HHS Public Access

Author manuscript

Birth Defects Res. Author manuscript; available in PMC 2018 July 17.

Published in final edited form as:

Birth Defects Res. 2017 July 17; 109(13): 1030–1038. doi:10.1002/bdr2.23605.

Identifying genetic sources of phenotypic heterogeneity in orofacial clefts by targeted sequencing

Jenna C. Carlson[1], **Margaret A. Taub**[2], **Eleanor Feingold**[1,3], **Terri H. Beaty**[4], **Jeffrey C. Murray**[5], **Mary L. Marazita**[3,6,7], and **Elizabeth J. Leslie**[7,*]

[1] Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA, 15261, USA

[2] Department of Biostatistics, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA

[3] Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA, 15261, USA

[4] Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore MD, USA

[5] Department of Pediatrics, Carver College of Medicine, University of Iowa, Iowa City, Iowa, 52242, USA

[6] Clinical and Translational Science, School of Medicine, University of Pittsburgh, Pittsburgh, PA, 15213, USA

[7] Center for Craniofacial and Dental Genetics, Department of Oral Biology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA, 15219, USA

Abstract

Background—Orofacial clefts (OFCs), including nonsyndromic cleft lip with or without cleft palate (NSCL/P), are common birth defects. NSCL/P is highly heterogeneous with multiple phenotypic presentations. Two common subtypes of NSCL/P are cleft lip (CL) and cleft lip with cleft palate (CLP) which have different population prevalence. Similarly, NSCL/P can be divided into bilateral and unilateral clefts, with unilateral being the most common. Individuals with unilateral NSCL/P are more likely to be affected on the left side of the upper lip, but right side affection also occurs. Moreover, NSCL/P is twice as common in males as in females. The goal of this study is to discover genetic variants that have different effects in case subgroups.

Methods—We conducted both common variant and rare variant analyses in 1,034 individuals of Asian ancestry with NSCL/P, examining four sources of heterogeneity within CL/P: cleft type, sex, laterality, and side.

Results—We identified several regions associated with subtype differentiation – cleft type differences in 8q24 ($p=1.00\times10^{-4}$), laterality differences in *IRF6*, a gene previously implicated

* Corresponding author: Elizabeth J. Leslie (ejl40@pitt.edu) 100 Technology Drive, Suite 500 Bridgeside Point, Pittsburgh, PA 15219, 412-648-1488.

with wound healing ($p=2.166\times10^{-4}$), sex differences and side of unilateral CL differences in *FGFR2* ($p=3.00\times10^{-4}$, $p=6.00\times10^{-4}$), and sex differences in *VAX1* ($p<1.00\times10^{-4}$) among others.

Conclusions—Many of the regions associated with phenotypic modification were either adjacent to or overlapping functional elements based on ENCODE chromatin marks and published craniofacial enhancers. We have identified multiple common and rare variants as potential phenotypic modifiers of NSCL/P, and suggest plausible elements responsible for phenotypic heterogeneity, further elucidating the complex genetic architecture of OFCs.

Keywords

orofacial cleft; complex trait; genetic epidemiology

INTRODUCTION

Orofacial clefts (OFCs) are common birth defects, affecting approximately 1 in 800 births worldwide (Leslie and Marazita, 2013). Approximately 30% of OFCs are syndromic, occurring in combination with some other structural, cognitive, or developmental anomalies. The remaining 70% of OFCs occur as isolated (i.e. nonsyndromic) defects. Nonsyndromic OFCs have complex etiology with multiple genetic and environmental factors interacting to influence risk.

Nonsyndromic OFCs are highly heterogeneous with multiple phenotypic presentations (Dixon *et al.*, 2011). OFCs are most commonly divided into three major subtypes: cleft lip (CL), cleft palate (CP), and cleft lip with cleft palate (CLP). CL and CLP share a defect of the lip and are commonly combined for analyses as cleft lip with or without cleft palate (CL/P) (Fogh-Andersen, 1942; Fraser, 1955). CL/P and CP have historically been considered distinct disorders with separate etiologies because of the different developmental origins of the lip and palate and markedly different prevalence rates in males and females (CP is twice as common in females as in males, while the opposite is true for CL/P (Mossey *et al.*, 2009)). However, they occasionally occur within the same family, an even known as “mixed clefting” commonly observed in syndromic OFCs, including Van der Woude syndrome (Leslie and Marazita, 2013).

The CL/P subgroup itself is quite heterogeneous and can be further subdivided into bilateral and unilateral clefts, affecting either the left or right side of the upper lip. Of these, left sided unilateral clefts are the most common and bilateral clefts are the least common (Gundlach and Maus, 2006). The causes of variability in phenotype are largely unknown, and may arise due to underlying genetic factors, different environmental exposures, or other unknown factors. There have been many studies investigating the genetic architecture of NSCL/P, most collapsing cleft subtypes into one larger group (primarily CL/P) for analysis (Dixon *et al.*, 2011; Leslie and Marazita, 2013). While this approach is powerful to identify sources of genetic variation that contribute to overall NSCL/P, any signal from genetic variation specific to only one subtype or that differentiates subtypes will be masked. Very few studies have explored genetic associations for clefting phenotypes beyond CL and CLP. There is some evidence that the 13q31 locus near *SPRY2* has a stronger effect in CLP (Jia *et al.*, 2015; Ludwig *et al.*, 2012). Similarly, variants in *IRF6* are more strongly associated with CL

than CLP (Marazita *et al.*, 2009; Rahimov *et al.*, 2008). Recent evidence suggests that *GREM1* is associated with clefts in the lip and soft palate (Ludwig *et al.*, 2016). Furthermore, variants in *GRHL3* are associated with CP and not with CL/P (Leslie *et al.*, 2016b; Mangold *et al.*, 2016; Wang *et al.*, 2016). Examining CL/P subtypes may elucidate more of the complex genetic architecture of OFCs by identifying genetic mechanisms that modify cleft subtype.

We hypothesized that genetic components of phenotypic heterogeneity, including any contribution of rare variants, can be found for recognized clefting loci. We performed association tests for four sources of phenotypic heterogeneity within CL/P: cleft type (CL vs. CLP), sex (male vs. female), laterality (unilateral vs. bilateral), and side (right unilateral vs. left unilateral) in targeted sequencing from the CleftSeq study (Leslie *et al.*, 2015).

