Identification of common non-coding variants at 1p22 that are functional for non-syndromic orofacial clefting

Huan Liu1,2, Elizabeth J. Leslie3, Jenna C. Carlson4, Terri H. Beaty5, Mary L. Marazita3,6, Andrew C. Lidral7 & Robert A. Cornell1

Genome-wide association studies (GWAS) do not distinguish between single nucleotide polymorphisms (SNPs) that are causal and those that are merely in linkage-disequilibrium with causal mutations. Here we describe a versatile, functional pipeline and apply it to SNPs at 1p22, a locus identified in several GWAS for non-syndromic cleft lip with or without cleft palate (NS CL/P). First we amplified DNA elements containing the ten most-highly risk-associated SNPs and tested their enhancer activity in vitro, identifying three SNPs with allele-dependent effects on such activity. We then used in vivo reporter assays to test the tissue-specificity of these enhancers, chromatin configuration capture to test enhancer-promoter interactions, and genome editing in vitro to show allele-specific effects on ARHGAP29 expression and cell migration. Our results further indicate that two SNPs affect binding of CL/P-associated transcription factors, and one affects chromatin configuration. These results translate risk into potential mechanisms of pathogenesis.

1Department of Anatomy and Cell Biology, College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA. 2State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory for Oral Biomedicine of Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan, Hubei 430079, China. 3Center for Craniofacial and Dental Genetics, Department of Oral Biology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15219, USA. 4Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA. 5Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA. 6Department of Human Genetics, Graduate School of Public Health and Clinical and Translational Science Institute, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15219, USA. 7Department of Orthodontics, College of Dentistry, University of Iowa, Iowa City, Iowa 52246, USA. Correspondence and requests for materials should be addressed to R.A.C. (email: robert-cornell@uiowa.edu).
left lip with or without cleft palate (CL/P) affects about 1 in 700 live births in the US, and has both genetic and environmental underpinnings. About 30% of cases of CL/P are syndromic and may be inherited in Mendelian fashion, whereas the remaining cases are non-syndromic (NS) and appear to be controlled by multiple genes and environmental factors. Collectively, eight independent genome-wide association studies (GWAS)\(^3\)–\(^10\), a linkage study\(^1\), meta-analyses of GWAS\(^12\)–\(^14\) and several replication studies (for example, ref. 13) provide statistical support achieving genome-wide significance for associations between single nucleotide polymorphism (SNP) markers and risk for NS CL/P. The GWAS approach has been unusually successful in identifying loci in which variation contributes significantly to risk for NS CL/P (ref. 8), in comparison to its degree of success for other complex diseases\(^15\).

There are several challenges to translating statistical associations revealed by GWAS into an understanding of the biological causes of NS CL/P. First, only a subset of SNPs in linkage disequilibrium (LD) with the lead GWAS SNP are likely directly pathogenic (that is, functional), but it is nearly impossible to distinguish these from functional SNPs within non-coding DNA are presumed, in most cases, to disrupt cis-regulatory modules. Because the connection between the sequence and function of enhancers is currently poorly understood, it is not immediately clear how a given change of a single base pair could alter the function of any given enhancer. Third, the identity of the ‘risk gene’ (that is, the one whose expression level influences risk for NS CL/P) is not necessarily obvious, because enhancers can lie at an arbitrary distance from the genes they regulate, skipping over intervening genes or lying within the introns of other genes\(^19\)–\(^20\). Finally, once a functional SNP is identified, the mechanism by which a single-base-pair difference influences enhancer activity must be examined experimentally.

To address these challenges, we applied an experimental pipeline to the 1p22 region associated with NS CL/P (refs 3,7,8,21,22). This locus was one of 13 regions selected for resequencing in NS CL/P case-parent trios. This effort discovered multiple SNPs strongly associated with NS CL/P that were in strong LD with the most significant SNP found in a GWAS, all within the introns of \(ABCA4\) (ref. 22). Craniofacial anomalies have been identified within the introns of \(ABCA4\) (ref. 23), but \(ABCA4\) gene is not a good candidate for the risk gene because, first, in mice, expression of \(ABCA4\) is not detectable in the developing lip or palate; second, in mice homoygous for targeted loss-of-function mutations in \(ABCA4\), no defects in craniofacial structure were reported\(^24\); and, finally, in humans, coding mutations in \(ABCA4\) that cause profound defects in retinal function (that is, Stargardt’s disease 1 (refs 25–27)) do not appear to cause CL/P. By contrast, a neighbouring gene, \(ARHGAP29\) is expressed in mouse palate and lip, its expression depends on IRF6, and coding variants in \(ARHGAP29\) are associated with CL/P (ref. 28).

We hypothesized that functional SNPs in 1p22 lie within enhancers that drive \(ARHGAP29\) expression in one or more oral tissue, and the risk alleles at these SNPs decrease \(ARHGAP29\) expression by altering the binding affinities of specific transcription factors. \(In vitro\) enhancer studies, chromosome-conformation capture, zebrafish-based \(in vivo\) enhancer assays, plus genome editing all provide evidence that three SNPs along with two haplotypes are likely to be functional. Finally, chromatin immunoprecipitation analyses indicate that the risk-associated alleles of these SNPs affect the activity of \(ARHGAP29\) enhancers by altering binding of specific transcription factors, and in one case disrupts the interaction of the enhancer with the \(ARHGAP29\) promoter.

**Results**

**Reporter assays reveal SNPs that affect enhancer activity.** We prioritized ten SNPs from the 1p22 locus for our experimental pipeline. All were strongly associated with risk for NS CL/P in Asian case-parent trios used in our resequencing project after correcting for multiple testing and all passed quality control filters\(^25\). The prioritized SNPs are listed in Supplementary Table 1 and depicted in Fig. 1a and Supplementary Fig. 1. We reasoned that functional SNPs will reside in enhancers, while SNPs with no functional effect but in LD with a causal SNP (sometimes called rider or hitch-hiking SNPs) may not. We first tested whether DNA elements containing these SNPs have enhancer activity in oral epithelium or palate mesenchyme cells, the two major cell types contributing to palate tissue. Eight of the ten SNPs lie within chromatin regions expressing marks indicative of enhancer activity in one or more of the 127 cell lines evaluated in the Roadmap Epigenomics project\(^29\) (Fig. 1a, middle track). For these SNPs, we amplified DNA elements that approximately matched the boundaries of such marks and contained one or more risk-associated SNP. For the two SNPs that did not fall into such chromatin regions, we amplified approximately 1 kb of DNA centred on each SNP (Fig. 1a). In total we cloned six enhancer candidates (E1–E6). The resequencing project revealed just two alleles each at all ten SNPs; the risk allele was the major allele at two SNPs and the minor allele at eight SNPs (Supplementary Table 1). As necessary, in each enhancer candidate we converted the riskallele to the non-risk allele by site-directed mutagenesis\(^22\).

