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Toward an Orofacial Gene Regulatory Network

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Abstract

Orofacial clefting is a common birth defect with significant morbidity. A panoply of candidate genes have been discovered through synergy of animal models and human genetics. Among these, variants in Interferon Regulatory Factor 6 (*IRF6*) cause syndromic orofacial clefting and contribute risk toward isolated cleft lip and palate (1/700 live births). Rare variants in *IRF6* can lead to Van der Woude Syndrome (1/35,000 live births) and Popliteal Pterygium Syndrome (1/300,000 live births). Furthermore, IRF6 regulates *GRHL3* and rare variants in this downstream target can also lead to Van der Woude Syndrome. In addition, a common variant (rs642961) in the *IRF6* locus is found in 30% of the world's population and contributes risk for isolated orofacial clefting. Biochemical studies revealed that rs642961 abrogates one of four AP-2alpha binding sites. Like *IRF6* and *GRHL3*, rare variants in *TFAP2A* can also lead to syndromic orofacial clefting with lip pits (Branchio-oculo-facial Syndrome). The literature suggests that AP-2alpha, IRF6 and GRHL3 are part of a pathway that is essential for lip and palate development. In addition to updating the pathways, players and pursuits, this review will highlight some of the current questions in the study of orofacial clefting.

Keywords

IRF6; *TFAP2A*; *GRHL3*; Van der Woude Syndrome; Popliteal Pterygium Syndrome; Branchio-Oculo-Facial Syndrome; Cleft lip and palate; embryonic development; mouse models

Historical Introduction

According to high-resolution imaging of Egyptian mummies, orofacial clefts have been a part of the human condition for over 3000 years (Hoffman and Hudgins, 2002). In 1845, Demarquay described orofacial clefts and lip pits as a "very rare" congenital malformation (as cited by Cervenka et al. (Cervenka J, 1967). Lip pits, or indentations, occurring with cleft lip and palate were subsequently described in five generations of an affected family (Test and Falls, 1947). Van der Woude reviewed the literature in 1954 and concluded that

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this entity traversed generations in an autosomal dominant pattern (Van Der Woude, 1954). As a result of this study, lip pits along with orofacial clefting became known as Van der Woude Syndrome (VWS OMIM #119300). In 1962, Levy highlighted the presence of symmetrically located lip pits on the medial edge of lower lips, now a nearly pathognomonic feature of VWS or “Lip Pit Syndrome” (Levy, 1962). The earliest recorded survey of VWS prevalence among all forms of cleft lip and palate took place in 1971 when Dronamraju reported that eight of 260 clefting families (3%) had VWS (Dronamraju, 1971). The incidence of VWS has been directly estimated at 3.6/100,000 live births (Burdick, 1986). Today, VWS is recognized as the most common syndromic orofacial clefting disorder.

Current clinical context and impact of orofacial clefting

Orofacial clefting results from defective palate and lip closure between the 6th and 12th week of human gestation. Current standard of care for children born with a cleft lip and plate includes surgical closure of a cleft lip by 4 months and closure of the palate by 12 months. Closure of alveolar clefts with bone grafts should be complete by 11 years of age, correction of residual abnormalities by 12 years of age and final nasal contours and treatment of breathing problems by 17 years of age. In addition to these surgeries, children born with CLP need to undergo speech therapy until the age of 11. According to the American Society of Plastic Surgeons, children born with CLP require a multi-disciplinary team to receive appropriate care. This team includes a pediatrician, pediatric dentist, otolaryngologist, auditory specialist, speech pathologist, genetic counselor and a social worker. Costs include surgical procedure, nursing, hospital stay, anesthesia, medication, devices and clinical tests. In total, each individual born with a cleft lip and/or palate (CLP) will require $200,000 for medical treatment (Wehby and Cassell, 2010). However, this extensive treatment regimen is not sufficient to prevent physical, psychological, social and neurological sequelae (Nopoulos et al., 2007b; Manna et al., 2009).

Despite interdisciplinary medical treatment and the enormous cost, there are numerous complications. These commonly include bleeding, infection, irregular healing of scars and puckering of tissues (contractures), asymmetries, remaining deformities, anesthesia risks, allergies to suture material and glue, damage to deep structures, such as blood vessels, nerves and muscles, and the possibility of surgery revision. In addition, changes in nose shape and teeth alignment may result after or from cleft repair. Teeth abnormalities associated with CLP may require additional repair (Aizenbud et al., 2011). Finally, because the mouth and palate are integral tissues, CLP morbidity also includes poor feeding, growth retardation and repeated ear infections.

Considering the complications and the number of healthcare providers needed for treatment, there has been a shift of CLP repair from the primary care office to teaching hospitals and an associated increase in cost (Basseri et al., 2011). While ongoing clinical investigation in CLP treatment has led to a dramatic decrease in the associated morbidity (Mulliken, 2004), the challenges are even greater for developing countries (Furr et al., 2010). In addition, individuals born with a CLP have an increased risk for cancer (Taioli et al., 2010) and neurological ((Shriver et al., 2006; Boes et al., 2007; Calzolari et al., 2007; Nopoulos et al., 2007b; Conrad et al., 2008; Conrad et al., 2009; Conrad et al., 2010; Nopoulos et al., 2010;

Rosen et al., 2011), musculoskeletal and cardiovascular diseases (Calzolari et al., 2007), surgical complications (Jones et al., 2010) and an increased risk of mortality between birth and 55 years of age (Christensen et al., 2004). However, we already know that syndromic clefting is responsive to *in utero* stimuli. For example, reduced maternal folate, alcohol consumption and maternal smoking increase clefting risk (Wu et al., 2010; Blanton et al., 2011). It follows that if the phenotype can be exacerbated, it can be ameliorated. These challenges highlight the emphasis on prevention, rather than treatment of CLP.

Variability of VWS suggests a personalized diagnostic and therapeutic intervention for iCLP

Variable expressivity and incomplete penetrance in VWS suggests unidentified genetic and/or environmental modifiers for isolated cleft lip and palate (iCLP, OMIM # 119530). Analysis of a single large VWS pedigree with multiple affected individuals showed the presence of lip pits with or without clefting, suggesting variable expressivity of a single rare variant (Baker, 1964). Janku et al. reported a penetrance of 96.7%, with lip pits present in 88% of affected individuals and clefting in 21% (Janku et al., 1980). Burdick et al. examined 864 individuals from 164 families and found that cleft lip and palate occurred more commonly than isolated cleft palate (Burdick et al., 1985). VWS is also associated with hypodontia, bifid uvula, hypernasal voice, lip mounds that secrete mucus, Hirshsprung disease, congenital heart defects, popliteal webs, limb anomalies and accessory nipples (Van Der Woude, 1954; Janku et al., 1980; Shprintzen et al., 1980; Nopoulos et al., 2007a; Nopoulos et al., 2007b). However, these associated anomalies are rare and 12–15% of individuals with VWS will present with orofacial clefting as an isolated clinical finding. As such, these individuals are phenotypically indistinguishable from the clinical diagnosis of isolated cleft lip and palate (iCLP). However, in contrast to the genetically simple, ‘one gene, one phenotype’ model of syndromes, iCLP is a common, complex disease with multiple genetic and environmental factors (Cobourne, 2004). Considering the phenotypic and clinical overlap, Murray et al. hypothesized that discovering the etiology of VWS would provide insights into iCLP (Murray et al., 1990).

