Published in final edited form as:
Birth Defects Res A Clin Mol Teratol. 2012 November ; 94(11): 934–942. doi:10.1002/bdra.23076.

Expression and mutation analyses implicate *ARHGAP29* as the etiologic gene for the cleft lip with or without cleft palate locus identified by genome wide association on chromosome 1p22

Elizabeth J Leslie[1], **M Adela Mansilla**[1], **Leah C Biggs**[1], **Kristi Schuette**[1], **Steve Bullard**[2], **Margaret Cooper**[3], **Martine Dunnwald**[1], **Andrew C Lidral**[2], **Mary L Marazita**[3], **Terri H Beaty**[4], and **Jeffrey C Murray**[1,*]

[1]Department of Pediatrics, University of Iowa, Iowa City, Iowa, 52242

[2]Department of Orthodontics, University of Iowa, Iowa City, Iowa, 52242

[3]Center for Craniofacial and Dental Genetics, Department of Oral Biology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, 15219

[4]Department of Epidemiology, School of Public Health, Johns Hopkins University, Baltimore, Maryland, 21205

Abstract

Background—Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common birth defect with complex etiology reflecting the action of multiple genetic and/or environmental factors. Genome wide association studies have successfully identified five novel loci associated with NSCL/P including a locus on 1p22.1 near the *ABCA4* gene. Since neither expression analysis nor mutation screening support a role for *ABCA4* in NSCL/P, we investigated the adjacent gene *ARHGAP29.*

Methods—Mutation screening for *ARHGAP29* protein coding exons was conducted in 180 individuals with NSCL/P and controls from the US and the Philippines. Nine exons with variants in *ARHGAP29* were then screened in an independent set of 872 cases and 802 controls. *Arhgap29* expression was evaluated using *in situ* hybridization in murine embryos.

Results—Sequencing of *ARHGAP29* revealed eight potentially deleterious variants in cases including a frameshift and a nonsense variant. *Arhgap29* showed craniofacial expression and was reduced in a mouse deficient for *Irf6*, a gene previously shown to have a critical role in craniofacial development.

Conclusion—The combination of genome wide association, rare coding sequence variants, craniofacial specific expression and interactions with *IRF6* support a role for *ARHGAP29* in NSCL/P and as the etiologic gene at the 1p22 GWAS locus for NSCL/P. This work suggests a novel pathway in which the *IRF6* gene regulatory network interacts with the Rho pathway via *ARHGAP29*.

Keywords

ARHGAP29; cleft lip and palate; candidate gene; complex traits; Rho signaling

Corresponding author: Jeffrey C. Murray, MD, Address: 2182 Medical Laboratories, Department of Pediatrics, University of Iowa, Iowa City, Iowa 52242, Phone: 1-319-335-6897, Fax: 1-319-335-6970, jeff-murray@uiowa.edu.

Introduction

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common birth defect occurring at a frequency of approximately 1 in 700 births (Dixon et al., 2011). The prevalence of clefting varies by geographic location with the highest rates observed in Asian and Native American populations and the lowest in populations of African descent. Orofacial clefts can be categorized as those affecting the lip with or without the palate and those affecting the palate only; it is generally accepted these are genetically and developmentally distinct disorders (Dixon et al., 2011). Although over 500 syndromes in which clefting is a primary feature have been identified (http://www.ncbi.nlm.nih.gov/OMIM), the majority of cleft cases lack additional structural or cognitive abnormalities, and are classified as isolated, or nonsyndromic clefts. The complex etiology of NSCL/P reflects the action of multiple genetic and/or environmental factors. The search for genetic contributors to NSCL/P has used a variety of approaches including candidate gene, genome wide linkage, and genome wide association studies (GWAS), but few of these have been convincingly replicated (Dixon et al., 2011). One gene consistently associated with NSCL/P is *IRF6*, a transcription factor critically involved in craniofacial development (Dixon et al., 2011). Other genes and loci with compelling statistical support from genome wide analysis for a role in NSCL/P include 8q24, 20q12 (*MAFB*), 10q25 (*VAX1*), and 1p22 (*ABCA4*) (Beaty et al., 2010; Birnbaum et al., 2009; Mangold et al., 2010).

Traditionally, linkage disequilibrium (LD) structure has been used to help in identifying etiologic variants after GWAS based on the concept that an etiologic variant will show a statistical association with a noncausal marker if the two are in strong LD in the population (Murray et al., 1987). We reported an association between markers near and in *ABCA4* (1p22) with NSCL/P reaching genome-wide significance, with the strongest signals coming from Asian populations (Beaty et al., 2010). Despite identifying a number of missense mutations in *ABCA4* in individuals with NSCL/P, expression of this gene was restricted to the retina (Beaty et al., 2010) and it has been extensively reported as having mutations that result in a spectrum of retinal disorders (Burke et al., 2011). Although we cannot exclude the possibility that misregulation of *ABCA4* expression could contribute to clefting, these data suggest *ABCA4* may not be the etiologic gene for clefting in this region.

The associated SNPs occurred in a relatively small region of low linkage disequilibrium (LD) (Figure 1), which would normally make it easy to identify the etiologic variant; however, given recent evidence suggesting GWAS signals can span multiple LD blocks (Dickson et al., 2010), and the width of the LD surrounding *ABCA4* that includes parts of adjacent genes, we hypothesized a neighboring gene may contain the true etiologic gene/locus. *GCLM*, encoding a glutamate-cysteine ligase subunit, is the closest gene telomeric to *ABCA4*. This gene is located 200kb and several LD blocks from the peak GWAS signal. Craniofacial expression of *Gclm* has not been detected (Diez-Roux et al., 2011) and the the *Gclm* knockout mouse has no obvious phenotype (Yang et al., 2002). We sequenced this gene in 180 individuals with NSCL/P and did not find any coding variants.

ARHGAP29 is located 47kb centromeric to *ABCA4* and encodes Rho GTPase activating protein (GAP) 29, a protein that mediates the cyclical regulation of small GTP binding proteins such as RhoA (Saras et al., 1997). RhoA is involved in many functions related to cellular shape, movement, cell-cell interactions, and proliferation, all critical for craniofacial development (Birnbaum et al., 2009). Rho is a downstream effector of both the Tgfb (Kardassis et al., 2009) and Wnt (Schlessinger et al., 2009) signaling pathways, which have been implicated in craniofacial development by *Wnt5a* (Moustakas et al., 2008), *Wnt9b* (Juriloff et al., 2006), and *Tfgb3* (Kaartinen et al., 1995) knockout mice with craniofacial

defects including clefts of the lip and/or palate. We hypothesized Arhgap29 could be a mediator of the Rho signaling implicated in craniofacial development.

Methods

Expression studies

Murine embryos were obtained from pregnant female mice at 10.5–14.5, or 17.5 d after conception (E10.5 to E14.5, E17.5). *In situ* hybridization was performed on C57BL/6 whole E10.5–14.5 embryos fixed in 4% formaldehyde overnight as described previously (Beaty et al., 2010) using a 747-nucleotide antisense probe transcribed from the T7 promoter from a pCRII-TOPO plasmid containing an insert spanning exons 2–10 of Arhgap29 mRNA as amplified with primers (forward primer: CTCCCAAGTAGAGGCTGCAC; reverse primer: GCTGGTGAATGATGTCAGGA). An Arhgap29 mRNA sense probe transcribed from the same clone served as a negative control.

