Disrupted IRF6-NME1/2 Complexes as a Cause of Cleft Lip/Palate

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Abstract

Mutations and common polymorphisms in interferon regulatory factor 6 (*IRF6*) are associated with both syndromic and nonsyndromic forms of cleft lip/palate (CLP). To date, much of the focus on this transcription factor has been on identifying its direct targets and the gene regulatory network in which it operates. Notably, however, *IRF6* is found predominantly in the cytoplasm, with its import into the nucleus tightly regulated like other members of the *IRF* family. To provide further insight into the role of *IRF6* in the pathogenesis of CLP, we sought to identify direct *IRF6* protein interactors using a combination of yeast 2-hybrid screens and co-immunoprecipitation assays. Using this approach, we identified *NME1* and *NME2*, well-known regulators of Rho-type GTPases, E-cadherin endocytosis, and epithelial junctional remodeling, as bona fide *IRF6* partner proteins. The *NME* proteins co-localize with *IRF6* in the cytoplasm of primary palatal epithelial cells in vivo, and their interaction with *IRF6* is significantly enhanced by phosphorylation of key serine residues in the *IRF6* C-terminus. Furthermore, CLP associated *IRF6* missense mutations disrupt the ability of *IRF6* to bind the *NME* proteins and result in elevated activation of *Rac1* and *RhoA*, compared to wild-type *IRF6*, when ectopically expressed in 293T epithelial cells. Significantly, we also report the identification of 2 unique missense mutations in the *NME* proteins in patients with CLP (*NME1* R18Q in an *IRF6* and *GRHL3* mutation-negative patient with van der Woude syndrome and *NME2* G71V in a patient with nonsyndromic CLP). Both variants disrupted the ability of the respective proteins to interact with *IRF6*. The data presented suggest an important role for cytoplasmic *IRF6* in regulating the availability or localization of the *NME1/2* complex and thus the dynamic behavior of epithelia during lip/palate development.

Keywords: orofacial clefts, interferon regulatory factor, epithelial adhesion, periderm, craniofacial biology, mutation

Introduction

Cleft lip with or without cleft palate (CLP) is the most common facial birth defect, occurring with a global incidence of ~1 in 700 live births (Dixon et al. 2011). Common variants in *interferon regulatory factor 6* (*IRF6*) are a major contributor to CLP incidence, accounting for ~12% of the genetic contribution to nonsyndromic forms of CLP (NS-CLP) (Zuccherro et al. 2004). More pathogenic *IRF6* mutations have also been shown to cause 2 allelic conditions: Van der Woude syndrome (VWS) and popliteal pterygium syndrome (PPS), which both present with orofacial clefts (Kondo et al. 2002). Recently, variants in *GRHL3* have been found in some *IRF6* mutation-negative VWS patients (Peyrard-Janvid et al. 2014).

Much attention has been paid to understanding the function of *IRF6* as a transcriptional regulator during lip morphogenesis. *IRF6* expression is detectable in the early orofacial ectoderm, a simple epithelium, and then becomes restricted to the outermost peridermal layer immediately prior to lip and palate fusion (Richardson et al. 2014). Loss of *Irf6* in mice results in a thickened and disorganized epithelium composed of rounded cells that have an undifferentiated appearance, reduced skin barrier function, and ectopic adhesions affecting, among other tissues, the oral cavity and esophagus (Ingraham et al. 2006; Richardson et al. 2006). Changes in other oral epithelia-derived

tissues, including tooth buds, ameloblasts, and salivary glands, have also been described (Blackburn et al. 2012; Chu et al. 2016; Tamasas and Cox 2017). Notably, the typically highly polarized ameloblasts showed reduced intercellular adhesion

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A supplemental appendix to this article is available online.

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and altered polarity, yet still differentiated, albeit with a slight delay (Chu et al. 2016).