While the phenotype suggests VWS can be a clinical model for iCLP, is there evidence that VWS can be a genetic model for iCLP? As a Mendelian disorder, familial studies are a critical component in VWS research (Cervenka et al., 1967; Vignale et al., 1998; Kondo et al., 2002). Houdayer et al. (2001) used a family design to ask if VWS and iCLP were associated in a parametric linkage analysis and transmission disequilibrium tests (TDT) (Houdayer et al., 2001). Although parametric linkage was not supportive, TDT provided evidence for a genetic link between VWS and iCLP (Houdayer et al., 2001). TDT measures the over-transmitted allele from parents to affected offspring and as such is robust to population structure, e.g., population stratification. However, there is no correlation between sex and phenotype in VWS, whereas males with iCLP tend to be more severely affected than females (Burdick et al., 1985; Calzolari et al., 2007; Huang et al., 2007).

Variable expressivity in VWS suggests that genetic and environmental modifiers could be leveraged to reduce disease burden. Known phenotypic modifiers within the VWS spectrum include locus heterogeneity (Peyrard-Janvid et al., 2014), different types (mis-sense vs.

truncation) (de Lima et al., 2009) and location of mutations (DNA Binding Domain vs. Protein Interaction Domain vs. Activation Domain) (Knight et al., 2006) and regulation of gene expression (enhancer, promoter) (Fakhouri et al., 2014). Significantly, a search for genetic modifiers using common variants at candidate loci did not yield a formally significant association (Leslie et al., 2013). However, prior work on a single pedigree identified a genetic modifier at 17p11.2–11.1 (Sertie et al., 1999). This suggests that a personalized, 'all of the above approach', will be necessary to map the road(s) to pathology. In contrast, at the cellular and biochemical levels, little is known about how IRF6 protein location (e.g. sequestration in sub-cellular organelle and exocytosis), activity and stability (e.g. resistance to degradation/turnover) contributes to the clinical spectrum of VWS.

Mutations in *IRF6* cause VWS and PPS

The first study into the etiology of VWS used the "red blood cell antigen" for genetic linkage as well as several biochemical assays, including electrophoretic studies of glucose-6-phosphate-dehydrogenase, haptoglobin, phosphoglucomutase, and hemoglobin (Schneider, 1973). An interstitial deletion of chromosome 1 at q32-q41 in a 41-month-old girl with lip pits refined the VWS locus (Bocian and Walker, 1987). A candidate-gene-and-region approach successfully identified linkage with the renin gene and the D1S65 locus in 1q using restriction fragment length polymorphisms (Murray et al., 1990). A microdeletion refined the VWS locus to 4.1 mega base pairs within 1q32-q41 (Sander et al., 1994). Cloning of the critical region allowed the production of a single YAC clone with an 850 kb segment containing the microdeletion and later a 900 kb gene map (Schutte et al., 1996; Schutte et al., 2000).

Several important twin studies have contributed to our understanding of VWS (Neuman and Shulman, 1961). Dizygotic twins discordant for VWS were described by Levy et al. (Levy, 1962). Cervenka et al. (1967) characterized the first published description of twins with lip pits, who were then identified as "probably monozygotic" based on facial features and blood typing (Cervenka et al., 1967). However, only one had a unilateral cleft lip. Monozygotic twins who are concordant for VWS were reported 30 years later when Hersh and Verdi (1992) showed siblings with unilateral cleft lip and palate, and lip pits (Hersh and Verdi, 1992). Currently, four monozygotic twins concordant for VWS have been reported (Tokat et al., 2005; Jobling et al., 2011). One of these reported on monozygotic twins who had VWS with varying levels of disease severity; i.e. one only had lip pits while the other had lip pits, a cleft lip and a cleft palate (Jobling et al., 2011). These findings are significant because they suggest monochorionic, diamniotic twins with VWS can have variable expressivity as a result of somatic mutations in *IRF6*, variants in the interacting genetic network, placentation or stochastic features.

Nearly 150 years after Demarquay first described a clefting syndrome with lip pits, Kondo et al. (2002) used discordant monozygotic twins to discover *Interferon Regulatory Factor 6* (*IRF6*) as the VWS gene (Kondo et al., 2002). Aided by advancing technology and a genetic map, targeted sequencing showed an *IRF6* mutation in the affected individual but not the unaffected sibling. Prevalence screening then showed that 68% of families with VWS had a mutation in *IRF6* (de Lima et al., 2009) (Fig. 1). With that discovery, the authors turned

their attention to popliteal pterygium syndrome (PPS OMIM #119500), a rarer form of syndromic clefting that had been reported in VWS pedigrees. PPS, like VWS, can include orofacial clefting, lip pits, hypodontia and skin anomalies. However, PPS is a more severe phenotype because it also includes webbing in the back of the knee (popliteal fossa), genital anomalies (hypoplasia of the labia majora, cryptorchidism or bifid scrotum), webbing between toes or fingers (syndactyly), triangular folds of skin over nails and tissue connecting the upper and lower eyelids (ankyloblepharon) (Bixler et al., 1973; Escobar and Weaver, 1978; Hammer et al., 1989). Furthermore, prior analysis of three families with PPS had already shown linkage to the Van der Woude Syndrome locus, at 1q32-q41 (Lees et al., 1999; Wong and Gustafsson, 2000). With linkage and phenotypic similarity, sequencing by Kondo et al. (2002) revealed that all 13 families with PPS had an *IRF6* mutation (Kondo et al., 2002). In contrast to VWS, more severely affected individuals who were diagnosed with PPS had a preponderance of single nucleotide substitution in exons 3 or 4, the DNA binding domain (Fig. 1) (Kondo et al., 2002; Leslie et al., 2012).

Since discovery of *IRF6*, case reports and case series from a wide geographical distribution have expanded the phenotypic spectrum of VWS (Kayano et al., 2003; Kim et al., 2003; Shotelersuk et al., 2003; Wang et al., 2003; Gatta et al., 2004; Ghassibe et al., 2004; Matsuzawa et al., 2004; Ghassibe et al., 2005; Item et al., 2005; Wang et al., 2005; Ye et al., 2005; Du et al., 2006a; Du et al., 2006b; Brosch et al., 2007; Paranaiba et al., 2008; Tan et al., 2008; de Lima et al., 2009; Yeetong et al., 2009; Malik et al., 2010; Scioletti et al., 2010; Birkeland et al., 2011; Minones-Suarez et al., 2011; Salahshourifar et al., 2011). While most studies of VWS have identified point mutations in *IRF6*, deletions as large as 2.98 Mb, involving 25 genes, have also been reported (Sander et al., 1994; Schutte et al., 1999; Salahshourifar et al., 2011). Considering that genomic deletions and premature stop codons can lead to VWS, the most likely etiology is genetic haplosufficiency. More severe phenotypes are associated with large deletions relative to point mutations, which suggests that additional genes within this locus are interacting with *IRF6*. de Lima et al. (2009) conducted a comprehensive study of *IRF6* mutations leading to VWS and found that while truncating mutations occurred throughout the open reading frame, point mutations are significantly over-represented in exons 3, 4, 7 and 8 (de Lima et al., 2009). From this, the authors concluded that exons 3 and 4 (DNA binding domain) and exons 7 and 8 (protein binding domain) coded for the functional machinery of the protein (Fig. 1). Mutations leading to PPS were more frequently found at residues predicted to bind DNA within exons 3 and 4 (discussed in “IRF6 structure-function in development and disease”). While this genotype-phenotype correlation was strong, it was not absolute because a single *IRF6* mutation can be associated with VWS or PPS within a family.