Immunodetection was performed on coronal sections of 4% formaldehyde-fixed, paraffin-embedded E14.5 heads. After blocking with 3% goat serum (Vector Laboratories, Burlingame, CA, USA), sections were incubated with rabbit anti-human Arhgap29 (Novus Biological, Littletown, CO), washed in PBS, and incubated in goat anti-rabbit 568 Alexa-Fluor (Molecular Probe, Eugene, OR). DAPI was used as a nuclear stain. Images were viewed with a Nikon Eclipse E800 and acquired with a SPOT RT Slider Diagnostic Instruments CCD camera using Spot Advanced software. Black and white images were pseudocolorized and merged.

Protein anaylsis was performed on skin from E17.5 *Irf6*-deficient embryos and wildtype littermates as previously described by Biggs et al. (2012). The *Irf6* mutant allele ($Irf6^{del1}$), on a C57BL/6 background, has been described previously (Biggs et al., 2012). Briefly, radioimmunoprecipitation assay (RIPA) extraction buffer was used for protein preparation. Equal amounts of protein were separated on 3–8% Tris-acetate SDS-Page gels (Invitrogen) under denaturing conditions. Proteins were transferred onto polyvinylidene fluoride membranes (BioRad Laboratories, Hercules, CA, USA), blocked in 10% non-fat dry milk and incubated in Arhgap29 or anti-mouse beta-actin (Sigma, St-Louis, MO) antibody. After incubation with HRP-conjugated secondary IgG antibodies (donkey anti-rabbit IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA), or donkey anti-mouse IgG HRP (GE Healthcare, Piscataway, NJ) antigen detection was performed with the chemiluminescent detection system ECL (GE Healthcare, Piscataway, NJ, USA). Histological analysis was performed on wild-type and mutant tissues as previously described by Ingraham et al. (2006). Additional details for expression studies are available upon request.

Samples

Informed consent was obtained for all participants (UI approval numbers 199804080, 199804081, 200008036). The case cohort consisted of 770 samples from individuals with NSCL/P from the Philippines and 280 US individuals with NSCL/P from Iowa collected without regard to a family history of clefting. Cases were separated into phenotypic subgroups based on individual cleft type where CL indicates that the individual has cleft lip only and CLP indicates cleft lip with cleft palate. Controls consisted of unrelated individuals without a history of clefting or other birth defect. 516 control samples were collected from the same sites in the Philippines as cases; 456 control samples from the US were collected at the University of Iowa Hospitals and Clinics.

Sequencing

Primers used to amplify coding exons and splice sites of *ARHGAP29* were designed with Primer 3 (Supplemental Table 1). PCR products were sent for sequencing using an ABI 3730XL (Functional Biosciences, Inc., Madison, WI). Chromatograms were transferred to a Unix workstation, base-called with PHRED (v.0.961028), assembled with PHRAP (v. 0.960731), scanned by POLYPHRED (v. 0.970312), and viewed with the CONSED program (v. 4.0). Polyphen 2 was used to evaluate missense mutations for possible functional significance (Adzhubei et al., 2010). Phase one sequenced the 22 protein coding exons in 90 cases from the Philippines, 90 cases from the US, and 170 Filipino controls. Phase two sequenced the nine exons with nonsynonymous variants from phase one in an independent set of 872 Filipino and US cases and 802 Filipino and US controls.

Statistics

To determine the contribution of sequencing variants to NSCL/P, we pooled variants as "very rare" and "rare", defined by the minor allele frequency (MAF) in the combined set of cases and controls to avoid selection bias (Pearson, 2011). "Very rare" defined variants with a MAF <1% and "rare" defined variants with a MAF<5%. We then compared the frequency of "very rare" and "rare" variants between cases and controls with Fisher's exact test using SAS. We analyzed the US and Filipino populations separately and in combined analyses. In these analyses, we excluded the set of US cases sequenced in phase 1 of the study to reduce bias (which resulted in p.Lys46Arg and p.Arg1142Gln being excluded). We did this for the failure of complete sequencing of *ARHGAP29* in phase 1 to not include US controls; therefore, exons harboring variants only in these controls would be missed and excluded from phase two.

Gene x gene interactions were tested using Cordell's likelihood ratio test (LRT) for the most associated SNP at the *ABCA4*/*ARHGAP29* locus (rs560426) and near candidate genes *IRF6* (rs2235371, rs642961), *MAFB* (rs13041247), and 8q24 (rs987525) using genotypes from the Beaty *et al*. GWA study (Beaty et al., 2010). Neither of the *IRF6* SNPs was directly genotyped on the Illumina 610 Quad panel, so called genotypes were used as imputed by BEAGLE (Browning et al., 2009).

Results

Arhgap29 is expressed in the developing face

Arhgap29 expression had not been previously described in the face, so we first characterized its expression in craniofacial development using a murine model. *Arhgap29* transcript was detected strongly in the medial and lateral nasal processes, while less intense expression was observed in the mandibular and maxillary processes at E10.5 (Figure 2A) and the shelves of the secondary palate at E13.5 (Figure 2C). Arhgap29 transcript and protein were identified in the epithelium and mesenchyme of coronal head sections at E10.5 and E14.5 (Figure 2E, 2F). This spatiotemporal expression profile of Arghap29 is highly consistent with a role in craniofacial development (Gritli-Linde, 2008; Jiang et al., 2006).

Arhgap29 may act downstream of Irf6 in craniofacial development

We previously identified *IRF6* as the gene causative for Van der Woude syndrome (Kondo et al., 2002) and strongly associated with NSCL/P (Rahimov et al., 2008; Zucchero et al., 2004). Extensive investigations using a mouse knockout model have further supported a critical role for *Irf6* in craniofacial development (Biggs et al., 2012; Ferretti et al., 2011; Ingraham et al., 2006; Richardson et al., 2006). Notably, Arhgap29 expression was decreased in all epithelia of *Irf6*-deficient embryos (Figure 2I), indicating *Arhgap29* may act downstream of *Irf6*. We used skin to quantify this decreased expression. Western blotting of

E17.5 cutaneous extracts indicated a significant decrease (2.8 fold, $p<0.05$) in Arhgap29 in *Irf6*-deficient skin compared to wildtype (Figure 2J, Supplemental Figure 1). These data support Arghap29 as a mediator of Irf6 signaling.

To determine if this molecular interaction translated to a genetic interaction in NSCL/P cases, we tested for an interaction between the most associated SNP (rs560426) near *ARHGAP29* and two SNPs near *IRF6* (rs2235271, rs642961). We tested both rs2235371, located in *IRF6*, and rs642961, located in an upstream enhancer (Fakhouri et al., 2012; Rahimov et al., 2008), because although rs642961 is etiologic and confers risk for CL, it does not completely account for the association with rs2235371 (Rahimov et al., 2008). We found a marginal interaction between these SNPs, ($p=0.04$ and $p=0.07$, respectively). Although these results are not significant, a true, positive association could be missed due to false negatives arising from imputation.