We also amplified four negative-control segments (C1–C4) from nearby chromatin that lacked marks indicative of enhancer or promoter function in the 127 cell lines (positions shown in Fig. 1a).

We engineered E1–E6 and C1–C4 into a standard firefly luciferase vector, electroporated each reporter construct, together with a plasmid that drives constitutive expression of renilla luciferase (transfection control), into a human fetal oral epithelial cell line (GMSM-K)\(^30\) and primary human embryonic palate mesenchymal (HEPM)\(^31\) cells, and monitored luciferase activity 72 h later. C1–C4 had similar low-level enhancer activity, whose average we defined as the baseline (separately for the two cell types). The activity of five of the six enhancer candidates in oral-epithelium cells was at least five-fold higher than baseline (Fig. 1c); the exception was E4, one of the two elements for which evidence of chromatin marks was lacking. Of the five with strong enhancer in oral epithelium, four also had enhancer activity in palate mesenchyme cells at least two-fold above baseline, although in each case much lower than in oral-epithelium cells. Based on these results, nine of ten SNPs passed the first criterion of residing within an active enhancer in an oral-epithelium cell line and or palate mesenchyme cells.

We next hypothesized that functional SNPs would have allele-dependent effects on the activity level of the enhancers containing them. To this end, we engineered the SNPs in each element from the non-risk allele to the risk-associated allele, and then repeated the \(in vitro\) luciferase reporter assay in both cell types (Fig. 2). For elements that contain more than one SNP (that is, E3 and E6), we altered one SNP at a time. We then prioritized SNPs based on whether they had allele-dependent effects on enhancer activity. For instance, the level of enhancer activity of E1 in oral epithelium cell lines and oral mesenchyme cells was independent of the allele of its resident SNP-of-interest, rs581244 (Fig. 2a, Supplementary Table 2), indicating this SNP is unlikely to be directly functional. By contrast, the enhancer activity of E2 in oral epithelium cells was...
In vivo enhancer tests reveal tissue specificity. The tissue specificity of enhancers containing these three putatively functional SNPs can reveal the basis of disease mechanisms, so we employed in vivo enhancer assays in zebrafish. To this end, we engineered the five elements passing the in vitro screening strategy using the cloned elements. (c) Relative luciferase activities of all the cloned candidate regulatory elements and control elements in GMSM-K (human oral keratinocyte cell line) and HEPM (human embryonic palatal mesenchymal cells). Black dash line indicates the average relative luciferase activity of control elements in GMSM-K, and grey dash line indicates the average relative luciferase activity of control elements in HEPM. \( n = 3 \) for each group, data represent means ± s.d., t-test, \( *P < 0.05, **P < 0.01 \) significant difference compared with average relative luciferase activity of control elements in respective cell type.
Figure 2 | Allele-specific effects of NS CL/P-associated SNPs on the in vitro activity of regulatory elements in GMSM-K cells and HEPM cells. (a) Relative luciferase activity of different elements with different alleles in GMSM-K cells. (b) Relative luciferase activity of different elements with different alleles in HEPM cells. n = 3 for each group, data represent means ± s.d., t-test, **P < 0.01 significant difference compared with enhancer activities of each element with non-risk allele in respective cell type.

The quantities of ligation product between the EcoR1 restriction fragment containing the ARHGAP29 promoter and (a) the one containing E2, (b) the one containing E3, (c) the one containing an element homologous to a craniofacial enhancer identified by p300 ChIP-SEQ in mouse facial tissue (mouse element 435) (ref. 23) were higher than those of it and other nearby fragments (Fig. 4b).

E4 and E5 exhibited relatively lower interaction frequency with the ARHGAP29 promoter. This was a surprising finding for E5, given the in vitro results presented above; it is noteworthy that the nearest EcoR1 site is over 4 kb from E5, perhaps limiting the efficiency of 3C in detecting interactions between the ARHGAP29 promoter and E5. By contrast, ligation products for the EcoR1 restriction fragment containing the ABCA4 and ABCD3 promoter and all fragments in those regions were similarly low (Fig. 4c).

Also, as expected, four EcoR1-digested fragments in the adjacent TAD exhibited very low interaction frequency with the promoters of ARHGAP29 (Supplementary Fig. 3b), ABCA4 (Supplementary Fig. 3c) and ABCD3 (Supplementary Fig. 3d). Similar outcomes were predicted using PreSTIGE, another method for evaluating intrachromosomal interactions, in NHEKs (ref. 36). These experiments strongly support ARHGAP29 as the NS CL/P risk gene in the 1p22 region.

The functional allele of an SNP could potentially affect the strength of an interaction between an enhancer and its corresponding promoter. We tested this possibility for rs4147828 (in E3). PCR and sequencing revealed that oral epithelium cells are heterozygous for the risk and non-risk alleles (Fig. 4c, INPUT). By contrast, in the amplicon of the 3C ligation products between the EcoR1-restriction fragment containing E3 and the ARHGAP29 promoter, only the non-risk allele at rs4147828 was detected (Fig. 4c). This result suggests the risk allele at rs4147828 disrupts the interactions between enhancer E3 and the ARHGAP29 promoter. It remains to be seen whether other functional SNPs have a similar effect or alter enhancer function by a distinct mechanism.
Genome editing shows SNPs affect expression of ARHGAP29.

We next tested whether deletion of E2 or E3 from the genome alters expression of ARHGAP29 in oral epithelium cells. We transfected the oral epithelium cell line with plasmids encoding Cas9 and a guide RNA (one on either side of the enhancer), performed selection and clonal isolation, and used PCR to identify cells in which E2, E3, or both had undergone bi-allelic deletion (Fig. 5a and Supplementary Fig. 4). In cells in which either E2 (3 isolated colonies) or E3 (3 isolated colonies) was eliminated, the expression of ARHGAP29 was significantly lower than in control cells ($P < 0.01$ by Student’s $t$-test); in cells lacking both elements (three isolated colonies), ARHGAP29 expression was less than 50% of normal levels (Fig. 5b). However, the expression of ABCD3 remained unchanged and that of ABCA4 was undetectable in cells of any genotype (Fig. 5b). These results confirm that both E2 and E3 are enhancers for ARHGAP29 in this cell line.