Like other IRF family members, IRF6 possesses an N-terminal helix-loop-helix DNA binding domain (DBD) and a C-terminal IRF association domain (IAD) (Takaoka et al. 2008). In studied IRF proteins, the C-terminus, which includes a serine-rich autoinhibitory region (SRR) in most family members, is required for interactions with other IRFs, nonnuclear proteins, and transcription factors. For example, IRF3 resides in the cytoplasm, but when phosphorylated following viral infection, it enters the nucleus to activate interferon expression (Qin et al. 2003). Phosphorylation also activates IRF5 transcriptional activity (Chang Foreman et al. 2012). Like IRF3 and IRF5, IRF6 is also predominantly found in the cytoplasm in most cell types, with very little observed in the nucleus at any stage of the cell cycle or at quiescence (Bailey et al. 2005). C-terminal serine phosphorylation has also been implicated in regulating the half-life (Bailey et al. 2008), dimerization, and transactivator functions of IRF6 (Kwa et al. 2014).

Around half of the CLP-associated missense mutations in IRF6 reside in the IAD or SRR portions of the protein (Leslie et al. 2013), highlighting the importance of interactions with other factors. However, almost all studies to date have focused on identifying transcriptional targets of IRF6 and the gene regulatory network in which it functions. Here we report that IRF6 physically interacts with the paralogs NME1 (nonmetastatic expressed 1) and NME2, so named because of their function in suppressing metastasis of various cancers (Hsu 2011). Among other functions, these 2 NME proteins are key regulators of RhoA and Rac1 activation, respectively, specifically controlling endocytosis of epithelial junctional proteins and concomitant cytoskeleton remodeling (Palacios et al. 2002; Hsu 2011). The data presented herein demonstrate that these interactions are regulated by C-terminal serine phosphorylation of IRF6, disrupted by CLP-linked IRF6 mutations, and that mutations in NME1/2 may also play a role in NS-CLP. These studies provide the first evidence for a cytoplasmic function for IRF6 and a direct mechanistic link to the observed changes in epithelial morphology that underlie susceptibility to CLP.

Materials and Methods

Generation of Constructs

Full-length and truncated human and chick IRF6 and NME1/2 complementary DNAs (cDNAs) were amplified by polymerase chain reaction (PCR) from different source DNA (see Appendix). Amplified IRF6 fragments were cloned into pCR8-TOPO, then into Gateway vectors pDEST32, pDEST-myc, and pcDNA-DEST53 (Invitrogen) for yeast 2-hybrid assays and co-immunoprecipitation studies. NME1 and NME2 were cloned into pDEST22, pDEST-myc, and pcDNA-DEST53 (or pEGFP-C2; Clontech) for the various studies.

Yeast 2-Hybrid Screens

Interactor screens were performed with the ProQuest Two-Hybrid System (Invitrogen) using chick IRF6 and a murine

E10.5 whole-embryo cDNA library as previously described (Short et al. 2002) (see Appendix). Due to strong self-activation of constructs containing both the DBD and SRR, including full-length IRF6, 3 truncated versions of IRF6 were used as independent baits (see Fig. 1).

Immunofluorescence on Whole and Sectioned Embryonic Tissue

Embryonic chick heads were collected at Hamburger-Hamilton stages (HH) 23, HH24, and HH25, representing pre-fusion and mid-fusion stages of primary palatogenesis. Heads were fixed and stained with the mouse anti-NME2 antibody and an AlexaFluor 488 goat anti-mouse secondary (1:300, A-11004; Invitrogen), then visualized by fluorescence microscopy. Ten-micron-thick cryosections of chick heads were also prepared and stained, with images acquired via confocal microscopy.

IRF6 Variant Selection and Mutagenesis

Twelve CLP-associated IRF6 mutations were chosen (see Appendix for criteria) and each generated by overlap PCR and independently cloned into the relevant 2-hybrid and mammalian expression vectors (described above). Analysis of putative serine/threonine phosphorylation sites in IRF6 was initially performed using NetPhos v2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>). Additional C-terminal serines were selected based on sequence conservation with those of functional importance in the related IRF5 and IRF3 proteins. Phosphoinhibitory (alanine substitutions) and phosphomimic (aspartate substitutions) mutations of each serine, individually and in combination, were created by PCR-based mutagenesis.

Co-Immunoprecipitation Studies

A total of 1 mg protein from HEK 293T cells transfected with wild-type and mutant myc-tagged IRF6 and green fluorescent protein (GFP)-tagged NME1/NME2 constructs was combined and immunoprecipitation performed using a Myc-Tag IP/co-immunoprecipitation (co-IP) kit (ThermoFisher). Western blotting was performed using various antibodies (see Appendix) and protein bands detected using Super Signal West Femto Chemiluminescent Substrate (ThermoFisher).