For families with VWS who did not have a mutation in *IRF6*, recent efforts have focused on finding targets of IRF6. In that regard, *Grhl3* was found to be downstream of IRF6 in zebrafish superficial epithelium and murine epidermis (de la Garza et al., 2013). Furthermore, prior analysis in a large Finnish family with VWS showed linkage to 1p34-p36, which contains the *GRHL3* locus (Koillinen et al., 2001). Exome sequencing in this family and targeted sequencing in others showed that 5% of families with VWS have mutations in *GRHL3*. Despite sequencing the open reading frames of both *IRF6* and

GRHL3, the etiology of VWS is unknown in the remaining 27% of affected families. Mutations in regulatory regions (Fakhouri et al., 2014), additional loci and/or combinations of mutations might be contributing to pathology in remaining families.

Variants within *IRF6* are associated with iCLP

Given that 10–15% of VWS appears as isolated Cleft Lip and Palate (iCLP) and that mutations in *IRF6* lead to VWS, it was predicted that common variants in *IRF6* could be contributing to the multifactorial risk of isolated, or non-syndromic orofacial clefting. Consistent with this rationale, three recent studies found a strong association between *IRF6* and isolated orofacial clefting (Zucchero et al., 2004; Rahimov et al., 2008; Beaty et al., 2010). The first, by Zucchero et al. (2004), showed that a non-synonymous substitution (V274I) within *IRF6* is associated with 12% of all orofacial clefting (Zucchero et al., 2004). Considering that clefting can be lethal in non-human primates and the ancestral allele confers risk, the association seemed counter-intuitive. However, despite the change in amino acid sequence, a substitution from valine to isoleucine is fairly conservative, i.e. is not predicted to alter protein structure/function. Instead, the authors predicted the ancestral allele was in linkage disequilibrium (LD) with the etiologic variant, i.e., the ancestral allele is on the same haplotype block as a disease predisposing, derived variant.

Consistent with this hypothesis, sequencing of highly conserved regions within the LD block that contains *IRF6* (140 kb in length) revealed an association to a non-coding variant (rs642961) 9.7 kb upstream of the *IRF6* transcription start site (Rahimov et al., 2008). Importantly, the disease-associated allele at rs642961 is derived. Furthermore, rs642961 lies within a 608 bp sequence (*MCS9.7*) that is highly conserved and has enhancer activity that recapitulates endogenous IRF6 expression *in vivo* (Fakhouri et al., 2012) (functional significance in “Transcriptional regulation of *IRF6* expression”). Importantly, rs642961 is also associated with the severity of iCLP (Kerameddin et al., 2015). While maternal and environmental factors did not modify the risk at rs642961, prenatal multi-vitamin supplementation reduces the risk of orofacial clefting for individuals carrying two additional *IRF6* variants (rs2076153 and rs17015218) (Wu et al., 2010). These data further support the role of IRF6 in iCLP and suggest that personalized intervention is on the horizon. With identification, genetic and environmental factors that alter *IRF6* expression or function may be leveraged to alter disease penetrance and/or expressivity in VWS and iCLP.

Murine *Irf6* alleles

Murine alleles have provided a wealth of information about the function of IRF6. A gene trap allele ($Irf^{gt/+}$), inserted 36 base pairs into intron 1, has several splice donor/acceptor sites and a stop codon that prematurely terminates IRF6 translation (Ingraham et al., 2006). Another allele (R84C) was made by targeted insertion of a human *IRF6* mutation that disrupts the DNA binding domain and leads to PPS ($Irf^{R84C/+}$) (Richardson et al., 2006). A more recent allele ($Irf6^{clft1/+}$) resulted from a forward genetic screen using *N*-ethyl-*N*-Nitrosourea (ENU) mutagenesis (Stottmann et al., 2010). Interestingly, affected embryos had a mutation at proline-39, which was previously reported in a VWS pedigree (Kondo et

al., 2002). With both deletions and human mutations, currently available murine models provide both fidelity and facility for understanding and intervening in this network.

The murine phenotypic spectrum includes cleft palate, microcephaly, tongue and mandibular defects, clubbed limbs, syndactyly, a bifid xiphoid and a shortened fused tail (Ingraham et al., 2006; Richardson et al., 2006; Boell et al., 2013; Goudy et al., 2013). Considering the expression pattern, affect in knockout mice and role in palatal development, the role of IRF6 in epithelium is a focus of many ongoing studies. Epithelial abnormalities include a hyperproliferative epidermis that fails to differentiate, a permeable skin barrier, esophageal adhesions and pervasive oral adhesions. Intraoral adhesions prevent palatal elevation and result in a cleft palate. It is currently unclear whether the pathological affects of adhesions are physical, e.g. restraining the shelves, and/or biological, e.g. downstream signaling. Loss of IRF6 also leads to evagination of tooth epithelium while a hypomorphic allele does not (Blackburn et al., 2012). Finally, over-expressing IRF6 using the *Krt14* promoter leads to absence of the skull and an open eye in 22% of embryos but rescues palatal defects caused by loss of *Tgfbr2* signaling (Iwata et al., 2013).

IRF6 structure-function in development and disease

Interferon Regulatory Factor 6 is a member of the IRF family of transcription factors, which share a high degree of sequence identity and have a common helix-turn-helix DNA binding motif (Taniguchi et al., 2001). The IRF family plays an important role in host defense by regulating the innate and/or adaptive immune systems (Honda and Taniguchi, 2006). In contrast, *Irf6* regulates embryonic development, including orofacial, skin, limb, tongue and brain morphogenesis (Ingraham et al., 2006; Biggs et al., 2011; Aerts et al., 2013; Goudy et al., 2013). *IRF6* is composed of nine exons, with a start codon in exon 3 and a stop codon in exon 9 (Fig. 1) (Bailey et al., 2005). Kondo et al. (2002) detected two *Irf6* transcripts from whole mouse embryos from E4.5 to E18.5 (Kondo et al., 2002). The smaller transcript (4.4 kb) is most common and includes an unspliced intron in the 3'UTR. The size and significance of a larger transcript is currently unknown. IRF6 expression was found in the brain, eyes, heart, liver, lung, placenta, skin, testes and tongue but not the spleen (Kondo et al., 2002). IRF6 is made up of 467 amino acids and Western blotting showed a band at 59 kDa (Bailey et al., 2005; Knight et al., 2006). Phosphorylation is required to activate IRF6 in cell culture and this form of the protein has been detected in murine mammary epithelium (Bailey et al., 2005; Bailey et al., 2009). Phosphorylation results in a second western blot band at 63 kDa (Bailey et al., 2005). Based on sequence identity and structure of IRF5 (Chen et al., 2008), IRF6 likely forms a dimer to function.