For E5, rather than delete the entire enhancer, we used CRISPR-Cas9-mediated homology-directed repair (HDR) to delete 10bp centred on its candidate functional SNP (rs560426), or to alter the allele of this SNP (see Methods). We identified at least five independent colonies with each engineered genotype, and used quantitative RT–PCR to monitor ARHGAP29 expression in each. Compared to unmanipulated cells, which were heterozygous for risk and non-risk alleles, cells engineered to be homozygous for the 10-bp deletion, or to be homozygous for the risk allele, had significantly lower average ARHGAP29 expression (10-bp deletion $P = 8.0E-06$, homozygous for risk allele, $P = 5.0E-04$, by Student’s $t$-test), whereas cells engineered to be homozygous for the non-risk allele had significantly higher average ARHGAP29 expression ($P = 2.8E-05$ by Student’s $t$-test) (Fig. 5f). In contrast, expression of ABCA4 was undetectable in all colonies tested, and ABCD3 expression was not affected by genome editing (Supplementary Fig. 7). These results confirm that expression of ARHGAP29 in this cell line is affected by the nucleotide at rs560426 (E5), supporting the candidacy of this SNP for being directly functional.

Next, we used HDR to engineer the alleles of rs2275035 (in E2) and rs4147828 (in E3). At both SNPs, unmanipulated GMSM-K oral epithelium cells are heterozygous for the risk and non-risk alleles (Fig. 5d). Using a two-step selection strategy, we first knocked in the desired allele in a template containing a neomycin-resistance (neo) cassette, and then removed the neo-
Figure 4 | Chromatin interactions at the 1p22 NS CL/P-associated region with ARHGAP29. (a) Topologically associated domains at the 1p22 NS CL/P-associated region showing candidate affected genes. (b) 3C-qPCR showing relative interactions frequency of 1p22 regions with promoter of ARHGAP29 and ABCA4 in GMSM-K and HEPM cells. Vertical grey boxes indicate EcoRI fragments interrogated. Purple box, craniofacial enhancer, identified in mouse genome23 and lifted over to human genome. n = 3 for each cell type. Points represent mean relative interaction frequency, while vertical lines indicate standard deviations. (c) Sanger-sequencing results of 3C products showing allele-specific chromatin looping between E3 and the ARHGAP29 promoter.

cassette, leaving behind a single loxP site of 34 base pairs (Supplementary Fig. 5a-d). As for HDR in E2, although the presence of the neo cassette modestly affected ARHGAP29 expression, the presence of the loxP site did not (that is, ARHGAP29 expression was not different in unedited ‘T/C’ cells and ‘loxP-T/loxP-C’ cells, P > 0.30 by Student’s t-test) (Fig. 5g). In three independent isolates of colonies of each genotype, average ARHGAP29 expression was higher in cells homozygous for the non-risk allele (P = 0.057 by Student’s t-test), and significantly higher in heterozygotes (P = 7.5E-3 by Student’s t-test), than in those homozygous for the risk allele (Fig. 5g). The results for rs4147828 were similar (Fig. 5h). Thus, the expression of ARHGAP29 appears to be affected by the nucleotide at rs2275035 and rs4147828, supporting that they are functional SNPs.

Since ARHGAP29 inactivates RhoA (ref. 37) and RhoA regulates keratinocyte migration38,39; gain and loss-of-function experiments have shown that ARHGAP29 positively regulate oral keratinocyte migration in vitro, while mutant ARHGAP29 identified in orofacial clefting patients is unstable and fails to accelerate cell migration40. We next asked whether the level of change of ARHGAP29 expression mediated by the allele of a potentially functional SNP is sufficient to alter cell migratory behaviour in vitro. We performed a scratch assay, using three independent colonies per engineered genotype and unedited colonies. We grew each culture to confluency, made several scratches in the cell lawn and monitored cell migration for 24 h. Cells homozygous for deletion of E2, E3 or E2 + E3 closed the scratches more slowly than unedited cells (Fig. 5i,j). Moreover, cells homozygous for risk alleles at SNPs rs2275035, rs4147828 and rs560426 migrated more slowly than those homozygous for the corresponding non-risk alleles (Fig. 5i,j and Supplementary Fig. 8). These findings indicate that variation at these SNPs is sufficient to alter the migratory behaviour of epithelial cells, presumably by altering ARHGAP29 expression, further supporting the candidacy of these SNPs as directly functional.

Engineered risk haplotypes affect ARHGAP29 expression. Having shown that the allele at single SNPs can affect enhancer activity and ARHGAP29 expression, we next sought to evaluate the effect of specific risk haplotypes. In our published targeted resequencing results, we reported two independent signals in Asian case-parent trios at the 1p22 locus: one tagged by rs560426 (in E5) and a second haplotype tagged by rs77179923 (in E6) (ref. 22). We revisited these data to examine haplotype frequencies with SNPs within or between enhancer candidates using phased parental data. Seven SNPs were in moderate to high LD with rs560426 (peak 1) and three SNPs were in moderate to high LD with rs77179923 (peak 2) (Supplementary Table 6).

In peak 1, rs2275035 (E2) and rs4147828 (E3) were in strong LD (r² = 0.61, D’ = 0.98) and, unsurprisingly, a haplotype comprised of both risk alleles (that is, the CA haplotype) was over-transmitted to NS CL/P offspring (P = 0.0015 by Student’s t-test), while a haplotype comprised of the non-risk alleles of both SNPs (that is, the TG haplotype) was under-transmitted (P = 8.00E-04 by Student’s t-test) (Supplementary Table 6). Phasing of the oral epithelium cells we used in this study
Figure 5 | Allele-specific effects of three SNPs in 1p22 on ARHGAP29 expression. (a) Schematic illustrations of the experimental strategy to generate homozygous deletion of E2 (ΔE2) and E3 (ΔE3) respectively or both (ΔE2 + E3) in GMSM-K cells. (b,c) Relative expression of ARHGAP29 and ABCD3 in ΔE2, ΔE3 and ΔE2 + E3 cells. Each box indicates average expression of three isolated colonies (n = 3) with same enhancer deletion. For each colony, ARHGAP29 and ABCD3 expression were measured for three times. Data represent means ± s.d., t-test, **P < 0.01. (d) Schematic showing the HDR of rs4147828 in E3. (e) Schematic showing the HDR of rs520426 in E5. (f–h) Relative expression of ARHGAP29 in cells with different genotypes. Each dot represents mean expression of ARHGAP29 in one isolated colony. (i) Representative photos showing migration of oral epithelium cell with different genotype (unedited, rs4147828_A allele (E3_A in figure), and ΔE3) in 0 h and 18 h after scratch. (j) Distance of migration of oral epithelial cells with different genotypes within 18 h after scratch. (E5_C/C indicates colony with homozygous C allele of rs4147828 in E5, and so forth.) Distances between two sides of scratch of each group at each time point were measured using ImageJ. n = 3 for each group, data represent means ± s.d., t-test, *P < 0.05, **P < 0.01.
revealed two rarer haplotypes (TA/CG), meaning each chromosome carried only one risk allele. The HDR strategy we used above yielded colonies with haplotypes CA/CG (three total risk alleles) or haplotypes TG/CQ, with only one risk allele (Fig. 6a). As expected, average ARHGAP29 expression in colonies with CA/CG were significantly lower than the colonies with TG/CQ and the original un-treated cells (Fig. 6b). The additive effect of combining risk alleles at both E2 and E3 was also observed in in vitro reporter assays where the enhancer was E2 fused to E3 (Fig. 6c).