Patients and Sequencing

A cohort of 222 CLP patients was available for screening that included 37 VWS patients who had been excluded for mutations in *IRF6* and *GRHL3*, 91 NS-CLP cases from the Philippines, and 94 NS-CLP cases from Iowa. Exons, including untranslated regions and intron-exon boundaries, of both *NME1* and *NME2* were sequenced. Samples for parents of individuals with missense mutations were sequenced to determine de novo status. Identified variants were filtered against 1000 Genomes and the exome variant server. All participating probands and parents were recruited after obtaining signed

Figure 1. Identification of NME1 and NME2 as interferon regulatory factor 6 (IRF6) interactors. **(A)** Three IRF6 constructs that did not exhibit any self-activation were used as baits in yeast 2-hybrid screens for identification of putative interactors. Only the construct expressing aa217-460 (containing the IAD and SRR) yielded putative interactors. -, no validated putative interactors; +, in-frame sequences that interacted with IRF6 when tested back in yeast cells; DBD, DNA binding domain; IAD, interferon association domain; SRR, serine-rich region. **(B)** Two independent clones representing NME2 were identified, activating 2 separate reporter genes, HIS (via 3AT selection) and LacZ (X-Gal assay). Negative (-) controls and positive (+) controls of increasing strength of interaction, provided as part of the ProQuest system. Co-transformation of IRF6 and NME2 into yeast MaV203 cells gave similar levels of growth on 3AT and intensity/color of X-Gal activation comparable to the strongest interacting control proteins (Control E). **(C)** Replacement of the NME2 ORF with NME1 in the 2-hybrid prey vector demonstrated that NME1 also interacted with IRF6 (aa217-460), which was subsequently verified by co-immunoprecipitation following expression of full-length IRF6 in HEK293T cells (data not shown). **(D)** The interaction of NME1 and NME2 with IRF6 was dependent on the SRR as confirmed by yeast 2-hybrid and co-immunoprecipitation assays. **(E)** Top row: Whole-mount immunohistochemical detection of NME2 in stage HH24 and HH25 chick faces (frontal view) showing the fusing facial processes that form the primary palate. Staining for NME2 is detected in all epithelia but appears more intense at the point of contact of the facial processes (red arrows) where epithelia are undergoing significant morphological change. However, as these were not planar confocal images, it is possible that this increased intensity is an artifact, reflecting stacked epithelia at the point of contact. Control embryos—those without either the primary or secondary antibody added—are not shown as there was no detectable fluorescence at the exposures and contrast used. Bottom row: Transverse sections through the converging maxillary and medial nasal processes (dashed lines in A) confirm NME2 is expressed in the cytoplasm of facial epithelia and the underlying mesenchyme (delimited by the red dashed line) before and during fusion of the maxillary and medial nasal processes. Staining appeared to be slightly stronger in the peridermal layer (white arrowhead) prior to contact, although this was not quantified. Ln, lateral nasal process; mn, medial nasal process; mx, maxillary process. This figure is available in color online.

informed consent in compliance with the institutional review board at the University of Iowa (IRB No. 199804080).

Results

Identification of NME1 and NME2 as Novel IRF6 Interactors

With the aim of identifying new IRF6 interactors pertinent to its function in the developing lip and palate, we conducted yeast 2-hybrid screens of a mouse E10.5 cDNA library—a developmental stage at which *Irf6* expression is maximal in the orofacial epithelia (Knight et al. 2006). Five distinct putative protein interactors were identified, all using IRF6 fragment aa217-460 (Fig. 1A, B). NME2, 1 of the 2 proteins described in this report, was represented by 2 independent full-length clones, which differed in length by 3 nucleotides. Initial screening was done using chick IRF6, although interactions were validated using human IRF6 constructs. Neither RIPK4 nor Maspin, 2 previously reported IRF6 interactors, were identified in our screen, and direct testing of Maspin, using both human and chick full-length clones, as well as both low and high copy number 2-hybrid vectors, also failed to reproduce the previously published data for this putative interactor. In contrast, we validated the interaction with NME2 using both truncated and full-length IRF6 using co-IP assays (Fig. 1D). Because NME2 shows >88% amino acid identity with NME1, we subsequently tested whether NME1 could also bind IRF6. Both yeast 2-hybrid and co-IP assays showed that NME1 and NME2 had similar affinities for IRF6 (Fig. 1C).

