</i>	(Elizabeth J. Leslie et al., 2017)
NSCL/P GWAS and replication analysis in Polish population	<i>DLG1</i>	(A. Mostowska et al., 2018)
GWAS and gene ontology analysis; 5 Chinese cohorts with CLO, CPO, and CLP groups	NSCPO: <i>IRF6</i> ; <i>POMGNT2</i> ; <i>NSD2</i> / <i>MSX1</i> ; <i>DOCK9</i> ; <i>PAX9</i> ; <i>DLK1</i> ; <i>FOXC2</i> - <i>FOXL1</i> ; <i>MAU2</i> . NSCLO: <i>IRF6</i> ; <i>MYCN</i> ; <i>VAX1</i> ; <i>GRM5</i> ; <i>ALX1</i> ; <i>DLK1</i> ; <i>MAFB</i>	(L. Huang et al., 2019)
NSCL/P GWAS and replication in patients of northeastern European ancestry	8q24.21; 12q23.1 (<i>ANO4</i>)	(van Rooij et al., 2019)

Table 2.

Summary of syndromic OFCs and candidate genes

SYNDROME	CLEFT GENE	NOTES ON CLEFT IF AVAILABLE	REFERENCES
3MC Syndrome	<i>COLEC11</i> , <i>MASPI</i>	CL/P reported in 40–68% of patients	(Gardner et al., 2017; Rooryck et al., 2011)
Andersen-Tawil Syndrome	<i>KCNJ2</i>	CPO in 8% of patients (n=36)	(Andelfinger et al., 2002; Tristani-Firouzi et al., 2002)
Apert Syndrome	<i>FGFR2</i>	CPO frequency depends on mutation type; 17–58%, 43% overall (n=85)	(Slaney et al., 1996; Wilkie et al., 1995)
Auriculocondylar Syndrome	<i>PLCB4</i>	Often includes CPO	(Rieder et al., 2012)
Bamforth-Lazarus Syndrome	<i>FOXE1</i>	Thyroid dysgenesis that includes CP	(Carre et al., 2014; Clifton-Bligh et al., 1998)
Baraitser-Winter Syndrome	<i>ACTB</i>	Very rare condition; <i>ACTB</i> G74S substitution in patient with CPO	(Di Donato et al., 2014)
Bartocas Papas/Recessive Popliteal Pterygium Syndrome	<i>RIPK4</i>	Severe phenotypes frequently include bilateral CLP	(Karen W. Gripp, Ennis, & Napoli, 2013; Mitchell et al., 2012)
Blepharochelodontic Syndrome	<i>CDH1</i> , <i>CTNND1</i>	Rare condition characterized by CL/P, usually bilateral	(Ghoumid et al., 2017)
Branchio-oculo-facial Syndrome	<i>TFAP2A</i>	Usually includes bilateral CL/P	(Milunsky et al., 2008)
Burn-McKeown Syndrome/ Oculo-oto-facial Dysplasia	<i>TXNL4A</i>	8/14 patients with compound heterozygous or homozygous mutations display CL/P or CPO	(Goos et al., 2017; Wieczorek et al., 2014)
Campomelic Dysplasia (PRS)	<i>SOX9</i>	Skeletal disorder that includes CPO in ~66% of patients	(Foster et al., 1994; Mansour, Hall, Pembrey, & Young, 1995; Wagner et al., 1994)
Carey-Fineman-Ziter Syndrome	<i>MYMK</i>	Myopathy that includes high-arched or cleft palate in most patients	(Di Gioia et al., 2017)
Catel-Manzke Syndrome	<i>TGDS</i>	PRS with hyperphalangy	(Ehmke et al., 2014)
CHARGE Syndrome	<i>CHD7</i>	25% of patients exhibit CL/P	(L. E. Vissers et al., 2004)
Chondroplasia with Joint Dislocations, GPAPP Type	<i>IMPAD1</i>	Includes posterior cleft palate	(L. E. L. M. Vissers et al., 2011)
Cleft Palate, Cardiac Defects, and Mental Retardation (CPCMR)	<i>MEIS2</i>	CLP or CPO among defects caused by <i>MEIS2</i> haploinsufficiency	(Johansson et al., 2014)
Cornelia de Lange Syndrome	<i>NIPBL</i>	Craniofacial phenotypes can include CP	(Krantz et al., 2004)
Craniofrontonasal Dysplasia	<i>EFNB1</i>	Characterized by midline defects that may include CL/P	(Twigg et al., 2004)
Crouzon Syndrome	<i>FGFR2</i>	Craniosynostosis with high-arched palate, occasional CP	(Reardon et al., 1994)
De la Chapelle Dysplasia/ Atelosteogenesis Type II	<i>SLC26A2</i>	Rare skeletal dysplasia includes CP	(Bonafé et al., 2008)