Consistent with human development, IRF6 expression is seen in murine oral epithelium (Fig. 2). In palatal epithelium, IRF6 expression is observed in both periderm and basal cells from E12.5 to E17.5 (Fig. 2). Periderm, marked by intermediate filaments KRT6 and KRT17, is a flat, squamous monolayer that coats the entire embryo. Periderm prevents pathological interepithelial adhesions between adjacent structures, including the palate, tongue and mandible (Richardson et al., 2014). Basal cells are cuboidal and are marked by intermediate filaments KRT5 and KRT14. Basal cells give rise to and anchor the periderm to the basal lamina (Richardson et al., 2009). At E12.5, palatal shelves start as mesenchymal

buds covered by periderm and basal cells. During the next 24 hours, the palatal shelves, including both the mesenchyme and epithelium, proliferate and expand, to inhabit the space between the tongue and mandible bilaterally. Loss of IRF6 arrests palatal development starting here, with the shelves wedged between the tongue and mandible until birth (Richardson et al., 2009). At E13.5, the palatal shelves elevate and pivot toward midline, ultimately apposing above the tongue. By E14.5, periderm cells are lost along the medial surface of the palatal shelves. Periderm cells that lack IRF6 have abnormal morphology and expression of KRT6 and KRT17 (Richardson et al., 2009; Peyrard-Janvid et al., 2014). At E14.5, the underlying basal cells adhere to each other and interdigitate to form the medial edge seam. Dissolution of this seam, in part via cell death, is paramount in fusion of palatal shelves. Without IRF6, basal cells continuously proliferate and express KRT14 (Ingraham et al., 2006). Between E15.5 and E17.5, the palate forms when a complete mesenchymal bridge separates the nasal and oral cavities (Knight et al., 2006). *Irf6* expression is also seen at the fusion point of the lateral and medial nasal processes and the maxillary processes, which fuse at E11.5 to form the upper lip (Knight et al., 2006). Loss of IRF6 in the mouse is not associated with a cleft lip (Ingraham et al., 2006; Richardson et al., 2006).

Based on the crystal structure of IRF1, IRF6 appears to contain a highly conserved penta-tryptophan winged-helix-loop-helix DNA binding domain in exons 3 and 4 (Kondo et al., 2002; Little et al., 2009). IRF6 is structurally characterized as transcription factor but it is mainly detected in the cytoplasm and rarely visualized in the nucleus with various antibodies. However, several lines of evidence suggest that IRF6 binds DNA and transcriptionally regulates gene expression in critical developmental pathways. First, injection of cDNA containing the IRF6 DNA binding domain (dominant negative construct) leads to more severe developmental defects than knocking down the transcript with a morpholino in zebrafish and xenopus embryos (Sabel et al., 2009; de la Garza et al., 2013). In humans, a mutation in the DNA binding domain, R84C, is associated with more severe developmental defects, including Popliteal Pterygium Syndrome (de Lima et al., 2009). Furthermore, R84C heterozygous embryos have more pervasive oral adhesions than embryos heterozygous for a gene trap (null) allele (Ingraham et al., 2006; Richardson et al., 2006). Biochemically, R84C appears to reduce IRF6 DNA-binding affinity (Little et al., 2009) with a concomitant reduction in transactivation of a luciferase reporter (Su, 2011). Together, these data suggest that R84C leads to gain-of-function during dimerization (protein-protein interaction) by sequestering protein from the other allele. Furthermore, these data suggest that transcriptional activity by IRF6 is fundamental for function.

In support of these data, Irf6 transcriptionally regulates *grhl3* via a highly conserved binding element. Furthermore, *grhl3* mRNA partially rescues zebrafish embryos injected with a dominant negative Irf6 (de la Garza et al., 2013) (Fig. 3). In primary human keratinocytes, a genome wide screen showed that IRF6 binds within this highly conserved *GRHL3* element and that knocking down *IRF6* leads to a reduction of *GRHL3* expression (Botti et al., 2011). During palatal development, IRF6 is required for GRHL3 expression in the epithelium and oral periderm (de la Garza et al., 2013). Like *Irf6*, loss of *Grhl3* leads to bilateral oral adhesions and palatal clefting (Peyrard-Janvid et al., 2014). IRF6 also transcriptionally regulates *OVOL1*, a transcription factor regulating epithelial differentiation and a repressor

of the oncogenic protein c-MYC (Nair et al., 2006; Botti et al., 2011). Importantly, the *MYC* locus (8q24) is a major factor in nonsyndromic cleft lip and palate (Birnbaum et al., 2009). Together, these data suggest that IRF6 transcriptionally regulates several epithelial factors.

However, perhaps the predominantly cytoplasmic expression pattern of IRF6 suggests a limited number of transcriptional targets. An illustrative comparison can be made with TP63, which is co-expressed with IRF6 throughout epithelial development and almost exclusively localized to the nucleus. TP63 binds over 7,500 genes and 1,213 (16%) of these targets are differentially expressed with knockdown studies (McDade et al., 2012). In contrast, a genome-wide survey showed that IRF6 binds nearly 2,200 genes and that only 2.6% (56/2177) of these putative targets were affected in a knockdown assay (Botti et al., 2011). Therefore, IRF6 binds fewer transcriptional targets and has an affect on a minority of these. Considering the importance of IRF6 in embryonic development, it follows that both transcriptional and post-translational targets are critical.

An important post-translational target of IRF6 is TP63. In humans, mutations in *TP63* can lead to Ectrodactyly, Ectodermal Dysplasia (EEC), which includes cleft lip and palate (OMIM #604292). While TP63 drives *IRF6* expression, IRF6 post-translationally targets TP63 for degradation via the proteasome (Moretti et al., 2010). This negative feedback loop is critical for palatal development (Thomason et al., 2010). In the mouse, embryos doubly heterozygous for *Trp63* and *Irf6* can develop a cleft palate. Considering cytoplasmic localization and regulation of TP63 protein stability, post-translational regulation seems critical. Protein-protein interactions by IRF6 that results in degradation of TP63 are most likely mediated by the protein-binding domain (exons 7 and 8) (Bailey et al., 2005) (Fig. 1). Another important protein target of IRF6 is the Mammary Serine Protease Inhibitor (Maspin, SERPINB5) (Bailey et al., 2005). In contrast to TP63, IRF6 cooperatively binds to Maspin to regulate differentiation in mammary epithelium. Transient re-expression of *IRF6* reduced breast cancer invasiveness and loss of IRF6 in skin is associated with squamous cell carcinoma (Botti et al., 2011; Stransky et al., 2011). Mutations in *IRF6* are also found in 5% of patients with head and neck squamous cell carcinoma (HNSCC) (Stransky et al., 2011). Regulatory partners and pathway of IRF6 in HNSCC are currently undetermined but an epithelial origin suggests a similar pathway. Aside from TP63 and Maspin, little is known about protein-protein interactions mediated by IRF6. Important targets for future work include identifying the E3 ubiquitin ligases that regulate and are regulated by IRF6.

The C-terminal domain (CTD) consists of a linker region and an α-helix encoded by exon 9, which appears to harbor the regulatory domain for IRF6 in mammary epithelium (Bailey et al., 2005) (Fig. 1). Important clues regarding function of the C-terminus can be gleaned from IRF5, which is most similar to IRF6. The C-terminal domain in IRF5 contains an activation/repression switch that is regulated by phosphorylation and is highly sensitive to mutagenesis (Chen et al., 2008). In human keratinocytes, converting serine and threonine to aspartic acid leads to constitutive activation and nuclear localization of IRF6 (Su, 2011). The factors regulating the on/off switch at this C-terminal domain are currently unknown.

Exons 5 and 6 appear to encode a less conserved proline-rich region. In contrast to frequent point mutations in the DNA binding domain, 30/34 disease-causing mutations in exons 5

and 6 are protein truncations (Fig. 1). Underrepresentation of missense mutations along with less conservation suggests that most coding changes in exons 5 and 6 are either innocuous or are associated with a different disease process. In support of the latter, whole-exome sequencing revealed an exon 5 mutation in a patient with Pierre Robin Sequence (Pengelly et al., 2014).