NME1 and NME2 Are Expressed in Prefusion Oral Epithelia

NME1 is ubiquitously and robustly expressed in all epithelia (Hsu 2011). To investigate the expression of NME2 during lip morphogenesis, we performed whole-mount and section-based immunofluorescence staining on chick embryos. These studies revealed expression throughout the head, with slightly stronger staining in the epithelium. Of note, staining appeared more intense in epithelia at the contact point between the converging facial processes in fusion stage embryos (Fig. 1E, top row). In sections, staining also appeared slightly more intense in the periderm near the contact site, and this cytoplasmic staining became more uniform following contact and initiation of epithelial dispersal (Fig. 1E, bottom row).

Serine 424 Phosphorylation Plays a Key Role in Regulating the IRF6-NME Interaction

Phosphorylation of C-terminal serines of many IRF proteins, including IRF6, has been shown to regulate numerous functions of these proteins (Kwa et al. 2014). For our studies, we initially focused on 2 serines within the SRR, S401 and S418, in chick IRF6 (Fig. 2A). As shown by 2-hybrid and co-IP

assays, individual or combinatory phosphomimic mutations (S>D) of each of these serines retained their ability to interact with the NME proteins, but the phosphoinhibitory cIRF6 S418A mutation alone and the S401-S418 double mutation largely abolished the interaction (Fig. 2B). Curiously, phosphoinhibitory mutations of the 2 equivalent human IRF6 (hIRF6) residues, S407 and S424, did not disrupt binding to the NME proteins (data not shown). We therefore investigated 2 other serines in hIRF6, S413 and S418, based on their conservation in IRF3 and IRF5 (Fig. 2A). Only when both phosphoinhibitory mutants of S413 and S418 were combined with S424A (S413-S418-S424AAA) was the hIRF6-NME interaction disrupted (Fig. 2C). Similar results were observed for the interaction with NME1 (data not shown).

CLP-Associated IRF6 Mutations Disrupt the NME Interaction and Rac1/RhoA Activation

Twelve VWS-associated IRF6 missense mutations in the IAD were tested for their ability to interact with the NME proteins. Notably, 9 of the 12 mutants showed markedly reduced interaction with the NME proteins (Fig. 3A). In support of the role of NME1 and NME2 in regulating RhoA and Rac1 activation, respectively, we previously found elevated activated Rac1 in *Irf6* knockout mice. Interestingly, cells ectopically expressing mutant IRF6 also exhibited higher levels of activated RhoA and to a lesser degree Rac1 compared to cells ectopically expressing wild-type IRF6 (Fig. 3B).

Identification of NME1 and NME2 Variants in NS-CLP

We consequently considered NME1 and/or NME2 as candidate CLP genes. To investigate this, all exons from each gene were amplified and sequenced in 222 patients with CLP. This analysis identified 2 coding variants (1 missense and 1 synonymous) in NME1, and 1 missense change was found in NME2 (Fig. 4). The NME1 missense variant was identified in a patient with VWS who was negative for coding variants in *IRF6* and *GRHL3*. The NME1 variant was inherited from an unaffected mother, while the NME2 variant was de novo. Neither was represented in 1000 Genomes or the Exome Variant Server. In the ExAC database, the NME2 variant was also not represented, while the NME1 variant was seen at a frequency of 0.004% (5/121,413 alleles globally). The NME1 missense change (G>A chr17:49,233,068) results in an arginine to glutamine substitution at residue 18 (RQ18) in the main NME1 isoform (isoform 2; NP_000260). The R18Q change is predicted as “possibly damaging” by PolyPhen2 and “damaging” by SIFT (Sorting Intolerant From Tolerant algorithm). The NME2 missense change (G>T chr17:49,245,688) results in a glycine 71 to valine substitution (G71V) predicted by PolyPhen2 as “probably damaging” and by SIFT as “deleterious.” Both arginine 18 of NME1 and glycine 71 of NME2 are conserved in NME1 and NME2 proteins across all eukaryotic species (Fig. 4A). Based on the