The E3 element contained rs4147828 and rs17111017, both among the top ten SNPs, as well as three other SNPs with significant TDT p-values that were not among the top ten SNPs. These five SNPs appear in the following order rs75017380, rs3789419, rs17111017, rs4147828 and rs147827 (Fig. 6d). The genotype of the unedited oral epithelium cell line is TGCGC/TGCAC (that is, heterozygous for the non-risk G allele and risk A allele at rs4147828). Analysis of phased haplotypes from the sequencing data revealed TGCGC is a non-risk haplotype, significantly under-transmitted to NS CL/P cases (\(P = \text{7E-04} \) by Student’s t-test) (Supplementary Table 7), while TGCAC is a neutral haplotype, neither under- nor over-transmitted to cases (\(P = 0.51 \) by Student’s t-test). This suggested that a particular combination of risk alleles, including the other E3 SNPs, may be required to affect ARHGAP29 expression. To test this possibility, we used appropriate homology arms to make the cells homozygous for TGGAG, a risk haplotype, significantly under-transmitted to NS CL/P cases (\(P = 0.0004 \) by Student’s t-test) (Supplementary Table 7). In experiments discussed above targeting rs4147828 we had already generated cells homozygous for the non-risk haplotype, TGCGC/TGCGC, and for the neutral haplotype, TGCAC/TGCAC. Contrary to the suggestion mentioned above, ARHGAP29 expression in cells homozygous for the risk-haplotype and for the neutral haplotype was not significantly different (Fig. 6e). Together these results support the idea SNP rs4147828 is functional while the other SNPs in E3 are carried along by linkage. They also imply the reason that TGGAG is a risk haplotype and TGCAC is a neutral one is merely due to information content and statistical power rather than functional effects of these other SNPs.

Finally, we tested for an association between all four SNPs with allele-specific effects: rs2275035 (in E2), rs4147828 (in E3) and rs560426 (in E5) from peak 1 and rs66515264 (in E6) from peak 2. Interestingly, the only haplotype that was significantly associated with NS CL/P was one including risk alleles at all four variants (Supplementary Table 7).

ChIP reveals SNP allele affects transcription factor binding. We hypothesized that the allele of functional SNP affects the binding affinity of developmental transcription factors. We used JASPAR (ref. 41) to predict binding event that would be affected by the allele of the functional SNP candidates. rs2275035 (in E2) lies within a binding site for KLF4 whose predicted quality is

---

**Figure 6 | Risk haplotypes affect ARHGAP29 expression.** (a-d) Schematic showing generation of oral epithelial cells of different haplotype. (b) Relative expression of ARHGAP29 in oral epithelial cells with different haplotypes of rs2275035 and rs4147828; each dot represents mean expression of ARHGAP29 in one isolated colony. \(n=3\) (colonies) for each group, ARHGAP29 expression were measured for three times, and data represent means ± s.d., t-test, **\(P<0.01\). (c) Relative luciferase activity of fused E2 and E3 with risk or non-risk haplotype of rs2275035 and rs4147828 in GMSM-K oral epithelial cells. \(n=3\) for each group, luciferase activity was measured for three times for each group, data represent means ± s.d., t-test, **\(P<0.01\). (a) Relative ARHGAP29 expression in oral epithelial cells with different haplotypes of rs75017380, rs3789419, rs17111017, rs4147828 and rs147827. Note that TGCAC/TGCAC is the same as ‘loxP-A/loxP-A’ in Fig. 5h. \(n=3\) (colonies) for each group, ARHGAP29 expression were measured for three times, and data represent means ± s.d., t-test, **\(P<0.01\). (f) Relative luciferase activity of E3 with different haplotype in GMSM-K oral epithelial cells. Haplotype of rs75017380, rs3789419, rs17111017, rs4147828 and rs147827 in E3 is (TGCGC), E3-risk-allele (TGCAC), E3-risk-haplotype (TGGAG). \(n=3\) for each group, data represent means ± s.d., t-test, ns, non-significant.
improved by the risk allele relative to the non-risk allele. This protein is known to act as a transcriptional activator or repressor depending on context\(^\text{42}\), and rare dominant-negative variants are present in Asian patients with NS CL/P (ref. \(^\text{43}\)). We carried out anti-KLF4 chromatin immunoprecipitation (ChIP) in lysates of GMSM-K cells, and found KLF4 binds E2 (Fig. 7a). Whereas the cell line is heterozygous for both risk and non-risk alleles at rs2275035, the ChIP-qPCR product was highly enriched for the risk-allele, showing KLF4 indeed binds this sequence more avidly (Fig. 7c). Given that E2 has lower enhancer activity when it harbours the risk allele at rs2275035 (Fig. 2a), we predicted that KLF4 binding inhibits the enhancer activity of E2. To test this, we transfected GMSM-K cells with the E2 luciferase reporter and an expression vector carrying human KLF4 cDNA. Luciferase levels were lower in the context of KLF4 over-expression, and the reporter was more sensitive to such inhibition when it harboured the risk allele at rs2275035 (Fig. 7d). These findings suggest a mechanism by which the risk allele of rs2275035 could lead to lower enhancer activity of E2.