Sequencing *ARHGAP29* identifies coding variants in NSCL/P patients

To determine if coding sequence variants in *ARHGAP29* contribute to NSCL/P, we used a two-phase design to sequence the protein coding exons of *ARHGAP29*. We identified nonsynonymous variants in 9 exons in cases and/or controls during the phase one. After the second phase, nine exons were sequenced in a total of 972 cases and 962 controls from the Philippines and the US, identifying a frameshift variant, a nonsense variant, and fourteen missense variants (Table 1, Figure 3). We categorized sequencing variants into three classes of allele frequencies: common alleles, with frequencies greater than 5%; rare alleles, with frequencies between 1% and 5%; and very rare alleles, with frequencies less than 1%.

Eight novel, very rare variants were identified in *ARHGAP29* in individuals with NSCL/P and were absent in controls. A two basepair deletion causing a frameshift (S21Yfs*20) was identified in an individual with bilateral cleft lip and palate (CLP), resulting in truncation of the *ARHGAP29* gene product after 20 amino acids. This deletion was also identified in an unaffected sibling. A nonsense variant, p.Lys326*, was identified in an individual with bilateral CLP, as well as the proband's unaffected mother and grandfather. Identifying these truncating variants in unaffected family members suggest they may not be completely penetrant but require interactions with other genetic and/or environmental predisposing covariates.

Two missense variants, p.Thr26Ala and p.Ala832Thr, were identified in individuals with cleft lip (CL) and were carried by an unaffected parent. Two additional variants, p.Thr622Met and p.Ile845Val, were identified in individuals with CLP. The former was also found in the proband's father, who also had CLP. In the US population, two novel, very rare variants, p.Lys46Arg and p.Arg1142Gln, were identified in individuals with CLP. Finally, two unreported, rare variants were found among Filipino cases and controls. The first, p.Val1202Leu was found at a frequency of 1.3% in Filipino cases and 1.4% in Filipino controls while p.Arg616His was found at frequencies around 4.0% in cases and 3.7% in controls.

ARHGAP29 contains four domains (Figure 3): a coiled-coil region know to interact with Rap2 (Myagmar et al., 2005), a C1 domain, the Rho GTPase domain, and a small C-terminal region that interacts with PTPL1 (Saras et al., 1997). The members of the cysteine-rich C1 domain family bind a diverse set of molecules or proteins including the second messenger diacylglycerol, phorbol esters, and RasGTP (Colon-Gonzalez et al., 2006). This domain also includes conserved cysteine and histidine residues suitable for binding zinc ions. Although the R616H variant does not directly affect these residues, the creation of another histidine could alter zinc binding. The highly conserved Rho GTPase domain is critical for the function of ARHGAP29. This domain contains a catalytic residue and seven residues that

make up the putative GTPase interaction site (Supplemental Figure 2). The I845V variant alters one of these interaction sites, though it is not clear what effect this variant would have on protein function given the similar biochemical properties of valine and isoleucine. The variants we identified were distributed throughout *ARHGAP29*, making it difficult to predict the specific effects of these variants on ARHGPA29 function. However, the two truncation variants suggest a loss of function paradigm.

Association of coding variants with NSCL/P

The cumulative frequency of very rare *ARHGAP29* variants was greater for cases than controls (Filipinos, 5.5% vs. 4.3%; US, 1.0% vs. 0.9%), but these differences were not statistically significant (p=0.36 and p=1.00, respectively). When both populations were combined, very rare variants were overrepresented in cases with NSCL/P (p=0.03, 4.6% vs. 2.7%). When stratified by cleft type, these variants were associated with cleft lip (CL) (p=0.02, 6.0% in cases and 2.7% in controls) but not with cleft lip with cleft palate (CLP) (Table 2). Similar results were found when considering all very rare and rare variants together (Table 2). Although these p-values are not significant after Bonferroni correction (20 tests, threshold p=0.0025), the power to detect strong effects with rare variants is limited. Therefore, we considered these results indicative of a trend toward association.

We also compared the variants identified in this study with published data from the 1000 Genomes Project (Consortium, 2010) and the NHLBI Exome Sequencing Project (ESP2500) which represents approximately 2500 European and African American individuals from cohorts sampled for heart, lung, and blood disorders (Supplemental Table 2). Although there were 12 missense variants in the Asian and European samples sequenced by the 1000 Genomes Project and 19 missense variants identified in the European American cohort of the ESP, none of the 8 nonsynonymous variants found exclusively in our cases with NSCL/P were present in either the 1000 Genome Project or ESP2500 data, strongly supporting these variants as potentially etiologic.

Discussion

In this study, we demonstrated clear craniofacial expression of *Arhgap29* and showed this expression to be decreased in *Irf6* deficient mice. We furthermore identified a nonsense variant, a frameshift variant, and fourteen missense variants in *ARHGAP29* which collectively are overrepresented in cases with NSCL/P compared to unaffected controls.

ARHGAP29 is located on chromosome 1p22, a locus that was identified by GWAS of NSCL/P. Despite the numerous successes of GWAS identifying novel loci associated with the tested disease or trait, one of its limitations is its inability to direct us to the causal gene or variant. In some cases, as in the association between prostate cancer and the 8q24 locus, the associated variant has a functional effect (Wasserman et al., 2010). However, for many studies the associated variant has no obvious biological connection to the disease or trait in question, leading to the notion that the associated variant is in LD with a causal one. However, GWAS typically implicate multiple SNPs within an LD block, which may include multiple genes. This study highlights the importance of considering other genes near GWA markers, rather than focus on the one nearest the peak signal. In the case of the 1p22 locus *GCLM* lies several LD blocks away from the peak signal, had no coding sequence variants detected and has not had craniofacial specific expression demonstrated; lowering, although not eliminating, its status as the etiologic gene. *ARHGAP29* is a more plausible candidate for clefting than *GCLM* or *ABCA4*, mutations in which cause several autosomal recessive retinal disorders (Burke et al., 2011). Although *ARHGAP29* and *ABCA4* are not located within the same LD block (Figure 1), it is possible that genome-wide significant associations are driven in part by multiple rare variants, possibly located in adjacent LD blocks (Dickson

et al., 2010). We also consider rs560426 a surrogate for the locus and hypothesize that this SNP is in LD with one or more common etiologic SNPs located within regulatory elements that act upon very distant targets, highlighting the importance of evaluating these associated regions broadly.

We identified a marginal association between rare variants in *ARHGAP29* and NSCL/P. Interest in rare variants is growing as one way to explain the "missing heritability" often occurring in the GWAS approach, but the analyses of these rare variants suffer from a lack of power due to very small numbers of observations. Larger sample sizes will be required to detect stronger associations between these rare variants and risk of NSCL/P. Although most of the variants we identified were inherited from an unaffected parent, unaffected family members of individuals with NSCL/P may have subepithelial defects of the *orbicularis oris* muscle (Marazita, 2007; Suzuki et al., 2009). Future studies may benefit from incorporating these sub-clinical phenotypes when available as they could increase our ability to detect genetic effects.