Biochemically, rapid turnover of IRF6 via the proteasome (Bailey et al., 2008) might be a way to regulate transcriptional and post-translational activity. Phosphorylated (active) IRF6 is also secreted by mammary epithelium into milk (Bailey et al., 2009). Within mammary ducts, alveolar cells use exocytosis to secrete proteins, which may contribute to sequestration or cytoplasmic localization. Considering presence in topologically distinct compartments (cytosol and extracellular medium) (Arnoys and Wang, 2007), IRF6 exhibits 'dual localization'. Fibroblast growth factor 3 (FGF3), among others in the FGF signaling pathway, also exhibits dual localization and contributes risk for orofacial clefting (Arnoys and Wang, 2007; Riley et al., 2007). A recent bioinformatic analysis shows IRF6 is related to several FGF pathway genes, among other important orofacial targets (Dai et al., 2015), and perhaps this dual localization is an explanation. Examining IRF6 expression in multiple tissues and time points along with inhibition of nuclear export with Leptomycin B may further elucidate the targets and functions of IRF6.

Transcriptional regulation of *IRF6* expression

IRF6 expression is implicated in multiple disease processes, including clefting and cancer. Therefore, understanding how this gene is regulated is critical. An enhancer (*MCS9.7*) recapitulates endogenous *IRF6* expression in skin and oral epithelium (Fakhouri et al., 2012). A common DNA variant (rs642961) in this enhancer is associated with isolated cleft lip and palate (CLP) but not cleft palate only (Rahimov et al., 2008). Biochemically, rs642961 abrogates one of four AP-2alpha binding sites within *MCS9.7*. The Transcription Factor Activating Protein 2 (AP-2) family of transcription factors is composed of five members that homo or heterodimerize to repress or activate gene expression through a common, conserved binding element. Like *IRF6*, mutations in *TFAP2A* can also lead to syndromic orofacial clefting with lip pits, known as Branchio-Oculo-Facial Syndrome (BOFS OMIM # 113620). BOFS is dominantly inherited and can present with malformation of the eyes, ears and skin (Li et al., 2013). Similar to *Irf6* knockout embryos (discussed above in "Murine *Irf6* alleles"), loss of AP-2alpha leads to severe craniofacial, limb and skin defects (Schorle et al., 1996; Zhang et al., 1996). However, *Tfap2a* knockout embryos are unique in the biomedical literature for absence of a thoracic and abdominal body wall as well as neural tube defects. Facial clefting, which results from failed neural tube closure, precludes analysis of palatal development in *Tfap2a*$^{-/-}$ embryos. However, tissue-specific deletion of *Tfap2a* supports a role in palatal development that is independent of neural tube closure (Brewer et al., 2004).

Recent work confirms that AP-2alpha binds to the *MCS9.7* enhancer and regulates *IRF6* expression (McDade et al., 2012), consistent with a functional role for rs642961. As such, rs642961 may reduce AP-2alpha trans-activation of *IRF6* expression, contributing to orofacial clefting risk. Alternatively, it is interesting to consider how loss of *IRF6* might be

contributing to the phenotypic spectrum of BOFS. In fact, numerous individuals with BOFS have mutations in the DNA binding domain of AP-2alpha, suggesting the importance of downstream transcriptional targets. Likewise, TP63 binds *MCS9.7* and regulates *IRF6* expression in primary keratinocytes (McDade et al., 2012) (Fig. 3). It is unknown whether IRF6 has a feedback function on AP-2alpha, as it has on TP63. However, recent data suggest that IRF6 binds upstream of *TFAP2C*, but not *TFAP2A* (Botti et al., 2011). *MCS9.7* also contains binding sites for *MAFB*, which was recently associated with CLP (Beaty et al., 2010; Moretti et al., 2010; Thomason et al., 2010). More recent work suggests additional *IRF6* enhancers are regulated by Notch signaling in keratinocytes (Restivo et al., 2011).

From morphology to molecule: Locus heterogeneity and the genotype-phenotype correlation in the mouse

The *Irf6* knockout phenotype is reproduced by knocking out genes at four additional loci (i.e., locus heterogeneity); *Stratifin* (*14-3-3σ*), *Ikka*, *Kdf1*, and, to a lesser extent, *Ripk4* (Herron et al., 2005; Rountree et al., 2010; Song et al., 2010; Lee et al., 2013). 14-3-3σ is a tumor suppressor protein that interacts with TP53 via a positive feedback loop to regulate the G2/M cell cycle checkpoint (Hermeking et al., 1997; Yang et al., 2003). 14-3-3σ also enhances Protein Kinase C activity and contains a Pleckstrin homology domain, critical in protein-protein interaction with serine/threonine phosphorylation (Dellambra et al., 1995; Yaffe et al., 1997; Rittinger et al., 1999). *Irf6* genetically interacts with *14-3-3σ* in skin, limb, craniofacial and oral cavity development. If the genetic interaction is direct, 14-3-3σ may be involved in phosphorylation and post-translational activation of IRF6 (Fig. 3). Mutations in *14-3-3σ* have not been associated with syndromic human disease. However, hypermethylation of a CpG regulatory island reduced *14-3-3σ* expression in 91% of breast carcinoma cells (Ferguson et al., 2000), and likely constitutes an early oncogenic event (Umbricht et al., 2001).

In humans, homozygous recessive mutations in Nuclear Factor Kappa-B Kinase subunit alpha (*IKKA*) leads to Severe Fetal Encasement Malformation, also called Cocoon Syndrome (OMIM # 613630). Cocoon Syndrome includes body wall, skin, limb and neural tube defects (Lahtela et al., 2010). IKKA, also known as CHUK, is a serine/threonine protein kinase that regulates the activation of NF-kB by marking its repressors (IkB Kinase) for ubiquitin-mediated degradation. In the skin, IKKA is a tumor suppressor protein and functions independently of NF-kB and IkB Kinase (Hu et al., 2001). IKKA is downstream of Transforming Growth Factor-β (TGFB) signaling in a complex with Smad2/3, which allows nuclear translocation independent of Smad4 (Descargues et al., 2008). Likewise, TGFB signaling regulates *IRF6* expression (Le et al., 2012; Iwata et al., 2013). Downstream, both IKKA and IRF6 regulate *OVOL1* expression (Descargues et al., 2008; Botti et al., 2011). In the palate, TGFB signaling regulates *Irf6* through SMAD4 (Iwata et al., 2013) but the molecular context of IKKA function in this tissue is less clearly delineated. Despite the phenotypic similarity in skin and palate, and the common upstream and downstream molecular targets, preliminary work does not support a genetic interaction between *Ikka* and *Irf6* (Richardson et al., 2006). Unfortunately, testing epistasis in the mouse is highly specific but not sensitive, i.e. absence of proof is not proof of absence. Additional clues may emerge

from an analysis of *IRF6* expression in *Ikka* knockout murine skin. As such, IKKA may be upstream of *IRF6* in skin and palate development but our current assays have not been sufficiently sensitive.