JASPAR predicted that rs4147828 (in E3) lies within a binding site of the transcription factor MAFB, and that the risk allele reduces the predicted quality of this site. MAFB promotes the differentiation of epidermis\(^\text{44}\), and MAFB is at a locus that is strongly associated with risk for NS CL/P in the Asian population\(^\text{3,22}\). Using anti-MAFB qPCR-ChIP, we confirmed MAFB binds at rs4147828 in E3 (Fig. 7b), and sequencing of the ChIP-PCR product revealed a significant enrichment of the non-risk allele (Fig. 7c). In reporter assays using GMSM-K cells, forced

---

**Figure 7** | Different alleles of rs2275035 and rs4147828 affect bindings of NS CL/P-related transcription factors in ARHGAP29 enhancers. (a,b) ChIP-qPCR validating KLF4 binding around rs2275035 (a) and MAFB binding around rs4147828 (b). \(n=3\) for each group, data represent means ± s.d., t-test, **\(P<0.01\). (c) Sanger Sequencing of anti-KLF4 and anti-MAFB ChIP-PCR product. (d) Relative luciferase activity of E2 with different alleles of rs2275035 upon overexpression of KLF4. \(n=3\) for each group, luciferase activity was measured for three times for each group, data represent means ± s.d., t-test, **\(P<0.01\). (e) Relative luciferase activity of E3 with different alleles of rs4147828 upon overexpression of MAFB. \(n=3\) for each group, luciferase activity was measured for three times for each group, data represent means ± s.d., t-test, **\(P<0.01\). (f) Illustration of a possible model of ARHGAP29 regulated by E2 and E3 with different genotypes.
embryonic stem cells have better colony-formation ability and are HDR in them. Although induced pluripotent stem cells and potential of the HEPM cell line, we did not manage to achieve an appropriate cell line. Because of the poor colony-forming of the risk gene. This power of this assay again depends on having simultaneously supporting the candidacy of functional SNPs and haplotype, could significantly alter the expression of risk allele at a single SNP, alone or in the context of a risk haplotype.

Discussion

We have demonstrated a systematic approach for investigating functional SNPs associated with NS CL/P, and it should be applicable to the investigation of other complex diseases (schematic of experimental pipeline shown in Supplementary Fig. 10). Starting with the ten SNPs in the 1p22 region that are most highly associated with NS CL/P in Asian case-parent trios, we conducted in vitro reporter assays in human oral epithelium and palate mesenchyme cells. Only nine of the candidate SNPs were found to reside within elements with enhancer activity, and only four were found to have allele-specific effects on enhancer activity. The utility of this assay, and that of the 3C-based method for identifying the best candidate as a directly causal gene, depends on having cell lines that are relevant to pathogenesis of the disorder in question. For instance, truly causal SNPs may be missed (false negative) if the disease-relevant enhancer in which it resides is inactive in the cell line model. Conversely, an inert SNP may be scored as causal (false positive) if by coincidence it has allele-dependent effects on an enhancer active in the cell line that is irrelevant to disease. Provided a relevant cell line is available, tests of allele-dependence of enhancer activity in vitro are a potent and relatively scalable assay for use at loci with large numbers of risk-associated SNPs associated (for example, IRF6 (ref. 22)).

Reporter assays in zebrafish revealed the tissue specificity of enhancers containing the functional SNP candidates. This assay and analogous ones in mouse have the potential to greatly streamline the investigation of pathogenicity of any given SNP, particularly if the risk gene has a complex expression pattern. For instance in the present case, E2 and E3 enhancers were active in only a subset of the tissues in which mouse Arhgap29 (refs 45, 46) and zebrafish arhgap29b (ref. 47) are expressed (for example, cranial epidermis, brain, oral mesenchyme and oral epithelium). Of note, expression was by far the strongest in cranial epidermis, and signalling from cranial epidermis might influence patterning of the underlying neural crest tissue48. The oral epithelium is also an excellent candidate tissue. Importantly, we can deduce that other domains of ARHGAP29 expression, for example, somites and endothelial cells, where E2 and E3 are inactive, are not involved in the pathogenicity of variation at 1p22. In principle, findings from such assays could strongly support one candidate as a causal gene for NS CL/P over another gene. However, in the present case it did not serve this purpose, as E2 and E3 were active in retina, where the ABCA4 gene is expressed. Also, E5, containing rs560426, exhibited low level enhancer activity in human oral epithelium cells in vitro, it did not do so, detectably, in zebrafish embryos, at least with the minimal promoters tested here.

CRISPR-Cas9-mediated HDR demonstrated that altering the risk allele at a single SNP, alone or in the context of a risk haplotype, could significantly alter the expression of ARHGAP29, simultaneously supporting the candidacy of functional SNPs and of the risk gene. This power of this assay again depends on having an appropriate cell line. Because of the poor colony-forming potential of the HEPM cell line, we did not manage to achieve HDR in them. Although induced pluripotent stem cells and embryonic stem cells have better colony-formation ability and are suitable for HDR (ref. 49), methods to convert such cells into oral epithelium and oral mesenchyme have not yet been reported.

Finally, ChIP revealed two SNPs affected the binding of specific transcription factors, MAFB and KLF4, potentially among others. Both proteins are members of gene regulatory networks active in epithelia, contribute to morphogenesis of the face (oral epithelium and cranial epidermis), and are associated with NS CL/P risk3,21,28,43. The evidence that the risk allele at a functional SNP affects MAFB binding, rs4147828, suggest there could be a gene-by-gene interaction between rs4147828 and the lead SNP at 20q12, where the MAFB gene resides. Detecting such interactions, unless they are unusually potent, requires very large sample sizes. We did not detect any statistical interaction between these loci in our recent large-scale GWAS (ref. 7). However, this required including 18 covariates in our regression model to account for population structure, limiting statistical power. This in no way limits the impact of identifying this biological interaction between ARHGAP29 and MAFB, two genes with limited research into their roles in craniofacial biology. The discovery of potential connections like these motivates further analyses of the gene regulatory networks controlling development of craniofacial tissues. Indeed despite mounting evidence that ARHGAP29 is the risk gene at 1p22, and that it participates in a network that includes KLF4, MAFB and IRF6 (refs 28, 50), the role of ARHGAP29 during craniofacial development remains unknown.

Methods

SNP selection. This study is based on association results from a targeted sequencing study of NS CL/P GWAS loci in case-parent trios from European and Asian ancestries22. The 1p22 locus was strongly associated with NS CL/P in Asian trios. There are several approaches for selecting SNPs for experimental pipelines, primarily based on LD with the lead SNP. Given that the 1p22 locus is within a region without strong LD patterns, we selected the top ten SNPs that had met all quality control metrics in our published study.

Haplotype analysis. Genotypes from the targeted sequencing were phased using BEAGLE (ref. 22). Phased haplotypes of 2–4 SNPs were analysed with a transmission disequilibrium test. Analyses were limited to case-parent trios from the Philippines, the subset of the Asian trios that drove the association signal. All analyses were implemented in R (v.3.3.0).

Zebrafish maintenance. D. rerio were maintained as described43 in the University of Iowa Animal Care Facility (protocol no. 6011616). Zebrafish embryos were staged according to ref. 52 at 28.5°C by hours or days post fertilization (h.p.f. or d.p.f.). And all the zebrafish experiments were performed in compliance with the ethical regulations in the Institutional Animal Care and Use Committee in the University of Iowa.