The very rare variants identified in *ARHGAP29* could contribute to the etiology of NSCL/P in individual families; however, GWAS data suggest one or more common markers (or more specifically an unobserved variant in LD with a common marker) could also contribute to the occurrence of clefting. Increasing evidence suggests etiologic variants, particularly for complex diseases, will occur in noncoding regions of the genome (Ernst et al., 2011; Visel et al., 2009). To date, only one common, etiologic variant (rs642961) has been identified in a recognized cleft candidate gene, *IRF6*, and it resides in an enhancer element near this gene (Fakhouri et al., 2012; Rahimov et al., 2008). We hypothesize common etiologic variants for the 1p22 locus may reside in regulatory elements of *ARHGAP29*. Sequencing of conserved and putative regulatory elements, followed by functional validation will be required to identify such causal variants.

ARHGAP29 is a Rho GTPase activating protein, involved in the regulation of small GTP binding proteins including Rho, Rac, and Cdc42 (Heasman et al., 2008). *ARHGAP29* is located in a region of chromosome 1 frequently deleted in Mantle cell lymphomas (MCL) (Ripperger et al., 2007; Schraders et al., 2008). Although there is reported increased risk of cancer in individuals with NSCL/P and their family members (Bille et al., 2005; Menezes et al., 2009; Taioli et al., 2010), a link between NSCL/P and MCL has not specifically been described, perhaps due to the rarity of this lymphoma subtype (Zhou et al., 2008). Prior to this study, the expression of *Arhgap29* was described in the heart of embryonic mice (Miller et al., 2008) and the vasculature of zebrafish (Gomez et al., 2009), which is consistent with its role in blood vessel development (Xu et al., 2011). In human tissues, *ARHGAP29* was present in skeletal muscle, heart, placenta, liver, and pancreas, with low expression in the brain, lung, and kidney (Saras et al., 1997); however, skin was not examined. A study of human fibroblasts cultured in folate-deficient and folate sufficient mediums, however, showed altered expression of *ARHGAP29* and other genes related to cell signaling, cytoskeleton, and extracellular matrix, including *WNT5A* (Katula et al., 2007). Folate deficiency has been suggested as a risk factor for NSCL/P, however convincing supporting evidence has been elusive (Dixon et al., 2011).

We demonstrated clear craniofacial expression of *Arhgap29* and showed this expression to be decreased in the palatal epithelium and skin of *Irf6* deficient mice. Our results suggest that Arhgap29 may act downstream of *Irf6*, which is also supported by a recent ChIP-seq study of human keratinocytes identifying an IRF6 binding site upstream of *ARHGAP29* (Figure 1)(Botti et al., 2011). The *Irf6* gene regulatory network includes both *p63* and *Tfap2a*, which regulate *Irf6* through an upstream enhancer element (Moretti et al., 2010; Rahimov et al., 2008; Thomason et al., 2010). Recently this pathway was expanded to

include a Pbx-dependent regulatory element controlling Wnt9b-Wnt3, which regulates *p63* and *Irf6* and is required for midface morphogenesis (Ferretti et al., 2011). Rho is a downstream effector of Wnt signaling (Schlessinger et al., 2009) and we suggest that the Rho signaling implicated in craniofacial development is mediated in part through the *IRF6* gene regulatory network via *ARHGAP29* (Figure 4).

Most of the variants identified in *ARHGAP29* were predicted to be damaging (including truncation variants) and suggest a loss-of-function paradigm. Support for this mechanism is provided by recent expression profiling of dental pulp stem cell cultures obtained from individuals with NSCL/P which showed a nearly 3-fold decrease in *ARHGAP29* compared to cultures from unaffected controls (Bueno et al., 2011). If this is the case, the loss of a GAP would maintain Rho in an active, GTP-bound form, effectively increasing Rho activity. This could negatively affect cellular migration throughout development, and may represent one possible mechanism by which ARHGAP29 could be involved in the etiology of NSCL/P. Recently, Kitase and Shuler (2012) showed that treatment of palate cultures with nocodazole, a drug that destabilizes microtubules, resulted in increased RhoA activity and caused formation of a multi-layered, hypertrophied medial edge epithelium (MEE) and failure of palatal fusion. This study demonstrated that the regulation of microtubule dynamics and actin microfilaments is required for the remodeling of the MEE during palatogenesis; and represents another mechanism by which *ARHGAP29* could be involved in NSCL/P. It also remains plausible the etiologic variants act through a gain-of-function mechanism and decrease Rho activity, the amount of Rho available, or disregulate the timing or position of crucial elements of craniofacial development. Recent work in autism suggests that there may be a zone of preferred expression for many genes and that shifts to either side can result in similar abnormal phenotypes (Auerbach et al., 2011).

In summary, our combination of rare, coding sequence variants which are potentially etiologic, and the clear evidence of gene expression in the facial processes forming the lip and palate during development make *ARHGAP29* the likely etiologic gene identified from a positive GWAS signal for NSCL/P on 1p22.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the many families who participated in this study; Jenni Mancuso, Brian Smith, Jamie L'Heureux, Kate Durda, and Jenni Rigdon for technical assistance; and Susie McConnell, Erin Brothers-Smith, and Nancy Davin for administrative assistance. The authors would like to thank the NHLBI GO Exome Sequencing Project and its ongoing studies which produced and provided variant calls for comparison: the Lung GO Sequencing Project [HL-102923], the WHI Sequencing Project [HL-102924], the Broad GO Sequencing Project [HL-102925], the Seattle GO Sequencing Project [HL-102926] and the Heart GO Sequencing Project [HL-103010]. This work was supported by grants from the National Institutes of Health [DE08559, DE020057].

Literature Cited

Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nature Methods. 2010; 7(4): 248–249. [PubMed: 20354512]

Auerbach BD, Osterweil EK, Bear MF. Mutations causing syndromic autism define an axis of synaptic pathophysiology. Nature. 2011; 480(7375):63–68. [PubMed: 22113615]

Beaty TH, Murray JC, Marazita ML, Munger RG, Ruczinski I, Hetmanski JB, Liang KY, Wu T, Murray T, Fallin MD, Redett RA, Raymond G, Schwender H, Jin SC, Cooper ME, Dunnwald M, Mansilla MA, Leslie E, Bullard S, Lidral AC, Moreno LM, Menezes R, Vieira AR, Petrin A,

Wilcox AJ, Lie RT, Jabs EW, Wu-Chou YH, Chen PK, Wang H, Ye X, Huang S, Yeow V, Chong SS, Jee SH, Shi B, Christensen K, Melbye M, Doheny KF, Pugh EW, Ling H, Castilla EE, Czeizel AE, Ma L, Field LL, Brody L, Pangilinan F, Mills JL, Molloy AM, Kirke PN, Scott JM, Arcos-Burgos M, Scott AF. A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. Nat Genet. 2010; 42(6):525–529. [PubMed: 20436469]

Biggs LC, Rhea L, Schutte BC, Dunnwald M. Interferon regulatory factor 6 is necessary, but not sufficient, for keratinocyte differentiation. J Invest Dermatol. 2012; 132(1):50–58. [PubMed: 21918538]

Bille C, Winther JF, Bautz A, Murray JC, Olsen J, Christensen K. Cancer risk in persons with oral cleft--a population-based study of 8,093 cases. Am J Epidemiol. 2005; 161(11):1047–1055. [PubMed: 15901625]