Figure 2. C-terminal serine phosphorylation-dependent interaction of NME1/2 with interferon regulatory factor 6 (IRF6). **(A)** Computer-assisted detection of potential serine phosphorylation sites in chick IRF6. Two serine residues in the IRF6 C-terminus SRR, at positions 401 and 418 (S407 and S424 in human IRF6 [hIRF6]), had very high predictive scores (0.930 and 0.924, respectively) of being phosphoserine sites. These and an additional two serines in hIRF6, S413 and S418, which were conserved in position between IRF3 and IRF5, were also investigated. Investigated serines are highlighted in red. DBD, DNA binding domain; IAD, interferon association domain; SRR, serine-rich region. **(B)** These serines were mutated, individually and in combination within the aa217-460 construct, to alanine (phosphoinhibitory) and aspartate acid (phosphomimic) and each tested for their impact on the IRF6-NME interaction by yeast 2-hybrid analyses. Results were confirmed by co-immunoprecipitation following expression of the mutant full-length IRF6 and NME1/2 proteins in HEK293T cells. Growth of the cIRF6 S418 (hIRF6 S424) phosphoinhibitory mutant was prevented in selective media. **(C)** In hIRF6, phosphoinhibitory mutations in S413, S418, and S424 were required to disrupt the NME1/2 interaction. Note the myc-tagged IRF6 doublet band at ~65 kDa. The upper band is considered the phosphorylated form of IRF6, suggesting that the protein is still phosphorylated elsewhere.

Figure 3. Most cleft lip and palate (CLP)-associated mutations in the interferon regulatory factor 6 (IRF6) interferon association domain (IAD) disrupt the interaction with the NME1/2 proteins. **(A)** Nine of 12 tested missense mutations in the IRF6 IAD disrupted the interaction with NME2 (and NME1) when tested in yeast 2-hybrid assays: R250Q, L251P, G267C, L294P, V321M, R339I, L373S, D381Y, and K388E. These results were confirmed by co-immunoprecipitation assays (data not shown). Two mutants, K320E and R400Q, showed no obvious reduction in interaction strength, while P258S showed a mild reduction in strength only as the stringency of the interaction conditions was increased to 100 mM 3AT (not shown). **(B)** In HEK293T cells, expression of mutant IRF6 constructs that fail to interact with NME1/2 exhibited elevated activation of Rac1 and RhoA, which are normally regulated by NME1/2.

X-ray structures of NME1 and NME2 (Webb et al. 1995; Min et al. 2002), arginine 18 resides at the hinge region between 2 α helices and situated at the interface between the 2 trimers of the functional hexameric structure (Fig. 4B). It forms a nonpolar interaction with the C-terminus, likely stabilizing the subunits, and a polar interaction with glutamine 30 (Fig. 4B) (Webb et al.

1995). Glycine 71 is positioned at the outer surface of the interface between the trimers, not far from arginine 18 (Fig. 4B). Importantly, both variants failed to interact with IRF6 when tested by yeast 2-hybrid assays and co-IP, suggesting they are pathogenic (Fig. 4C). Four unique intronic variants of unknown significance were also identified in *NME1* and 3 unique intronic

Figure 4. Rare missense variants in *NME1* and *NME2* identified in patients with cleft lip and palate (CLP) disrupt the interaction of the respective encoded proteins with interferon regulatory factor 6 (IRF6). **(A)** The identified *NME1* and *NME2* variants, R18Q and G71V, alter residues that are otherwise completely conserved among vertebrates. **(B)** Location of the affected (or *NME2* equivalent) residues in the *NME2* hexamer structure (PDBe 1nue). **(B')** Lateral view of the hexameric structure with each *NME2* monomer highlighted in a different color. **(B'')** Zoomed-in view (boxed region in B') showing that R18 and G71 reside in close proximity to each other at the interface of the 2 trimers, raising the possibility that they may destabilize the hexameric structure. **(C)** Yeast 2-hybrid assays demonstrate that both mutations disrupt the interaction with IRF6 (aa217-460), when tested with 2 independent reporter genes. Loss of interaction with IRF6 was also confirmed by co-immunoprecipitation using full-length IRF6.