Plasmid constructs. All the candidate elements in 1p22 locus were cloned using the BAC RP11-109C4 as template and primers are documented in Supplementary Table 9. Products were cloned into pENTR/D-TOPO plasmid (Life Technologies, Carlsbad, CA, USA) for Sanger Sequencing validation. After comparing the genotypes of the cloned elements with the risk or non-risk genotype for each SNPs reported in our previous study47, site-directed mutagenesis was employed to get the elements with non-risk or risk allele. After sequencing confirmation, candidate elements were shuttled into cFos-FFLuc plasmid for in vitro luciferase assay, and cFos-GFP (ref. 53) or ZED (ref. 54) plasmids for zebrafish in vivo enhancer assay. Homology arms used for HDR-mediated by CRISPR-Cas9 were cloned using the elements verified in the pENTR-D-TOPO plasmids mentioned above as templates, then digested and inserted to the upstream and downstream of loxP-neor-loxp cassette of pEasy-Flox (a gift from Klaus Rajewsky (Addgene plasmid #11725)).

KLF4 cDNA (RefSeq: NM_004235.4) was obtained from Stratagene. The coding sequence of human MAFB was cloned directly from human genomic DNA. The coding sequence of human KLF4 and MAFB was cloned into pENTR-D-TOPO plasmid for Sanger sequencing validation, after which they were shuttled into pCS2 + destination plasmid for over-expression assay.

Cell culture and transfections. GM0-S human embryonic oral epithelial cell line (a kind gift from Dr Daniel Grenier)36 was maintained in keratinocyte serum-free medium (Life Technologies) supplemented with EGF and bovine pituitary extract (Life Technologies). All cells were incubated at 37 °C in 5% CO₂, Human
embryonic palatal mesenchyme cells (HEPM)\(^31\) were purchased from ATCC (ATCC: CRL-1486) and maintained in ATCC-formulated Eagle’s minimum essential medium (ATCC) supplemented with 10% fetal bovine serum (Life Technologies) and 1% antibiotic-antimycotic (Life Technologies). The 293FT cell line (human embryonic kidney cell line) was purchased from Invitrogen and maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies) and 1% antibiotic-antimycotic (Life Technologies). Early passage of 293FT cells were used for lentivirus production. All the cell lines used in this study were tested to be free of mycoplasma contamination and authenticated by genetic profiling using polymorphic short tandem repeats.

To generate lentivirus coding Cre recombinase, lentivector pLOX-CW-CRE (ref. \(^{55}\), c-fos-EF1-GFP vector with pmD2G2 and mPA2 (kind gift of Dr Didier Trono) into 293FT cells\(^{36}\) using Lipofectamine 3000 (Life Technologies). Lentivirus was collected and purified everyday from second day to fourth day after transfection.

**Dual luciferase activity assay.** For dual luciferase activity assay, each reporter construct was co-transfected with Renila Luciferase plasmid for three biological replicates. Briefly, plasmids were electroporated into GMSM-K cells (1 × 10\(^6\) per transfection) with Amaza Cell Line Nucleofector Kit V (Lonza, Cologne, Germany) using Nucleofector II (Lonza) (program: X-005), and plasmids were electroporated into HEP3B cells (1 × 10\(^6\) per transfection) with Amaza Basic Nucleofector Kit for Primary Mammalian Fibroblasts (Lonza) using Nucleofector II (Lonza) (program: U-020). The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and 20/20n Luminometer (Turner BioSystems, Sunnyvale, CA, USA) were employed to evaluate the luciferase activity when cells were approximately 95% confluent at 72 h post-transfection following the manufacturer’s instructions.

**Relative luciferase activities were calculated by the ratio between the value for firefly and Renila luciferase activities.** Three measurements were made for the lysis from each transfection. All the quantified results were presented as mean ± s.d. Student t-test was used to determine statistical significance.

**Zebrafish enhancer in vitro reporter assay.** For transient transgenic assay, the c-fos-GFP vector with each candidate regulatory element was injected along with the tol2 mRNA (20–30 ng ml\(^{-1}\)) into at least 200 one-cell stage embryos. The embryos were screened at 6 h.p.f. 24 h.p.f., 48 h.p.f. and 4 d.p.f. for documenting transfection efficiency in different embryo development stages. A consistent GFP expression pattern in a minimum of 10% of injected fish was the criterion for tissue-specific enhancer activity (recorded in Supplementary Table 3), which will be sufficient to predict their pattern in F1. For E2 and E3 that exhibit consistent craniofacial enhancer activity, we shuttled them into ZED vector to establish stable transgenic line. F0 adults of E2 and E3 were outcrossed with NHGRI-1 line\(^57\), F1 embryos were screened at 6 h.p.f., 24 h.p.f., 48 h.p.f. and 4 d.p.f. for documenting transfection. Embryo was collected and purified everyday from second day to fourth day after transfection. For transient transgenic assay, the tol2 mRNA (20–30 ng ml\(^{-1}\)) into 293FT cells\(^{56}\) using Lipofectamine 3000 (Life Technologies). Lentivirus construct was co-transfected with Renila Luciferase plasmid for three biological replicates. Briefly, plasmids were electroporated into GMSM-K cells (1 × 10\(^6\) per transfection) with Amaza Cell Line Nucleofector Kit V (Lonza, Cologne, Germany) using Nucleofector II (Lonza) (program: X-005), and plasmids were electroporated into HEP3B cells (1 × 10\(^6\) per transfection) with Amaza Basic Nucleofector Kit for Primary Mammalian Fibroblasts (Lonza) using Nucleofector II (Lonza) (program: U-020). The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and 20/20n Luminometer (Turner BioSystems, Sunnyvale, CA, USA) were employed to evaluate the luciferase activity when cells were approximately 95% confluent at 72 h post-transfection following the manufacturer’s instructions. Relative luciferase activities were calculated by the ratio between the value for firefly and Renila luciferase activities. Three measurements were made for the lysis from each transfection. All the quantified results were presented as mean ± s.d. Student t-test was used to determine statistical significance.

**Chromatin immunoprecipitation and genotype of 3C product.** Primers for 3C-qPCR were designed using Primer3 Plus online software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and tested against EcoRI-digested fragment flanking CFOS (NM_001328667.1) for 3 days, and serial dilution in 96-well plates were used to clone the genome DNA sequence of GMSM-K cells with StrataClone Blunt PCR Cloning Kit (Aglent Technologies, Santa Clara, CA, USA). Sanger sequencing of ten resulting plasmids identified the phase of the haplotypes.

There is a high-scoring gRNA target site just 17 bp away from 5’ end of rs560426 (chr1:42330208, hg19) of CFOS. We co-transfected cells with a single stranded donor template and a plasmid encoding the relevant gRNA and Cas9 (that is, the gRNA and CAS9 expression plasmid). We applied selection, isolated single cells and grew colonies from them, and used polyacrylamide gel (PAGE)-gel-based genotyping\(^{61}\) or high-resolution melting analysis\(^{62,63}\) followed by Sanger sequencing to identify colonies that were homozygous for this 10-bp deletion, the risk allele or the non-risk allele (Supplementary Fig. 4).