METHODS

Sample

We compared subtypes within clefting cases from the CleftSeq study to investigate the potential genetic contribution to clefting heterogeneity. CleftSeq is a targeted sequencing study of 13 previously reported loci associated with NSCL/P (Leslie *et al.*, 2015). These 13 regions, totaling 6.3 Mb, were comprised of 9 “high-priority” candidates from previous GWAS and/or genome-wide linkage studies and 4 regions containing candidate genes with prior evidence of rare variants contributing to NSCL/P (Table 1). Sequencing was performed on 1,498 case-parent trios from Europe, the United States, China and the Philippines.

From the 1,489 trios, we extracted 1,034 probands with NSCL/P of Asian (i.e. Chinese or Filipino) ancestry for analysis and cross-classified them using the four clefting subtype definitions (Table 2). Among the 1,034 cases, 33 with unknown laterality were excluded from the analysis of laterality and side of cleft lip groups.

Common Variant Analysis

For each factor (i.e. cleft type, sex, laterality, and side), we performed a case vs. case analysis, directly comparing allele frequencies at each SNP between the two groups (e.g. CL vs. CLP, male vs. female, etc.). This type of analysis has very high power to find genetic risk factors that differ between the two groups, but it has no power to find factors that are important in both groups. Thus this design is strictly a test for heterogeneity in the genotype/phenotype relationship, not an overall test of genetic effect. Ideally, this test will discover new loci for which there is an effect in only one subgroup; such loci may be masked in an overall scan when groups are combined.

We analyzed the association between the four cleft subtype phenotypes and 19,982 – 20,089 common SNPs (MAF > 0.01) in the thirteen candidate regions by directly comparing the two case subtypes using traditional Chi-Square tests for association. Each Asian population (Chinese and Filipino) was analyzed separately to account for any population stratification. Low-quality SNPs (missing genotypes > 5% or HWE $p < 0.0001$) were excluded from analyses.

Inverse-variance effects-based meta-analysis of the two population-specific scans was performed on 13,183 – 13,427 SNPs to detect any signal common to Asian populations. SNPs were excluded from the meta-analyses if they were flagged as low-quality in at least one population-specific analysis, or if effects were heterogeneous between populations (Cochran's Q $p < 0.05$). Statistical significance was determined using a Bonferroni threshold adjusting for four scans of thirteen regions of 9.615×10^{-4} (i.e. 0.05/52). This threshold allows for the generation of hypotheses regarding the genetic mechanisms of clefting subtypes and thus is not as strictly conservative as a Bonferroni correction for the number of markers tested (5200 tests, p-value threshold of 1×10^{-5} (Leslie *et al.*, 2015)). Thus, the suggestive associations found in this study should be followed up rigorously. Common variant analyses were performed using PLINK software (Purcell *et al.*, 2007).

Rare Variant Analysis

Rare variants (MAF < 0.01) were also interrogated for association with subtype differentiation using the same phenotype definitions as in the common variant analysis.

First, variants within exons of canonical transcripts of each gene were examined using gene-based versions of the Collapsed Multivariate and Combining (CMC) test (Li and Leal, 2008) and the Sequence Kernel Association Test (SKAT) (Wu *et al.*, 2011).

Secondly, two window-based approaches were used to investigate burdens of all rare variants. SNPs were combined into regions using two window-based methods – 2,662 windows using a fixed window size of 5Kb with 2.5Kb overlap between windows, and 14,232 windows using exactly 20 SNPs per window with 10 SNP overlap between windows (windows at the end of each region contained at least 14 SNPs). Each window was comprised of SNPs from only one of the candidate regions. Windows are highly correlated within each candidate region, so statistical significance was again determined using a Bonferroni threshold of 9.615×10^{-4}. Rare variants were analyzed with the SKAT option in RVTESTS software (Zhan *et al.*, 2016).

Functional Annotation of Rare Variant Windows

The CleftSeq project sequenced 6.3Mb of largely noncoding DNA around these GWAS and OFC candidate genes. We failed to identify significant associations in analyses of coding variants (results not shown), so we hypothesized that functional variants would be regulatory. We examined intervals containing overlapping windows for functional elements based on ENCODE chromatin marks (Consortium *et al.*, 2011; Rosenbloom *et al.*, 2013) and published craniofacial enhancers (Attanasio *et al.*, 2013; Brinkley *et al.*, 2016; Rada-Iglesias *et al.*, 2012).

RESULTS

Cleft Type

In the common variant meta-analysis, 20 SNPs from 4 loci were significantly associated with CL v. CLP differentiation (Figure 1A). These associations were seen in SNPs on 9q22 near *PTCH1* and *FOXE1*, on 17p22 near *NOG*, and on 20q12 near *MAFB*. Specifically, a set

of variants in and near PTCH1 were more strongly associated with CL than with CLP (lead SNP: rs202111971 $p = 6.484 \times 10^{-4}$, Figure 1B). A neighboring set of variants did not show formally significant differences by cleft type, but tended to be more strongly associated with CLP (Figure 1B). In the 9q22 region, a set of SNPs downstream of the *FOXE1* transcription start site were more strongly associated with CLP than with CL (lead SNP: rs73492791 $p = 1.138 \times 10^{-4}$, Figure 1C). Moreover, minor alleles in the 17p22 regions and 20q12 regions were more strongly associated with CLP (lead SNPs: rs7208145 $p = 9.041 \times 10^{-4}$, rs6129626 $p = 5.039 \times 10^{-4}$, Figure 1D-E). Notably, none of these SNPs associated with cleft type differentiation (CL vs. CLP) was significantly associated with risk of OFC overall (Leslie *et al.*, 2015).

Twenty-five windows of rare variants in the *PAX7*, *ARHGAP29*, *8q24*, *FOXE1*, *VAX1*, *NTN1*, and *NOG* sequencing regions were significantly associated with cleft type differentiation (CL vs. CLP) (Supplementary Material, Table S1). Of these, two sets of three overlapping windows (8:129790677-129795772 [min $p = 4.50 \times 10^{-4}$] and 8:130298273-130305772 [min $p = 1.00 \times 10^{-4}$]) on 8q24 are particularly interesting because they contain SNPs that individually show strong association with NSCL/P in Europeans. Furthermore, one of these intervals (8:129790677-129795772) consisting of three overlapping windows was located adjacent to a putative regulatory element as defined by H3K27Ac marks in multiple cell types from ENCODE (Figure 4A).