Figure 5. Simplified model for the role of the interferon regulatory factor 6 (IRF6)-NME1/2 interaction in oral epithelia. Phosphorylation of human IRF6 (hIRF6) C-terminal serines (S424 and, to a lesser degree, S413), presumably by RIPK4, promotes interaction with, and sequestering of, the NME1-NME2 hexamer in the cytosol. Sequestering of the NME complex helps promote epithelial polarity and junctional complex stability. Some phosphorylated IRF6 (at S413, but enhanced by S424) enters the nucleus to activate transcription. Together, these events promote epithelial differentiation. In cleft lip and palate (CLP), mutations in IRF6 or of the NME-IRF6 interaction interface result in destabilization of adhesion complexes, a change in epithelial shape, and an increase in proliferation. DBD, DNA binding domain; IAD, interferon association domain; SRR, serine-rich region.

variants in *NME2*. All other identified variants were present in similar allelic proportions in the 1000 Genomes database. In 16 patient samples, we were unable to amplify 1 or more exons of *NME2*, although the significance of this was not pursued.

Discussion

Structural abnormalities in, and reduced levels of, IRF6 are a major contributor to CLP incidence. To date, 2 IRF6-interacting proteins have been reported: Maspin and RIPK4 (Bailey et al. 2005; Kwa et al. 2014). Maspin is a serine protease inhibitor and tumor suppressor whose expression, like IRF6, is inversely correlated with breast cancer invasiveness (Bailey et al. 2005). As a predicted secreted protein, it is unclear what role, if any, Maspin has in the developing lip or even how it physiologically

relates to the function of IRF6. In contrast, RIPK4 is a cytoplasmic kinase that has been shown to phosphorylate cytoplasmic IRF6, resulting in its increased nuclear import and transcriptional activation (Kwa et al. 2014). Most research on IRF6 has focused on identifying its transcriptional targets and dissecting the gene network in which it participates (Richardson et al. 2006; Thomason et al. 2010; Ferretti et al. 2011; Dougherty et al. 2013; Kwa et al. 2014). However, the majority of both endogenous and ectopically expressed IRF6 remains in the cytoplasm in most cell types even after phosphorylation by RIPK4 (Bailey et al. 2005; Little et al. 2009; Kwa et al. 2014), raising the possibility of a cytoplasmic role for IRF6 or, at least, a mechanism by which it is sequestered in the cytoplasm until needed in the nucleus.

Here we describe 2 additional IRF6 interactors: the paralogous NME1 and NME2 proteins. In humans, the *NME1* and *NME2* genes reside in a head-to-tail arrangement on chromosome 17q11.33, and their encoded proteins function as homo- and hetero-hexamers (Hsu 2011), consistent with the notion they arose through gene duplication. The NME proteins encode nucleoside diphosphate kinases that regulate production of nucleoside triphosphates (particularly GTP) for a variety of cellular processes, including membrane trafficking and cytoskeleton remodeling (Hsu 2011). NME1 interacts with numerous cellular proteins. The best characterized are TIAM1, a Rho-type guanine nucleotide exchange factor (Rho-GEF) that specifically activates the GTPase, Rac1, to facilitate cytoskeletal changes (Otsuki

et al. 2001), and ADP ribosylation factor 6 (ARF6), which regulates dynamin-dependent endocytosis (Palacios et al. 2002). In epithelia, recruitment of NME1 to the membrane promotes the endocytosis of E-cadherin and the deconstruction of the apical junctional complex (Palacios et al. 2002). Despite its significant sequence identity with NME1, NME2 does not reportedly bind TIAM1. Although less characterized, it interacts with the RhoA-specific GEF, AKAP-LBC (Hsu 2011) to regulate cytoskeletal dynamics that underlie cell shape change, and with plakoglobin, a binding partner of E-cadherin with which it co-immunoprecipitates (Aktary et al. 2010). Through the latter, it has been proposed to regulate epithelial adhesion and polarity (Aktary et al. 2010). In each case, binding of the NME proteins to the GEFs inhibits the exchange activity. In line with this, elevated levels of active Rac1 have been reported

in ameloblasts in vivo (Chu et al. 2016) and elevated RhoA in cultured keratinocytes (Biggs et al. 2014) when *Irf6* is ablated. Interestingly, ectopic expression of mutant IRF6 that cannot bind the NME proteins also leads to elevated activated Rac1.