Like 14-3-3σ, the Receptor-Interacting serine/threonine Protein Kinase 4 (RIPK4) regulates keratinocytes differentiation and interacts with Protein Kinase C (Chen et al., 2001). Like IKKA, RIPK4 activates NF-kB (Meylan et al., 2002). However, *Ripk4* knockout embryos appear to be the least severely affected of the cohort (Holland et al., 2002). In contrast, human mutations in *RIPK4* can lead to a lethal type of Popliteal Pterygium Syndrome, called Bartsocas-Papas Syndrome (BPS OMIM # 263650). Like PPS, caused by mutation in *IRF6*, BPS is associated with popliteal webbing, ankyloblepharon, cleft lip and palate and syndactyly (Mitchell et al., 2011; Kalay et al., 2012). Like Cocoon Syndrome (OMIM #613630), caused by mutations in *IKKA*, BPS is associated with severe craniofacial defects, leading to superficial visualization of the nasal cavity, in what may be a form of facial clefting. *In vivo* assays of epistasis between *Ripk4* and *Irf6*, *Ikka* or *14-3-3*σ have not been reported. However, recent work shows that RIPK4 activates IRF6 (Kwa et al., 2015). A recently discovered gene, Keratinocytes Differentiation Factor 1 (*Kdf1*), like *Irf6*, appears to interact with *Trp63* and *14-3-3*σ in skin, limb and craniofacial development (Lee et al., 2013). Cytoplasmic localization and association with the cellular membrane suggest a signaling function for KDF1.

Is there also evidence for locus heterogeneity in humans? Like *IRF6*, mutations in *RIPK4* can lead to PPS. Like *RIPK4*, mutations in *IKKA* can lead to BPS (Leslie et al., 2015). Therefore, for the 27% of families without a known genetic mutation leading to VWS, *14-3-3*σ, *IKKA*, *KDF1* and *RIPK4* appear to be good candidate genes. For the remaining 73% of families with either a mutation in *IRF6* or *GRHL3*, common or rare variants in *14-3-3*σ, *IKKA*, *KDF1* and *RIPK4* may be acting as genetic modifiers. In addition to cellular and biochemical assays, a systematic analysis for epistasis in the mouse, including double and triple mutants, may contribute to our knowledge of this complex and seemingly redundant network.

Translating Therapies

Ongoing preventative efforts have focused on folate and multi-vitamin supplementation to reduce birth defects (van Rooij et al., 2004; Wu et al., 2010). Associations between orofacial clefting and variants in genes of folate metabolism provide a context for this approach (Mills et al., 2008; Blanton et al., 2011). A recent review suggests folate supplementation affects gene expression through methylation reactions (Blom et al., 2006). Bisulfite sequencing showed a ~300 bp CpG island in the *IRF6* promoter was methylated. Methylation of the *IRF6* promoter reduced expression and increased risk for squamous cell carcinoma (Botti et al., 2011). If supplementation affects methylation and epigenetic regulation at the *IRF6* promoter, a therapeutic intervention might be possible by titrating folate and *IRF6* levels based on personal risk. However, if folate intake increases methylation and reduces *IRF6* expression, mass fortification may not reduce risk for everyone. For *Grhl3*, while folate had no affect, inositol supplementation rescued neural tube defects (Ting et al., 2003). The oral cavity was not evaluated with inositol supplementation.

Given widespread epithelial pathology, several studies predicted rescuing epidermal cells would prevent the knockout phenotype. Consistent with this rationale, using the *KRT14* promoter to drive *Ikka* in basal epithelial cells of *Ikka* knockout embryos led to rescue of skin, skeletal and limb defects (Sil et al., 2004). However, the pups did not feed and died due to persistent esophageal adhesions that occluded the gastrointestinal tract. In contrast to wildtype embryos (Vassar et al., 1989; Takahashi et al., 1995), the *KRT14* promoter was inactive in the esophagus of *Ikka* knockout embryos. Skeletal and limb rescue is intriguing because it involves both cartilaginous and bony structures that lie beneath the epidermal cells, strongly suggesting non-cell autonomous function. Unlike skin and limb rescue, a curled tail persisted, suggesting additional cell autonomous function for *Ikka* in neural tube development. In an analogous experiment using the *KRT5* promoter, only a super-physiological dose of IKKA completely rescued tail development (Liu et al., 2011). This is intriguing for two reasons. First, it suggests IKKA and epidermal cells are necessary for tail development. Second, it suggests a sufficiently high dose of epithelial IKKA can compensate for extra-epithelial IKKA. The compensation might be through non-cell autonomous signaling or direct action of IKKA at distant cells.

Similarly, using the *KRT14* promoter to drive *Ripk4* in *Ripk4* knockout pups rescued cutaneous defects. As seen with *Ikka*, *KRT14* spatio-temporal regulation of *Ripk4* was not sufficient to rescue esophageal adhesions (Rountree et al., 2010). In a test for epistasis, epithelial expression of *Ripk4* using the *KRT14* promoter did not rescue *Ikka* and *14-3-3σ* knockout embryos. Considering less severely affected knockout embryos and failure to rescue loss of *Ikka* and *14-3-3σ*, *RIPK4* may be in a parallel, but converging pathway or require both IKKA and 14-3-3σ for function. Rescue of *Irf6* and *Kdf1* knockout embryos using the *KRT14-Ripk4* transgene has not been reported.

In addition to genetic rescue, experimental embryonic gene therapy protocols to prevent disease in animal models have been developed for cystic fibrosis (Keswani et al., 2011), Duchenne muscular dystrophy (Koppanati et al., 2010), Herlitz junctional epidermolysis bullosa (Muhle et al., 2006; Endo et al., 2011), thrombotic thrombocytopenic purpura (Niiya et al., 2009) and congenital blindness (Dejneka et al., 2004). Likewise, gene delivery to oral epithelium and developing epidermis is possible during development (Wu et al., 2012). In mature skin, epithelial stratification (cornified layer) and keratin secretion forms a physical barrier that prevents entry by viral and bacterial pathogens. However, during early embryonic development, a cornified layer is not present, leaving the tissue highly susceptible to transduction. As such, intra-aminotic injection of a viral vector with epithelial tropism may provide robust targeting. Like developing epidermis, oral epithelium can be transduced. Circulation of amniotic fluid in and through the embryo also ensures delivery of viral vectors into the oral cavity. As lip and palate development occurs between the 6^{th} and 10^{th} week of human gestation, ultrasound may be used to visualize fetal anatomy. Considering mouse models discussed here, *in vivo* assays seem feasible. Furthermore, transduction of periderm, a cell type lost before birth, limits untoward long-term affects. Finally, the immune-privileged status of amniotic fluid limits innate and adaptive blunting of gene delivery. Like many orofacial clefting genes, *Irf6*-related pathogenesis results from

insufficient epithelial expression. As such, *in utero* gene delivery may provide a therapeutic modality for single gene clefting disorders.

Future Directions

Orofacial clefting is a common birth defect, with multiple genetic and environmental factors. Among these, *IRF6* variants result in two orofacial clefting syndromes (VWS and PPS) and increase risk for isolated cleft lip and palate. Crucially, the genes that regulate and are regulated by *IRF6* also cause syndromic orofacial clefts (*TFAP2A* and BOFS, *GRHL3* and VWS, *TP63* and EEC). Furthermore, recent literature suggests an emerging phenotypic spectrum from mutations in *IRF6*, *RIPK4* and *IKKA* and locus heterogeneity for PPS, BPS and Cocoon Syndrome, all of which confer significant morbidity and mortality and involve epidermal development. More broadly still, variants within *IRF6* are associated with squamous cell carcinoma. Currently available murine models include a genetrap, human mutations, a hypomorph and an over-expresser, providing robust resources for additional investigation. This network is critical, the tools are available and the system appears to be amenable to interventions.