Laterality

In the common variant meta-analysis, 27 SNPs from 2 loci were significantly associated with laterality differences (Figure 2A). These associations were seen for 26 SNPs on 1q32 near *IRF6* (lead SNP: rs6540559 $p = 2.166 \times 10^{-4}$, Figure 2B) and a single SNP on 17p22 near *NOG* (rs184942776 $p = 5.262 \times 10^{-5}$, Figure 2C). SNPs in *IRF6* were associated with differentiation between bilateral and unilateral CL/P. Specifically, minor alleles of SNPs in *IRF6* were associated with unilateral CL/P. The minor alleles at these SNPs also are significantly protective against overall OFC risk (Supplementary Material, Table S5).

Differences in CL/P laterality were observed in 17 windows of rare variants (Supplementary Material, Table S2). Despite having many overlapping windows of rare variants, there was no evidence of known regulatory or enhancer elements within these intervals.

Sex

While no significant associations for sex differences were observed in the common variant analysis (Figure 3A), 28 windows of rare variants were significantly associated with sex differences (Supplementary Material, Table S3).

Eight windows defining three larger intervals (10:118624030-118629029 [min $p = 5.00 \times 10^{-4}$], 10:118638519-118644029 [min $p < 1.00 \times 10^{-4}$], and 10:118851530-11885725 [min $p < 1.00 \times 10^{-4}$]) near *VAX1* were significantly associated with sex differences in Filipinos. One of these intervals (10:118851530-11885725), comprised of three windows near *VAX1*, overlapped a craniofacial regulatory element identified from p300 ChIP-Seq in craniofacial tissue in mouse embryos (Attanasio *et al.*, 2013; Visel *et al.*, 2009) (Figure 4B). It is unclear what gene is regulated by this element, as the activity pattern of the enhancer resembles the

endogenous expression of both adjacent genes *VAX1* and *SHTN1* (Armit *et al.*, 2012; Diez-Roux *et al.*, 2011). Interestingly, other significant windows in this region occurred immediately downstream of *SHTN1*.

Two non-overlapping windows near *FGFR2* (10:123368869-123373868 [$p = 3.00 \times 10^{-4}$] and 10:123479803-123483275 [$p = 8.00 \times 10^{-4}$]) were also significantly associated with sex differences in Filipinos. The first of these windows overlapped multiple regulatory annotations including a binding site for p63, a transcription factor known to regulate *FGFR2* (Ferone *et al.*, 2012; Fomenkov *et al.*, 2003) (Figure 4C). The second window overlaps more regulatory annotations characteristic of epithelial enhancers (Figure 4C).

Side of Lip

We did not observe any significant associations with right unilateral vs. left unilateral CL/P in the common variant analysis (Figure 3B). However, 13 windows of rare variants were significantly associated with side of cleft lip differentiation (Supplementary Material, Table S4). Interestingly, one window near *FGFR2* (10:123431369-123436368 [$p = 6.00 \times 10^{-4}$]) was significantly associated with side of cleft lip differentiation in Filipinos and was adjacent to active enhancers from human neural crest cell lines and a putative palate enhancer from p300 ChIP-seq of mouse palatal tissue (Figure 4C).

DISCUSSION

NSCL/P is a complex disorder with many different anatomical forms. GWASs have identified dozens of genetic associations with NSCL/P (Beaty *et al.*, 2010; Leslie *et al.*, 2016a; Ludwig *et al.*, 2012; Mangold *et al.*, 2010); however, a small number of studies have identified cleft subtype specific associations, most of which are reflect differences between CL and CLP (Ludwig *et al.*, 2012; Marazita *et al.*, 2009; Rahimov *et al.*, 2008). The current study adds to these findings by identifying both common and rare variants that are associated with subtype differentiation in cleft type, laterality, sex, and side of unilateral CL. We performed common and rare variant association testing with four cleft subtypes (cleft type: CL vs. CLP; laterality: unilateral vs. bilateral; sex; and side: right vs. left CL/P) to further interrogate OFC-associated regions from the CleftSeq targeting sequencing study. We identified several regions associated with cleft subtype differentiation – common variants in *IRF6* and rare variants in 8q24, *FGFR2*, and *VAX1* among others. Notably, these associations are found with both previously known clefting-associated variants and variants that were not significantly associated with overall clefting (CL/P). Multiple associations with regulatory (non-coding) elements and differences in clefting subtypes, contributing to the evidence that non-coding variants have a significant role in the genetic causes of NSCL/P (Leslie and Marazita, 2013; Leslie *et al.*, 2015; Rahimov *et al.*, 2008). However, it is not clear from the association results which alleles are relevant to these phenotypes; systematic studies in model systems will likely be required to identify functional SNPs and a possible mechanism.

We identified 26 SNPs within *IRF6* associated with differences between unilateral and bilateral CL/P. Specifically, individuals with unilateral CL/P had higher frequencies of minor alleles in these 26 variants than did bilateral CL/P individuals. *IRF6* has been previously

implicated in wound healing (Biggs *et al.*, 2014; Biggs *et al.*, 2012; Jones *et al.*, 2010), so these cleft laterality differences are particularly interesting. The same alleles showing a protective effect for overall cleft risk were more strongly associated with unilateral CL/P than bilateral. If we consider unilateral CL/P as a less severe presentation of clefting than bilateral CL/P, our finding that OFC-protective variants are associated more strongly with unilateral CL/P and previous evidence that IRF6 is associated with CL (Rahimov *et al.*, 2008) together suggest that the IRF6 locus is associated with decreased risk of severe clefting.

Rare variants on 8q24 were found to significantly differ between CL and CLP, including an interval adjacent to a putative regulatory element. This provides strong evidence for a regulatory role of variants within 8q24 on the presentation of NSCL/P. Furthermore, SNPs on 8q24 have previously shown very strong association with cleft risk in European GWAS (Beaty *et al.*, 2011; Birnbaum *et al.*, 2009; Grant *et al.*, 2009; Murray *et al.*, 2012), but are not associated with cleft risk in Asian GWAS(i.e. in common variant analyses). This may be due to population-specific differences in SNP informativeness within 8q24, which reflects haplotype diversity (Murray *et al.*, 2012). SNPs within 8q24 have markedly higher heterozygosity in Europeans than Asians, making common-variant associations within this region far more powerful among Europeans. We hypothesize that this region also is associated with clefting risk in other populations although the statistical evidence from analyses of common variants is lacking. The association with cleft type differentiation within windows of 8q24 rare variants observed in the Filipino population here may be evidence that some SNPs within 8q24 have with clefting risk in Asian populations.