Desmosterolosis	<i>DHCR24</i>	33–40% CPO reported	(Andersson, Kratz, & Kelley, 2002; Rohanizadegan & Sacharow, 2018)
Diamond-Blackfan Anemia (DBA)	<i>RPL5</i> , <i>RPL19</i> , <i>RPL26</i>	Associated with at least 8 ribosomal protein genes, CP observed in patients with <i>RPL5</i> , <i>RPL19</i> , and <i>RPL26</i> mutations	(Hanna T. Gazda et al., 2012; H. T. Gazda et al., 2008; Konno et al., 2010)
DiGeorge and 22q11.2 Deletion Velo-cardio-facial Syndromes	22q11.2, includes <i>TBX1</i>	55–100% of patients exhibit palatal anomalies, 7.3–11% exhibit overt CPO.	(Yagi et al., 2003)

SYNDROME	CLEFT GENE	NOTES ON CLEFT IF AVAILABLE	REFERENCES
Ectrodactyly, Ectodermal Dysplasia, and Orofacial Clefts (EEC) Syndrome	<i>TP63</i>	Characterized by the presence of CLO, CPO, or CLP	(Barrow et al., 2002; Celli et al., 1999)
Ehlers-Danlos Syndrome	<i>FKBP14</i>	May include mild palate defects such as bifid uvula, submucous CP, or cleft soft palate	(Baumann et al., 2012)
Emanuel Syndrome	Supernumary chromosomal derivative	Malsegregation of 11;22 translocation; 54% CP reported	(Carter, St Pierre, Zackai, Emanuel, & Boycott, 2009)
Femoral-facial Syndrome	Unknown	CL/P reported in 63% of patients	(Luisin et al., 2017)
Fraser Syndrome	<i>FRAS1</i>	CL/P reported in 7–13% of patients	(Hoeftle et al., 2013; van Haelst, Scambler, Fraser Syndrome Collaboration, & Hennekam, 2007)
Frontonasal Dysplasia	<i>ALX1, ALX3, ALX4</i>	Midline facial defects can include nasal cleft, CL, and CP	(Kayserili et al., 2009; Twigg et al., 2009; Uz et al., 2010)
Goltz-Gorlin Syndrome/Focal Dermal Hypoplasia	<i>PORCN</i>	Can include CL/P, ~15%	(Bornholdt et al., 2009; X. Wang et al., 2007)
Gordon Syndrome/Distal Arthrogryposis Type 3	<i>PIEZO2</i>	Most cases include CPO	(Alisch et al., 2017; McMillin et al., 2014)
Gorlin-Goltz Syndrome/Nevoid Basal Cell Carcinoma	<i>PTCH1</i>	CL/P reported in ~8.5% of patients	(Hahn et al., 1996; Lambrecht & Kreusch, 1997)
Hay-Wells/Ankyloblepharon-Ectodermal Dysplasia-Clefting Syndrome	<i>TP63</i>	Typified by presence of OFCs	(McGrath et al., 2001)
Hereditary Neuralgic Amyotrophy	<i>SEPT9</i>	May include CP	(Laccone et al., 2008)
Holoprosencephaly + CL/P	<i>SIX3, TGIF1, SHH, PTCH1, GLI2, ZIC2, DISP1</i>	Prevalence and severity of OFC varies with associated gene and causal mutation	(Aguilella et al., 2003; K. W. Gripp et al., 2000; Ribeiro et al., 2006; E. Roessler et al., 1996; Erich Roessler et al., 2003; Erich Roessler et al., 2009; Solomon et al., 2010; Wallis et al., 1999)
Hyperphosphatasia Mental Retardation Syndrome	<i>PGAP3, PIGL, PIGV</i>	Can include CP, frequency depends on associated gene	(Abdel-Hamid et al., 2018; Altassan, Fox, Poulin, & Buhas, 2018; Horn, Krawitz, Mannhardt, Korenke, & Meinecke, 2011)
Kabuki Syndrome	<i>KMT2D (MLL2)</i>	CL/P is observed in ~40% of patients	(Hannibal et al., 2011)
Kallmann Syndrome/Idiopathic Hypogonadotrophic Hypogonadism	<i>FGF8, FGFR1</i>	Occasionally includes CPO	(Dode et al., 2003; Falardeau et al., 2008; Pitteloud et al., 2006)
Klippel-Feil Syndrome	<i>MEOX1, GDF6</i>	May include CP	(Mohamed et al., 2013; May Tassabehji et al., 2008)
Larsen Syndrome	<i>FLNB</i>	Osteochondroplasia with ~15% CPO	(Bicknell et al., 2007)
Loeys-Dietz Syndrome	<i>TGFBR1, TGFBR2</i>	Commonly includes bifid uvula, occasional CPO	(Loeys et al., 2005)