Birnbaum S, Ludwig KU, Reutter H, Herms S, Steffens M, Rubini M, Baluardo C, Ferrian M, Almeida de Assis N, Alblas MA, Barth S, Freudenberg J, Lauster C, Schmidt G, Scheer M, Braumann B, Berge SJ, Reich RH, Schiefke F, Hemprich A, Potzsch S, Steegers-Theunissen RP, Potzsch B, Moebus S, Horsthemke B, Kramer FJ, Wienker TF, Mossey PA, Propping P, Cichon S, Hoffmann P, Knapp M, Nothen MM, Mangold E. Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. Nat Genet. 2009; 41(4):473–477. [PubMed: 19270707]

Botti E, Spallone G, Moretti F, Marinari B, Pinetti V, Galanti S, De Meo PD, De Nicola F, Ganci F, Castrignano T, Pesole G, Chimenti S, Guerrini L, Fanciulli M, Blandino G, Karin M, Costanzo A. Developmental factor IRF6 exhibits tumor suppressor activity in squamous cell carcinomas. Proc Natl Acad Sci U S A. 2011; 108(33):13710–13715. [PubMed: 21807998]

Browning BL, Browning SR. A unified approach to genotype imputation and haplotype-phase inference for large data sets of trios and unrelated individuals. Am J Hum Genet. 2009; 84(2):210–223. [PubMed: 19200528]

Bueno DF, Sunaga DY, Kobayashi GS, Aguena M, Raposo-Amaral CE, Masotti C, Cruz LA, Pearson PL, Passos-Bueno MR. Human stem cell cultures from cleft lip/palate patients show enrichment of transcripts involved in extracellular matrix modeling by comparison to controls. Stem Cell Reviews. 2011; 7(2):446–457. [PubMed: 21052871]

Burke TR, Tsang SH. Allelic and phenotypic heterogeneity in ABCA4 mutations. Ophthalmic Genet. 2011; 32(3):165–174. [PubMed: 21510770]

Colon-Gonzalez F, Kazanietz MG. C1 domains exposed: from diacylglycerol binding to protein-protein interactions. Biochim Biophys Acta. 2006; 1761(8):827–837. [PubMed: 16861033]

Consortium TGP. A map of human genome variation from population-scale sequencing. Nature. 2010; 467(7319):1061–1073. [PubMed: 20981092]

Dickson SP, Wang K, Krantz I, Hakonarson H, Goldstein DB. Rare variants create synthetic genome-wide associations. PLoS Biol. 2010; 8(1):e1000294. [PubMed: 20126254]

Diez-Roux G, Banfi S, Sultan M, Geffers L, Anand S, Rozado D, Magen A, Canidio E, Pagani M, Peluso I, Lin-Marq N, Koch M, Bilio M, Cantiello I, Verde R, De Masi C, Bianchi SA, Cicchini J, Perroud E, Mehmeti S, Dagand E, Schrinner S, Nurnberger A, Schmidt K, Metz K, Zwingmann C, Brieske N, Springer C, Hernandez AM, Herzog S, Grabbe F, Sieverding C, Fischer B, Schrader K, Brockmeyer M, Dettmer S, Helbig C, Alunni V, Battaini MA, Mura C, Henrichsen CN, Garcia-Lopez R, Echevarria D, Puelles E, Garcia-Calero E, Kruse S, Uhr M, Kauck C, Feng G, Milyaev N, Ong CK, Kumar L, Lam M, Semple CA, Gyenesei A, Mundlos S, Radelof U, Lehrach H, Sarmientos P, Reymond A, Davidson DR, Dolle P, Antonarakis SE, Yaspo ML, Martinez S, Baldock RA, Eichele G, Ballabio A. A high-resolution anatomical atlas of the transcriptome in the mouse embryo. PLoS biology. 2011; 9(1):e1000582. [PubMed: 21267068]

Dixon MJ, Marazita ML, Beaty TH, Murray JC. Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet. 2011; 12(3):167–178. [PubMed: 21331089]

Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, Epstein CB, Zhang X, Wang L, Issner R, Coyne M, Ku M, Durham T, Kellis M, Bernstein BE. Mapping and analysis of chromatin state dynamics in nine human cell types. Nature. 2011; 473(7345):43–49. [PubMed: 21441907]

Fakhouri WD, Rhea L, Du T, Sweezer E, Morrison H, Fitzpatrick D, Yang B, Dunnwald M, Schutte BC. MCS9.7 Enhancer activity is highly, but not completely, associated with expression of Irf6 and p63. Dev Dyn. 2012; 241(3):i.

Ferretti E, Li B, Zewdu R, Wells V, Hebert JM, Karner C, Anderson MJ, Williams T, Dixon J, Dixon MJ, Depew MJ, Selleri L. A Conserved Pbx-Wnt-p63-Irf6 Regulatory Module Controls Face Morphogenesis by Promoting Epithelial Apoptosis. Dev Cell. 2011; 21(4):627–641. [PubMed: 21982646]

Gomez GA, Veldman MB, Zhao Y, Burgess S, Lin S. Discovery and characterization of novel vascular and hematopoietic genes downstream of etsrp in zebrafish. PLoS ONE. 2009; 4(3):e4994. [PubMed: 19308258]

Gritli-Linde A. The etiopathogenesis of cleft lip and cleft palate: usefulness and caveats of mouse models. Curr Top Dev Biol. 2008; 84:37–138. [PubMed: 19186243]

Heasman SJ, Ridley AJ. Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat Rev Mol Cell Biol. 2008; 9(9):690–701. [PubMed: 18719708]

Ingraham CR, Kinoshita A, Kondo S, Yang B, Sajan S, Trout KJ, Malik MI, Dunnwald M, Goudy SL, Lovett M, Murray JC, Schutte BC. Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (Irf6). Nat Genet. 2006; 38(11):1335–1340. [PubMed: 17041601]

Jiang R, Bush JO, Lidral AC. Development of the upper lip: morphogenetic and molecular mechanisms. Dev Dyn. 2006; 235(5):1152–1166. [PubMed: 16292776]

Juriloff DM, Harris MJ, McMahon AP, Carroll TJ, Lidral AC. Wnt9b is the mutated gene involved in multifactorial nonsyndromic cleft lip with or without cleft palate in A/WySn mice, as confirmed by a genetic complementation test. Birt Defects Res A Clin Mol Teratol. 2006; 76(8):574–579.

Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. Nat Genet. 1995; 11(4):415–421. [PubMed: 7493022]

Kardassis D, Murphy C, Fotsis T, Moustakas A, Stournaras C. Control of transforming growth factor beta signal transduction by small GTPases. FEBS J. 2009; 276(11):2947–2965. [PubMed: 19490100]

Katula KS, Heinloth AN, Paules RS. Folate deficiency in normal human fibroblasts leads to altered expression of genes primarily linked to cell signaling, the cytoskeleton and extracellular matrix. J Nutr Biochem. 2007; 18(8):541–552. [PubMed: 17320366]

Kitase Y, Shuler CF. Multi-layered hypertrophied MEE formation by microtubule disruption via GEF-H1/RhoA/ROCK signaling pathway. Dev Dyn. 2012

Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, Howard E, de Lima RL, Daack-Hirsch S, Sander A, McDonald-McGinn DM, Zackai EH, Lammer EJ, Aylsworth AS, Ardinger HH, Lidral AC, Pober BR, Moreno L, Arcos-Burgos M, Valencia C, Houdayer C, Bahuau M, Moretti-Ferreira D, Richieri-Costa A, Dixon MJ, Murray JC. Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. Nat Genet. 2002; 32(2):285–289. [PubMed: 12219090]