Additionally, rare variant associations with potential regulatory elements were observed when examining sex differences and markers near *VAX1* and *FGFR2* and those near *FGFR2* and the left vs. right side of unilateral CL/P. While it is not immediately clear how *VAX1* and *FGFR2* specifically contribute to sex differences in NSCL/P, biological hypotheses regarding sex differences in other disorders (e.g. autism) involve a multiple-threshold multifactorial liability model in which females have a higher threshold than males. In other words, affected females are hypothesized to carry a higher mutational burden than affected males. The same would hold for NSCL/P, where there are more affected males than females. Under this hypothesis, relatives of affected females are at increased risk for CL/P, which is supported by population-based recurrence risk estimates from Denmark (Grosen *et al.*, 2010). A similar threshold model may also pertain to differences in laterality and severity of NSCL/P.

Contrary to the common disease-common variant hypothesis, we observed clear contributions from both common and rare variants in this study of the genetic underpinnings of NSCL/P and the potential differences within NSCL/P subtypes. This work adds to a growing body of evidence implicating rare variants in risk of NSCL/P (Al Chawa *et al.*, 2014; Leslie and Murray, 2012; Leslie *et al.*, 2015). Importantly, this work highlights the impact of rare variants as potential phenotypic modifiers, an area that needs larger studies in additional populations that are expanded to the entire genome. As costs of whole genome sequencing decrease, these studies will be more feasible for NSCL/P and will continue to improve our understanding of the genetic architecture of NSCL/P.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We are grateful to the families who participated in this study, recruitment staff, and the Operations Group and the Bioinformatics Group at the McDonnell Genome Institute. This work was supported by grants from the NIH (HG005925 [J.C.M., M.L.M.], DE008559 [J.C.M., M.L.M.], DE009886 [M.L.M.], DE016930 [M.L.M.], DE016148 [M.L.M.], DE014581 [T.H.B.], DE018993 [T.H.B.], and DE025060 [E.J.L].

LITERATURE CITED

Al Chawa T, Ludwig KU, Fier H, Potzsch B, Reich RH, Schmidt G, Braumann B, Daratsianos N, Bohmer AC, Schuencke H, Alblas M, Fricker N, Hoffmann P, Knapp M, Lange C, Nothen MM, Mangold E. Nonsyndromic cleft lip with or without cleft palate: Increased burden of rare variants within Gremlin-1, a component of the bone morphogenetic protein 4 pathway. Birth defects research Part A, Clinical and molecular teratology. 2014

Armit C, Venkataraman S, Richardson L, Stevenson P, Moss J, Graham L, Ross A, Yang Y, Burton N, Rao J, Hill B, Rannie D, Wicks M, Davidson D, Baldock R. eMouseAtlas, EMAGE, and the spatial dimension of the transcriptome. Mammalian genome : official journal of the International Mammalian Genome Society. 2012; 23(9-10):514–524. [PubMed: 22847374]

Attanasio C, Nord AS, Zhu Y, Blow MJ, Li Z, Liberton DK, Morrison H, Plajzer-Frick I, Holt A, Hosseini R, Phouanenavong S, Akiyama JA, Shoukry M, Afzal V, Rubin EM, FitzPatrick DR, Ren B, Hallgrimsson B, Pennacchio LA, Visel A. Fine tuning of craniofacial morphology by distant-acting enhancers. Science. 2013; 342(6157):1241006. [PubMed: 24159046]

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. Nature genetics. 2010; 42(6):525–529. [PubMed: 20436469]

Beaty TH, Ruczinski I, Murray JC, Marazita ML, Munger RG, Hetmanski JB, Murray T, Redett RJ, Fallin MD, Liang KY, Wu T, Patel PJ, Jin SC, Zhang TX, Schwender H, Wu-Chou YH, Chen PK, Chong SS, Cheah F, Yeow V, Ye X, Wang H, Huang S, Jabs EW, Shi B, Wilcox AJ, Lie RT, Jee SH, Christensen K, Doheny KF, Pugh EW, Ling H, Scott AF. Evidence for gene-environment interaction in a genome wide study of nonsyndromic cleft palate. Genetic epidemiology. 2011; 35(6):469–478. [PubMed: 21618603]

Biggs LC, Naridze RL, DeMali KA, Lusche DF, Kuhl S, Soll DR, Schutte BC, Dunnwald M. Interferon regulatory factor 6 regulates keratinocyte migration. J Cell Sci. 2014; 127:2840–2848. Pt 13. [PubMed: 24777480]

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]

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. Nature genetics. 2009; 41(4):473–477. [PubMed: 19270707]

Brinkley JF, Fisher S, Harris MP, Holmes G, Hooper JE, Jabs EW, Jones KL, Kesselman C, Klein OD, Maas RL, Marazita ML, Selleri L, Spritz RA, van Bakel H, Visel A, Williams TJ, Wysocka J, FaceBase C, Chai Y. The FaceBase Consortium: a comprehensive resource for craniofacial researchers. Development. 2016; 143(14):2677–2688. [PubMed: 27287806]

Consortium EP, Myers RM, Stamatoyannopoulos J, Snyder M, Dunham I, Hardison RC, Bernstein BE, Gingeras TR, Kent WJ, Birney E. A user's guide to the encyclopedia of DNA elements (ENCODE). PLoS biology. 2011; 9(4):e1001046. [PubMed: 21526222]

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. Nature reviews Genetics. 2011; 12(3):167–178.

Ferone G, Thomason HA, Antonini D, De Rosa L, Hu B, Gemei M, Zhou H, Ambrosio R, Rice DP, Acampora D, van Bokhoven H, Del Vecchio L, Koster MI, Tadini G, Spencer-Dene B, Dixon M, Dixon J, Missero C. Mutant p63 causes defective expansion of ectodermal progenitor cells and impaired FGF signalling in AEC syndrome. EMBO Mol Med. 2012; 4(3):192–205. [PubMed: 22247000]

Fogh-Andersen, P. Inheritance of Harelip and Cleft Palate. Munksgaard; Copenhagen: 1942.