Marshall's/Stickler Syndrome	<i>COL2A1, COL11A1, LOXL3</i>	Variable phenotypic spectrum, can include CP	(Alzahrani et al., 2015; L. Guo et al., 2017)
Meckel-Gruber Syndrome	<i>RPGRIP1L, TCTN2, TMEM67, TMEM216</i>	Ciliopathy with many causal genes, can include CL/P	(Delous et al., 2007; Shaheen et al., 2011; U. M. Smith et al., 2006; Valente et al., 2010)
Moebius Syndrome	Likely combination of Genetic and Environmental Influences	Affects cranial nerves VI/VII, can include CPO	(Rizos, Negrón, & Serman, 1998)

SYNDROME	CLEFT GENE	NOTES ON CLEFT IF AVAILABLE	REFERENCES
Mohr-Majewski Syndrome/ Oral-facial-digital (OFD) Syndrome Type IV	<i>TCTN3</i>	Severe ciliopathy which typically includes CL/P	(Thomas et al., 2012)
Mowat-Wilson Syndrome	<i>ZEB2 (ZFHX1B)</i>	Occasional CPO or mild palatal anomalies	(Dastot-Le Moal et al., 2007; M. Wilson et al., 2003)
Muenke Syndrome	<i>FGFR3</i>	Craniosynostosis that occasionally includes CPO or CLP	(Anderson et al., 2013)
Multiple Epiphyseal Dysplasia	<i>SLC26A2</i>	Out of 6 known causal genes, CPO only reported in cases with recessive <i>SLC26A2</i> mutations	(Briggs & Chapman, 2002; Mäkitie et al., 2015)
Multiple Pterygium Syndrome	<i>MYH3, CHRNA</i>	Occasional cleft palate	(Chong et al., 2015; Morgan et al., 2006)
Myhre Syndrome	<i>SMAD4</i>	Missense <i>SMAD4</i> SNPs in patients with CL/P and CPO	(Caputo et al., 2012)
Nager Syndrome	<i>SF3B4</i>	CP is present in most patients	(Czeschik et al., 2013)
Native American/Bailey-Bloch Myopathy	<i>STAC3</i>	Patients exhibit CPO or high arched palate	(Horstick et al., 2013; Stamm et al., 2008)
Oculo-dento-digital Dysplasia	<i>GJA1</i>	Uncommon CPO or CLP observed in ~3% of reported cases	(Amano et al., 2012; Paznekas et al., 2009)
Oculo-facio-cardio-dental Syndrome	<i>BCOR</i>	Palate defects from bifid uvula to overt cleft observed in 31% of patients (n=26)	(Davoody, Chen, Nanda, Uribe, & Reichenberger, 2012; Hilton et al., 2009)
OFD Syndrome Type IX	<i>TBC1D32, SCLT1</i>	Ciliopathy; commonly includes midline CLP	(Adly, Alhashem, Ammari, & Alkuraya, 2014)
OFD Syndrome Type XIV	<i>C2CD3</i>	Ciliopathy includes CPO	(Thauvin-Robinet et al., 2014)
Opitz G/BBB Syndrome	<i>MID1, SPECC1L</i>	Often includes CL/P	(Kruszka et al., 2015; Quaderi et al., 1997)
Osteopathia striata with cranial sclerosis	<i>AMER1 (WTX)</i>	52% of patients with <i>AMER1</i> mutation exhibited OFC	(Jenkins et al., 2009)
Otopalatodigital Syndrome	<i>FLNA</i>	Both types 1 and 2 caused by <i>FLNA</i> mutations and commonly include CPO	(Robertson et al., 2003)
Papillon-Leage-Psaume/OFD Syndrome Type I	<i>OFD1</i>	CPO or CL/P frequency > 80%	(Ferrante et al., 2001)
Peters-Plus Syndrome	<i>B3GLCT</i>	CL/P is reported in nearly half of patients	(Lesnik Oberstein et al., 2006)
Pfeiffer Syndrome	<i>FGFR1, FGFR2</i>	Most patients have high arched palate, occasional CP	(Stoler et al., 2009)
Pierre Robin Sequence (Isolated)	<i>SOX9, BMPR1B</i>	PRS is typified by cleft palate	(Benko et al., 2009; Jakobsen et al., 2007; Y. Yang et al., 2017)
Popliteal Pterygium Syndrome	<i>IRF6</i>	Phenotypic overlap with the allelic VWS, usually including CLP or CPO	(Kondo et al., 2002)
Rapp-Hodgkin Syndrome	<i>TP63</i>	Ectodermal Dysplasia that includes CLP	(Kantaputra, Hamada, Kumchai, & McGrath, 2003; van Straten & Butow, 2013)