Currently, the cause of VWS is unknown in more than 25% of affected families. Determining the etiology in these families may reveal additional risk factors in isolated orofacial clefting. Considering locus heterogeneity and evolving phenotypic spectrums, identifying environmental and genetic modifiers seems critical in understanding, and perhaps one day managing, risk. Work thus far suggests that a personalized approach will be necessary. It is also not clear how specific cellular and biochemical processes contribute to disease penetrance and severity. In that regard, identifying the post-translational targets of and E3 ligases that regulate and are deployed by IRF6 seems critical. Molecularly, describing transport of IRF6 in and out of the nucleus may aid in designing small-molecule targets. Despite locus heterogeneity in mice, *Ikka* and *Irf6* do not interact and either our *in vivo* assay is not sufficiently sensitive or there are two, or more, converging pathways leading to the same phenotype. While the former is simpler, the latter is supported by interaction between *Irf6* and *14-3-3σ*. Furthermore, *Ripk4* expression did not rescue *Ikka* and *14-3-3σ* knockout mice. As such, it will be important to test for epistasis among *Irf6*, *14-3-3σ*, *Ikka*, *Kdf1* and *Ripk4*. Finally, identification of *GRHL3* mutations in families with VWS and inositol rescue of a birth defect in *Grhl3* knockout mice suggests that testing the role of supplements may be reasonable in orofacial clefts.

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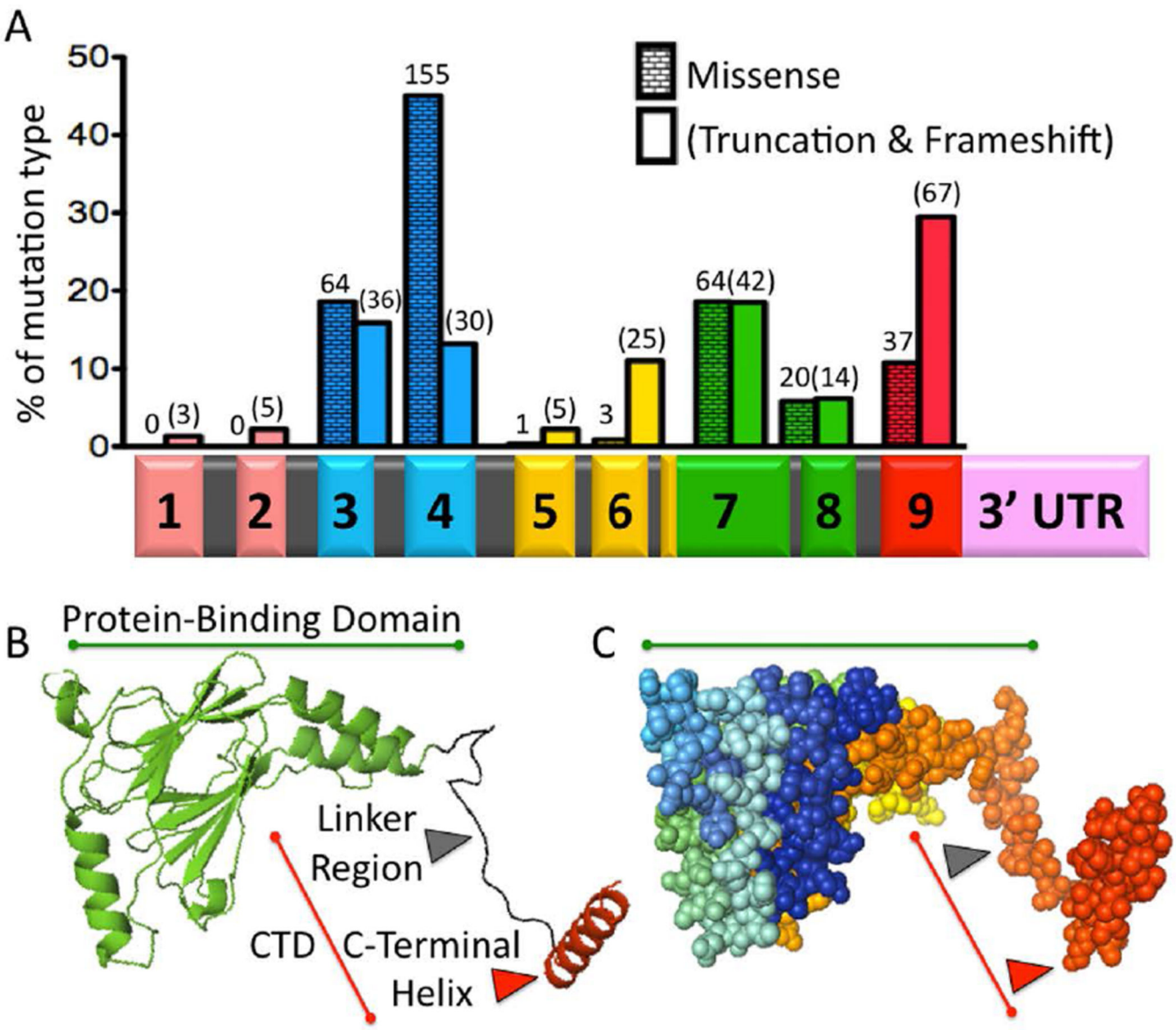

Figure 1. Syndromic Mutations and the Structure of *IRF6*

A) *IRF6* Mutations in VWS and PPS. Histogram showing the percent of either missense (running bond pattern) or truncation/frameshift (blank pattern) mutations contained in each exon. Number of probands is shown above each bar, for a total of 571 (Leslie et al., 2012; Leslie et al., 2015). Beneath the histogram is the *IRF6* primary transcript with a bar graph color code to indicate functional protein regions. Exons 1 and 2 (peach color) constitute the 5' Untranslated Region. Exons 3 and 4 are the DNA binding domain (blue). Exons 5 and 6 are less conserved (yellow). The majority of exon 7 and all of exon 8 are the Protein-Binding Domain (also known as the Interferon Association Domain) (green). Exon 9 contains the C-Terminal Domain (CTD) (red). The 3'Untranslated Region is also illustrated (pink). Introns are not drawn to scale. **B)** Protein Structure. A cartoon of IRF6 Protein-Binding Domain (PBD) (green) and C-Terminal Domain (CTD) (red), based on the structure of IRF5. The PBD consists of two beta-pleated sheets that form a central pore and are

surrounded by three helices. The CTD consists of the Linker Region (black) and the C-Terminal Helix (red), which controls dimerization and activation/repression of IRF5. **C)** A sphere model of IRF6 in the same orientation as the cartoon model. The PBD is highlighted with a green line, while the CTD is highlighted with a red line. Different regions of the protein are highlighted using a spectrum to label the carbon backbone. Both the cartoon and sphere models were created using PyMol.