Mangold E, Ludwig KU, Birnbaum S, Baluardo C, Ferrian M, Herms S, Reutter H, de Assis NA, Chawa TA, Mattheisen M, Steffens M, Barth S, Kluck N, Paul A, Becker J, Lauster C, Schmidt G, Braumann B, Scheer M, Reich RH, Hemprich A, Potzsch S, Blaumeiser B, Moebus S, Krawczak M, Schreiber S, Meitinger T, Wichmann HE, Steegers-Theunissen RP, Kramer FJ, Cichon S, Propping P, Wienker TF, Knapp M, Rubini M, Mossey PA, Hoffmann P, Nothen MM. Genome-wide association study identifies two susceptibility loci for nonsyndromic cleft lip with or without cleft palate. Nat Genet. 2010; 42(1):24–26. [PubMed: 20023658]

Marazita ML. Subclinical features in non-syndromic cleft lip with or without cleft palate (CL/P): review of the evidence that subepithelial orbicularis oris muscle defects are part of an expanded phenotype for CL/P. Orthod Craniofac Res. 2007; 10(2):82–87. [PubMed: 17552944]

Menezes R, Marazita ML, Goldstein McHenry T, Cooper ME, Bardi K, Brandon C, Letra A, Martin RA, Vieira AR. AXIS inhibition protein 2, orofacial clefts and a family history of cancer. J Am Dent Assoc. 2009; 140(1):80–84. [PubMed: 19119171]

Miller RA, Christoforou N, Pevsner J, McCallion AS, Gearhart JD. Efficient array-based identification of novel cardiac genes through differentiation of mouse ESCs. PLoS ONE. 2008; 3(5):e2176. [PubMed: 18478100]

Moretti F, Marinari B, Lo Iacono N, Botti E, Giunta A, Spallone G, Garaffo G, Vernersson-Lindahl E, Merlo G, Mills AA, Ballaro C, Alema S, Chimenti S, Guerrini L, Costanzo A. A regulatory feedback loop involving p63 and IRF6 links the pathogenesis of 2 genetically different human ectodermal dysplasias. J Clin Invest. 2010; 120(5):1570–1577. [PubMed: 20424325]

Moustakas A, Heldin CH. Dynamic control of TGF-beta signaling and its links to the cytoskeleton. FEBS Lett. 2008; 582(14):2051–2065. [PubMed: 18375206]

Murray JC, Buetow KH, Donovan M, Hornung S, Motulsky AG, Disteche C, Dyer K, Swisshelm K, Anderson J, Giblett E, et al. Linkage disequilibrium of plasminogen polymorphisms and assignment of the gene to human chromosome 6q26–6q27. Am J Hum Genet. 1987; 40(4):338–350. [PubMed: 2883890]

Myagmar BE, Umikawa M, Asato T, Taira K, Oshiro M, Hino A, Takei K, Uezato H, Kariya K. PARG1, a protein-tyrosine phosphatase-associated RhoGAP, as a putative Rap2 effector. Biochem Biophys Res Commun. 2005; 329(3):1046–1052. [PubMed: 15752761]

Pearson RD. Bias due to selection of rare variants using frequency in controls. Nat Genet. 2011; 43(5): 392–393. author reply 394–395. [PubMed: 21522173]

Rahimov F, Marazita ML, Visel A, Cooper ME, Hitchler MJ, Rubini M, Domann FE, Govil M, Christensen K, Bille C, Melbye M, Jugessur A, Lie RT, Wilcox AJ, Fitzpatrick DR, Green ED, Mossey PA, Little J, Steegers-Theunissen RP, Pennacchio LA, Schutte BC, Murray JC. Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. Nat Genet. 2008; 40(11):1341–1347. [PubMed: 18836445]

Richardson RJ, Dixon J, Malhotra S, Hardman MJ, Knowles L, Boot-Handford RP, Shore P, Whitmarsh A, Dixon MJ. Irf6 is a key determinant of the keratinocyte proliferation-differentiation switch. Nat Genet. 2006; 38(11):1329–1334. [PubMed: 17041603]

Ripperger T, von Neuhoff N, Kamphues K, Emura M, Lehmann U, Tauscher M, Schraders M, Groenen P, Skawran B, Rudolph C, Callet-Bauchu E, van Krieken JH, Schlegelberger B, Steinemann D. Promoter methylation of PARG1, a novel candidate tumor suppressor gene in mantle-cell lymphomas. Haematologica. 2007; 92(4):460–468. [PubMed: 17488656]

Saras J, Franzen P, Aspenstrom P, Hellman U, Gonez LJ, Heldin CH. A novel GTPase-activating protein for Rho interacts with a PDZ domain of the protein-tyrosine phosphatase PTPL1. J Biol Chem. 1997; 272(39):24333–24338. [PubMed: 9305890]

Schlessinger K, Hall A, Tolwinski N. Wnt signaling pathways meet Rho GTPases. Genes Dev. 2009; 23(3):265–277. [PubMed: 19204114]

Schraders M, Jares P, Bea S, Schoenmakers EF, van Krieken JH, Campo E, Groenen PJ. Integrated genomic and expression profiling in mantle cell lymphoma: identification of gene-dosage regulated candidate genes. Br J Haematol. 2008; 143(2):210–221. [PubMed: 18699851]

Suzuki S, Marazita ML, Cooper ME, Miwa N, Hing A, Jugessur A, Natsume N, Shimozato K, Ohbayashi N, Suzuki Y, Niimi T, Minami K, Yamamoto M, Altannamar TJ, Erkhembaatar T, Furukawa H, Daack-Hirsch S, L'Heureux J, Brandon CA, Weinberg SM, Neiswanger K, Deleyiannis FW, de Salamanca JE, Vieira AR, Lidral AC, Martin JF, Murray JC. Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. Am J Hum Genet. 2009; 84(3):406–411. [PubMed: 19249007]

Taioli E, Ragin C, Robertson L, Linkov F, Thurman NE, Vieira AR. Cleft lip and palate in family members of cancer survivors. Cancer Invest. 2010; 28(9):958–962. [PubMed: 20569073]

Thomason HA, Zhou H, Kouwenhoven EN, Dotto GP, Restivo G, Nguyen BC, Little H, Dixon MJ, van Bokhoven H, Dixon J. Cooperation between the transcription factors p63 and IRF6 is essential to prevent cleft palate in mice. J Clin Invest. 2010; 120(5):1561–1569. [PubMed: 20424327]

Visel A, Rubin EM, Pennacchio LA. Genomic views of distant-acting enhancers. Nature. 2009; 461(7261):199–205. [PubMed: 19741700]

Wasserman NF, Aneas I, Nobrega MA. An 8q24 gene desert variant associated with prostate cancer risk confers differential in vivo activity to a MYC enhancer. Genome Res. 2010; 20(9):1191–1197. [PubMed: 20627891]

Xu K, Sacharidou A, Fu S, Chong DC, Skaug B, Chen ZJ, Davis GE, Cleaver O. Blood vessel tubulogenesis requires Rasip1 regulation of GTPase signaling. Dev Cell. 2011; 20(4):526–539. [PubMed: 21396893]