Renpenning Syndrome	<i>PQBP1</i>	10% reported CPO	(Stevenson et al., 2005)
Richieri-Costa-Pereira	<i>EIF4A3</i>	78.5% reported CPO; Often includes mandibular cleft	(F. P. Favaro et al., 2014; Francine Pinheiro Favaro et al., 2011)
Roberts/SC Phocomelia Syndrome	<i>ESCO2</i>	CLP in >50% of patients	(H. Vega et al., 2010; Hugo Vega et al., 2005)
Robinow Syndrome	<i>ROR2, WNT5A, DVL1, DVL3</i>	Characteristic facial features, sometimes include CP	(Person et al., 2010; van Bokhoven et al., 2000; J. White et al., 2015; J. J. White et al., 2016)

SYNDROME	CLEFT GENE	NOTES ON CLEFT IF AVAILABLE	REFERENCES
SATB2-Associated Syndrome/ Glass Syndrome	<i>SATB2</i>	59% of patients include CPO overall, varies by mutation type.	(Zarate & Fish, 2017)
Saethre-Chotzen Syndrome	<i>TWIST1</i>	Craniosynostosis can include CP	(Howard et al., 1997)
Schilbach-Rott Syndrome	<i>9q22.32–33 dup./ PTCH1</i>	<i>PTCH1</i> overexpression may contribute; commonly includes CL/P	(Prontera et al., 2019)
Simpson-Golabi-Behmel Syndrome	<i>GPC3</i>	Cleft palate in 13% of patients	(Tenorio et al., 2014)
Smith-Lemli-Opitz Syndrome	<i>DHCR7</i>	Approximately half of patients have CP	(Fitzky et al., 1998; Wassif et al., 1998; Waterham et al., 1998)
TARP Syndrome	<i>RBM10</i>	Characterized by PRS	(Johnston et al., 2010)
Tetra-Amelia Syndrome	<i>WNT3, RSPO2</i>	Often includes bilateral CLP	(Niemann et al., 2004; Szenker-Ravi et al., 2018)
Thurston/OFD Syndrome Type V	<i>DDX59</i>	Includes CPO/bifid uvula	(Shamseldin et al., 2013)
Treacher-Collins Syndrome	<i>TCOF1, POLR1C, POLR1D</i>	93% of cases attributed to TCOF1; high- arched or cleft palate in 28%	(Dauwerse et al., 2011; Group, 1996; Trainor, Dixon, & Dixon, 2009)
Van der Woude Syndrome	<i>IRF6, GRHL3</i>	CL/P or CPO that includes usually lower lip pits and hypodontia	(Kondo et al., 2002; Peyrard-Janvid et al., 2014)
Varadi-Papp/OFD Syndrome Type VI	<i>CPLANE1 (C5orf42), TMEM216</i>	Very rare form of OFDS which can include CLP or CPO; Can be caused by <i>CPLANE1</i> mutations, 2 patients with <i>TMEM216</i> mutations were reported with OFD6	(Valente et al., 2010; Váradi, Szabó, & Papp, 1980)
Vici Syndrome	<i>EPG5</i>	CP observed in 6% (n=50) of patients	(Byrne et al., 2016)
Waardenburg Syndrome	<i>PAX3</i>	High degree of genetic heterogeneity, Type I associated with <i>PAX3</i> , occasionally includes CL/P	(Da-Silva, 1991; M. Tassabehji et al., 1992)
Walker-Warburg Syndrome/ Muscular Dystrophy- Dystroglycanopathy	<i>POMT1</i>	Type of muscular dystrophy with occasional CLP; Many subtypes and causal genes, <i>POMT1</i> , <i>POMT2</i> mutations identified in CLP patients	(Vajsar et al., 2008; van Reeuwijk et al., 2005)
Wolf-Hirschhorn Syndrome	<i>NSD2, WHSC2, LETM1</i> (deletion)	CL/P commonly observed among associated craniofacial malformations	(Rutherford & Lowery, 2016)
Wolff-Parkinson-White/20p12.3 Microdeletion Syndrome	<i>BMP2</i>	PRS/CPO common characteristic; <i>BMP2</i> likely key gene contributing to craniofacial phenotype	(Lalani et al., 2009; Sahoo et al., 2011; Williams, Uhas, Bunke, Garber, & Martin, 2012)
X-Linked Mental Retardation + CL/P	<i>PQBPI, PHF8</i>	Several dozen genes are implicated in XLMR; <i>PQBPI</i> , <i>PHF8</i> mutations identified in cases that included CL/P	(Kalscheuer et al., 2003; Laumonnier et al., 2005)