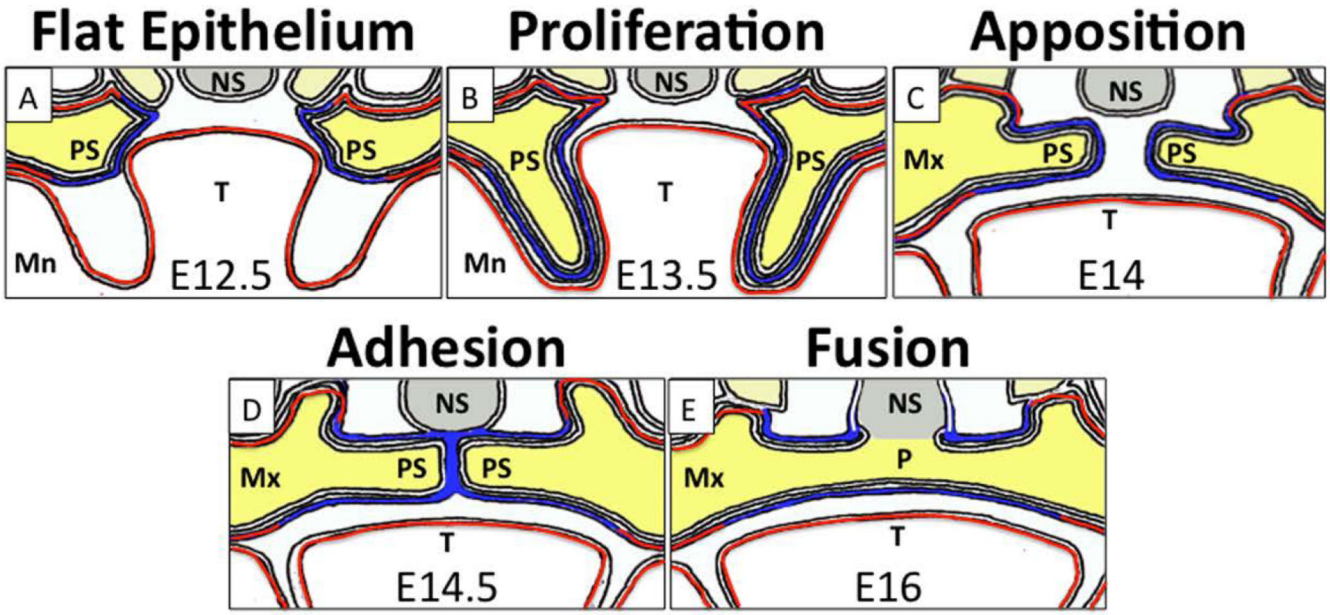

Figure 2. IRF6 expression during stages of palatal development

A) Palatal development begins at E12.5 with a flat palatal epithelium (blue) that expresses Irf6, and an underlying mesenchyme (yellow). **B)** A period of rapid proliferation leads to formation of palatal shelves alongside the tongue (T) and mandible (Mn). Irf6 is also expressed in mandibular, lingual and maxillary epithelium (red). **C)** Reorientation of the palatal shelves leads to a midline pivot and a horizontal suspension above the tongue. **D)** Apposition of the palatal shelves, followed by loss of periderm, allows adhesion, or interdigitation of the epithelial cells to produce the medial edge seam. Irf6 is expressed in both periderm and basal cells and is required for their function. **E)** Dissolution of the medial edge seam, which is composed of basal cells, leads to fusion of the shelves. After fusion, a mesenchymal bridge separates the nasal cavity from the oral cavity. Mn: mandible; Mx: Maxilla; NS: nasal septum; P: palate; PS: palatal shelves; T: tongue.

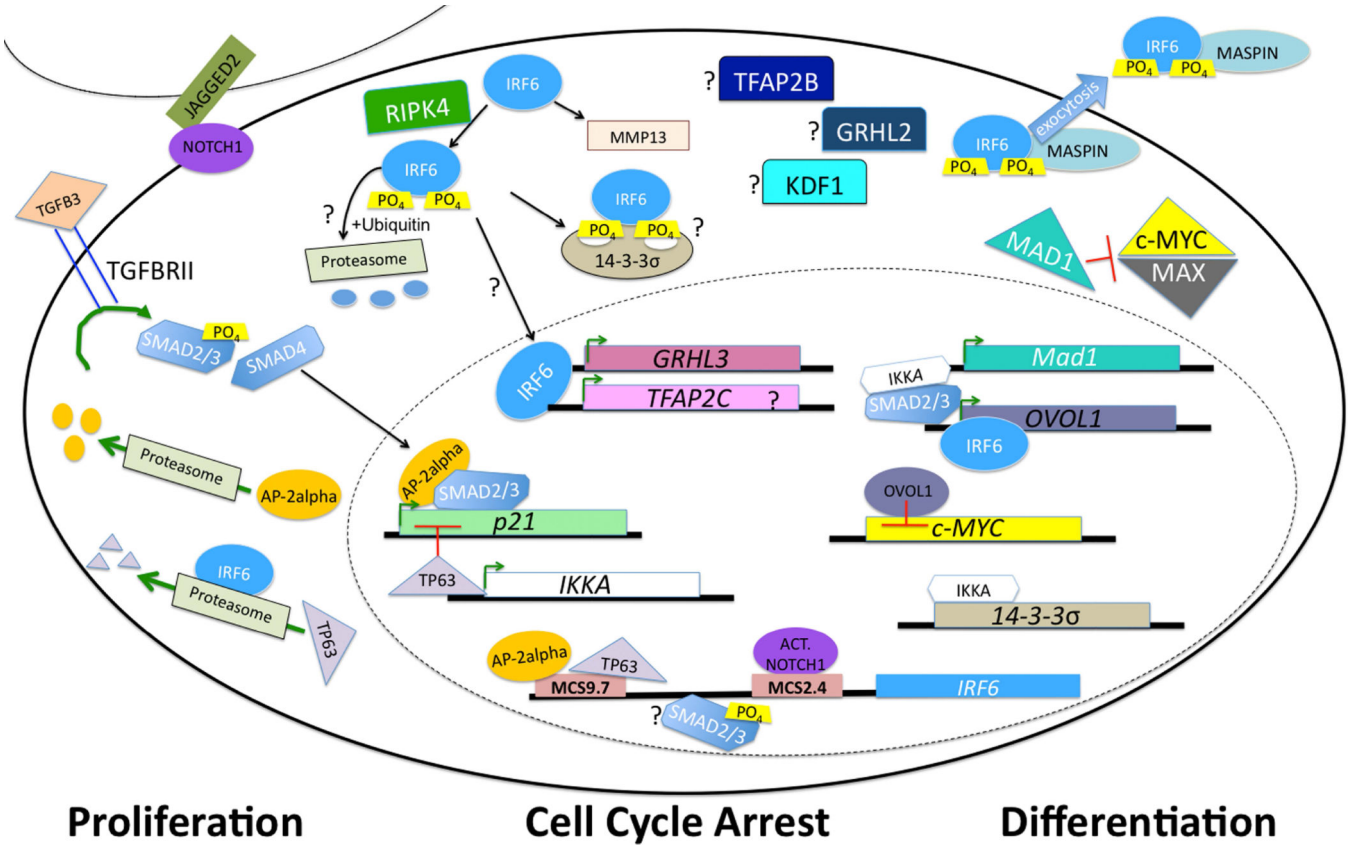

Figure 3. An Orofacial Gene Regulatory Network

IRF6 regulates the switch between proliferation and differentiation, and loss of function mutations lead to orofacial clefting. Current data support interaction with TGFB, NOTCH and OVOL1, among other factors. However, many questions remain, including how IRF6 is targeted to the proteasome, whether it regulates *TFAP2A*, *TFAP2B* or *GRHL2*, and where KDF1 fits in this pathway.