Fomenkov A, Huang YP, Topaloglu O, Brechman A, Osada M, Fomenkova T, Yuriditsky E, Trink B, Sidransky D, Ratovitski E. P63 alpha mutations lead to aberrant splicing of keratinocyte growth factor receptor in the Hay-Wells syndrome. J Biol Chem. 2003; 278(26):23906–23914. [PubMed: 12692135]

Fraser FC. Thoughts on the etiology of clefts of the palate and lip. Acta genetica et statistica medica. 1955; 5(4):358–369. [PubMed: 13339079]

Grant SF, Wang K, Zhang H, Glaberson W, Annaiah K, Kim CE, Bradfield JP, Glessner JT, Thomas KA, Garris M, Frackelton EC, Otieno FG, Chiavacci RM, Nah HD, Kirschner RE, Hakonarson H. A genome-wide association study identifies a locus for nonsyndromic cleft lip with or without cleft palate on 8q24. The Journal of pediatrics. 2009; 155(6):909–913. [PubMed: 19656524]

Grosen D, Chevrier C, Skytthe A, Bille C, Molsted K, Sivertsen A, Murray JC, Christensen K. A cohort study of recurrence patterns among more than 54,000 relatives of oral cleft cases in Denmark: support for the multifactorial threshold model of inheritance. Journal of Medical Genetics. 2010; 47(3):162–168. [PubMed: 19752161]

Gundlach KK, Maus C. Epidemiological studies on the frequency of clefts in Europe and world-wide. Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 2006; 34(Suppl 2):1–2.

Jia Z, Leslie EJ, Cooper ME, Butali A, Standley J, Rigdon J, Suzuki S, Gongorjav A, Shonkhuuz TE, Natsume N, Shi B, Marazita ML, Murray JC. Replication of 13q31.1 association in nonsyndromic cleft lip with cleft palate in Europeans. American journal of medical genetics Part A. 2015; 167(5): 1054–1060.

Jones JL, Canady JW, Brookes JT, Wehby GL, L'Heureux J, Schutte BC, Murray JC, Dunnwald M. Wound complications after cleft repair in children with Van der Woude syndrome. The Journal of craniofacial surgery. 2010; 21(5):1350–1353. [PubMed: 20856020]

Leslie E, Murray J. Evaluating rare coding variants as contributing causes to non-syndromic cleft lip and palate. Clinical genetics:n/a-n/a. 2012

Leslie EJ, Carlson JC, Shaffer JR, Feingold E, Wehby G, Laurie CA, Jain D, Laurie CC, Doheny KF, McHenry T, Resick J, Sanchez C, Jacobs J, Emanuele B, Vieira AR, Neiswanger K, Lidral AC, Valencia-Ramirez LC, Lopez-Palacio AM, Valencia DR, Arcos-Burgos M, Czeizel AE, Field LL, Padilla CD, Cutiongco-de la Paz EM, Deleyiannis F, Christensen K, Munger RG, Lie RT, Wilcox

A, Romitti PA, Castilla EE, Mereb JC, Poletta FA, Orioli IM, Carvalho FM, Hecht JT, Blanton SH, Buxo CJ, Butali A, Mossey PA, Adeyemo WL, James O, Braimah RO, Aregbesola BS, Eshete MA, Abate F, Koruyucu M, Seymen F, Ma L, de Salamanca JE, Weinberg SM, Moreno L, Murray JC, Marazita ML. A multi-ethnic genome-wide association study identifies novel loci for non-syndromic cleft lip with or without cleft palate on 2p24.2, 17q23 and 19q13. Hum Mol Genet. 2016a

Leslie EJ, Liu H, Carlson JC, Shaffer JR, Feingold E, Wehby G, Laurie CA, Jain D, Laurie CC, Doheny KF, McHenry T, Resick J, Sanchez C, Jacobs J, Emanuele B, Vieira AR, Neiswanger K, Standley J, Czeizel AE, Deleyiannis F, Christensen K, Munger RG, Lie RT, Wilcox A, Romitti PA, Field LL, Padilla CD, Cutiongco-de la Paz EM, Lidral AC, Valencia-Ramirez LC, Lopez-Palacio AM, Valencia DR, Arcos-Burgos M, Castilla EE, Mereb JC, Poletta FA, Orioli IM, Carvalho FM, Hecht JT, Blanton SH, Buxo CJ, Butali A, Mossey PA, Adeyemo WL, James O, Braimah RO, Aregbesola BS, Eshete MA, Deribew M, Koruyucu M, Seymen F, Ma L, de Salamanca JE, Weinberg SM, Moreno L, Cornell RA, Murray JC, Marazita ML. A Genome-wide Association Study of Nonsyndromic Cleft Palate Identifies an Etiologic Missense Variant in GRHL3. American journal of human genetics. 2016b; 98(4):744–754. [PubMed: 27018472]

Leslie EJ, Marazita ML. Genetics of cleft lip and cleft palate. American journal of medical genetics Part C, Seminars in medical genetics. 2013; 163C(4):246–258.

Leslie EJ, Taub MA, Liu H, Steinberg KM, Koboldt DC, Zhang Q, Carlson JC, Hetmanski JB, Wang H, Larson DE, Fulton RS, Kousa YA, Fakhouri WD, Naji A, Ruczinski I, Begum F, Parker MM, Busch T, Standley J, Rigdon J, Hecht JT, Scott AF, Wehby GL, Christensen K, Czeizel AE, Deleyiannis FW, Schutte BC, Wilson RK, Cornell RA, Lidral AC, Weinstock GM, Beaty TH, Marazita ML, Murray JC. Identification of Functional Variants for Cleft Lip with or without Cleft Palate in or near PAX7, FGFR2, and NOG by Targeted Sequencing of GWAS Loci. American journal of human genetics. 2015

Li B, Leal SM. Methods for detecting associations with rare variants for common diseases: application to analysis of sequence data. American journal of human genetics. 2008; 83(3):311–321. [PubMed: 18691683]

Ludwig KU, Ahmed ST, Bohmer AC, Sangani NB, Varghese S, Klamt J, Schuenke H, Gultepe P, Hofmann A, Rubini M, Aldhorae KA, Steegers-Theunissen RP, Rojas-Martinez A, Reiter R, Borck G, Knapp M, Nakatomi M, Graf D, Mangold E, Peters H. Meta-analysis Reveals Genome-Wide Significance at 15q13 for Nonsyndromic Clefting of Both the Lip and the Palate, and Functional Analyses Implicate GREM1 As a Plausible Causative Gene. PLoS Genet. 2016; 12(3):e1005914. [PubMed: 26968009]

Ludwig KU, Mangold E, Herms S, Nowak S, Reutter H, Paul A, Becker J, Herberz R, AlChawa T, Nasser E, Bohmer AC, Mattheisen M, Alblas MA, Barth S, Kluck N, Lauster C, Braumann B, Reich RH, Hemprich A, Potzsch S, Blaumeiser B, Daratsianos N, Kreusch T, Murray JC, Marazita ML, Ruczinski I, Scott AF, Beaty TH, Kramer FJ, Wienker TF, Steegers-Theunissen RP, Rubini M, Mossey PA, Hoffmann P, Lange C, Cichon S, Propping P, Knapp M, Nothen MM. Genome-wide meta-analyses of nonsyndromic cleft lip with or without cleft palate identify six new risk loci. Nature genetics. 2012; 44(9):968–971. [PubMed: 22863734]