To knockout E2 and E3, we cotransfected GMSM-K cells with two CRISPR-Cas9 donor plasmids expressing two gRNAs flanking risk and non-risk allele in vivo. (E2 or E3) with risk or non-risk allele were injected into more than 200 embryos respectively for three times. Twenty-four hours after injection, unhealthy or dead embryos were excluded from this study. Every healthy embryo was examined under the fluorescent microscope to compare the tissue-specific GFP pattern in an unblended way. The investigator was not blinded to group allocation during experiment. The results are recorded in Supplementary Table 4.
rs604026 were synthesized by IDT. Five microliters single strand DNA (10 μM) with PX330 expressing gRNA targeting rs604026 were co-transfected into GMSM-K by electroporation as described above. Forty-eight hours after transfection, serial dilution approach was used to get single cell clone. When single cell colonies were formed, colony was screened by PAGE to obtain homozygous rs604026 or the colonies with homozygous deletion. If the genotype of rs604026 in the colonies was heterozygous, at least three bands will be seen in PAGE, but PCR product from colonies with homozygous rs604026 will have only one band. After screening, genotypes of the homozygous colonies were confirmed using Sanger Sequencing.

For the other two SNPs and haplotypes were performed using plasmids as donor templates. Donor plasmid with different genotypes or haplotypes were cotransfected with PX330 expressing the relevant gRNA. The nucleoexposed GMSM-K cells were plated in 10 cm dishes, and 48 h after transfection, were treated with G418 (Sigma-Aldrich) (800 ng ml⁻¹) and Ganciclovir (InvivoGen, San Diego, CA, USA) (4 μM) for 10 days. After drug selection, colonies were manually picked from each dish, screened and expanded. We avoided picking colonies growing close to each other. For screening the colonies with neomycin cassette in both alleles, we employed high-resolution-melting-curve-assay (CFX-Connect, Bio-rad, Hercules, CA, USA) with primers flanking the cassette. Sanger sequencing was employed to confirm the genotype. In order to delete the neomycin cassette, we delivered lentiviruses expressing Cre recombinase. After infection, colonies were screened using PCR. Sanger sequencing was used to confirm the genotype.

**In vitro wound healing assay.** For each genotype, same number of cells from three colonies of GMSM-K cells were plated independently in six-well plate. Scratch assay were performed according to standard protocol.²² Briefly, scratches were generated with a P200 tip, and static images of three fixed spots along each scratch on 0 h (right after scratch), 3, 6, 12, 18, and 24 h after scratching. Static images were recorded on a Leica DMR2A compound microscope with a colour 12 bit ‘QIClick’ camera (Qimaging). Intervals of each spot were measured for three times with ImageJ (https://imagej.nih.gov/ij/). Since the scratches in some wells almost closed at 18 h, we only included the distance of cell migration within 18 h after scratch was made. Distances were presented as mean ± s.d. Representative photos are presented in Supplementary Fig. 8.

**Statistical analysis.** For luciferase assay and RT-PCR, each group includes three biological replicates, and for each biological replicate, three technical replicates were measured to ensure the statistical power. For CRISPR-engineered cells, at least three independent colonies were picked up for experiment (detailed numbers are recorded in Supplementary Fig. 6). Statistical analyses were carried out using two-tailed, unpaired Student’s t-test. Before t-test, normal distribution of all the data were checked using normality test and equality of variances were checked using F-test implemented in R (v.3.2.5).

**Data availability.** The data that support the findings of this study are available from the corresponding author on reasonable request.

**References**

1. Rahimov, F., Jugessur, A. & Murray, J. C. Genetics of nonsyndromic orofacial clefts. Cleft Palate Craniofac. J. 49, 73–91 (2012).

2. Dixon, M. J., Marazita, M. L., Beaty, T. H. & Murray, J. C. Cleft lip and palate: understanding genetic and environmental influences. Nat. Rev. Genet. 12, 167–178 (2011).

3. Beaty, T. H. et al. A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near IRAK1, ADAMTS20 and all orofacial clefts, and nonsyndromic cleft lip with or without cleft palate locus identified by targeted sequencing of FOXE1. Am. J. Hum. Genet. 96, 397–411 (2015).

4. Attanasio, C. et al. Fine tuning of craniofacial morphology by distant-acting enhancers. Science 342, 1241006 (2013).

5. Handoko, L. et al. CTCF-mediated functional chromatin interactome in pluripotent cells. Nat. Genet. 43, 630–638 (2011).

6. Li, G. et al. Extensive promoter-centered chromatin interactions provide a topological basis for transcription regulation. Cell 148, 84–98 (2012).

7. Ludwig, K. U. et al. Genome-wide meta-analyses of nonsyndromic cleft lip with or without cleft palate identify six new risk loci. Nat. Genet. 44, 968–971 (2012).

8. Leslie, E. J. et al. Identification of functional variants for cleft lip with or without cleft palate in or near PAX7, FGFR2, and NOG by targeted sequencing of clefts. Nat. Genet. 45, 237–241 (2013).

9. Wang, H. P., Jalali, S., Heitmann, J. F. & Kannabiravan, C. Homozygous null mutations in the ABCA4 gene in two families with autosomal recessive retinal dystrophy. Am. J. Ophthalmol. 141, 906–913 (2006).

10. Leslie, E. J. et al. 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. Birth Defects Res. A. Clin. Mol. Teratol. 94, 934–942 (2011).

11. Roadmap Epigenomics, C. et al. Integrative analysis of 111 reference human epigenomes. Nature 518, 317–330 (2015).

12. Gilchrist, E. P., Moyer, M. P., Shillito, E. J., Clare, N. & Murrah, V. A. Establishment of a human paloeyonic oral epithelial cell line. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 90, 340–347 (2000).

13. Kusuda, T. & Pratt, R. M. Mesenchymal cells from the human embryonic palate are highly responsive to epithelial growth factor. Science 213, 563–565 (1981).

14. Dixson, J. R. et al. Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature 485, 376–380 (2012).

15. Smith, E. M., Lajoie, B. R., Jain, G. & Dekker, J. Invariant TAD boundaries constrain cell-type-specific looping interactions between promoters and distal elements around the CFTR locus. Am. J. Hum. Genet. 98, 185–201 (2016).

16. Li, Y. et al. Characterization of constitutive CTCF/cohesin loci: a possible role in establishing topological domains in mammalian genomes. BMC Genom. 14, 559 (2013).