Yang Y, Dieter MZ, Chen Y, Shertzer HG, Nebert DW, Dalton TP. Initial characterization of the glutamate-cysteine ligase modifier subunit Gclm(−/−) knockout mouse. Novel model system for a severely compromised oxidative stress response. J Biol Chem. 2002; 277(51):49446–49452. [PubMed: 12384496]

Zhou Y, Wang H, Fang W, Romaguer JE, Zhang Y, Delasalle KB, Kwak L, Yi Q, Du XL, Wang M. Incidence trends of mantle cell lymphoma in the United States between 1992 and 2004. Cancer. 2008; 113(4):791–798. [PubMed: 18615506]

Zucchero TM, Cooper ME, Maher BS, Daack-Hirsch S, Nepomuceno B, Ribeiro L, Caprau D, Christensen K, Suzuki Y, Machida J, Natsume N, Yoshiura K, Vieira AR, Orioli IM, Castilla EE, Moreno L, Arcos-Burgos M, Lidral AC, Field LL, Liu YE, Ray A, Goldstein TH, Schultz RE, Shi M, Johnson MK, Kondo S, Schutte BC, Marazita ML, Murray JC. Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. N Engl J Med. 2004; 351(8):769–780. [PubMed: 15317890]

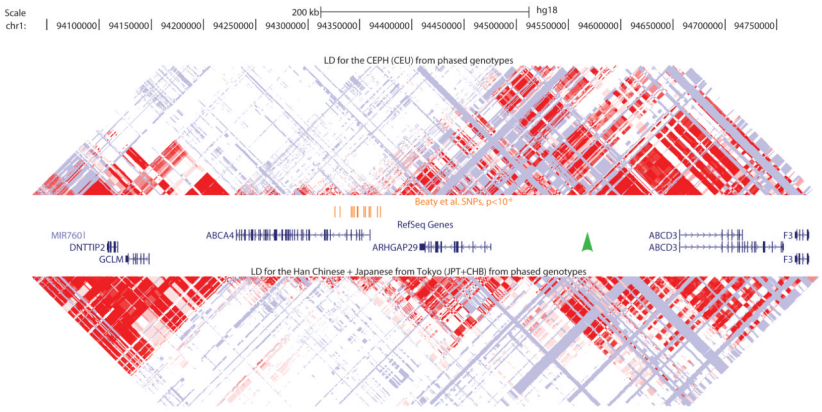

Figure 1. Details of the 1p22.1 locus

Linkage disequilibrium structure from CEU (top) and CHB+JPT (bottom) HapMap samples. Orange lines mark the positions of genome wide associated SNPs from Beaty *et al.* with a p-value of 10^{-6} or better. The green triangle marks the location of the IRF6 binding site identified by ChipSeq in human keratinocytes by Botti *et al.*

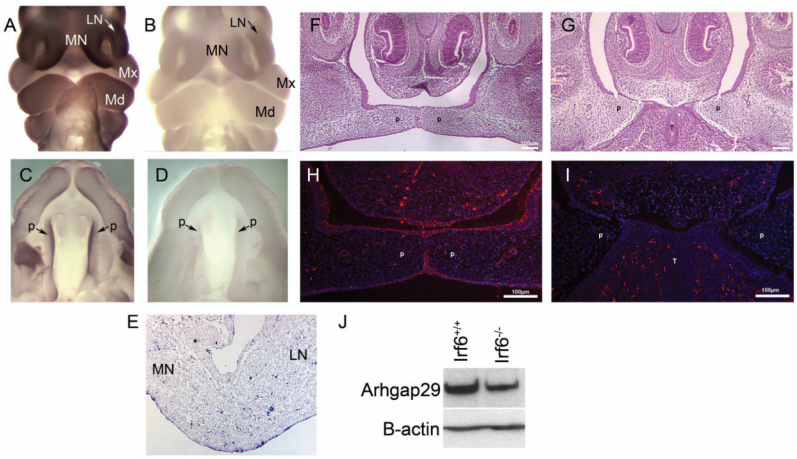

Figure 2. Expression of Arhgap29 during craniofacial development

(A–D) *In situ* hybridization of whole mount embryos at E10.5 (A, B) and E13.5 (C, D) for *Arhgap29* (A, C) and sense control (B, D). (A) and (B) are frontal views of the embryo, (C) and (D) are views of the roof of the mouth. (E) *In situ* hybridization of coronal sections at E10.5 showing the nasal processes after fusion. (F–I) Coronal sections of the secondary palate of wild-type (F, H) and Irf6$^{-/-}$ (G, I) embryos. (F) and (G) are histological sections and (H) and (I) show immunofluorescence for Arhgap29 (red); nuclear DNA is labeled with DAPI (blue). (J) Western blot for Arhgap29 and beta-actin of wild-type and *Irf6*$^{-/-}$ E17.5 skin protein extract. MN, medial nasal process; LN, lateral nasal process; Mx, maxillary process; Md, mandibular process; p, palatal shelf; T, tongue.

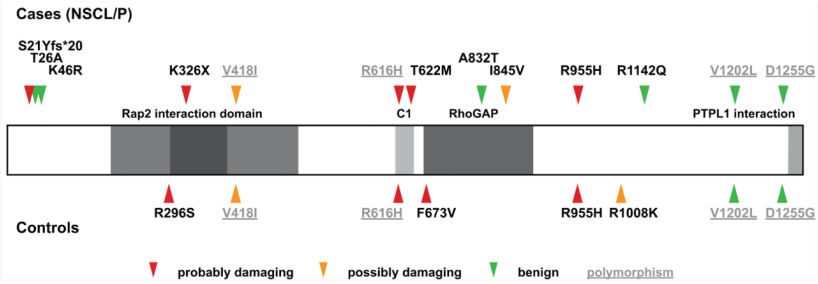

Figure 3. Schematic of the ARHGAP29 protein and sequencing variants

Protein domains are depicted by grey boxes and labeled above. Positions of the variants identified in the mutation screen are indicated by colored arrows (cases- top; controls- bottom). Arrows are color coded by Polyphen-2 prediction (green- benign, orange-possibly damaging, red- probably damaging). Polymorphisms (variants identified in more than one control individual) are denoted by an underline.