Mangold E, Bohmer AC, Ishorst N, Hoebel AK, Gultepe P, Schuenke H, Klamt J, Hofmann A, Golz L, Raff R, Tessmann P, Nowak S, Reutter H, Hemprich A, Kreusch T, Kramer FJ, Braumann B, Reich R, Schmidt G, Jager A, Reiter R, Brosch S, Stavusis J, Ishida M, Seselgyte R, Moore GE, Nothen MM, Borck G, Aldhorae KA, Lace B, Stanier P, Knapp M, Ludwig KU. Sequencing the GRHL3 Coding Region Reveals Rare Truncating Mutations and a Common Susceptibility Variant for Nonsyndromic Cleft Palate. American journal of human genetics. 2016; 98(4):755–762. [PubMed: 27018475]

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. Nature genetics. 2010; 42(1):24–26. [PubMed: 20023658]

Marazita ML, Lidral AC, Murray JC, Field LL, Maher BS, Goldstein McHenry T, Cooper ME, Govil M, Daack-Hirsch S, Riley B, Jugessur A, Felix T, Morene L, Mansilla MA, Vieira AR, Doheny K, Pugh E, Valencia-Ramirez C, Arcos-Burgos M. Genome scan, fine-mapping, and candidate gene analysis of non-syndromic cleft lip with or without cleft palate reveals phenotype-specific differences in linkage and association results. Human heredity. 2009; 68(3):151–170. [PubMed: 19521098]

Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. Cleft lip and palate. Lancet. 2009; 374(9703): 1773–1785. [PubMed: 19747722]

Murray T, Taub MA, Ruczinski I, Scott AF, Hetmanski JB, Schwender H, Patel P, Zhang TX, Munger RG, Wilcox AJ, Ye X, Wang H, Wu T, Wu-Chou YH, Shi B, Jee SH, Chong S, Yeow V, Murray JC, Marazita ML, Beaty TH. Examining markers in 8q24 to explain differences in evidence for association with cleft lip with/without cleft palate between Asians and Europeans. Genetic epidemiology. 2012; 36(4):392–399. [PubMed: 22508319]

Pruim RJ, Welch RP, Sanna S, Teslovich TM, Chines PS, Gliedt TP, Boehnke M, Abecasis GR, Willer CJ. LocusZoom: regional visualization of genome-wide association scan results. Bioinformatics. 2010; 26(18):2336–2337. [PubMed: 20634204]

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. American journal of human genetics. 2007; 81(3):559–575. [PubMed: 17701901]

Rada-Iglesias A, Bajpai R, Prescott S, Brugmann SA, Swigut T, Wysocka J. Epigenomic annotation of enhancers predicts transcriptional regulators of human neural crest. Cell stem cell. 2012; 11(5): 633–648. [PubMed: 22981823]

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. Nature genetics. 2008; 40(11):1341–1347. [PubMed: 18836445]

Rosenbloom KR, Sloan CA, Malladi VS, Dreszer TR, Learned K, Kirkup VM, Wong MC, Maddren M, Fang R, Heitner SG, Lee BT, Barber GP, Harte RA, Diekhans M, Long JC, Wilder SP, Zweig AS, Karolchik D, Kuhn RM, Haussler D, Kent WJ. ENCODE data in the UCSC Genome Browser: year 5 update. Nucleic acids research. 2013; 41:D56–63. Database issue. [PubMed: 23193274]

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

Wang Y, Sun Y, Huang Y, Pan Y, Jia Z, Ma L, Ma L, Lan F, Zhou Y, Shi J, Yang X, Zhang L, Jiang H, Jiang M, Yin A, Cheng J, Wang L, Yang Y, Shi B. Association study between Van der Woude Syndrome causative gene GRHL3 and nonsyndromic cleft lip with or without cleft palate in a Chinese cohort. Gene. 2016; 588(1):69–73. [PubMed: 27129939]

Wu MC, Lee S, Cai T, Li Y, Boehnke M, Lin X. Rare-variant association testing for sequencing data with the sequence kernel association test. American journal of human genetics. 2011; 89(1):82–93. [PubMed: 21737059]

Zhan X, Hu Y, Li B, Abecasis GR, Liu DJ. RVTESTS: An Efficient and Comprehensive Tool for Rare Variant Association Analysis Using Sequence Data. Bioinformatics. 2016