The C-terminal SMAD-like interaction domain, or IAD, of IRF family members is responsible for their homo- and heteromeric interactions as well as interaction with transcriptional coactivators (Qin et al. 2003; Panne et al. 2007; Chen and Royer 2010). Structural analyses of IRF3 and IRF5 have demonstrated that phosphorylation within the adjacent SRR introduces negative charges that relieves autoinhibition and facilitates protein interactions with the IAD (Qin et al. 2003; Chen et al. 2008). Our data indicate that interaction between NME1/2 and IRF6 is largely dependent on phosphorylation within the IRF6 SRR. Specifically, we find that the interaction is regulated principally by phosphorylation of serine 424, although phosphorylation of other serines may be required at least in mammals. Serine 424 is 1 of 2 serines targeted by RIPK4 (Kwa et al. 2014). Previously, Bailey and colleagues (2008) showed that phosphorylation of IRF6 increased in proliferating cells and that this phosphorylation promoted its ubiquitylation and degradation, although they did not identify the specific phosphorylated residues that were mediating this. More recently, Kwa et al. (2014) provided evidence in support of C-terminal serine phosphorylation of hIRF6 by RIPK4 that influenced its dimerization and subsequent transactivation of chemokine gene expression as part of the innate immune response. Although interaction between NME1/2 and the main protein interaction interface of IRF6, the IAD, could barely be detected when the entire SRR was removed, we found that the majority of CLP-associated IAD mutations, in the context of the full-length protein, still disrupted NME binding. Either these specific IAD mutations disrupt the overall conformation of IRF6 or the interaction between the IAD and SRR is critical even following phosphorylation. Further work is required to dissect the complex role of phosphorylation in regulating the functions of IRF6 in the cytoplasm and nucleus.

In support of a bona fide role for the IRF6-NME complex during morphogenesis of the lip, we found intense staining for NME2 in the contact zone epithelia at the time of lip fusion when the epithelia are undergoing dynamic shape changes. Although we did not observe any obvious alteration in subcellular localization of the NME proteins in the thin peridermal cells, we have recently shown that the subcellular distribution of NME2 in highly polarized, columnar ameloblasts is dramatically altered following loss of *Irf6* (Chu et al. 2016). In these cells, the normal cytoplasmic distribution shifted to the apico-lateral membrane, and this correlated with a weakening of intercellular adhesion and a more rounded appearance (Chu et al. 2016), similar to the cellular changes in the lip and palatal epithelia reported in earlier other knockout models (Ingraham et al. 2006; Richardson et al. 2006). An important role for the IRF6-NME interaction is also supported by our identification of 2 rare missense mutations (1 in *NME1* and 1 in *NME2*) in patients with *IRF6*- and *GHRL3*-negative VWS and NS-CLP, respectively. The variants (*NME1* R18Q and *NME2* G71V) altered

residues that are completely conserved across all species and, when tested, both mutants failed to interact with wild-type IRF6, suggesting pathogenicity. These findings indicate that additional evaluation of *NME1* and *NME2* in NS-CLP cohorts is warranted and that mutations in *NME1* may be rare causes of VWS, adding a third gene to a common Mendelian form of clefting.

Based on these data, we propose that disruption of the IRF6-NME complex, as well as the concomitant activation of Rac1 and RhoA, significantly contributes to epithelial changes that are thought to lead to the presentation of CLP (Fig. 5). A role for aberrant activation of RhoA, a process regulated by the NME proteins, has previously been implicated in CLP susceptibility with the finding of potentially pathogenic variants in *ARHGAP29*, which encodes a Rho-GTPase activating protein, in NS-CLP (Leslie et al. 2012). These data therefore raise the possibility that IRF6 has a dual function: a cytoplasmic role with a direct impact on epithelial adhesion and a nuclear role as a transcriptional regulator affecting epithelial differentiation. Dissecting these contributions will be important to better understand the contribution of *IRF6* to CLP susceptibility.

Author Contributions

M.T. Parada-Sanchez, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; E.Y. Chu, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript; L.L. Cox, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; S.S. Undurty, contributed to data acquisition and analysis, critically revised the manuscript; J.M. Standley and J.C. Murray, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; T.C. Cox, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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