17. Rao, S. S. P. et al. A 3D map of the human genome at kilobase resolution reveals principles of chromosome looping. Cell 159, 1665–1680 (2014).

18. Corradin, O. et al. Combinatorial effects of multiple enhancer variants in linkage disequilibrium dictate levels of gene expression to confer susceptibility to common traits. Genome Res. 24, 1–13 (2014).

19. Saras, J. et al. A novel GTPase-activating protein for Rho interacts with a PDZ domain of the protein-tyrosine phosphatase PTPPL1. J. Biol. Chem. 272, 24333–24338 (1997).

20. Jackson, B. et al. RhOa is dispensable for skin development, but crucial for contraction and directed migration of keratinocytes. Mol. Biol. Cell 22, 593–605 (2011).

21. Zhang, Z. et al. Migration of epithelial cells on laminins: RhOa antagonizes directionally persistent migration. Eur. J. Cell Biol. 90, 1–12 (2011).

22. Liu, H. et al. Exome sequencing provides additional evidence for the involvement of ARHGAP29 in Mendelian orofacial clefting and extends the phenotypic spectrum to isolated cleft palate. Birth Defects Res. Part A. Clin. Mol. Teratol 109, 27–37 (2017).
41. Mathelier, A. et al. JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res.* 44, D120-D125 (2016).

42. Rowland, B. D. & Peeper, D. S. KLF4, p21 and context-dependent opposing forces in cancer. *Nat. Rev. Cancer* 6, 11–23 (2006).

43. Liu, H. et al. Irf6 directly regulates Klf17 in zebrafish periderm and Klf4 in murine oral epithelium, and dominant-negative KLF4 variants are present in patients with cleft lip and palate. *Hum. Mol. Genet.* 25, 766–776 (2016).

44. Lopez-Pajares, V. et al. A LincRNA-MAF:MAFB transcription factor network regulates epidermal differentiation. *Dev. Cell* 32, 693–706 (2015).

45. Finger, J. H. et al. The mouse gene expression database: new features and how to use them effectively. *Genes* 53, 510–522 (2015).

46. Eppig, J. T. et al. The Mouse Genome Database (MGD): facilitating mouse as a model for human biology and disease. *Nucleic Acids Res.* 43, D726–D736 (2015).

47. Sprague, J. et al. The Zebrafish Information Network: the zebrafish model organism database provides expanded support for genotypes and phenotypes. *Nucleic Acids Res.* 36, D768–D772 (2008).

48. Knight, R. D., Javidan, Y., Zhang, T., Nelson, S. & Schilling, T. F. AP2-dependent signals from the ectoderm regulate craniofacial development in the zebrafish embryo. *Development* 132, 3127–3138 (2005).

49. Soldner, F. et al. Parkinson-associated risk variant in distal enhancer of alpha-synuclein modulates target gene expression. *Nature* 533, 95–99 (2016).

50. Biggs, L. C. et al. Interferon regulatory factor 6 regulates keratinocyte migration. *J. Cell Sci.* 127, 2840–2848 (2014).

51. Westerfield, M. The Zebrafish Book (University of Oregon Press, 1993).

52. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* 203, 253–310 (1995).

53. Fisher, S. et al. Evaluating the biological relevance of putative enhancers using Tol2 transposon-mediated transgenesis in zebrafish. *Nat. Protoc.* 1, 1297–1305 (2006).

54. Bessa, J. et al. Zebrafish enhancer detection (ZED) vector: a new tool to facilitate transgenesis and the functional analysis of cis-regulatory regions in zebrafish. *Dev. Dyn.* 238, 2409–2417 (2009).

55. Salmon, P. et al. Reversible immortalization of human primary cells by lentivector-mediated transfer of specific genes. *Mol. Ther.* 2, 404–414 (2000).

56. Barde, I., Salmon, P. & Trono, D. Production and titration of lentiviral vectors. *Curr. Protoc. Neurosci.* 53, 4.21.1–4.21.23 (2010).

57. LaFave, M. C., Varshney, G. K., Vemulapalli, M., Mullikin, J. C. & Burgess, S. M. A defined zebrafish line for high-throughput genetics and genomics: NHGRI-1. *Genetics* 198, 167–170 (2014).

58. Hagege, H. et al. Quantitative analysis of chromosome conformation capture assays (3C-qPCHR). *Nat. Protoc.* 2, 1722–1733 (2007).

59. Hitchler, M. J. & Rice, J. C. Genome-wide epigenetic analysis of human pluripotent stem cells by ChIP and ChIP-Seq. *Methods Mol. Biol.* 767, 253–267 (2011).

60. Sen, G. L. et al. ZNF750 is a p63 target gene that induces KLF4 to drive terminal epidermal differentiation. *Dev. Cell* 22, 669–677 (2012).

61. Zhu, X. et al. An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. *Sci. Rep.* 4, 6420 (2014).

62. Talbot, J. C. & Amacher, S. L. A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles. *Zebrafish* 11, 583–585 (2014).

63. Vossen, R. H., Aten, E., Roos, A. & den Dunnen, J. T. High-resolution melting analysis (HRMA): more than just sequence variant screening. *Hum. Mutat.* 30, 860–866 (2009).

64. Liang, C. C., Park, A. Y. & Guan, J. L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat. Protoc.* 2, 329–333 (2007).

Acknowledgements

We are grateful for excellent technical assistance and animal husbandry from Greg Bonde. We are grateful to Johann Eberhart from the University of Texas at Austin for helping us interpret zebrafish craniofacial anatomy. We thank Martine Dunnwald for discussion of the in vitro scratch assay, Adam Dupuy for discussion of CRISPR experiment design, and Jeffery Murray, supported by DE08559, for permitting analysis of unpublished targeted resequencing results. We thank Axel Visel and Cailyn Spurrell for preliminary analyses of enhancer activity in mouse embryos of a subset of elements discussed here. Research reported in this publication was supported by the National Institute of Health grants DE023575 (R.A.C.), DE025060 (E.I.L.), DE016418 (M.L.M.), HG005925 (M.L.M.), DE014581 and DE018493 (T.H.B.), and by the National Natural Science Foundation of China 81400477 (H.L.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions

H.L. planned experiments, performed the cell-based and fish-based experiments and prepared the manuscript. E.I.L., J.C.C. and M.L.M. performed analysis of human genetic data. T.H.B. contributed to GWAS and resequencing experiments that identified the risk-associated SNPs at 1p22. A.C.L. planned experiments. R.A.C. planned experiments, supervised the project and prepared the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Liu, H. et al. Identification of common non-coding variants at 1p22 that are functional for non-syndromic orofacial clefting. *Nat. Commun.* 8, 14759; doi: 10.1038/ncomms14759 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2017