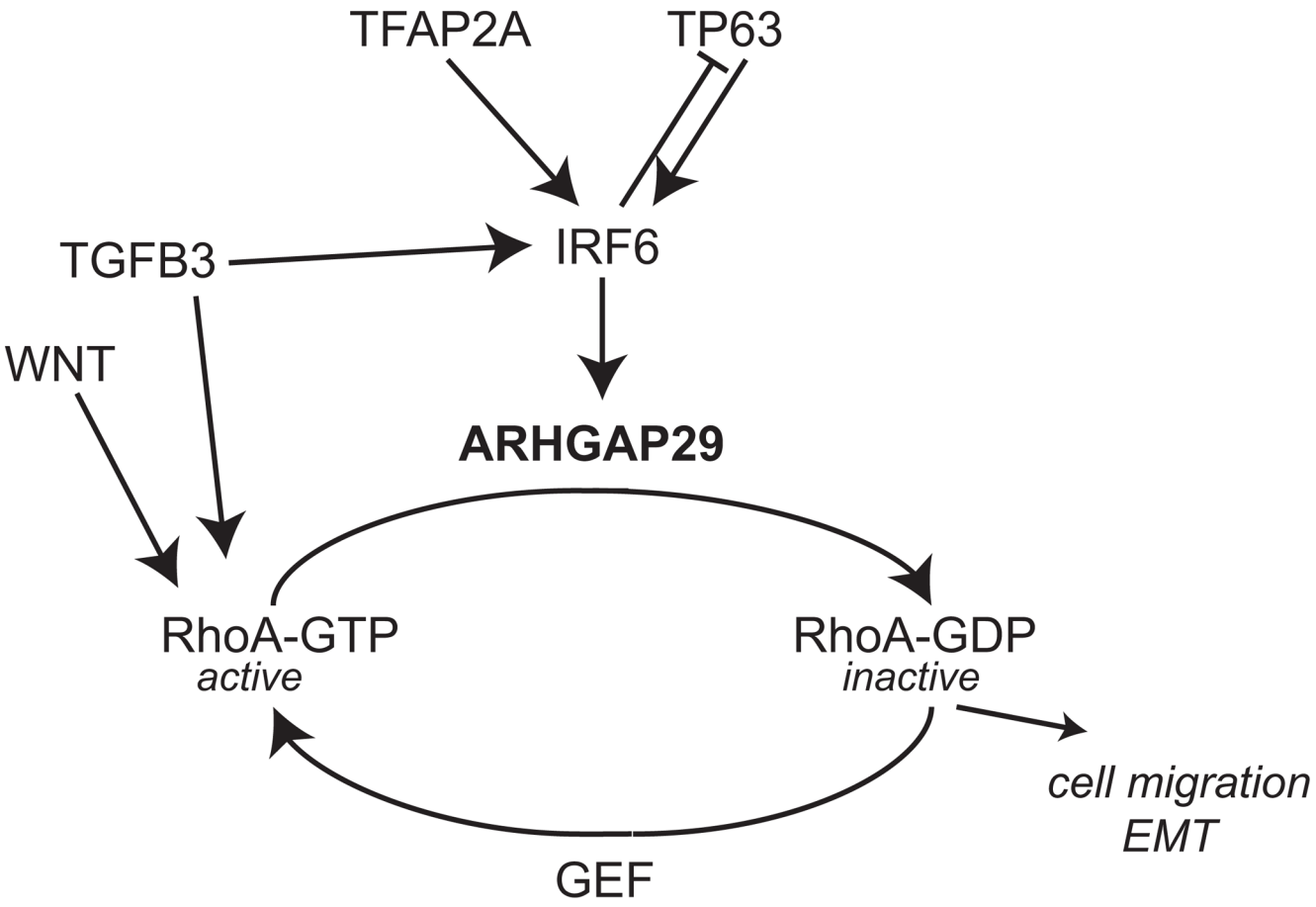

Figure 4. Model of hypothetical pathway involving IRF6, ARHGAP29, and the Rho signaling pathway

The hypothetical pathway links the IRF6 gene regulatory network (IRF6, TP63, TFAP2A, and TGFB3, all previously implicated in craniofacial development) with the Rho signaling pathway through ARHGAP29. This pathway may also involve WNT and TGFB3, which can signal through the Rho pathway, and are required for craniofacial development. We propose that decreased ARHGAP29 would maintain Rho in an active state, which would negatively regulate processes critical for craniofacial development such as cellular migration. GEF, guanine nucleotide exchange factor.

Table 1

Nonsynonymous mutations identified in cases with NSCL/P and controls

Mutation (DNA)[a]	Mutation (protein)[b]	rs	Philippines		US		Conservation[d]	Variant Category[e]	Polyphen 2 prediction
			Cases[c] N=1440	Controls[c] N=1032	Cases[c] N=362	Controls[c] N=912			
c.62_63delCT	p.S21Yfs*20		1	0	0	0	n/a	VR	*Truncation*
c.76A>G	p.Thr26Ala		1	0	0	0	9/11	VR	Benign
c.137A>G	p.Lys46Arg		0	0	1	0	11/14	VR	Benign
c.888G>C	p.Arg296Ser		0	1	0	0	13/13	VR	Probably Damaging
c.976A>T	p.Lys326X		1	0	0	0	12/14	VR	*Truncation*
c.1252G>A	p.Val418Ile	rs148959325	17	13	2	0	12/14	VR	Possibly Damaging
c.1847G>A	p.Arg616His		55	34	0	0	14/14	R	Probably Damaging
c.1865C>T	p.Thr622Met		1	0	0	0	10/14	VR	Probably Damaging
c.2017T>G	p.Phe673Val		0	0	0	1	12/12	VR	Probably Damaging
c.2494G>A	p.Ala832Thr		1	0	0	0	10/14	VR	Benign
c.2533A>G	p.Ile845Val		1	0	0	0	14/14	VR	Possibly Damaging
c.2864G>A	p.Arg955His	rs113546321	0	0	1	1	9/9	VR	Probably Damaging
c.3023G>A	p.Arg1008Lys	rs140638899	0	0	0	1	10/12	VR	Possibly Damaging
c.3425G>A	p.Arg1142Gln		0	0	1	0	11/14	VR	Benign
c.3604G>T	p.Val1202Leu		18	8	0	0	6/13	VR	Benign
c.3764G>A	p.Asp1255Gly	rs1999272	1	0	2	1	11/14	VR	Benign

[a] NM_004815.3

[b] NP_004806.3

[c] Counts are for minor alleles; N=total number of chromosomes

[d] Conservation of amino acids determined from Human, Chimp, Rhesus, Mouse, Rat, Guinea Pig, Rabbit, Cow, Horse, Dog, Hedgehog, Chicken, Stickleback, and Zebrafish sequences

[e] Variant category for analysis; VR=very rare (MAF<1%), R=rare (MAF<5%)

Table 2

Association analysis of rare, nonsynonymous variants with types of clefts in US and Filipino cases versus controls

	MAF < 1.0%		MAF < 5.0%	
Population (N)	***P value***	***OR (95% CI)***	***P value***	***OR (95% CI)***
US				
CLO (70)	0.18	3.32 (0.60,18.49)	0.18	3.32 (0.60,18.49)
CLP (122)	0.58	NA (no case mutation)	0.58	NA (no case mutation)
CL/P (192)	1.00	1.19 (0.22, 6.55)	1.00	1.19 (0.22, 6.55)
Controls (456)				
Philippines				
CLO (180)	0.16	1.75 (0.86, 3.55)	0.18	1.60 (0.79, 3.21)
CLP (590)	0.67	1.16 (0.66, 2.07)	0.78	1.10 (0.63, 1.90)
CL/P (770)	0.36	1.30 (0.76, 2.20)	0.52	1.21 (0.73, 2.02)
Controls (516)				
Combined				
CLO (250)	0.02	2.32 (1.21, 4.45)	0.03	2.15 (1.13, 4.09)
CLP (712)	0.13	1.54 (0.90, 2.65)	0.18	1.48 (0.88, 2.51)
CL/P (962)	0.03	1.74 (1.06, 2.86)	0.04	1.65 (1.02, 2.67)
Controls (972)				

CL/P, cleft lip with or without cleft palate; CLP, cleft lip with cleft palate; CLO, cleft lip only