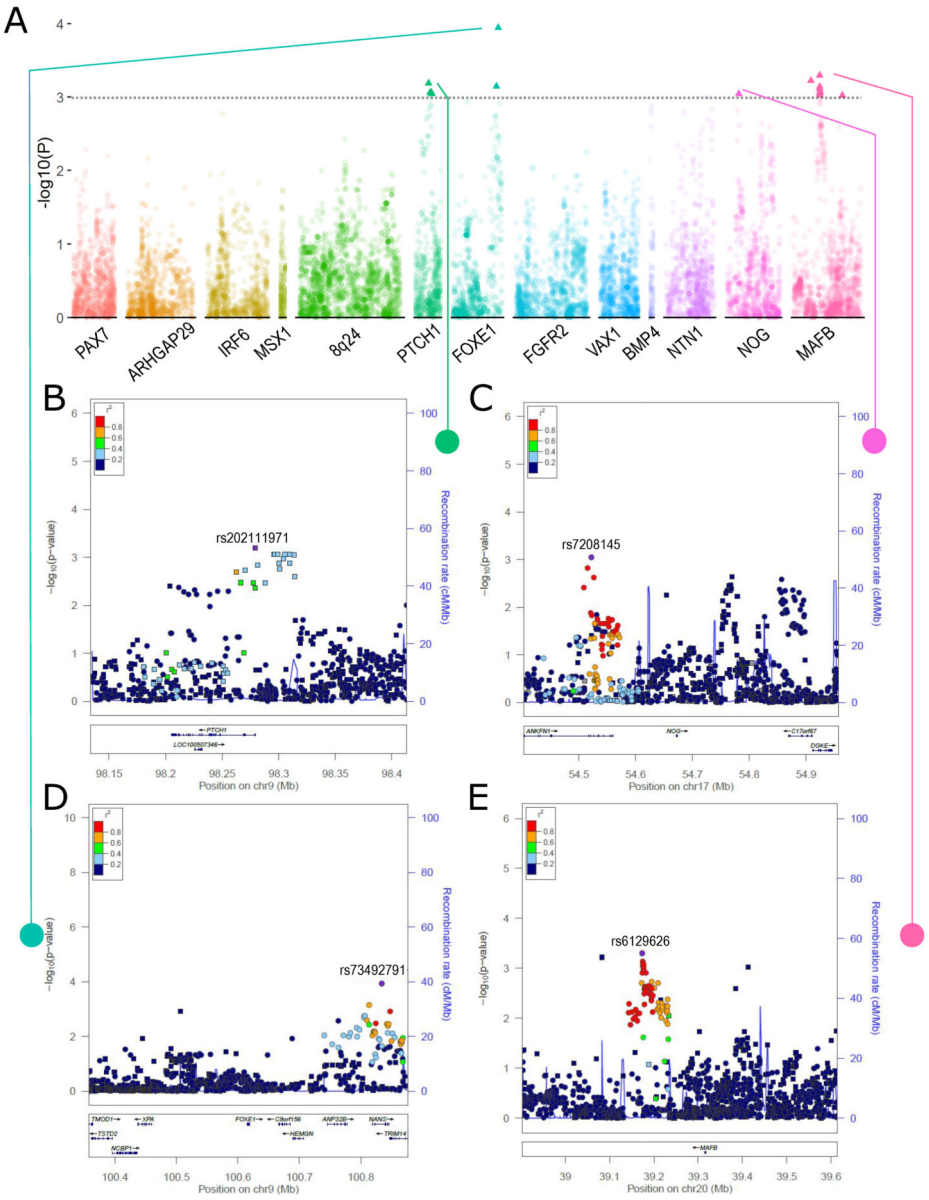

Figure 1. CL vs. CLP cleft type modifiers

A) Cleft type (CL vs. CLP) association results from the common-variant meta-analysis of Filipino and Chinese populations. (B) – (E) Regional association plots for 9q22 (x2), 17q22, and 20q12 showing $-\log_{10}$(P-values) for SNPs with stronger association with CL (squares) and stronger association with CLP (circles) based on the direction of the odds ratio. Plots were generated using LocusZoom (Pruim *et al.*, 2010). The recombination overlay (blue line, right y-axis) indicates the boundaries of the LD-block. Points are color coded according to pairwise linkage disequilibrium (r2) with the index SNP.

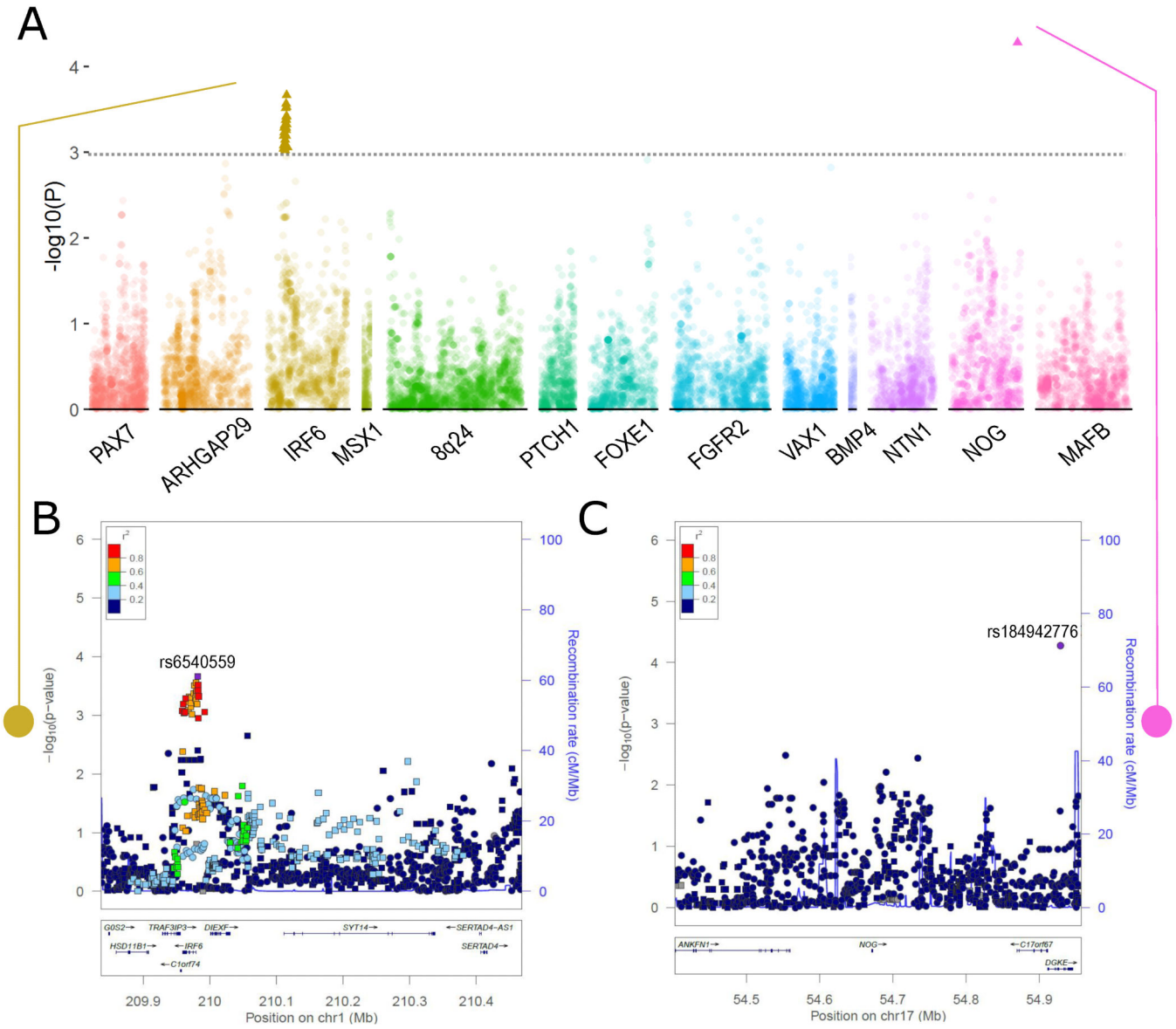

Figure 2. Unilateral vs. bilateral CL/P modifiers

(A) Laterality (unilateral vs. bilateral) association results from the common-variant meta-analysis of Filipino and Chinese populations. (B) – (C) Regional association plots for 1q32 and 17q22 showing $-\log_{10}$(P-values) for SNPs with stronger association with unilateral CL/P (squares) and stronger association with bilateral CL/P (circles) based on the direction of the odds ratio. Plots were generated using LocusZoom (Pruim *et al.*, 2010). The recombination overlay (blue line, right y-axis) indicates the boundaries of the LD-block. Points are color coded according to pairwise linkage disequilibrium (r2) with the index SNP.

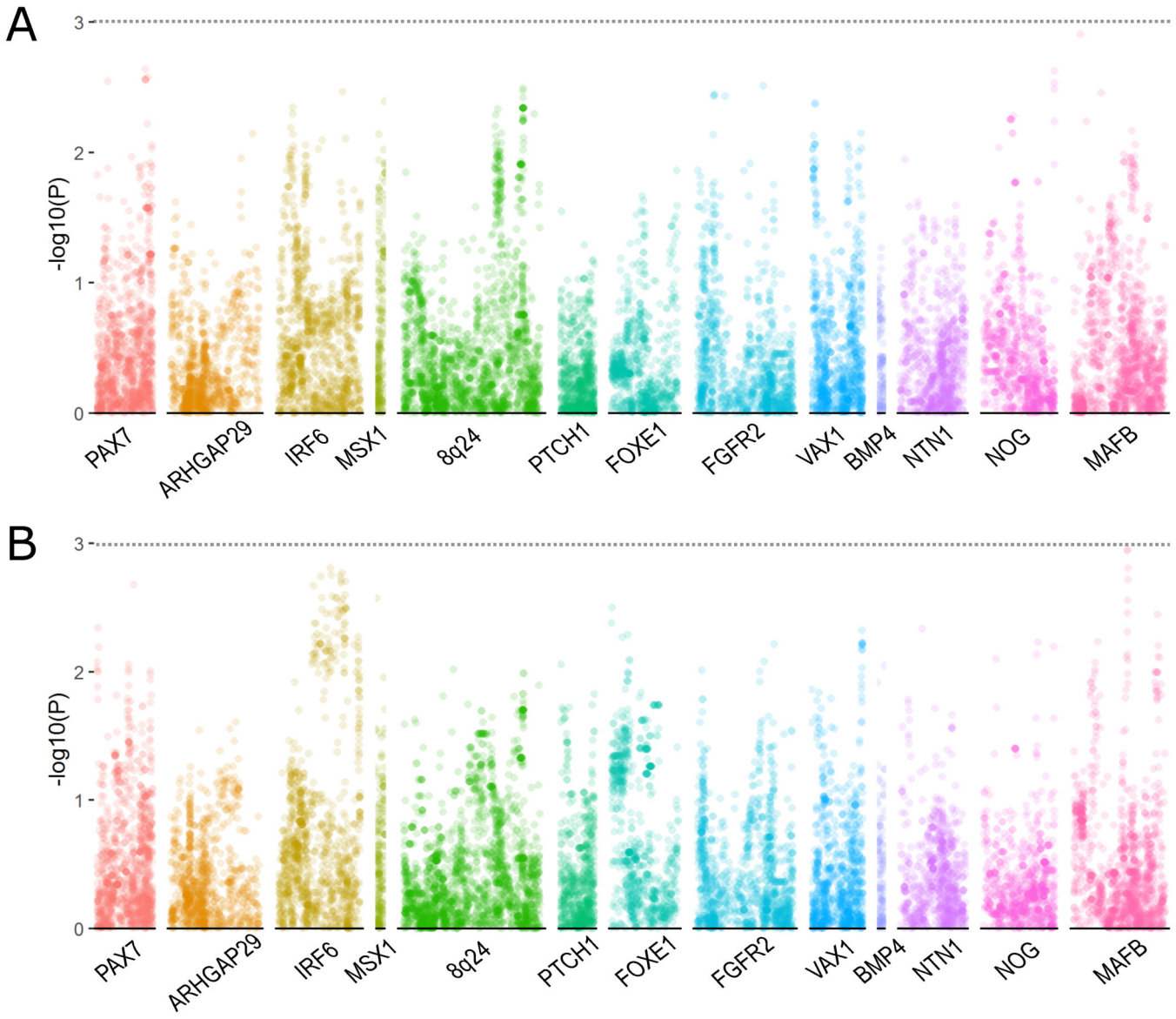

Figure 3. Sex-specific and side modifiers of CL/P

(A) Sex (male vs. female) association results from the common-variant meta-analysis of Filipino and Chinese populations. (B) Side (right unilateral vs. left unilateral) association results from the common-variant meta-analysis of Filipino and Chinese populations.

Figure 4. Significant rare variant windows with potential regulatory effects

(A) 8q24 for cleft type, (B) VAX1 for sex, and (C) FGFR2 for sex and side.

Table 1

CleftSeq regions.

	Region	Candidate Gene in Region	Target Region (GRCh37)	Size (kb)
previous GWAS hits	1p36	*PAX7*	chr1: 18,772,300 – 19,208,054	435.8
	1p22	*ARHGAP29*	chr1: 94,324,660 – 95,013,109	688.4
	1q32	*IRF6*	chr1: 209,837,199 – 210,468,406	631.2
	8q24	--	chr8: 129,295,896 – 130,354,946	1059.1
	10q25	*VAX1*	chr10: 118,421,625 – 119,167,424	745.8
	17p13	*NTN1*	chr17: 8,755,114 – 9,266,060	510.9
	17p22	*NOG*	chr17: 54,402,837 – 54,957,390	554.6
	20q12	*MAFB*	chr20: 38,902,646 – 39,614,513	711.9
previous linkage hit	9q22	*FOXE1*	chr9: 100,357,692 – 100,876,841	519.1
candidate gene regions (evidence for rare variants)	4p16	*MSX1*	chr4: 4,825,126 – 4,901,385	76.3
	14q22	*BMP4*	chr14: 54,382,690 – 54,445,053	62.4
	10q26	*FGFR2*	chr10: 123,096,374 – 123,498,771	402.4
	9q22	*PTCH1*	chr9: 98,133,647 – 98,413,162	279.5

Table 2

Sample used for modifier analyses by population.

	Cleft Type		Sex		Laterality		Side of CL	
	CL	*CLP*	*Female*	*Male*	*Unilateral*	*Bilateral*	*Right unilateral*	*Left unilateral*
China	117	284	126	275	278	101	112	166
Philippines	171	462	219	414	440	182	147	293
Total	***288***	***746***	***345***	***689***	***718***	***282***	***259***	***459***